PhD-FSTM-2023-015
The Faculty of Science, Technology and Medicine

DISSERTATION

Defence held on 24/01/2023 in Belvaux

to obtain the degree of

DOCTEUR DE L'UNIVERSITÉ DU LUXEMBOURG

EN BIOLOGIE

by

Perrine FLORENT
Born on 15 July 1994 in Béthune (France)

SOIL VIRAL PARTICLES AS TRACERS OF SURFACE WATER SOURCES AND FLOW PATHS

Dissertation defence committee

Dr Leslie OGORZALY, dissertation supervisor
Doctor, Luxembourg Institute of Science and Technology (LIST)

Dr Jean-Luc BUEB
Professor, Université du Luxembourg

Dr Laurent PFISTER, Chairman
Doctor, Luxembourg Institute of Science and Technology (LIST)
Affiliate Professor, Université du Luxembourg

Dr Christophe GANTZER
Doctor, Laboratoire de Chimie Physique et Microbiologie pour les Matériaux et l'Environnement (LCPME)
Professor, UMR 7564 CNRS-Université de Lorraine

Dr Anne-Claire BAUDOUX
Doctor, Station biologique de Roscoff, Centre National de la Recherche Scientifique (CNRS)

Affidavit

I hereby confirm that the PhD thesis entitled "**Soil viral particles as tracers of surface water sources and flow paths**" has been written independently and without any other sources than cited.

Luxembourg, 09/03/2023 _____

Name Perrine FLORENT

Summary

Albeit recent technological developments (e.g. field deployable instruments operating at high temporal frequencies), experimental hydrology is a discipline that remains measurement limited. From this perspective, trans-disciplinary approaches may create valuable opportunities to enlarge the number of tools available for investigating hydrological processes. Tracing experiments are usually performed in order to investigate the water flow pathways and water sources in underground areas. Since the 19th century, researchers have worked with hydrological tracers to do this. Among them, the fluorescent dyes and the isotopes are the most commonly used to follow the water flow while others like salts or bacteriophages are employed as additional tracers to those mentioned above. Bacteriophages are the least known of all, but it has been studied since the 1960s as hydrological tracers, especially in karstic environments. The purpose is to evaluate the potential for bacteriophages naturally occurring in soils to serve as a new environmental tracer of hydrological processes. We hypothesize that such viral particles can be a promising tool in water tracing experiments since they are safe for ecosystems. In both fields of hydrology and virology, the knowledge regarding the fate of bacteriophages within the pedosphere is still limited. Their study would not only allow proposing potential new candidates to enlarge the hydrological tracers available, but also improving the current knowledge about the bacteriophage communities in soil and their interactions with certain environmental factors. For this purpose, we aim at describing the bacteriophage communities occurring in the soil through shotgun metagenomics analysis. Those viruses are widely spread in the pedosphere, and we assume that they have specific signatures according to the type of soil. Then, bacteriophage populations will be investigated in the soil water to analyse the dis/similarities between the two communities as well as their dynamics in the function of the precipitation events. This way, with a relatively high abundance of soil and soil water and a capacity of being mobilised, good bacteriophage candidates could be selected as hydrological tracers.

Acknowledgements

All right… After four years of working on this PhD project and months of writing this manuscript, as surprising as it may sound “all right” is the perfect word to express what I am currently feeling. Writing the acknowledgements is what I consider the ultimate conclusion of a PhD work. It is the moment when you realise that you are concluding four years of hard, intense and passionate work. As I wrote the first words of the acknowledgements, "all right" was the first that came to mind. A way of pausing and reflecting on what I wanted to say. The past few years have clearly been a mixture of many different feelings. The excitement about the project and its execution, the pride of publishing the results, and the satisfaction of accomplishment. However, I also experienced a lot of obstacles and disappointments. The long hours in the laboratory to optimise methods that will unfortunately not be used as planned, the numerous rejections for publications... far too many by the way, or the pressure to redo numerous analyses in a very short time following errors that were noticed much too late. Despite the excitement of research, doubt and stress often took over, leading to the belief that one would fail. I was overcome by these emotions many times during my PhD, often wondering if I was really up to the task. But in reality, looking back on these four years, I have had an incredible experience that forced me to surpass myself. This journey has allowed me to learn a lot more about my abilities, about what I am capable of doing. In the end, I am proud of the opportunity I was given, and everything ended up **all right**. This achievement would not have been possible without the help of many people whom I would like to take some time to deeply thank.

I would like to acknowledge first the funding received from the **Luxembourg National Research Fund (FNR)** without whom the project would have never been possible.

I would like to thank **Dr Stéphan JACQUET** as well as **Dr Jean-Luc BUEB** for accepting to participate in my annual review committee. Your feedback and expertise have helped me enormously to evolve in this PhD project year after year.

I am very grateful to **Dr Anne-Claire BAUDOUX** and **Dr Christophe GANTZER** for their participation in my thesis jury as external members. Your presence, your attentive reading of my thesis as well as our discussions during this defence were truly appreciated and helpful to me.

I am also grateful to **Prof**. **Laurent PFISTER** who coordinates the **HYDRO-CSI Doctoral Training Unit (DTU)**, in which my PhD project was embedded with 13 other PhD projects. The DTU gave me the incredible opportunity to share and work together with people from everywhere and with different backgrounds. Thank you, Laurent, for your kind advice all along my PhD and for giving me your expertise in hydrology. It was a great pleasure to have discussions with you. In the same line, I would like to thank all the PhDs from the DTU, Jasper, Anne, Jie, Concetta, Alessandro, Tessa, Enrico, Henry, Dhruv, Judith, Carol, Niels and Nicolaus, for sharing all or part of this adventure and also the ones who were not involved in the DTU but remained significant to this PhD journey, Gitanjali, Vita, Guilhem, Ginevra, Samuele, Adnan, and Emmanuella.

A very special thank goes to my supervisor, **Dr Leslie OGORZALY**, for… everything actually. Thank you greatly for trusting me with this PhD project and for giving me the opportunity to work on this subject. You passed on to me your great interest in virology, you have clearly changed my vision of viruses. You have guided me and accompanied me all along this incredible adventure, throughout the methodological obstacles and publication rejection. You have been available and supportive; I could have never achieved any of this without your help.

Another special thank you to my co-supervisor, **Dr Henry-Michel CAUCHIE**, for trusting me too with this PhD project. Thanks for all the discussions we had about ecology and statistics, I felt much less alone in my passion for statistics, which apparently seems to be totally crazy to "normal people" (I don't get it…). You have definitely played a complementary role, with Leslie, absolutely valuable for my PhD; the fusion of your two expertise helped me to forge my own, and thus to grow professionally.

I would like to thank the entire Microbiology team: **Dr Christian PENNY**, **Dr Jean-Baptiste BURNET**, **Dr Louise HOCK**, **Cécile WALCZAK**, **Delphine COLLARD**, **Dr Manon CHASSAING**, **Victoria STOPA** and **Dr Nathalie GEODERS** for all these team meetings eating and laughing (and working too, of course). It was truly a pleasure working and exchanging with you all.

I would like to thank **Dr Magdalena CALUSINSKA** for introducing me to bioinformatics and helping me to understand this field, which was still unknown to me before my PhD. I will always be grateful to **Dr Malte HEROLD** for his expertise in bioinformatics, and especially for improving my pipelines and solving issues I did not know existed; you have clearly been a "PhDsaver".

My next thanks go to **Jérôme Juilleret** and **Jean-François Iffly** for their valuable help in the set-up and guidance of the field experiments, especially on the Weierbach. I really enjoyed discussing with you.

I spent four years at the same desk in the same office, but the office A106 was clearly a mill where some incredible people came through, and whom I would like to thank, from the oldest, Valentin, Mariem, Mahmood, Brice, Jie, Dominika and Jasper to the newest, Delphine, Cécile, Manon, Victoria, Manu, Jennyfer and Thierry. **Dominika**, I loved sharing this space and discussing with you. I will truly miss our ingenious entrepreneurial ideas mainly based on coloured lab coats. **Victoria**, thank you for your joie de vivre, and of course, long live tiramisu and amaretto! **Jenny**, I didn't think I would find someone who can be as both calm and crazy as me. Thanks for all the crazy laughs. The best for last and I'm sure you'll agree with that. **Thierry**, my twin ch'timi with whom I have a scary shared fate, thank you for our discussions mostly focusing on one subject (tmtc) and all the laughter. I don't think I'm wrong when I say that we'll meet again, fate will take care of itself. A l'arvoyure min t'chio!

I could not write these acknowledgements without deeply thanking **Dr Maureen FEUCHEROLLES**. I see you laughing when you read "Dr." and I know you like it. We have been on this PhD adventure together almost from the beginning to the end. We lived everything, every emotion, every exasperation, every joy, every doubt. I loved when we complained together about everything, we were the Vamps of the LIST. So, thank you for being my friend.

I would like to thank **Louis KRIEGER**, the PhD warrior. You have been my friend and confidant since we met. It has always been a pleasure to talk freely with you. Our coffee breaks have been a real escape. We still have a thanksgiving to do together, that's a promise, especially for all those pumpkin pies and carrot cakes that you make so well (which are also the main reason for my friendship with you, of course). Thank you for everything.

My friend and my co-pilot, **Benjamin DEY**. Thanks for all the discussions, both serious and crazy. Going to work has never been as much fun for me as it was when we made the trip together. Fun fact, we had so much to talk about, that even 45 minutes was not enough time to address everything. I wish you the best for you and your incredible family with Florence et Momo. Thank you two for the dinners and those moments outside the PhD.

I don’t know how to thank them, my best friends, the ones who have been there for me over the years. **Ziming**, **Amal**, **Yoann**, **Sarah** and **Mickael**, believe me, it brings tears to my eyes to write these words. You have been and still are the family I have chosen. I grew up by your side, I discovered so many cultures and traditions. We shared madness, food, tears, laughs and incredible adventures. They were my most beautiful years. We are geographically far away today but we will never lose sight of each other, I promise! I also would like to thank my best friend for the last… 17 years, my sister, **Marianne**. Our friendship has been a given for so long. We share this love for travel and so we have been together very little for the last couple of years, but it has never taken anything away from the value of our friendship. It has always been the most incredible. Finally, **Arnaud** and **Aurore** (also Neix and Skadi), you have been my roots, each time I came back to "My North" as I like to call it. Thank you for being there all the time.

Here comes the biggest thank you I would like to give. To my family. To my mom and my dad, **Nathalie** and **Alain FLORENT**, to my brother, **Nicolas FLORENT** and my grandparents, **Rina** and **Pierre CAUCHY**. Vous m'avez toujours supporté, depuis le début. Les mots ne sont clairement pas suffisants pour vous dire à quel point sans vous je n'y serai jamais arrivé. Vous avez été mes piliers, vous m'avez fait confiance et m'avez encouragé. Vous avez toujours cru en moi, alors même que je doutais de moi-même. Je vous aime fort, Vi amo molto !

My love, **Beni**, how can I thank you for everything? Thank you for sharing your life with me, you are an incredible, kind and loving human being. You have been unconditionally supportive. You have lived this adventure with me, undergoing my stress, my madness and my stubbornness. Thank you so much for your patience. Köszönök mindent, túlságosan szeretlek.

Finally, I would like to thank all the people I have not mentioned in these acknowledgements, but who have participated in some way in this project; nothing could have been done without the collaboration of absolutely everyone.

Table of content

INTRODUCTION 1

CHAPTER 1: STATE-OF-THE-ART 5

1.1 FUNDAMENTALS OF BACTERIOPHAGES 5

1.1.1 Morphological and genomic diversity 5

1.1.2 Reproduction cycles 6

1.1.3 Taxonomical diversity 8

1.2 BACTERIOPHAGE ECOSYSTEM ECOLOGY 13

1.2.1 Environmental diversity of bacteriophages 13

1.2.2 Impact of environmental factors on bacteriophage dynamics 19

1.2.3 Detection methods for bacteriophages in environments 22

1.3 THE APPLICATION OF BACTERIOPHAGES IN HYDROLOGY 28

1.3.1 Hydrological tracers and requirements 28

1.3.2 Fundamentals and limitations of the artificial tracers 30

1.3.3 Emerging of innovative tracers: and if they were already there? 31

1.4 BACTERIOPHAGES AS HYDROLOGICAL TRACERS: PAST, PRESENT AND FUTURE 35

CHAPTER 2: OBJECTIVES AND METHODOLOGIES 67

2.1 RESEARCH AIMS AND OBJECTIVES 67

2.2 METHODOLOGY 69

2.2.1 Studied areas 70

2.2.2 Sample collection and selected data 71

2.2.3 Viral identification and diversity analyses 72

2.2.4 Optimisation of the bioinformatics pipeline 74

CHAPTER 3: RESULTS 79

3.1 WP1: BACTERIOPHAGE DIVERSITY IN THE SOILS OF LUXEMBOURG 79

3.1.1 Optimisation methodology 1: Elution and concentration of viruses and improvement of DNA yield and library construction from soil samples 80

3.1.2 Soil pH, calcium content and bacteria as major factors responsible for the distribution of the known fraction of the DNA bacteriophage populations in soils of Luxembourg *93*

3.2 WP2: MOBILISATION OF THE BACTERIOPHAGES FROM THE SOIL MATRIX TO THE SOIL WATER **119**

3.2.1 Optimisation methodology 2: Investigation of the passage of bacteriophages through the porous candles *120*

3.2.2 Mobilisation of the bacteriophages from the soil matrix to the soil water *135*

CHAPTER 4: DISCUSSION AND PERSPECTIVES **189**

4.1 LIMITATIONS OF THE CURRENT METHODOLOGY **190**

4.2 DYNAMICS OF NATURALLY OCCURRING DNA-BACTERIOPHAGES IN THE TERRESTRIAL COMPARTMENT **193**

4.2.1 Characterisation of natural populations of DNA-bacteriophages in various types of soil *193*

4.2.2 Mobilisation of natural populations of DNA-bacteriophages in the soil of Weierbach *196*

4.3 WITHIN THE PROCESS OF SELECTING BACTERIOPHAGE CANDIDATES AS HYDROLOGICAL TRACERS **198**

4.3.1 Understand the catchment of interest to improve the use of natural bacteriophages as hydrological tracers *198*

4.3.2 Best-suited bacteriophage candidates according to the criteria of an ideal tracer *202*

4.4 METHODOLOGICAL PROPOSALS FOR FURTHER ANALYSES **209**

4.4.1 Development of detection methods specific to the selected bacteriophage *209*

4.4.2 Towards a better understanding of the fate of RSL1 for future applications in tracing experiments *210*

CONCLUSION **215**

REFERENCES **217**

List of Figures

FIGURE 1: THE DIFFERENT BACTERIOPHAGE FAMILIES AND THEIR RESPECTIVE SHAPES (ELBREKI ET AL., 2014) 6
FIGURE 2: LYTIC AND LYSOGENIC CYCLES OF BACTERIOPHAGES (KHAN, 2019). 8
FIGURE 3: GENERAL SCHEME OF THE RESEARCH AIM AND OBJECTIVES OF THE PHD PROJECT. 69
FIGURE 4: WORKFLOW OF THE METHODOLOGY DESIGNED DURING THE PHD PROJECT TO IDENTIFY NATURALLY OCCURRING BACTERIOPHAGE POPULATIONS. 70
FIGURE 5: MAP OF THE STUDY SITE, THE ATTERT RIVER BASIN, WITH THE LOCATION OF THE EIGHT SELECTED SAMPLING SITES (MODIFIED FROM GEOPORTAIL.LU). THE PLAIN BLACK LINE REPRESENTS ROUGHLY THE BORDER BETWEEN THE LUXEMBOURG AND BELGIUM SIDES OF THE BASIN, AND THE DASHED RED LINE IS THE LIMIT BETWEEN THE TWO LUXEMBOURG REGIONS: OESLING AND GUTLAND. 71
FIGURE 6: WORKFLOWS FOR THE OPTIMISATION OF THE BIOINFORMATICS PIPELINE TO OBTAIN THE MICROBIAL TAXONOMIC CLASSIFICATION. 75
FIGURE 7: (A) GENERALISED SCHEME OF THE RELATIONSHIP BETWEEN SOIL TEXTURE AND SOIL WATER CONTENT (%) WITH THE DASHED LINES REPRESENTING THE RANGE OF SOIL WATER CONTENT MEASURED ON THE SOIL OF WEIERBACH (≅ 20-26 %) (O'GREEN, 2013). (B) DIFFERENT STEPS OF THE DRAINAGE ACCORDING TO THE INCREASING SOIL WATER CONTENT (%), PORE SPACE AND SOIL MOISTURE POTENTIAL (MPA) (O'GREEN, 2013). 199
FIGURE 8: STRUCTURE OF THE BACTERIOPHAGE RSL1 WITH (A) THE CAPSID ORGANISATION IN 3D RECONSTRUCTION AND ITS CONCENTRIC DNA GENOME INSIDE, (B) A 3D RECONSTRUCTION OF THE HELICAL TAIL (EFFANTIN ET AL., 2013). (C) MORPHOLOGY OF THE BACTERIOPHAGE RSL1 WITH AN ELECTRON MICROGRAPH (YAMADA ET AL., 2007) (SIZE OF BAR = 100 NM). 206
FIGURE 9: MORPHOLOGY OF THE GENUS *OMEGAVIRUS*, TO WHICH THE SPECIES *OMEGAVIRUS THIBAULT* BELONGS (SWISS INSTITUTE OF BIOINFORMATICS, 2015). 208

List of Tables

TABLE 1: CLASSIFICATION OF BACTERIOPHAGES ACCORDING TO THE UPDATED TAXONOMY PROPOSED BY THE ICTV (AS OF MARCH 2022) 10
TABLE 2: CONCENTRATION OF VIRAL PARTICLES FOUND IN THE AQUATIC ECOSYSTEMS, (A) MARINE AND (B) FRESHWATER ECOSYSTEMS, WITH SOME SPECIES OF BACTERIOPHAGES RESPECTIVELY IDENTIFIED. 15
TABLE 3: CONCENTRATION OF VIRAL PARTICLES FOUND IN THE TERRESTRIAL ENVIRONMENTS WITH SOME SPECIES OR TYPES OF BACTERIOPHAGES RESPECTIVELY IDENTIFIED 18
TABLE 4: ISOELECTRIC POINTS OF SOME BACTERIOPHAGES (MODIFIED FROM MICHEN AND GRAULE, 2010) ... 22
TABLE 5: SUMMARY OF THE METHODS USED TO DETECT BACTERIOPHAGES WITH THEIR RESPECTIVE ADVANTAGES AND DISADVANTAGES. 26
TABLE 6: RELEVANT HYDROLOGICAL TRACERS (LEIBUNDGUT AND SEIBERT, 2010) 29

TABLE 7: ALL THE EIGHT CRITERIA PREDEFINED TO BEST MEET THE REQUIREMENTS OF AN IDEAL TRACER, FOR THE CONVENTIONAL TRACERS (I.E. DYES, SALTS AND ISOTOPES) AND BACTERIOPHAGES AS BOTH ARTIFICIAL (I.E. INJECTED) AND ENVIRONMENTAL (I.E. NATURAL POPULATIONS) TRACERS. 34
TABLE 8: SUMMARY OF THE MOST IMPORTANT CHARACTERISTICS OF ALL SITES AS PART OF THE WP1, WHERE THE 8 SITES ARE DISPLAYED WITH THEIR VEGETATION, GEOLOGICAL REGION LOCATION, SOIL PH, CALCIUM CONTENT, CARBON/NITROGEN (C/N) RATIO AND THE BACTERIOPHAGE AND BACTERIAL DIVERSITIES 195
TABLE 9: THE EIGHT CRITERIA PREDEFINED TO BEST MEET THE REQUIREMENTS OF AN IDEAL TRACER, FOR THE CONVENTIONAL TRACERS (I.E. DYES, SALTS AND ISOTOPES) AND BACTERIOPHAGES AS BOTH ARTIFICIAL (I.E. INJECTED) AND ENVIRONMENTAL (I.E. NATURAL POPULATIONS) TRACERS. 204

List of abbreviations

AFM	Atomic Force Microscope
cDNA	complementary DeoxyriboNucleic Acid
C/N	Carbon to Nitrogen ratio
CEC	Cation Exchangeable Capacity
CFM	Chemical Force Microscopy
CPG	Casamino acid-Peptone-Glucose
DNA	DeoxyriboNucleic Acid
DTU	Doctoral Training Unit
FCM	Flow CytoMetry
GC	Genomic Copies
HIM	Helium-Ion Microscopy
ICTV	International Committee on Taxonomy of Viruses
kbp	kilobase pairs
NCBI	National Center for Biotechnology Information
NGS	Next-Generation Sequencing
nr	non redundant database
ORF	Open Reading Frame
OTU	Operational Taxonomic Unit
(RT)-qPCR	RetroTranscription - quantitative Polymerase Chain Reaction
ddPCR	digital droplet Polymerase Chain Reaction
pH	potential Hydrogene
pI	Isoelectric point
PFU	Plaque Forming Unit
RNA	RiboNucleic Acid
TEM	Transmission Electron Microscopy
UV	UltraViolet
VLP	Virus-Like Particles
WP	WorkPackage

INTRODUCTION

Today, the long-term impacts of global change are well recognized, with multiple effects on the environment having been observed and documented. Water, energy, transportation, wildlife, agriculture, ecosystems as well as human health are all consequently damaged by global change. Water resources are particularly under threat due to the continuous degradation of water quality. Consequently, there is a pressing need to improve our understanding of the fundamental hydrological and biological functions of catchments to manage, protect and restore the aquatic environments.

The current PhD project, called HydroPhage is embedded into the FNR PRIDE HYDRO-CSI DTU (Doctoral Training Unit) program that is based on the hypothesis that the stationarity of environmental systems is a limitation to the development of new knowledge, forecasting and prediction tools for further understandings of hydrological processes. The fourteen individual PhD projects of the DTU shall contribute to reducing the knowledge gaps inherent to the complex rainfall-runoff transformation processes and feedback mechanisms within hydrological systems. The PhD projects are wired around four complementary thematic clusters: (1) Innovative field-deployable instruments for high-frequency monitoring; (2) New tracers and approaches for investigating hydrological processes; (3) Remote sensing applied to hydrology – New vistas from faster revisit times and high-resolution imagery; (4) Hydrological forecasts and projections under global change.

This PhD project is part of the second cluster and proposes to explore the potential of viruses, and more specifically bacteriophages, as new and alternative tracers for investigating surface water sources and flow paths. As a first step, a history of the use of bacteriophages as hydrological tracers since the 1960s has been compiled. This has enabled us to highlight the numerous advantages of using these bacteriophages to understand hydrological processes, but also to note the presence of certain limitations. As an artificial tracer, bacteriophages were introduced at a specific point into the environment studied to follow its transport within the water cycle processes. The major limitation that attracted our attention is the volume of bacteriophage solution injected into the environment, leading to a potentially negative environmental impact that remains poorly understood. In line with the current environmental

context, this project aims to develop a tracer from the natural population of bacteriophages. As a matter of fact, ecosystems contain a large diversity of bacteriophages naturally present, particularly in the terrestrial compartment. For this reason, the main objective of this thesis project is to **assess the potential of natural soil bacteriophages to serve as environmental tracers of hydrological processes, with the ambition of identifying the bacteriophage communities and proposing relevant and sustainable environmental tracer candidates**. The ambition of this project is to demonstrate that soil bacteriophages can be transported from one compartment to another within the water cycle. Bacteriophages originating from the terrestrial compartment would be used as new environmental tracers with the interest of understanding the connectivity from soil to potentially groundwater or surface water. However, as they have never been used in this way to date, the use of these tracers requires in-depth studies prior to their application. This pre-application exploration consists of identifying natural populations of bacteriophages and then studying their dynamics on a small scale. Therefore, two work packages (WPs) have been built around these two important issues, as follows:

1. **WP1** is focused on studying DNA-bacteriophage communities in several types of soils in Luxembourg in relation to biotic and abiotic factors
2. **WP2** investigates the mobilisation of DNA-bacteriophages from the soil to the soil water, potentially triggered by precipitations

The results of this research allowed us to rule on the potential of bacteriophages to become environmental tracers but also to ideally highlight bacteriophage species among the diversity identified. This manuscript will therefore be a guide in the exploration of these new tracers with, in a first step, a state-of-the-art with description of the diversity of bacteriophages at the structural, genomic, taxonomic and ecological levels, their relationship with the environment and their past involvement in tracing experiments. This will be followed by a more detailed explanation of the objectives of the work and the methodology applied, and the results obtained will be provided in the form of scientific articles (published or in preparation to be shortly submitted). Finally, these results will be discussed in depth and perspectives will be proposed for further developments to better understand the relevance of the potentially selected tracers.

Chapter 1:

STATE-OF-THE-ART

Chapter 1:

STATE-OF-THE-ART

1.1 Fundamentals of bacteriophages

Bacteriophages are defined as viruses that specifically infect and replicate in bacterial cells (Moineau, 2013). With an estimated total of 10^{31} viral particles on the planet (Keen, 2015), they are considered the most abundant biosphere biological entities and ubiquitous (Clokie et al., 2011). Besides their significant abundance, bacteriophages are very diverse in terms of shape, size, and genome (Grose and Casjens, 2014; Jacobs-Sera et al., 2020; Yuan and Gao, 2017). Their basic morphological structure consists of a protein capsid surrounding the genome and can sometimes be supplemented with a lipidic envelope.

1.1.1 Morphological and genomic diversity

The capsid is organised into repetitive structural protein subunits and can have different specific shapes: icosahedral, prolate or helical (Louten, 2016; Luque and Reguera, 2010). For some bacteriophages, the structure can become more complex with the addition of extra elements such as a tail, fibres, collar or even spikes (Figure 1) (Jończyk et al., 2011; Wommack et al., 1992). While their morphological size range is commonly reported to be 20 - 200 nm in length (Rossi and Käss, 1998), the capsid diameter varies from 50 nm, i.e., bacteriophages λ and T4, to 150-180 nm, i.e., bacteriophage G (Ageno et al., 1973; Hua et al., 2017). Bacteriophages survive differently in the environment depending on their morphology and their size. Investigations on bacteriophage survival during storage revealed that tailed and icosahedral bacteriophages showed better survival at 4°C and -80°C, with however individual variations (Dawson et al., 2005; Mercanti et al., 2012). For example, the tailed bacteriophages P2 and Mu have been found to be sensitive at 4°C through the loss of the tail sheaths, suggesting a higher sensitivity due to the fragility of the tails (Ackermann et al., 2004). Finally, bacteriophages with a lipid envelope around their capsid showed lower persistence than those without under environmental stress (e.g. temperature, UV) (Ceballos et al., 2012; Silverman and Boehm, 2020).

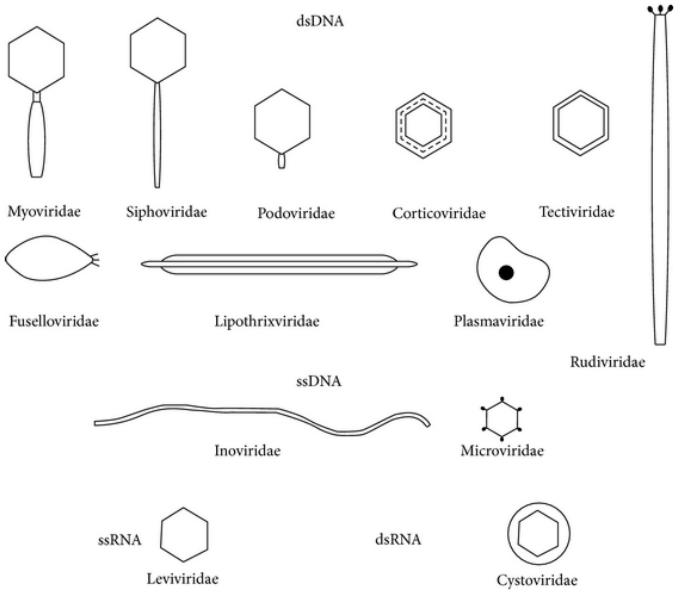


Figure 1: The different bacteriophage families and their respective shapes (Elbreki et al., 2014)

The development of molecular biology has allowed knowledge of the genetic diversity of bacteriophages in the environment to evolve by increasingly elucidating new genomic sequences (Batinovic et al., 2019). Thus, the bacteriophage genome has shown a great variation in shape and size. It can be composed of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), being linear or circular and single- (ss) or double-stranded (ds) (Hatfull and Hendrix, 2011). In addition, ssRNA bacteriophage genomes can exist in two variants; positive sense and negative sense (Callanan et al., 2018; Li et al., 2015; Wolf et al., 2018). Its size ranges from 3.4 kilobases (kb) for the bacteriophage MS2 (Friedman et al., 2009) to nearly 500 kilobase pairs (kbp) for the bacteriophage G (Hatfull and Hendrix, 2011). Among the bacteriophages currently known and described those with double-stranded DNA (dsDNA) are commonly considered to be the most represented (Clokie et al., 2011; Mavrich and Hatfull, 2017; Dion et al., 2020).

1.1.2 Reproduction cycles

Bacteriophages are not capable of self-reproduction. To replicate, they must infect their bacterial host and propagate either through a lytic replication cycle or a lysogenic one (Figure 2) (Clokie et al., 2011; Haq et al., 2012). In both cases, virions, complete infectious virus

particles, initiate replication by attaching to the outermost layer of the bacterial envelope or one of its structures, such as a flagellum or pili (Cota-Robles and Coffman, 1963; Tars, 2020). Then, they introduce their genome through the bacterial membrane, into the cytoplasm of the cell (Kasman and Porter, 2021; Maghsoodi et al., 2019). The steps following the introduction of the viral genomes depend on the reproduction cycles initiated by the bacteriophages. Most bacteriophages replicate through a lytic cycle (Moon, 2017). In this cycle, the bacteria's cellular machinery is used to initiate the biosynthesis of the new viral proteins and genomes within the bacterial cell. The new materials then assemble to create new bacteriophages, which are released, causing the lysis of the host bacteria, and seeking out new bacteria to infect (Salmond and Fineran, 2015). In comparison, the lysogenic cycle does not result in the degradation of the host cell. Instead, the bacteriophage genome integrates into the bacterium's genome, as a prophage. This cycle will remain stable and follow classical cell division until the prophage enters a lytic cycle as a result of DNA damage caused by environmental stressors (e.g. presence of antibiotics, UV, mitomycin C) (Davies et al., 2016; Fortier and Sekulovic, 2013; Howard-Varona et al., 2017). Both lytic and lysogenic viruses can be observed in the environment, for which the respective role must be distinguished. Temperate bacteriophages, which follow a lysogenic cycle, play significant roles in bacterial evolution, notably being a source of genetic variation through horizontal gene transfer or even by increasing bacterial competitiveness in the survival process (Harrison and Brockhurst, 2017). Temperate bacteriophages are rather found in less stable environments, where bacterial populations fluctuate the most (i.e. marine systems, high latitude) (Angly et al., 2006; Davies et al., 2016).

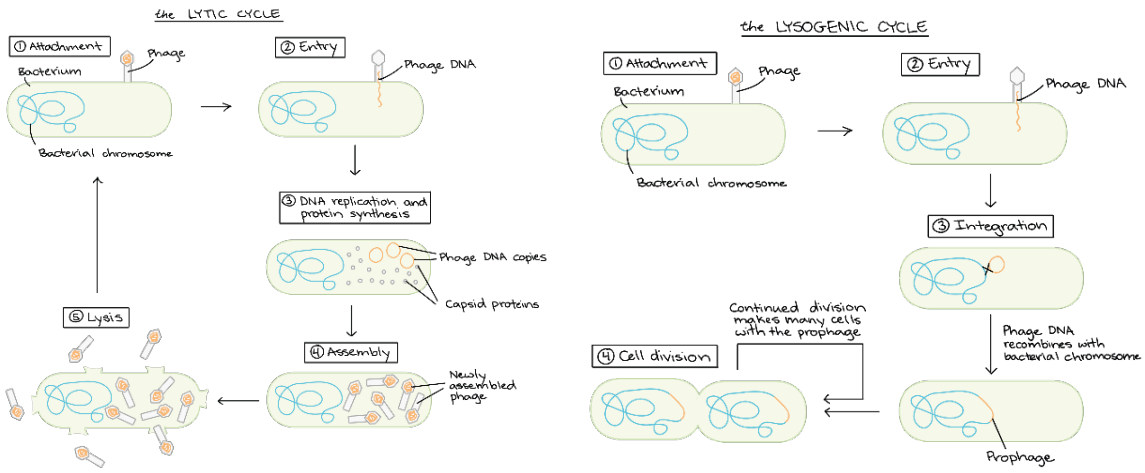


Figure 2: Lytic and lysogenic cycles of bacteriophages (Khan, 2019).

1.1.3 Taxonomical diversity

Bacteriophage taxonomy is presented by the International Committee on Taxonomy of Viruses (ICTV), which classifies them according to their morphology and the type of nucleic acid (Lefkowitz et al., 2018). Before the development of molecular biology, bacteriophages were organised within the taxonomy according to their morphology based on electron microscopy observations (e.g., presence of contractile or non-contractile tail, lemon-shape, filamentous, and so on) (Lopes et al., 2014a; White and Orlova, 2019). The order *Caudovirales* was created to gather tailed bacteriophages, including especially the three most common families, *Siphoviridae*, *Myoviridae* and *Podoviridae*. Then, other families of bacteriophages appeared, encompassing bacteriophages with similar morphotypes, such as *Inoviridae*, *Microviridae*, *Tectiviridae*, *Corticoviridae*, *Plasmaviridae*, *Leviviridae*, and *Cystoviridae* (Novik et al., 2004; Ackermann, 2011; Tolstoy et al., 2018; ICTV, 2020). With the advent of sequencing techniques, knowledge of bacteriophages has constantly been growing and thus, the viral taxonomy has continually been updated (Walker et al., 2020). Today, viral taxonomy no longer depends solely on morphology but is updated according to genomics and phylogeny (Harris and Hill, 2021; Turner et al., 2021). With the development of viral databases, bioinformatic tools revealed the absence of monophyly between the members of the families *Siphoviridae*, *Myoviridae* and *Podoviridae*. Therefore, as of March 2022, the order *Caudovirales* was dissolved as well as the three families *Siphoviridae*, *Myoviridae* and *Podoviridae* (Turner et al., 2021). All members of the former order *Caudovirales* were then transferred to the class *Caudoviricetes*, and four new orders were

created: *Crassvirales*, *Kirjokansivirales*, *Methanobavirales*, *Thumleimavirales* (Koonin and Yutin, 2020). Most of the traditional families are now assigned to unclassified *Caudoviricetes* and members of the families are either reassigned to new families or affiliated with other already known families. However, the distinction between these three former families remains discernible, with the creation of the morphotypes Myovirus, Siphovirus and Podovirus. The morphotype Myovirus, also called morphotype A, has a head-tailed structure with a tubular tail and additional structures such as a baseplate and collar. Siphovirus displays an icosahedral capsid with a noncontractile tail for morphotype B1 or a single prolate capsid for morphotype B2. Finally, Podovirus, or morphotype C, presents an icosahedral with a short noncontractile tail and stacked disks with fibres (Turner et al., 2021). For the rest of the manuscript, the names of the three former families (i.e. *Siphoviridae*, *Myoviridae* and *Podoviridae*) will be kept, as well as the order *Caudovirales*, since the databases that gather the bacteriophage sequences and the respective taxonomy used in the PhD project, did not include the last update of the ICTV yet. This new taxonomy of bacteriophages can be summarised as reported in Table 1.

Table 1: Classification of bacteriophages according to the updated taxonomy proposed by the ICTV (as of March 2022)

Order	Family	Structure	Genome	Examples	References
Halopanivirales	*Matshushitaviridae*	≅ 100 nm capsid	Circular dsDNA	ΦIN93	(Matsushita and Yanase, 2009, 2008)
Kalamavirales	*Tectiviridae*	Inner lipid vesicle, pseudo-tail	dsDNA	PRD1	(Schulz et al., 2019)
Ligamenvirales	*Lipothrixviridae*	Rod-shaped capsid	dsDNA	SIVF	(Prangishvili and Krupovic, 2012)
	Rudiviridae	Rod-shaped capsid	dsDNA	SIRV1	
Mindivirales	*Cystoviridae*	Segmented, lipid envelope	dsRNA	f6	(Poranen and Mäntynen, 2017)
Norzivirales (former *Levivirales*)	*Atkinsviridae*	icosahedral capsid	ssRNA	-	(Callanan et al., 2021)
	Duiniviridae	Icosahedral capsid	ssRNA		
	Fiersviridae (former *Leviviridae*)	icosahedral capsid	ssRNA	MS2	
	Solspiviridae	Icosahedral capsid	ssRNA	-	
Petitvirales	*Microviridae*	Icosahedral capsid	ssDNA	ΦX174	(Roux et al., 2012)
Timlovirales	*Blumeviridae*	-	ssRNA	-	(Callanan et al., 2021)
	Steitzviridae	-	ssRNA	-	
Tubulavirales	*Inoviridae*	filamentous	ssDNA	M13	(Knezevic et al., 2021)
	Paulinoviridae	filamentous	ssDNA		
	Plectroviridae	filamentous	ssDNA		

Vinavirales		*Corticoviridae*	Icosahedral capsid with an internal lipid membrane	dsDNA	PM2	(Oksanen and Consortium, 2017)
Durnavirales		*Picobirnaviridae (proposal)*[1]	Icosahedral capsid	dsRNA	-	(Delmas et al., 2019)
Crassvirales		*Crevaviridae*	Icosahedral capsid with tail	dsDNA	-	(Dutilh et al., 2012; Koonin and Yutin, 2020)
		Intestiviridae	Icosahedral capsid with tail	dsDNA	-	
		Steigviridae	Icosahedral capsid with tail	dsDNA	-	
		Suoliviridae	Icosahedral capsid with tail	dsDNA	-	
Unclassified *Caudoviricetes*	Morphotype A: Myovirus (former *Myoviridae*)	*Ackermannviridae*	contractile tail	dsDNA	MAM1	(Turner et al., 2021)
		Chaseviridae	Icosahedral capsid, contractile tail	dsDNA	sv4HA13	
		Straboviridae	-	dsDNA	-	
		…[2]				
	Morphotype B: Siphovirus (former *Siphoviridae*)	*Demerecviridae*	non-contractile tail	dsDNA	T5	
		Drexlerviridae	Icosahedral capsid, non-contractile tail	dsDNA	T1	
		Herelleviridae	contractile tail	dsDNA	SPO1	
		…[2]				
	Morphotype C: Podovirus (former *Podoviridae*)	*Autographiviridae*	Icosahedral capsid, head-tail structure	dsDNA	T7	
		Schitoviridae	Icosahedral capsid, head-tail structure	dsDNA	N4	

	Zobellviridae	Icosahedral capsid, head-tail structure	dsDNA	CVT22	
	…[2]				
Unassigned	*Autolykiviridae*	Icosahedral capsid	dsDNA	viph1249a	(Kauffman et al., 2018)
	Finnlakeviridae	Icosahedral capsid	ssDNA	FLIP	(Laanto et al., 2017)
	Fuselloviridae	Lemon-shaped, envelope	dsDNA	SSV1	(Ceballos et al., 2012)
	Plasmaviridae	pleomorphic, Lipid envelope	dsDNA	L2	(Krupovic and Consortium, 2018)

[1] it has been suggested that the members might infect bacteria and not mammals; [2] only the most popular families are listed for each morphotype, knowing that the lists are not exhaustive

1.2 Bacteriophage ecosystem ecology

1.2.1 Environmental diversity of bacteriophages

Most viruses found in the environment have been characterised as bacteriophages in terms of morphology and genome (Muniesa et al., 2011). Bacteriophages rely on their hosts to replicate. Therefore, they can be found wherever bacteria are present, and in relatively high proportions (Clokie et al., 2011). Their abundance and diversity have been characterised mainly in marine environments, with the oceans recognised as the most bacteriophage-dense ecosystem (Luo et al., 2017; Perez Sepulveda et al., 2016). Research on soil viral diversity has developed since the 1970s but remains poorly documented to date (Bi et al., 2022; Wu et al., 2021).

In aquatic environments

Bacteriophages are a tremendously abundant biological entity in marine environments because their hosts are numerically dominant in the sea waters (Breitbart et al., 2018; Luo et al., 2017). In both marine and freshwater environments, the concentration of viral particles varies from 10^5 to 10^7 per millilitre, with viruses being more abundant in marine ecosystems than in freshwater (Bergh et al., 1989; Suttle, 2005). Studies of aquatic compartments have mainly focused on the diversity of DNA viruses, resulting in the interpretation of a predominantly DNA virus community. However, with the growing interest in molecular techniques, the exploration of viral communities has been extended to the discovery of RNA viruses. Their presence in the aquatic compartment has proved to be more important than previously thought, with an abundance of ssRNA viruses (e.g. order *Picornavirales*) (Kolundžija et al., 2022). Marine ecosystems have been much more explored compared to freshwaters. Among these freshwaters, lakes have been the most studied water bodies (Bruder et al., 2016). The abundance of viruses was the highest in the wetlands with more than 1 billion VLPs in one millilitre of water, significantly correlated with the soil water content (Williamson et al., 2017). The viral compositions of the estuaries were shown to be mainly characterised by unidentified viruses and most of the known fraction included pelagiphages (infecting bacterial order Pelagibacterales) and cyanophages (infecting cyanobacteria). In addition, the composition was composed of a high proportion of *Myoviridae*, *Podoviridae*, *Siphoviridae* and *Phycodnaviridae* was highlighted (Aishwarya et al., 2021; Cai et al., 2022; Sun et al., 2021). (Batinovic et al., 2019; Børsheim, 1993; Sime-Ngando, 2014). Bacteriophages are indeed present in a wide variety of aquatic environments

(Jończyk et al., 2011; Massali and Bouaninba, 2016). In terms of the genome, the average genome size of aquatic bacteriophages has been reported to range from 10 to 660 kb in both marine and freshwater environments (Kavagutti et al., 2019; Sime-Ngando, 2014). The abundance of bacteriophages in the aquatic environment was found to be correlated with bacterial activities. Indeed, in winter, bacteriophage concentration showed a similar behaviour as bacteria since its concentration decreased when bacterial productivity was low. It has been shown that viral abundances vary more strongly in freshwater environments from one season to another due to a higher variation in seasonal cycles compared to marine environments (Sime-Ngando and Colombet, 2009). In marine ecosystems, the most abundant bacteriophages were found to be members of *Myoviridae*, *Siphoviridae*, *Podoviridae* and *Inoviridae* (Kimura et al., 2008; Parmar et al., 2018). Most marine bacteriophages were morphologically characterised as tails with icosahedral or octahedral heads, such as the *Pseudoalteromonas phage vB_PspS-H40/1* or *Alteromonas virus vB_PspP-H4/4* (Goldscheider et al., 2007; Kallies et al., 2017). The literature reports the dominance of *Podoviridae* and *Siphoviridae* on freshwaters as well as a high abundance of cyanophage (e.g., *Microcystis myocyanophages*) (Jaskulska et al., 2022; Skvortsov et al., 2016). As far as bacteriophages are concerned, those with an RNA genome are far from being the most representative of aquatic environments, since many species of RNA-bacteriophages are still unknown and among the order of bacteriophages listed by the ICTV, only two have an RNA genome (Adriaenssens et al., 2018; Krishnamurthy et al., 2016).

Table 2: Concentration of viral particles found in the aquatic ecosystems, (A) marine and (B) freshwater ecosystems, with some species of bacteriophages respectively identified.

(A)

Marine ecosystems	Country	Concentrations	Bacteriophages	References
Seas	Germany	-	*Phage H106/1, H100/1, H105/1*	(Wichels et al., 1998)
	USA	10^5-10^7 virus/mL	-	(Ortmann and Suttle, 2005)
	Germany	10^6 VLP/mL	*Myoviridae* > *Podoviridae* > *Siphoviridae* Including mainly *cyanophage*	(Garin-Fernandez et al., 2018)
	USA	10^3-10^8 virus/mL	*Cyanophage, tailed phages*	(Proctor and Fuhrman, 1990)
	Spain	-	*Myoviridae, Podoviridae*	(López-Pérez et al., 2017)
Oceans	Australia	-	*Myoviridae* > *Podoviridae* > *Siphoviridae*	(Focardi et al., 2020)
	Hawaii	-	*Prochlorococcus phage, Synechococcus phage, cyanophage*	(Luo et al., 2017)
	Antarctic	10^6-10^7 VLPs/mL	-	(Guixa-Boixereu et al., 2002)
	Antarctic	10^6 virus/mL	*dsDNA bacteriophages*	(Sotomayor-Garcia et al., 2020)
	Arctic	-	*T4-like phages*	(Filée et al., 2005)
	Norway	10^5-10^7 virus/mL	Domination of non-tailed phages	(Bratbak et al., 1990)
Estuaries	USA	10^6-10^8 virus/mL	Phage with single head structure	(Wommack et al., 1992)
	China	10^5-10^7 virus/mL	-	(Cai et al., 2022)
	USA	10^5-10^8 virus/mL	*Acinetobacter phages*	(Sun et al., 2021)

> means 'more abundant than'; VLP = virus-like particles

(B)

Freshwater ecosystems	Country	Concentrations	Bacteriophages	References
Rivers	France	-	*Myoviridae* > *Siphoviridae* > *Podoviridae* Including Prophage *LambdaSo*, Phage *phiE125*	(Leroy et al., 2008)
	Ireland	-	*Myoviridae*, *Podoviridae*, *Siphoviridae*	(Moon et al., 2020a)
	Austria	10^7 VLP/mL	Viruses with 60 to 90 nm head	
	India	-	*Klebsiella pneumoniae* specific phages	(Sundaramoorthy et al., 2021)
Lakes	Germany	10^5-10^7 virus/mL	*Myoviridae* > *Siphoviridae* > *Podoviridae* *Dan18/9, Gps18/13-15, Gps18/17*	(Wolf et al., 2003)
	USA	10^6 GC/mL	*Myoviridae* >> *Podoviridae* > *Siphoviridae* > *Phycodnaviridae*	(Mohiuddin and Schellhorn, 2015)
	Quebec	10^7-10^8 virus/mL	*>100 nm viruses, 50-70nm viruses*	(Maranger and Bird, 1995)
	USA	-	Phages ϕFenriz, ϕHabibi, ϕMoody, ϕVader	(Malki et al., 2015)
	South Korea	-	*Myoviridae* > *Podoviridae* > *Siphoviridae*	(Moon et al., 2020b)
	Switzerland	10^7-10^8 VLP/mL	Hexagonal or spherical heads without tails or no tails	(Filippini et al., 2006)
	Germany	10^7 phage/mL	Mainly tailed phages	(Hennes and Simon, 1995)
Wetlands	USA	10^5-10^8 virus/mL	-	(Williamson et al., 2017)
	USA	-	*Siphoviridae* > *Podoviridae* > *Myovridiae*	(Dalcin Martins et al., 2018)
	France	10^6-10^8 virus/mL	*Microviridae, Circoviridae, Caudovirales*	(Ballaud et al., 2016)
	USA	10^5 virus/mL	Planktonic viruses	(Farnell-Jackson and Ward, 2003)

> means 'more abundant than'; VLP = virus-like particles; GC = genomic copies

In terrestrial environments

As bacteria are numerous and play a significant role in soil life (Buerkert et al., 2012; Smith et al., 1985), bacteriophages should be particularly abundant in such environments. They can interact indirectly within the biogeochemical cycling by modifying the bacterial communities (Han et al., 2022; Williamson et al., 2005). Moreover, some soil characteristics have an important role in the distribution of bacteriophages. The abundance of bacteriophages increases with increasing soil moisture and organic content, while ploughing reduces the abundance of bacteriophages through bacteria (Armon, 2011; Walshe et al., 2010). In a comparative study led by Williamson *et al.* (2017), bacteriophages are found mainly in agricultural soils, forest soils (5.8×10^9 viral particles per gram dry weight) or even cold deserts (2.2×10^3 viral particles per gram dry weight) (Williamson et al., 2017). Then, their abundance is lower in forests because the soil is drier than in wetlands, while the abundance of bacteriophages in forests is higher than in agricultural soils due to higher soil temperatures in forests. We can thus conclude the important influence of both the water content and the temperature on the abundance of bacteriophages in soils. Regarding morphology, the most common bacteriophages found in soils are dsDNA tail-head bacteriophages (Ashelford et al., 2003), which include *Siphoviridae*, *Myoviridae* and *Podoviridae* (Han et al., 2022; Williamson et al., 2003). Trubl et al. (2019) reported identifying more dsDNA than ssDNA viruses in soils, with approximately 13 ssDNA OTUs (Operational taxonomic unit) vs 582 dsDNA vOTUs (Trubl et al., 2019). Another study highlighted 34% of ssDNA viruses and then 66% of dsDNA viruses among the identified populations in soils (Kim et al., 2008). However, various soils from Scotland and Ireland were reported to be dominated by small ssDNA viruses over dsDNA-tailed bacteriophages in the soil (< 2% of dsDNA sequences vs 70-80% ssDNA sequences), especially with the presence of the family *Microviridae* (Grujcic et al., 2018; Kim et al., 2008; Reavy etal., 2015). Regarding RNA-bacteriophages, the same observation as for the aquatic environment is made where bacteriophages with an RNA genome are also the less representative viruses of the terrestrial compartment in terms of ecological and taxonomical diversity (Krishnamurthy et al., 2016). As part of the RNA-bacteriophage diversity investigated in various grassland soils, bacteriophages belonging to the class *Leviviricetes* and the family *Fiersviridae* were identified and dominantly present (Hillary et al., 2022; Wu et al., 2021).

Table 3: Concentration of viral particles found in the terrestrial environments with some species or types of bacteriophages respectively identified

Terrestrial ecosystems	Country	Concentrations	Bacteriophages	References
Forest	USA	10^7 virus/g	-	(Helsley et al., 2013)
	USA	10^9 VLP/g	*Podoviridae* > *Siphoviridae*/*Myoviridae*	(Williamson et al., 2005)
Agricultural soil	USA	10^7-10^{10} VLP/g	*Podoviridae, Myoviridae, Siphoviridae, Microviridae, Mimiviridae,* and *Phycodnaviridae*	(Liang et al., 2019)
	UK	10^7-10^8 virus/g	*Pseudomonas* and *Serratia* phages	(Ashelford et al., 2003)
	UK	10^9 virus/g	*Podoviridae, Myoviridae, Siphoviridae Fiersviridae, Inoviridae*	(Swanson et al., 2009)
	China	10^7 virus/g	*Podoviridae, Myoviridae, Siphoviridae*	(Chen et al., 2014)
	USA	10^8-10^9 VLP/g	Mainly tailless phages *Podoviridae* > *Siphoviridae*/*Myoviridae*	(Williamson et al., 2005)
	USA	10^8 VLP/g	*Podoviridae, Myoviridae, elongate capsid phages*	(Williamson et al., 2003)
Grasslands	USA	-	*Caudovirales, Kalamavirales, Petitvirales*, and *Tubulavirales*	(Wu et al., 2021)
Deserts	South Africa	-	*Siphoviridae* >> *Podoviridae* > *Myoviridae*	(Adriaenssens et al., 2015)
	Morocco/USA/Saudi Arabia/Afghanistan/ Kuwait/UAE	10^3-10^7 virus/g	-	(Gonzalez-Martin et al., 2013)
	Antarctica	10^7-10^9 virus/g	-	(Williamson et al., 2007)

1.2.2 Impact of environmental factors on bacteriophage dynamics

Biotic relationship virus-bacteria

The abundance and survival of bacteriophages in the environment depend on that of their bacterial hosts, as they require a host to replicate (Haq et al., 2012). The impact of bacteriophages on the bacterial community is acknowledged and has been more studied (Braga et al., 2020; Naureen et al., 2020). As agents of bacterial mortality, bacteriophages are likely to have a significant influence on bacterial community dynamics, structure and function, as well as biogeochemical cycling, horizontal gene transfer processes and the pre-decomposition of the organic matter (Fazzino et al., 2020; Sullivan et al., 2017). Indeed, in the aquatic environment, it was reported that bacteriophages caused 10-20% of bacterial mortality, and ultimately limited bacterial populations (Fuhrman and Noble, 1995; Suttle, 1994; Weinbauer et al., 1999). The death of the bacteria engenders the release of the nutrients that were comprised in it, increasing the nutrient concentration of the natural media or their bioavailability (Gobler et al., 1998; Tran and Anantharaman, 2021).

Abiotic impact on the inactivation of bacteriophages

In general, in the absence of external environmental stresses (e.g. UV, temperature), bacteriophages show persistence for weeks to months in their own habitat (Clokie et al., 2011; Jończyk et al., 2011). Several environmental factors (e.g. pH, radiation, temperatures) can control this persistence of bacteriophages in different ways. First, the capsid can be damaged by enzymatic or chemical reactions, and the bacteriophage receptors (i.e., proteins) can be altered which prevents the recognition of the bacteria by the bacteriophages (Langlet et al., 2007; Nasser and Oman, 1999). Secondly, chemical or physical influences can affect the genetic material and proteins (Harvey and Ryan, 2004; O'Brien and Newman, 1977).

Temperature and sunlight are the two most significant environmental factors influencing the inactivation of bacteriophages. The temperature has an influence on viral inactivation through the chemical reactions with proteins or nucleic acids (Bertrand et al., 2012; Feng et al., 2003; Jończyk et al., 2011). Generally, the optimal temperature for better survival of bacteriophages is around 4-10°C, while above this temperature, they can be inactivated (Corapcioglu et al., 2006; Fauvel et al., 2019; Jurczak-Kurek et al., 2016; Yates et al., 1985). In terms of physical damages, the capsid is altered above 40°C due to protein denaturation or microbial degradation, whereas under 40°C, the damages rather concern the nucleic acid

(Jończyk et al., 2011; Nasser and Oman, 1999; O'Brien and Newman, 1977). The inactivation rate has been observed lower during winter months, not only because of lower temperatures but also due to lower sunlight exposure (Sinton and Ching, 1987). Indeed, it has been shown that sunlight increased the inactivation of bacteriophage T7 (Kinnunen, 1978). These radiations, more particularly the UVBs ($320 < \lambda < 400$ nm), attack and photodamage the viral nucleic acids (Silverman et al., 2013; Weinbauer et al., 1999).

Additionally, pH showed affecting bacteriophage survival. Indeed, at an acidic pH (≈ 2), bacteriophages are all inactivated due to the denaturation of the proteins or nucleic acids (Feng et al., 2003; Jurczak-Kurek et al., 2016; Nishide et al., 2011). The natural pH of water is around 5-9 and bacteriophages are stable under this range of pH values. At a high pH (> 11), most bacteriophages show sensitivity to those pH values (e.g. SSV1, T7 …) (Feng et al., 2003; Jończyk et al., 2011; Taj et al., 2014). Bacteriophage inactivation according to the pH will also depend on the genome. Indeed, DNA-bacteriophages would be less affected by environmental pH than RNA-bacteriophages (le Vay et al., 2020). In general, DNA is more stable than RNA due to the presence of hydroxyl groups that are sensitive to hydrolysis (Fordyce et al., 2013). Therefore, RNA-bacteriophages can be more easily inactivated compared to DNA-bacteriophages.

Abiotic impact on the fate of bacteriophages

Adsorption strongly influences the fate of bacteriophages in the environment (Kimura et al., 2008). In gravel and sandy soil, bacteriophages tend to be retained especially because of a high adsorption rate and the presence of vegetation (Sinton and Ching, 1987). The adsorption on substratum or particles protects the bacteriophages against their inactivation (Bitton, 1975; Rossi and Aragno, 1999). Indeed, the inactivation rate of the bacteriophage Φ11M15 was reduced owing to their adsorption on clay surfaces (Babich and Stotzky, 1980). The bacteriophages T2 and T7 also underwent a reduction of their inactivation rate by adsorption respectively in the kaolinite (Bitton, 1975) and the montmorillonite (Bitton and Mitchell, 1974), two kinds of clays. The mechanism of adsorption is driven by either van der Waals electrostatic forces or hydrophobic interactions (Rossi and Käss, 1998). The van der Waals forces consist in an interaction between atoms or molecules that depends on the distance. They are weak and interact most at shorter distances. The adsorption through electrostatic forces will be influenced by the pH of the surrounding environment and by charges present

on the surface of the molecule (Germann et al., 2002). The hydrophobic interactions concern the aggregation of hydrophobic molecules in hydrophobic compounds of a media (Chattopadhyay and Puls, 2000). This process especially occurs between the bacteriophages and the organic matter content in the soil (Pieper et al., 1997). Bacteriophages display different degrees of hydrophobicity, where for example bacteriophage Qβ were found more hydrophobic than MS2, and thus, this means that bacteriophages will not interact in the same manner depending on their hydrophobicity (Sautrey et al., 2018).

The adsorption rate is mainly influenced by pH. Each bacteriophage has an attributed pI (isoelectric point), which is the pH at which a bacteriophage carries no net electrical charge on its surface (Michen and Graule, 2010) (Table 4). Bacteriophages are negatively charged when the pH is higher than the pI whereas they are positively charged when the pH is lower (Michen and Graule, 2010; Rossi and Käss, 1998). For pH values superior to 7, the net surface charge of most bacteriophages is negative, leading to repulsion forces that prevent attachment to some environmental matrices. Regarding the soil compartment, the transport of viruses is highly dependent on the soil pH, and it mostly increases their survival by a higher adsorption rate (Hurst et al., 1980).

Besides influencing bacteriophage adsorption, pH showed to play a role in the persistence of the bacteriophage genome. Environmental DNA was found to persist longer at neutral pH than at acidic pH, with a persistence of 143 days in the latter case, while RNA only survived 57 hours at neutral pH (Kagzi et al., 2022). Not only does the impact of pH on the genetic information concern the bacteriophage as a complete particle but can also affect the genetic information in the free state after the inactivation of the bacteriophage.

Table 4: Isoelectric points of some bacteriophages (modified from Michen and Graule, 2010)

Bacteriophage species	Family	pI
Emesvirus zinderi (MS2)	*Fiersviridae*	3.9
Alphatectivirus PRD1	*Tectiviridae*	3.8-4.2
Qubevirus durum (Qβ)	*Fiersviridae*	5.3
Tequatrovirus T4	*Straboviridae*	4
Lambdavirus λ	*Unclassified Caudoviricetes*	3.8
Sinsheimervirus ΦX174	*Microviridae*	6.6-7
Corticovirus PM2	*Corticoviridae*	7.3
Pepevirus rubrum	*Fiersviridae*	4.3-4.9

More details regarding the impact of environmental factors on the survival and fate of bacteriophages are given in the review paper, presented in section 4 of this manuscript's state of the art.

1.2.3 Detection methods for bacteriophages in environments

Several methods exist to detect bacteriophages from environmental samples, however, they target bacteriophages differently and provide distinct information, as reported in Table 5. The standard method for detecting bacteriophages is the plaque assay in a double agar layer (Abedon and Yin, 2009; Anderson et al., 2011). In a Petri dish, the bottom layer allows bacterial growth while the top layer contains both host bacteria and bacteriophages. After incubation, lysis plaques appear on the agar once the bacteriophages have infected the host bacteria. These plaques are then counted to determine the number of bacteriophages in the plaques-forming unit (PFU) (Andersson and Lood, 2019; Francis et al., 2017). This simple and effective method is considered the gold standard for bacteriophage enumeration, but it detects infectious bacteriophages and requires knowledge of the bacterial host of the bacteriophage in question (Bae et al., 2003; Furiga et al., 2011; Gallet et al., 2011).

Additional methods are available to allow the detection of bacteriophages, notably with the visualisation of the whole bacteriophage particles. Whole bacteriophage particles, and particularly unknown bacteriophages, are characterised and identified based on their morphology, which has allowed the development of viral taxonomy (Ackermann, 2012). Transmission electron microscopy (TEM) not only quantifies them but also identifies

bacteriophage families (Richert-Pöggeler et al., 2019; Roingeard et al., 2019). However, in addition to being laborious and costly, this method reaches its limits when dealing with complex samples, such as soils (Ács et al., 2020). Moreover, it requires expertise in its operation, which makes it often difficult to get the right viral density on the samples for reliable enumeration (Kaletta et al., 2020). Also, in the field of microscopy, Helium-Ion Microscopy (HIM) has made its way into the visualisation of bacteriophages by providing high-resolution images (Schmidt et al., 2021). This technique pushes the limits of simple morphological identification of bacteriophages, allowing in particular the visualisation of the interaction of the bacteriophage with its host and those at different stages of its cycle (Lappänen et al., 2017). Flow cytometry is a technique based on the detection of fluorescence commonly used for the enumeration of viral-like-particle (VLPs) in the environment (Duhamel and Jacquet, 2006; Jacquet et al., 2010). In environmental samples, these VLPs are defined as particles of the size of viruses including nucleic acid and could be as functional as viruses (Hyman et al., 2021). Fluorescence is produced by adding a dye (e.g., SYBR™ Green), which binds to the nucleic acid and is excited by a laser (Heinrichs et al., 2021; Maltseva and Langlois, 2022). However, this analysis does not allow identifying viruses or discriminating bacteriophages from the rest of the VLPs (Colombet et al., 2019). Moreover, the dye employed will bind to DNA, viral or not, which can result in an overestimation of the viral density (Brown et al., 2015; Nunez, 2001; Renner et al., 2020).

With the advent of molecular techniques, methods have been developed to enumerate viral nucleic acids or proteins. These techniques allow the detection of all bacteriophages regardless of their infectivity status (Ho et al., 2016). For this purpose, real-time polymerase chain reaction (PCR) is used to determine bacteriophage genome concentrations (Antonelli et al., 2016; Duyvejonck et al., 2019; Kłopot et al., 2017; Refardt, 2012). This method can either use a non-specific fluorescent dye that intercalates into the dsDNA, or a sequence-specific DNA probe coupled to a fluorescent dye (Lind et al., 2006; Zipper et al., 2004). The fluorescence emitted is then detected to provide concentrations of the bacteriophage genome. The quantitative real-time polymerase chain reaction (or qPCR) will determine the copy number of amplified DNA, while the qualitative real-time polymerase chain reaction will give a presence or absence of a sequence (Han et al., 2021). It also allows quantifying RNA-bacteriophages by performing a reverse transcription (RT)-(q)PCR, where an initial reverse transcription step is followed by qPCR carried out on the complementary DNA (cDNA) created (Flannery et al., 2013; Miranda and Steward, 2017). This technique has demonstrated

high detection sensitivity, with the use of fluorescent-labelled probes being more accurate than intercalating dyes, which is less selective for quantification (Anderson et al., 2011). Finally, Droplet Digital PCR (ddPCR) is the latest addition to PCR methods for viral detection. The principle of this technique is the same as real-time PCR, except that the PCR solution is divided into several nanolitre-sized droplets, in which a separate PCR takes place (Morella et al., 2018). The ddPCR is an absolute quantification of the amplified DNA since each droplet represents one DNA molecule. Not only has the method shown higher sensitivity than real-time PCR, but it does not require the construction of an internal standard (Kim et al., 2014). However, if the primer or probe selected does not show specificity to the target, then the amplification of irrelevant DNA could lead to an overestimation of the viral population (Martinez-Hernandez et al., 2019).

Since 1977 and for more than 40 years, Sanger sequencing has been the reference technology for deciphering genomes, which was particularly the case for the bacteriophage ΦX174, the first DNA-genome bacteriophage sequenced (Sanger et al., 1977). Although this technology is still used for viral detection, it is not the most suitable for complex samples (e.g. environmental samples) with a need for high sensitivity (Daniels et al., 2021). Next-Generation Sequencing (NGS) then appeared with the development of new technologies allowing the detection and identification of bacteriophages from various habitats, notably using metagenomics. Metagenomics is a powerful method to reveal microbial diversity in environmental samples based on the reconstruction of DNA sequences. The classic metagenomic protocol involves the first step of DNA extraction followed by sequencing and a final step of bioinformatics analysis (Parmar et al., 2018; Zhang et al., 2018). Among the NGS technology, Illumina sequencing is the most frequently used and is based on identifying DNA bases emitted by fluorescent signal, after sequence cleavage (e.g. 100 – 150 bp) and allows sequencing short reads. Additionally, long-read sequencing has been developed, such as PacBio or Nanopore technologies, which allows the contribution to viral genome reconstruction from metagenomes (Hiraoka et al., 2016). Metagenomics is not a quantitative method but allows the detection of bacteriophage species in complex environments (Fernandez-Cassi et al., 2018; Hayes et al., 2017; Mohiuddin and Schellhorn, 2015). Bioinformatics analysis assembles reads and contigs obtained from sequenced genomes, information that is then used to determine a microbial abundance of a sample. In this way, the method is often considered to be semi-quantitative (Gong et al., 2018; Roux et al., 2015b, 2014). On one hand, the use of bioinformatics analyses using databases referencing

previously characterised viral genomes provides information regarding the taxonomic assignment of the viral sequenced obtained (Lischer and Shimizu, 2017; Sierra et al., 2020). On the other hand, analyses conducted independently of available databases allow the investigation of as-yet unknown viruses thus the potential discovery of new species (Dutilh et al., 2012; Sedlar et al., 2017). NGS technology presents the advantage of being highly reproducible and requiring very low DNA input (Lee et al., 2013; Slatko et al., 2018). Moreover, it provides information about the known and especially the unknown fraction of the microbial populations. However, despite the progress made in the development of increasingly user-friendly bioinformatics tools, these post-sequencing analyses remain laborious and still require programming expertise (Ács et al., 2020). Moreover, the field of virology continuously needs further analysis of unknown viruses to expand the databases, which are still largely incomplete to date. Many databases are available, with more particularly nr database, which is a non-redundant database and gather several databases, such as RefSeq, Genbank. The content of this database consisted in 74% bacteria, 23% Eukaryota, 2.21% Archeae and 0.79% viruses, reflecting the poor representation of viruses in databases (Bagheri et al., 2020; Zhulin, 2015). It is interesting to note that both the reference-based and independent-reference bioinformatics analyses can be used in a complementary manner to obtain the most complete picture possible of the viral or microbial communities in an environment and thus, overcome those limitations (Valiente-Mullor et al., 2021).

Table 5: Summary of the methods used to detect bacteriophages with their respective advantages and disadvantages.

Methods	Aim	Advantages	Disadvantages	References
DETECTION OF INFECTIOUS BACTERIOPHAGES				
Plaque assay	Quantification of infectious bacteriophages	Simple and efficient Gold standard for bacteriophage quantification	Detects only infectious bacteriophages Requires knowledge of the bacterial host	(Andersson and Lood, 2019; Francis et al., 2017)
DETECTION OF WHOLE BACTERIOPHAGE PARTICLES				
Transmission Electron Microscopy (TEM)	Identification and quantification of bacteriophage	Direct visualisation of bacteriophages Structural analyses Interesting for unknown bacteriophages	Requires specific expertise Very expensive Time-consuming	(Richert-Pöggeler et al., 2019 ; Roingeard et al., 2019)
Helium Ion Microscopy (HIM)	Identification of bacteriophage	Highest magnification image Visualisation of virus-virus or virus-host interactions	No elemental quantification Requires specific expertise	(Lappänen et al., 2017 ; Frese et al., 2021 ; Schmidt, Byrne and Maasilta, 2021)
DETECTION OF NUCLEIC ACID AND PROTEINS				
Flow cytometry	Quantification of VLPs	Fast analysis of heterogeneous populations of cells Measure many detectable parameters of fluorescence	Analyses one cell at a time Requires specific expertise No discrimination of bacteriophages from the rest of the VLPs	(Duhamel and Jacquet, 2006)

Quantitative and/or qualitative PCR	Quantification and/or detection of genome of bacteriophages (Nucleic acid)	High detection sensitivity Fluorescent labeled probes is more precise	No discrimination between viable and non-infectious bacteriophages Intercalating dyes is less selective for quantification	(Anderson et al., 2011; Ogorzaly and Gantzer, 2006)
Droplet Digital PCR (ddPCR)	Quantification of genome of bacteriophages (Nucleic acid)	High detection sensitivity No need for internal standards Can be used in complex environments	Overestimate viral abundance	(Kim et al., 2014; Martinez-Hernandez et al., 2019; Morella et al., 2018)
Next generation sequencing (NGS)	Identification and quantification of bacteriophage (Nucleic acid)	No prior isolation needed Identification of unknown bacteriophages High reproducibility Less nucleic acid input required (around some nanograms)	No discrimination between viable and non-infectious bacteriophages Semi-quantitative data Underestimation of bacteriophage abundance Need for laborious bioinformatic analysis Loss of data during post-sequencing analysis Largely incomplete genomic databases	(Ács et al., 2020; Hiraoka et al., 2016; Parmar et al., 2018; Zhang et al., 2018)

1.3 The application of bacteriophages in hydrology

1.3.1 Hydrological tracers and requirements

Hydrological tracers have been used for centuries to characterise water flow paths and estimate groundwater velocities. In the late 1800s, the first experiments using tracers (e.g. chloride, fluorescein) were conducted in European karst aquifers (Divine and McDonnell, 2005; Kinnunen, 1978; Rossi and Käss, 1998). Tracing experiments allow the characterisation of water flow paths and involve the monitoring of constituents in hydrological catchments to improve our understanding of water cycle processes (Mangin, 1984). Tracers can be used for studying water in all its phases, behaviours and characteristics within contrasted environments ultimately shedding new light on questions related to flow pathways, velocities and travel times, hydrodynamic dispersion, recharge and discharge (Flury and Wai, 2003a; Leibundgut and Seibert, 2010). They mainly help to answer the following questions: Where does the water flow? Where does it come from? Is there a hydrological network between two given points? How do substances spread in the water?

The two main categories of tracers are the so-called environmental and artificial tracers (Table 6). An environmental tracer exists naturally or accidentally in the environment, such as the stable isotopes (e.g. hydrogen or oxygen) (Behrens et al., 2001; Schudel et al., 2002) and radioactive ones (e.g. tritium), respectively. An artificial tracer is a compound intentionally introduced into the environment (e.g. salts or dyes) (Berryman, 2005; Leibundgut and Seibert, 2010). In hydrology so far, the main used tracers are isotopes and dyes and thus they are considered conventional tracers. Other less common tracers, also called alternative tracers, are colloidal particles such as bacteriophages, and microspheres (Schudel et al., 2002).

Table 6: Relevant hydrological tracers (Leibundgut and Seibert, 2010)

Environmental tracers	**Artificial tracers**	
	Solute tracers	Non solute tracers
Stable isotopes *Deuterium (^{2}H)* *Oxygen-18 (^{18}O)* *Carbon-13 (^{13}C)* *Nitrogen-15 (^{15}N)* *Sulfur-34 (^{34}S)*	***Fluorescent dyes*** *Naphthionate* *Pyranine* *Uranine* *Eosine* *Rhodamines*	***Dissolved gas tracers*** *Helium (He)* *Neon (Ne)* *Stable isotopes of Krypton (Kr)* *Sulfur hexafluoride (SF_6)*
Radioactive isotopes *Tritium (^{3}H)* *Carbon-14 (^{14}C)* *Argon-39 (^{39}Ar)* *Krypton-85 (^{85}Kr)* *Radon-222 (^{222}Rn)* *Radium-226 (^{226}Ra)* *Silicium-32 (^{32}Si)* *Chlorine-36 (^{36}Cl)*	***Salts*** *Sodium/potassium chloride* *Sodium/potassium bromide* *Lithium chloride* *Potassium iodide* *Sodium borate (borax)*	***Particulates tracers*** *Lycopodium spores* *Bacteria* *Viruses & bacteriophages* *DNA* *Synthetic microspheres* *Phytoplankton* *Diatoms*
Noble gases	***Non-fluorescent dyes*** *Brilliant blue*	
Anthropogenic trace gases *Chlorofuorocarbons (CFCs)* *Sulfur hexafluoride (SF_6)*	***Radionucleids*** *Tritium (^{3}H)* *Chrome-51 (^{51}Cr)* *Bromide-62 (^{62}Br)* *Inidum-131 (^{131}I)*	
Geochemical compounds *Silicate* *Chloride* *DOC* *Heavy metals*	***Fluorobenzoic acids***	
Physicochemical parameters *Electrical conductivity* *temperature*		

Regarding the use of artificial tracers, their use must meet some prior requirements. The ideal tracer must be representative of the water movement. Therefore, its behaviour must be close to that of the water itself. Moreover, it is important to limit the amount and number of tracers injected during a tracing experiment. This means that we have to target the lowest possible concentrations and residence times (Schudel et al., 2002). To do this, a list of eight criteria has been developed: (1) Low sorptivity and sorption, (2) Good chemical and biological stability, (3) Good solubility, (4) Low background concentration, (5) Insensitive to changes (e.g. light, pH, temperature), (6) An efficient detection limit, (7) A low toxicity and environmental impact and (8) A low cost (Leibundgut and Seibert, 2010).

1.3.2 Fundamentals and limitations of the artificial tracers

The number of artificial tracers significantly increased after the Second World War, which allowed over years and especially from the 1960s onwards to have a better perception of the flow path of rainfall to the water table (Zimmermann et al., 1966). The tracing studies performed with artificial tracers are becoming more frequently used as additional tracers for the development, parametrization as well as validation of models for solute transport in the ground- and surface waters (Leibundgut and Seibert, 2010). They may be used to study water movement, but they are also capable of investigating the effects of sorption, biodegradation and storage of an aquifer (Berryman, 2005). The most known tracers involved in this category are fluorescent dyes and salts.

Fluorescent dyes have been commonly used to trace the flow of groundwater and pollutants in karst areas (Field et al., 1995). The most typical dyes are uranine, fluorescein, uosine or even rhodamine WT (Labat and Mangin, 2015; Smettem and Trudgill, 1983; Wolkersdorfer and LeBlanc, 2012). Despite some qualities such as high detectability, low cost, good solubility, and low adsorption rate, the fluorescent dyes can be toxic for humans and the ecosystems (e.g. Sulforhodamine B, Rhodamine WT) and tend to adsorbed in acid environments and to be damaged by oxidants or light (Behrens et al., 2001; Schudel et al., 2002). Commonly used as a complement tracer to fluorescent dyes since the 1950s, salts have been used in karstic environments and porous media and include chloride or bromide (Bastviken et al., 2007; Kirchner et al., 2010). However, this tracer has shown some flaws in its use, like high background noise from geology and human activity (e.g. road salting), an increase in chloride concentration in soils due to evaporation, or microbial uptake as well as ion exchanges (Bastviken et al., 2007; Levy and Chambers, 1987; Schudel et al., 2002; Whitmer et al., 2000).

The limitations described just above raised the need for new hydrological tracers, including DNA, microspheres and bacteriophages. The theoretically unlimited number of synthetic DNA tracers appeared as a major advantage, especially for the multi-tracer experiments (Foppen et al., 2011). They were used in fractured hard rock aquifers to explain flow patterns and velocities of water in hydrological environment investigations (Sabir et al., 2000). However, DNA can interact with their surrounding environment, which can be overcome through encapsulation (made of silica or even polylactic acid), also called microspheres (Liao et al., 2018). This encapsulation material is biodegradable and therefore does not remain for a long time in the ecosystems. However, the fate of the released genetic material

is not well known, and this brings us back to the inconvenience of using free DNA (Barnes and Turner, 2015; Nielsen et al., 2007). In the late 1960s, the first hydrological tracing experiments using bacteriophages were performed by Kinnunen and his team (Kinnunen, 1978). The bacteriophages can be efficient notably within groundwater pathways or even in a karstic environment, whereas their transport was little studied within the soil matrix (McKay et al., 1993; Skilton and Wheeler, 1988). Their host-specificity is the main advantage since it allows to use of different bacteriophages in the same tracer experiment without hindering their detection (Ghanem et al., 2016). In addition, they are not pathogenic for humans, and they represent a low ecological risk (Drury and Wheeler, 1982; Martin, 1988; Martin and Thomas, 1974). However, bacteriophages tend to be more retained especially because of a high adsorption rate and the presence of vegetation (Sinton and Ching, 1987). This process of adsorption on substratum or particles revealed some protection for bacteriophages against their inactivation (Bitton, 1975).

1.3.3 Emerging of innovative tracers: and if they were already there?

Environmental tracers include both natural and accidental substances already present in ecosystems. While natural tracers are elements from natural systems (e.g. rocks), accidental substances are present in environments due to anthropogenic activities (Berryman, 2005). The most commonly used environmental tracers in the application of tracing experiments are isotopes. There are two types of isotopic tracers commonly used in hydrological tracer studies, the stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{18}O and ^{34}S) and the radioisotopes (^{3}H, ^{51}Cr, ^{60}Co, ^{82}Br and ^{131}I) (Flury and Wai, 2003b; Selleck and Koff, 2008). It is naturally present in ecosystems, then the input concentrations are difficult to define. In that way, it is not possible to have a clear view of where the overland flow originated. Isotopes are employed as artificial tracers, but above all, they are mostly used as environmental tracers (Cook, 2020). Since the late 1940s, they have allowed a better understanding of the hydrological processes by determining runoff generation, runoff component separation, transit times, recharge and groundwater flow (Gibson et al., 2005; Leibundgut and Seibert, 2010). The investigation of the major processes of the hydrological cycle (evaporation, and precipitation…) has mainly been carried out by combining two water isotopes, hydrogen (^{2}H) and oxygen (^{18}O) (Gibson et al., 2005). Despite their proven effectiveness to date, they are not considered to be conservative as they can be fractionated by evaporation (Kirchner et al., 2010). Furthermore,

they cannot give evidence about certain hydrological connectivity such as hillslope-riparian-stream connectivity (Martínez-Carreras et al., 2015).

In the development of new hydrological tracers, terrestrial diatoms have been recently added to the list as environmental tracers. They are one of the most common algal groups in freshwater as well as marine environments (Serôdio and Lavaud, 2020). They have attracted the hydrologists' attention because of their ubiquity in aquatic and terrestrial environments. Therefore, they enable us to provide evidence about the connectivity between those two compartments by using tracing experiments (Pfister et al., 2009). In addition, they are easily transported within the hydrological cycle by water flow. Moreover, their species diversity highly depends on physio-geographical factors such as temperature or even pH as well as anthropogenic pollution (Martínez-Carreras et al., 2015).

As described in the previous section of the manuscript, bacteriophages are numerously abundant in soils (Armon, 2011) and thus, in line with the use of environmental tracers, it was consistent to find a way to use the natural populations of bacteriophages. Moreover, since bacteriophages have already been used as artificial tracers, some data are available regarding their fate and transport (McKay et al., 1993; Skilton and Wheeler, 1988). The main reason for using the natural populations of bacteriophages was the impressive volume and concentration of artificial bacteriophage tracers injected during the tracing experiments. In a climatic context like the one we have nowadays, some environmental regulations regarding the introduction of external entities into the environment exist and must be followed (Environment Agency, 2021; Wolkersdorfer and LeBlanc, 2012).

The use of naturally occurring soil bacteriophages is a first for hydrology and it is therefore important to learn more about this new tracer concept. On the face of it and following the criteria set up to match the ideal tracer, naturally occurring bacteriophages seem to be a good option as an environmental tracer. However, there is still a whole world to discover about them to ensure their reliability (Table 7). To fill the information gap, the availability of existing data on bacteriophages used as artificial tracers will allow us to transfer this knowledge to bacteriophages as environmental tracers and thus help us to develop them for the purpose of applying them in hydrological studies. Thus, the next section is dedicated to the history of bacteriophages used as hydrological tracers, their advantages and disadvantages to know better about these entities. In addition, some perspectives for future

tracing experiments will be proposed to optimise the use of bacteriophages, with a virological point of view.

Table 7: All the eight criteria predefined to best meet the requirements of an ideal tracer, for the conventional tracers (i.e. dyes, salts and isotopes) and bacteriophages as both artificial (i.e. injected) and environmental (i.e. natural populations) tracers.

Criteria	Dyes	Salts	Radioactive isotopes		Stable isotopes		Bacteriophages	
			Injected	Natural	Injected	Natural	Injected	Natural
Low sorption	No	No	No	No	No	No	No	?
Good chemical and biological stability	No	No	No	No	No	No	No	?
Good solubility	Yes	Yes	Yes	Yes	Yes	Yes	No	?
Low background concentration	No	No	Yes	NC	Yes	NC	Yes	?
Insensitive to changes	No	No	No	No	No	No	No	?
Efficient detection limit	Yes	No	Yes	Yes	Yes	Yes	Yes	?
Low toxicity and environmental impact	No	No	No	Yes	Yes	Yes	No	?
Low cost	Yes	Yes	No	Yes	No	Yes	Yes	?
Total of criteria met	3	2	3	5	4	5	3	?

NC = Not Concerned

1.4 Bacteriophages as hydrological tracers: past, present and future

Despite the proven qualities of bacteriophages as tracers, their use and especially their injection into the environment are important points that need to be discussed from a virological point of view, as they are very different from that of a hydrologist. Indeed, hydrologists see bacteriophages as biological entities that specifically infect bacteria, thus giving them the advantage of being environmentally friendly, and non-pathogenic for humans and animals. Furthermore, this host specificity allows hydrologists to use different bacteriophages simultaneously without causing interference during their detection. Finally, the gold standard detection method used in tracing experiments, the plaque assay, is highly appreciated for its high sensitivity, low application cost and ease of execution. From a virological point of view, this method has limitations in the detection of bacteriophages, which can however be overcome by applying complementary methods. Beyond detection methods, the aspect of being environmentally safe must be discussed, especially for the application of bacteriophages as artificial tracers for which large volumes and concentrations are injected. Finally, bacteriophages are entities that are simple in their structure, but complicated in their dynamism in environments that can affect their transport and therefore very important to consider. This section is presented as a review paper, which has been published in the *Water* Journal. The objective of this review, in addition to reporting the history of the use of these bacteriophages as hydrological tracers, is also to note the limitations and to propose solutions to optimise the results obtained during the tracing experiments.

Review article available at:

Florent, P.; Cauchie, H.-M.; Ogorzaly, L. A Virological Perspective on the Use of Bacteriophages as Hydrological Tracers. *Water* **2022**, *14*, 3991.

https://doi.org/10.3390/w14243991

water

MDPI

Review

A Virological Perspective on the Use of Bacteriophages as Hydrological Tracers

Perrine Florent [1,2], Henry-Michel Cauchie [1] and Leslie Ogorzaly [1,*]

[1] Environmental Research and Innovation Department (ERIN), Luxembourg Institute of Science and Technology (LIST), 4422 Belvaux, Luxembourg
[2] Faculté des Sciences, de la Technologie et de la Communication (FSTC), Doctoral School in Science and Engineering (DSSE), University of Luxembourg, 4365 Esch-sur-Alzette, Luxembourg
* Correspondence: leslie.ogorzaly@list.lu; Tel.: +352-275-8885-5069

Abstract: Hydrological tracers, commonly used for characterizing water flow paths and sources, do not often meet all the requirements of an ideal tracer. Trans-disciplinary approaches are advocated as the way forward to enlarge the number of tracers available for investigating hydrological processes. Since the 19th century, hydrological tracers have been increasingly used, particularly in underground areas. The tracer toolbox at hand includes a large variety of options, including fluorescent dyes, isotopes, salts or bacteriophages, with each tracer offering specific qualities and complementarities. While their potential for hydrological studies has been studied in karstic environments since the 1960s, bacteriophages remain insufficiently understood. According to the selection methodology used in this review, more than thirty experiments have been listed, involving in total around seventeen different bacteriophages. These have facilitated the investigation of groundwater, surface water (i.e., river, lake and marine water), wetland and wastewater hydrological processes. The tracing experiments have also highlighted the possible interaction between bacteriophages and the surrounding environments. Bacteriophages have successfully helped researchers to understand the water flow within watersheds. Certain advantages, such as the sensitivity of detection, the ease of producing high concentrations of bacteriophages to be injected, their specificity for a host and their non-pathogenicity for human and animal cells, make bacteriophages appreciable tracer candidates for tracing experiments. However, the adsorption process or environmental factors such as temperature, pH and UV light considerably impact the fate of bacteriophages, thereby leading to an attenuation of the phage signal. Considering both the flaws and the qualities of bacteriophages, their use as hydrological tracers requires new insight and further discussions regarding experimental tracing conditions.

Keywords: bacteriophages; hydrological tracers; aquatic ecosystems; transport; fate; alternative methodological approaches

check for updates

Citation: Florent, P.; Cauchie, H.-M.; Ogorzaly, L. A Virological Perspective on the Use of Bacteriophages as Hydrological Tracers. *Water* **2022**, *14*, 3991. https://doi.org/10.3390/w14243991

Academic Editor: Constantinos V. Chrysikopoulos

Received: 8 November 2022
Accepted: 1 December 2022
Published: 7 December 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Hydrological tracers have been used for more than a century and have allowed the study of water in all its phases in a wide range of contrasting environments [1]. They also contribute to addressing fundamental questions on hydrological processes, such as the sources of water and matter, flow paths and transit times [2,3], velocities and travel times, hydrodynamic dispersion, groundwater recharge and discharge generation [1,4]. The first known use of tracers dates from the late 1800s, when they were used to investigate groundwater recharge and velocity in European karst aquifers [1,5].

Currently, the tracing tools at hand can be divided into two categories, namely tracers that are naturally or accidentally present in the environment (i.e., environmental tracers) and those that are artificially and intentionally introduced into the environment (i.e., artificial tracers). For example, stable (e.g., hydrogen or oxygen) and radioactive (e.g., tritium) isotopes are environmental tracers [6,7], while salts or dyes are artificial tracers [1,8].

In experimental hydrology, the most commonly used tracers, defined as conventional tracers, are isotopes and fluorescent dyes. In parallel to the use of the previous tracers, additional tracers can be employed, either by considering available natural resources such as diatoms or by introducing colloidal particles such as bacteriophages and microspheres [7,9]. However, the use of these tracers is less widespread than that of conventional tracers. To choose the right tracer among a large set of options, eight criteria are usually considered: (i) low sorptivity and sorption, (ii) good chemical and biological stability, (iii) good solubility, (iv) low background concentration, (v) insensitive to changes (light, pH, temperature), (vi) efficient detection limit, (vii) low toxicity and environmental impact and (viii) low cost. Above all, the perfect tracer should be conservative, i.e., stable and non-reactive with the surrounding environment [7]. Unfortunately, such an ideal tracer does not exist. Although hydrogen or oxygen-stable isotopes are often considered as such, since they are the basic constituents of the water molecule [4,10], recent studies have refuted this fundamental statement [11]. For instance, the initial enthusiasm for the use of stable oxygen and hydrogen isotopes for hydrograph separation has been gradually replaced by substantial concerns about their limitations in, for example, determining flow paths to reach streams during storms [12]. While the analytical techniques used to measure the different types of tracers are generally robust and reliable, the interpretation of tracer signals is far from straightforward. The non-conservative behaviour of most tracers is a major concern in this respect [13]. Fluorescent dyes are best suited to sufficiently transparent waters, but they are quickly attenuated by sunlight and, like rhodamine B, are easily adsorbed on the surrounding materials (e.g., soil constituents) [4,6,14]. In addition, the determination of dye mixtures and dye residuals from the initial spiking can be challenging [15]. Salts, such as bromide or chloride, have also shown their limitations when it comes to differentiating their presence from the natural background [14]. Geochemical elements and isotopes of hydrogen and oxygen in water are cardinal tools for investigating the time source (i.e., event vs. pre-event water), runoff generation mechanisms (e.g., saturation overland flow, groundwater flow, perched saturated flow) and geographic origins (i.e., location of the water before it arrived at the stream) of storm runoff components [16]. Despite considerable knowledge having been gained from countless studies in small experimental catchment areas over the past four decades, theoretical research has gradually replaced field observations and empirical understanding, mainly due to budgetary constraints on the one hand and difficulties in identifying suitable sampling sites in larger catchment areas on the other [17,18]. When considering larger catchment areas, runoff generation mechanisms have also been increasing and form a highly heterogeneous mixture of water types [19]. Consequently, isotope tracers have been preferentially used in small catchment areas [12], and their application to larger scales remains highly challenging [20]. For overcoming this status quo, complementary tools have been proposed, such as distributed fibre-optic temperature sensing [21,22], thermal infrared (IR) imagery [23,24], the use of terrestrial diatoms as environmental biological tracers of hydrological connectivity across multiple scales [25,26], artificial DNA tracers [27,28] or the injection of bacteriophages [14,29]. Bacteriophages are viruses that specifically infect bacterial hosts. They are almost exclusively composed of a nucleic acid molecule (DNA or RNA) carrying the genetic information, which is protected by a protein capsid [30,31]. They exhibit a wide range of shapes, sizes (20 to 200 nm) and structures. Bacteriophages were introduced as hydrological tracers in the late 1960s, and their suitability for such applications has been investigated since then. Three main reasons have motivated hydrologists in the use of bacteriophages as hydrological tracers: (i) the sensitivity of the detection method, (ii) their host specificity and (iii) their so-called eco-friendly properties [32–36]. Yet, the reasons mentioned above must be carefully considered because, if not properly investigated before using bacteriophages as tracers, these aspects may unknowingly hinder the efficient conduct of the tracing experiments.

The objective of this review was to provide a synthetic overview of past and current uses of bacteriophages as hydrological tracers to identify the advantages and limitations of such an approach from an original virological perspective. To achieve this objective, all

facets of the use of bacteriophages in the previously reported tracing experiments were reviewed according to the methodology set up and described in the first part of the review. A description of the used bacteriophages and their effectiveness was then carried out to further highlight the limitations encountered when using them. Finally, we provide solutions, improvements, and guidance for future applications, based on our understanding of the fate and transportation of viruses in the environment.

2. Methodology

2.1. Search Strategy

To identify all relevant scientific studies concerning the use of bacteriophages as a hydrological tracer over the years, the literature review was conducted using six different online bibliographic databases, namely Web of Science (https://mjl.clarivate.com/search-results, accessed on 7 November 2022), Science Direct (https://www.sciencedirect.com/, accessed on 7 November 2022), PubMed (https://pubmed.ncbi.nlm.nih.gov/, accessed on 7 November 2022), Wiley Online Library (https://onlinelibrary.wiley.com/, accessed on 7 November 2022), Google Scholar (https://scholar.google.com/, accessed on 7 November 2022) and SpringerLink (https://link.springer.com/, accessed on 7 November 2022). The literature review was conducted until June 2022. For each bibliographic database, the first phase of the search consisted of identifying and counting the number of articles matching the keywords (1) "Phage*" or "Bacteriophage*" and (2) "Hydrological tracer*" or "tracer*", with the linguistic annotation required by each database. All articles meeting the above-mentioned keyword combination were retained for the second selection phase. It should be noted that book chapters and conference abstracts were also included.

After the initial screening of the bibliographic database using the above-mentioned keywords, all duplicates were removed, and an additional manual screening based on titles and abstracts was performed in order to select only publications using bacteriophages as hydrological tracers and to eliminate studies which conducted tracing experiments for any purpose other than hydrology (e.g., microbial source tracking). No restrictions were placed on the type of experiments conducted, whether in the laboratory, in the field or using modelling, if the bacteriophages were used in the context of understanding water transportation in ecosystems. All experiments using bacteriophages for other purposes, such as microbial source tracking, studies on the behaviour of bacteriophages in the environment or environmental pollution, were eliminated.

2.2. Data Extraction and Analysis

All selected manuscripts were downloaded and sorted using Mendeley v1.19.8 as the bibliographic reference manager software (http://www.mendeley.com, accessed on 7 November 2022). For each scientific paper selected, various data were extracted: the year of experiments, the country where the experiments were conducted, the bacteriophage species used, the environmental compartments investigated (e.g., groundwater, surface water), the environmental characteristics of the compartment investigated and the types of experiments performed (i.e., laboratory or field). In addition, for the most used bacteriophages, technical details of the experiments were also recorded where available: the initial concentrations of the bacteriophage solutions, the location of the injection point(s), the distance travelled by the injected bacteriophages between the injection site and downstream sampling site, the time and speed of bacteriophage transport, the parallel uses of other tracers and the percentage recovery of bacteriophages. All figures produced from bibliographic data were generated using the 'ggplot2' package with RStudio v1.4.1106 and R v4.0.4 [37].

3. Bacteriophages as Hydrological Tracers: An Overview

According to our eligibility criteria, thirty-two hydrological tracing experiments using bacteriophages were chosen. They were all conducted between 1968 and the present day (Figure 1A). Kinnunen and his team were the pioneers, with seven experiments

performed between 1968 and 1976 [38]. Usually working with conventional tracers, they started working with bacteriophages to study the transportation of groundwater, surface water and wastewater (Figure 1B). At the same time, other researchers turned to the use of bacteriophages for their tracing experiments, albeit scarcely [39–41]. All types of water have been subject to experiments using bacteriophages, with groundwater and surface waters being the most investigated compartments. While only a small number of works were carried out in the 1980s [14,15,32,35], a significant increase in this type of experimentation was noticed in the late 1990s [42–45]. In the 2000s, tracing experiments using bacteriophage became less frequent to the point that they were no longer reported in 2015 [46]. In the 2000s until today, more and more scientific papers using bacteriophages for hydrological tracing purposes have turned to laboratory analyses with the aim of better understanding the fate of bacteriophages in the different compartments of the ecosystem. Indeed, as their properties were not well known at the time, their use as hydrological tracers could have been a limitation.

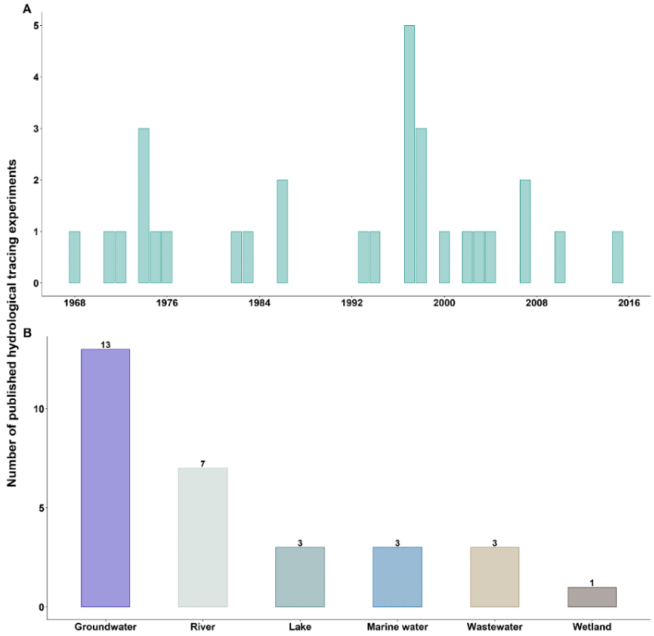


Figure 1. Total number of scientific papers reporting the use of bacteriophages during hydrological tracing experiments according to the year of experiments (**A**) and categorised by the water type investigated (**B**).

3.1. First Hydrological Tracing Experiments Using Bacteriophages

In October 1968, the first hydrological tracing experiments using the *Escherichia coli* bacteriophage F52 (or F52 for short) were performed by Kinnunen and Niemelä [38]. The experiment was the first of a series of tracing experiments conducted until 1975, where the objective was to study the water travel speed of a Kymikoki river (Finland), which extends from a power plant. During these experiments, four to five litres of F52 phage suspensions (10^{10} to 10^{11} plaque-forming unit [pfu]/mL) were injected directly into the river, where the main sewer is discharged, and then water was sampled at 17 km further down the stream. The bacteriophages travelled 8700 m in 7.7 h on average with a velocity ranging from 839 to 1872 m per hour, and an average discharge of 345 m^3/s was determined. The objectives

of this first experiment were not fully achieved, but the results were considered promising enough to warrant further experiments with bacteriophages.

3.2. Objectives of Hydrological Tracing Experiments Using Bacteriophages

Bacteriophages have been used primarily to study water flow velocity in various aquatic environments, such as surface waters (i.e., marine and fresh) [15,35,44] and groundwater [38,41,47,48], and secondly to investigate the connectivity between different aquatic compartments [44,49]. Paul et al. were able to determine the movement of domestic wastewater in the surface waters of the Florida reef using *Enterobacteria phage PRD1* (*Tectiviridae*) and *Listonella phage phiHSIC* (*Siphoviridae*) bacteriophages. Overall, the use of bacteriophages has proven to be effective in hydrological tracing experiments, with a better detection sensitivity than conventional tracers, particularly in karst aquifers and saturated porous environments [42,45,48]. The use of bacteriophages was also efficient in studying water flow in fractured strata when used in conjunction with fluorescent dyes [47,49,50]. Despite the strong attenuation of the bacteriophage signal due to the rapid movement of groundwater, the potential of such an artificial tracer in tracing and identifying the water source has been demonstrated for short-term experiments [41,47,48]. In the application of wastewater tracing, bacteriophages have been used to explore potential deficiencies within sewage treatment plants [15,38,51,52]. Bacteriophage *Escherichia virus T7* (*Autographiviridae*), used for leak detection in wastewater treatment systems, was found to be sensitive to changing conditions in sewage treatment plants and was therefore not a suitable tracer for this application. In contrast, the use of the bacteriophage F52 successfully detected the shortcut in a sewage lagoon and allowed for its repair [38]. In 1988, another sewer leak was successfully investigated using *Emesvirus zideri* (*Fiersviridae*), formerly known as the bacteriophage MS2 (*Leviviridae*), where the fluorescent dyes initially used to detect the leak had not given conclusive results [15]. All the compartments studied in such tracing experiments were aquatic environments, and no experiments have been conducted in the soil to potentially investigate the infiltration processes or the preferential water paths in soil pores.

4. Bacteriophages Commonly Used as Hydrological Tracers

4.1. Description of the Bacteriophages Used in the Tracing Experiments

In total, seventeen different bacteriophage strains have been used as hydrological tracers in our set of selected publications. The most commonly used bacteriophages belong to the families *Fiersviridae*, *Siphoviridae* and *Autographiviridae* (Figure 2A). At the species level, the two most widely used are *Escherichia virus T7* (*Autographiviridae*) and the marine bacteriophage *Pseudoalteromonas phage vB_PspS-H40/1* (H40/1 for short, *Siphoviridae*). In equal third place are *Serratia marescens* phages (unknown families), *Emesvirus zideri* (*Fiersviridae*) (former MS2, *Leviviridae*) and the bacteriophage F52 (unknown family) (Figure 2B). However, the bacteriophage F52 was specifically used in a single study conducted in surface water and wastewater in the very first series of tracing experiments [38].

The viral taxonomy built by the International Committee on Taxonomy of Viruses (ICTV) has recently been updated based on genetic similarities between bacteriophages, thus involving a reclassification of the entire order *Caudovirales*. For the bacteriophages of this review whose strain and family names have been changed, both the old and new names are mentioned. However, it should be noted that only the names employed in the articles have been retained for convenience.

The detailed characteristics of all bacteriophages used as hydrological tracers are summarised in Table 1. Briefly, most of them have a DNA genome with a head-tail structure, except PRD1, phiX174 and MS2, which all have a single capsid. In addition, MS2 is the only one with an RNA genome. Of all the bacteriophages used in the tracing experiments, MS2, T7, PRD1 and phiX174 are the best known and the most widely described in the literature (i.e., genome, structure, pI, host). In particular, the MS2 phage is used as a study model in many experiments in virology, and a large amount of information is therefore

available on it [53–55]. F-specific RNA bacteriophages, including MS2, have also been proposed as a microbial source tracking tool or as indicators of faecal and viral pollution of waters [56–58], which has led to numerous studies on its behaviour and fate in aquatic environments [59,60]. In contrast, very little is known about the bacteriophages F52, F46 and F137 apart from their bacterial host *Escherichia coli*. This was isolated from a Finnish river and was used exclusively in the very first tracing experiments [38]. Similarly, for Serratia phages (Figure 2B), the strain was not specified in three of the five experiments reported, while the remaining bacteriophage, S24VA (NCIMB 10645), infecting the bacterial strain *Serratia marcescens* subsp. *marcescens* (NCIMB 10644), was used as a tracer. Up until 2020, 25 different genomes of Serratia phages had been described. All described Serratia phages are virulent bacteriophages, except for two of them, bacteriophages Eta and Parlo, which are temperate bacteriophages [61,62]. Little is known about the bacteriophages infecting *Serratia marcescens*, almost all of which were isolated from human and/or animal specimens (e.g., faeces) [63,64].

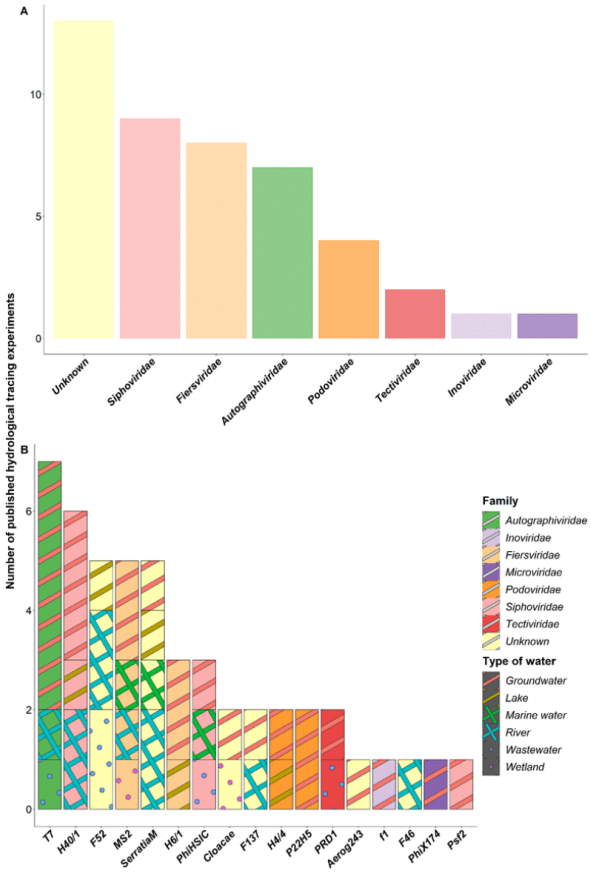


Figure 2. Distribution of bacteriophages commonly used in hydrological tracing experiments, classified according to (**A**) the bacteriophage families and (**B**) the bacteriophage species. Bacteriophage families are distinguishable by the background colours of the bar chart, and the type of water studied is represented by the coloured pattern textures. Unknown = bacteriophage strains (Cloacae phage, Serratia phage, Aerog243 phage, F137, F52, and F46) for which the families are not known.

Table 1. Characteristics of all bacteriophages used as hydrological tracers.

Bacteriophages	Family	Structure	Genome	Isoelectric Point	Bacterial Host	References
THE FIVE MOST USED BACTERIOPHAGES AS HYDROLOGICAL TRACERS						
Enterobacteria phage T7 *(Teseptimavirus T7)*	*Autographiviridae*	55-nm capsid with 19-nm tail	dsDNA of 40 kb	4.85	*Most strains of Escherichia coli*	[65–67]
Pseudoalteromonas phage vB_PspS-H40/1	*Siphoviridae*	Non-contractile 68-nm tail and an icosahedral capsid of 45-nm diameter	dsDNA of 45 kb	Unknown	*Pseudoalteromonas sp.*	[68]
Coliphage F52	*Unknown*	Unknown	Unknown	Unknown	*Escherichia coli*	[38]
Lessievirus MS2 *(Emesvirus zinderi)*	*Fiersviridae*	Small icosahedral virus (28 nm)	(+) ssRNA of 3 kb	3.9	*Escherichia coli*	[59,69]
Serratia phage strains *	*Myoviridae, Siphoviridae, Podoviridae, Ackermannviridae*	Head-tailed structure	dsDNA of 44–350 kb	Unknown	*Strains of Serratia marcescens*	[63,70]
ALL THE OTHER BACTERIOPHAGES USED AS HYDROLOGICAL TRACERS						
Listonella phage phiHSIC	*Siphoviridae*	47-nm capsid diameter with a non-contractile tail of 146 nm	dsDNA genome of 37,966 bp	Unknown	*Vibrio pelagius*	[71]
Pseudoalteromonasvirus vB_PspP-H6/1	*Podoviridae*	56-nm diameter icosahedral capsid and a short (15 nm) tail	linear dsDNA genome of a size of 36,753 bp	<4	*Pseudoalteromonas sp.*	[72,73]
Alteromonasvirus vB_AspP-H4/4	*Podoviridae*	Icosahedral capsid of 41 nm with a short tail of 6.6 nm	dsDNA genome of 47,631 bp	Unknown	*Alteromonas sp.*	[68]
Enterobacteria phage PRD1 *(Alphatectivirus PRD1)*	*Tectiviridae*	62-nm capsid	dsDNA genome of 15 kb	3.8–4.2	Gram-negative bacterial species (i.e., *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*)	[74,75]
***Enterobacter cloacae* phage strains** *	Unknown	Isometric head of about 55–93 nm with a 103 nm long	DNA genomes	Unknown	*Enterobacter cloacae*	[76,77]
Coliphages F137 and F46	Unknown	Unknown	Unknown	Unknown	*Escherichia coli*	[38]
Salmonella phage P22H5	*Podoviridae*	T7-like structure	dsDNA	Unknown	*Salmonella typhimurium*	[49]
Enterobacteria phage f1	*Inoviridae*	Filamentous 850 nm long	ssDNA	Unknown	*Escherichia coli*	[36]
Pseudomonas phage Psf2 *(Tunavirus Psf2)*	*Drexlerviridae* (former *Siphoviridae*)	T1-like structure (60-nm head and 151-nm tail)	Circular genome of about 50 kb	Unknown	*Pseudomonas fluorescens*	[42]
Enterobacteria phage phiX174 *(Sinsheimervirus phiX174)*	*Microviridae*	Icosahedral capsid	Circular ssDNA	6.6–7	*Escherichia sp.*	[78]
***Aerobacter aerogenose* 243 phages**	Unknown	Unknown	Unknown	Unknown	*Aerobacter aerogenes NCTC 243*	[41]

Note: * None of the articles clarified which bacteriophage strain was used. The names in brackets are the latest scientific names updated by the ICTV, and those in bold are the names used in the various papers in which bacteriophages were used as hydrological tracers.

4.2. Effectiveness of Bacteriophages as Hydrological Tracers

All the characteristics regarding the transportation of bacteriophages observed during the various tracing experiments selected in this review are reported in Table 2. It should be noted that all ranges in this table have been purely reported from the literature taking into account the lowest and highest values recorded for each characteristic. The bacteriophage T7 was successfully used to study groundwater flow conditions within more or less permeable aquifers, over distances of hundreds of meters [36,42,45]. Marine bacteriophages are particularly appreciated when investigating freshwater flow since they do not display any background concentrations in this type of water. The bacteriophage H40/1 was used to study groundwater and surface water (i.e., lake and river) flow. It was noticed during surface water tracing experiments that both bacteriophages T7 and H40/1 were found to reach the sampling well faster or in a much lower concentration than the conventional tracer (i.e., uranine) [45,79]. It has been suggested that these processes may be due to the use of preferential pathways through the aquifers by the bacteriophages before reaching the sampling wells. While T7 and H40/1 were transported faster through porous materials, they were on the contrary found as being attenuated in lower permeable media, such as in clay and silt aquifers [42,45]. In karst aquifers, all marine bacteriophages were shown to follow shallow flow systems, while at the same time, the bacteriophage H6/1 displayed lower recovery rates than H40/1 due to a higher adsorption on clay [42,45]. In addition, a multi-tracer experiment using the three marine bacteriophages was recognised in an investigation of water circulation in lakes, especially to overcome the detection issue caused by the dilution [80]. *Listonella* phage ΦHSIC was successfully used from 1995 to 2000 to investigate the wastewater movement from a septic system to surface waters and groundwater. The bacteriophage ΦHSIC, isolated from marine waters, showed particularly high stability in marine subsurface water, where it travelled over long distances (hundreds of meters), and exhibited a notably higher stability than the bacteriophages MS2 and PRD1 that were used as complements [44,51].

While investigating groundwater flow, MS2 concentrations were found to be strongly attenuated, which could be explained by the adsorption of the bacteriophage onto the fracture walls or other material of the subsurface environments [44,46,48]. In experiments using PRD1 as a complement to MS2 to investigate water migration through fractured saprolite aquifer, PRD1 was detected over longer distances than MS2 (18 m vs. 13.5 m) [81]. Both bacteriophages remained viable for hundreds of days after injection, and PRD1 even reappeared in the sampling wells several months after injection, suggesting the remobilization of PRD1 after its adsorption to the clay particles (i.e., saprolite) in the aquifer. This adsorption–desorption process with geological media has indeed been demonstrated for PRD1, with a potential impact on its persistence during its transportation in aquifers, either by slowing it down or accelerating it [29]. *Enterobacter cloacae* phage showed similar behaviour to other bacteriophages in wetlands and aquifers, with a strong attenuation of its concentration in the aquifer, but better recovery rates than the chemical tracer (i.e., rhodamine WT) in the constructed wetlands. P22H5 also underwent adsorption and dilution events during its transportation through groundwater, despite its efficiency in determining channels within aquifers [50], while *Aerobacter aerogenose* 243 phages were successfully used without displaying adsorption on clay particles [41].

Table 2. Characteristics of the transportation of bacteriophages during the various tracing experiments.

Bacteriophages	Reservoirs	Distance Travelled	Time Travelled	Velocity	Loss at the End of the Experiment	Number of Experiments	Other Tracers Used in Complement	References
THE FIVE MOST USED BACTERIOPHAGES AS HYDROLOGICAL TRACERS								
Enterobacteria phage T7 *(Teseptimavirus T7)*	Groundwater and lake	1–160 m (24 km in one experiment)	20–70 h	1–350 m/h	99.9%	7	H40/1, f1, F52, F137, F46, naphthionate	[36,38,45]
Pseudoalteromonas phage vB_PspS-H40/1	Freshwater (especially lakes)	64 m–6 km	8–48 h	8–2000 m/h	40–99%	6	T7, H6/1, uranine	[45]
Coliphage F52	River and lake	8–62 km	4–119 h	900–2000 m/h	-	5	T7, F137, F46	[38]
Lessievirus MS2 *(Emesvirus zinderi)*	Water surface treatment wetlands, aquifers	4–15 m	4 h–5 d	0.2–19 m/h	10–77%	5	PhiHSIC, PRD1, bromide	[44,46,48]
Serratia phage strains *	Surface water	3–10 km	1–6 h	-	30–62%	5	MS2, fluorescein, Enterobacteria phage, *Bacillus* spores, Lithium	[15,35]
ALL THE OTHER BACTERIOPHAGES USED AS HYDROLOGICAL TRACERS								
Listonella phage phiHSIC	From wastewater to surface water and groundwater	10 m–4 km	3–10 h	0.1–2 m/d 1–140 m/h	-	3	PRD1, MS2	[44,51]
Pseudoalteromonasvirus vB_PspP-H6/1	Freshwater (especially lakes)	6 km	5–26 h to 4 d	23–210 m/h	75–99.9%	3	H40/1, T7, Psf2, H4/4	[42,45,79]
Alteromonasvirus vB_AspP-H4/4	Freshwater (especially lakes)	-	1–48 h	-	99.9%	2	H40/1, T7, Psf2, H6/1	[42,79]
Enterobacteria phage PRD1 *(Alphatectivirus PRD1)*	Low clay-content media	4 m–4 km	6 h–5 d	0.2–5 m/d 1–57 m/h	50%	2	MS2, bromide, PhiHSIC	[48,51]
***Enterobacter cloacae* phage strains** *	Constructed wetlands and groundwater	-	3–14 d	0.8–4 L/s	9–64% in wetlands 100% in groundwater	2	Photine, fluorescein, Serratia phage	[47]
Coliphages F137 and F46	River and groundwater	31–46 km	78–111 h	300–400 m/h	-	2 and 1	T7, F52	[38]
Salmonella phage P22H5	Karstic aquifer, groundwater	-	4–28 d	4–36 m/d	99.2–99.9%	2	Deuterium, bromide, chloride, sulfate, pyranine, naphthionate, uranine, sulforhoramine, microspheres	[49,50]
Enterobacteria phage f1	Permeable aquifers	11–110 m	20–70 h	-	100%	1	T7, naphthionate	[36]
Pseudomonas phage Psf2 *(Tunavirus Psf2)*	Groundwater	-	11 d	-	-	1	T7, H6/1, H40/1, H4/4	[42]
Enterobacteria phage phiX174 *(Sinsheimervirus phiX174)*	Karstic aquifer	-	10–14 d	-	99%	1	Photine, fluorescein, Serratia phage, Enterobacteria phage	[47]
***Aerobacter aerogenose* 243 phages**	Groundwater	200–700 m	2–8 d	1–8 m/h	99.9%	1	-	[41]

Note: * None of the articles clarified which bacteriophage strain was used. The names in brackets are the latest scientific names updated by the ICTV, and those in bold are the names used in the various papers in which bacteriophages were used as hydrological tracers. h = hours; d = days; s = seconds; m = meters; km = kilometers; L = liters.

For the investigation of groundwater flow, the bacteriophage F137, like bromide, was shown to have successfully travelled within the whole groundwater system to determine the velocity of the water travel, while the other used tracers used (i.e., the bacteriophage T7, rhodamine and salts) were lost during transport. In addition, in contrast to the bacteriophage T7, the bacteriophage f1 was shown to be suitable for use in permeable aquifers [36]. Despite a higher attenuation of the bacteriophage phiX174 compared to dyes in a karstic aquifer, it showed less adsorption and more stability than the two other bacteriophages used in the same tracing experiment, Serratia phage and *Enterobacter cloacae* [47]. The bacteriophage Psf2 also showed its effectiveness when used to complement marine bacteriophages and the bacteriophage T7 and helped explore groundwater conduits [42]. However, the article did not specify the detailed results obtained after the tracing experiments, such as the velocity, the recovery rate or even the distance travelled. Finally, while determining the pathways into a porous limestone aquifer, Serratia phage managed to highlight the potential presence of fractures, notably because its concentration was found to be attenuated during transportation [47].

4.3. Combination of Hydrological Tracers in Multi-Injection Experiments

The specificity of bacteriophages for their bacterial hosts is characteristic that is well-known and appreciated by hydrologists since several bacteriophages can be used simultaneously without interfering with the detection of the others. For instance, MS2 phage, various strains of Serratia phage or even T7 phage are commonly deemed complementary to other tracers and can be used simultaneously with other bacteriophages [15,42,75] or conventional tracers (e.g., salts, fluorescein, rhodamine WT, microspheres) [33,38,46,80]. Experiments relying on the marine bacteriophage H40/1 always additionally included either other marine bacteriophages (i.e., H6/1, H4/4) [42,45,79], non-marine bacteriophages (i.e., T7, MS2) [42,45] or fluorescent dyes (i.e., uranine, fluorescein) [45,78].

Multi-injection experiments helped researchers compare the fate of multiple tracers and then put forward their best properties, depending on the objectives and the studied ecosystems. In the studies selected for this review, MS2, PRD1 and T7 were preferably used for short-scale experiments where the travelled distances ranged from 1 to 15 m, 3 to 100 m and up to 160 m [33,36,48,75]. Studies using MS2 demonstrated that its travel times varied from 1 h to several days in surface treatment wetlands and it was found to be no longer detectable after spending 17 h in the aquifers [44,75]. Other works compared MS2 to conventional tracers such as bromide, fluorescein and rhodamine WT. In most studies, MS2 phage reached the sampling stations with a delay compared to conventional tracers. The authors assumed that this behaviour was due to adsorption–desorption processes and concluded that this may hamper MS2-based estimations of water travel velocities [15,48]. Moreover, the recovered concentration of MS2 phage in bedrock fractures was ten times lower than that of PRD1, suggesting that narrower pathways were preferentially used by MS2 due to its smaller size (MS2—26 nm < PRD1—62 nm), and thus MS2 was not recovered in the sampling site. On the contrary, small bacteriophages (i.e., 20–30 nm diameter) have a velocity similar to that of water, better fitting the flow of water in fractured aquifers [48]. Moreover, when the permeability of the aquifer increases, the bacteriophage moves faster [36]. F137 and bromide had a similar velocity (0.125 m/h) and were the only tracers detectable during the entire experiment, while the signal of rhodamine, chloride and T7 were lost [38].

The contrasting performances of tracers were also noticeable in non-hydrological tracing experiments that were not reported in this review but might offer transferable explanations regarding the potential factors impacting these differences. In studies which aim at tracing microbial contamination or the fate of bacteriophages in water, marine bacteriophages (e.g., H40/1) have shown a higher transportation rate [81,82], a lower degree of dispersion [45,83] and a more sensitive detection limit than conventional tracers such as bromide.

5. Towards a More Adequate Use of Bacteriophages in Hydrological Tracing Experiments

In the selected studies, bacteriophages were valued by hydrologists in particular for their specificity toward their bacterial host, their eco-friendly property and the sensitivity of their detection methods. However, in regard to the ecological equilibrium of the ecosystem of interest, the eco-friendly property remains controversial, and the sensitivity of their detection methods requires further discussion from a virological point of view. In addition, the interaction between bacteriophages and the environment studied is not yet sufficiently understood. This important aspect needs to be further explored, even before the implementation of tracing experiments. The transportation of bacteriophages in the aquatic environment can be strongly influenced by the physicochemical characteristics of the ecosystem as well as their intrinsic properties (e.g., morphology, isoelectric point, size) [30]. Therefore, bacteriophages to be used as tracers must be properly selected according to the studied watershed to ensure their efficiency as hydrological tracers (Table 3).

Table 3. The three main issues in the use of bacteriophages as hydrological tracers with their limitations and the proposed solutions.

	Status Quo	Encountered Problems	Proposed Solutions
Detection methods	Use of culture-based methods only	This only detects the infectious bacteriophages, which mean they need to be intact to be detected.	The complementary use of cultured-based methods with molecular techniques, which would allow for the detection of the nucleic acid. Thus, all bacteriophages, intact and damaged, can be detected.
Eco-friendly property	Bacteriophages are biological entities and thus are safe for the environment. In addition, they can be rapidly eliminated from the environment.	The injection of large volumes of highly concentrated bacteriophage solutions into the environment. Their genetic material can be released and persist for a long period in the environment.	The natural populations of bacteriophages could be considered since they are numerously abundant.
Surrounding environment	Few of the reported tracing experiments using bacteriophages considered the characteristics of the ecosystems of interest and the environmental conditions before tracer injection.	Bacteriophage inactivation is highly dependent on environmental factors, which can lead to their detection using cultured-based methods being missed.	Characterise the catchment of interest before launching the tracing experiment and select the bacteriophage species according to the relationship between the virus and the catchment properties.

5.1. Methodological Optimisation for Bacteriophage Detection

5.1.1. Limitations of the Current Detection Methods

In most studies, the detection and quantification of bacteriophages are carried out using the plaque assay. Briefly, the plaque assay is based on the incorporation of host bacterial cells in log-phase growth into agar medium. This creates a dense and turbid layer of bacteria able to sustain viral growth. A bacteriophage suspension can subsequently infect, replicate within and lyse cells. With each lysed cell, multiple adjacent ones become immediately infected by newly produced bacteriophages. After several cycles, a clear zone (a plaque) can be observed in the turbid plaque, indicating the presence of what was initially a single bacteriophage particle. The number of plaque-forming units per volume (i.e., PFU/mL) of a sample can thus be determined from the number of plaques generated on the agar layer [84–86]. The plaque assay is one of the easiest, fastest, most cost-effective and most reliable methods for the direct quantification of infectious bacteriophages and therefore the only detection method used in tracing experiments [87]. The sensitivity is appreciable, as one plaque can be detected in a sample volume of 1 to 5 mL [14,36,48,88]. However, only infectious bacteriophages can be detected. As previously

mentioned, bacteriophages can be altered and/or inactivated during transportation in the environment, leaving the particles defective or even resulting in them becoming free nucleic acid. Defective particles are no longer infectious, which makes it impossible to detect them by plaque assay. Therefore, the disappearance of bacteriophages described in certain tracing experiments may be due to the use of an inappropriate detection method, rather than the actual loss of the tracer. Furthermore, by using such a methodological approach (plaque assay), only culturable bacteriophages for which the bacterial host is known and cultivable can be used as hydrological tracers for injection, which greatly limits the number of bacteriophages available.

5.1.2. The Interest in Using Several Complementary Methods for Bacteriophage Detection

To overcome the limitations of the plaque assay, the detection of the nucleic acid of a bacteriophage using molecular biology tools should also be considered (Figure 3). In this way, both infectious and non-infectious (damaged, inactivated and free DNA/RNA) bacteriophages can be detected simultaneously. Nowadays, molecular methods are well-developed, not excessively expensive (i.e., qPCR is about 5–10 €/sample) and widely implemented in all microbiology laboratories. A quantitative (real-time) polymerase chain reaction (qPCR) is a genomic amplification coupled with a fluorescent detection system. During the course of the DNA amplification, the fluorescence is detected and recorded in real-time [58,88–90]. In the case of RNA-viruses detection, a quantitative reverse transcription PCR (RT-qPCR) is used which transcribes RNA into complementary DNA (cDNA) by reverse transcriptase before carrying out the qPCR [91–93]. Beyond detection, such methods also allow for the quantification of the viral nucleic acids originally present in the analysed sample [94]. Moreover, using this molecular technique can promote multi-tracer experiments since the simultaneous detection of different bacteriophage species from an environmental sample is possible thanks to the high specificity of the primers and probes used in such an approach [95,96]. Among the articles selected in this review, only one tracing experiment involved the detection of bacteriophages (F-specific RNA bacteriophages) in stream water samples by applying RT-qPCR [46]. The detection method was successfully used to reveal several patterns such as the better performance of one bacteriophage strain (i.e., GA-phage), a dilution effect caused by precipitations and the loss of all bacteriophages, most likely due to a preferential pathway or bacteriophage behaviour (e.g., adsorption). Given the new information provided by molecular techniques, certain conclusions drawn from hydrological tracing experiments exclusively using the plaque assay would certainly be questionable. Indeed, the loss of bacteriophages described in these studies would most likely be the result of the specific detection of infectious bacteriophages, thus omitting the detection of the non-infectious ones. The two methods should therefore be considered complementary to each other to provide a complete picture of the concentration of bacteriophages recovered during a tracing experiment.

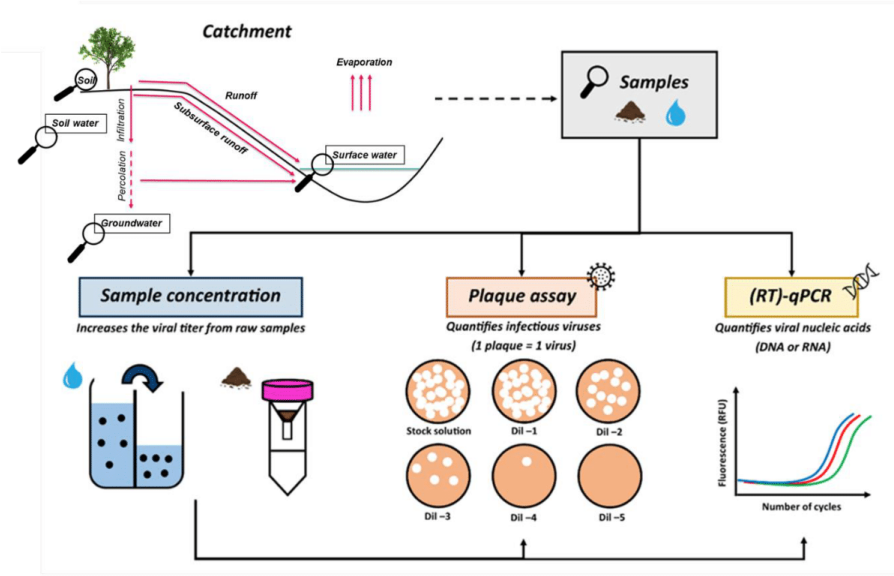


Figure 3. Methodology for the detection of bacteriophages from environmental samples. (Dil = dilution).

The detection of bacteriophages from water samples can be improved by including a viral concentration step before the analytical step. The purpose of this step is to artificially increase the concentration of the bacteriophage of interest in a water sample by reducing the volume of the sample. Thus, it allows for the detection of small amounts of bacteriophage which would not be quantifiable without the concentration step. The viral concentration of water samples can be achieved by using either adsorption/elution methods (e.g., electronegative or electropositive filters), ultrafiltration with polyethylene glycol (PEG) precipitation, centrifugation or centrifugal ultrafiltration, methods that are well-described in the vast number of reviews available [46,97–99].

5.2. "Eco-Friendly" Property of Bacteriophages

5.2.1. The Controversy of This Safe Biological Entity

The main advantage of using bacteriophages as hydrological tracers reported in the literature is their harmless effect on humans and environments since they specifically infect bacteria. Bacteriophages are considered safe for the environment, which has been proven by standard ecological toxicity tests [33–35,49]. Bacteriophages were therefore considered suitable for applications where non-polluting tracers were needed [41]. However, considering the ecosystem equilibrium, the claim that bacteriophages are environmentally friendly is partly flawed. Each tracing experiment requires the injection of large volumes (10–20 L) of highly concentrated bacteriophage solutions (10^4 to 10^{17} particles per litre) into the environment [33,45,48]. Although bacteriophages are natural biological entities, the introduction of a compound into an environment that is not their natural habitat can cause biological contamination by disturbing the equilibrium of ecosystems. Little is known about the impact of injected bacteriophages on existing bacterial communities, so it is reasonable to assume that these exogenous entities can disrupt the ecological diversity of bacteria [100,101]. Bacterial populations are naturally able to adapt to bacteriophage infection by developing resistance mechanisms mainly through genetic mutations but also through adaptative immunity or surface receptor modification [102,103]. The addition of exogenous bacteriophages in large volumes and concentrations can therefore increase

either the mortality or the mutation rate of natural bacteria. The ecological functions of bacteria may also be impacted. Indeed, bacteriophages play a role in the biogeochemical cycles of bacteria, including the global carbon and nitrogen cycles [104,105]. Altering the natural bacterial community could cause disruptions or interruptions in biogeochemical cycles, which could impact nutrient availability (i.e., carbon, nitrogen) and generate faulty processes on a larger scale (i.e., the water cycle) [106,107].

One of the arguments often put forward to support the eco-friendly property of bacteriophages is their rapid elimination from the environment into which they have been introduced. However, although many environmental factors (e.g., temperature, radiation, pH) may be responsible for the inactivation (the loss of their ability to infect bacteria) of bacteriophages in the environment [30], their genetic material (DNA or RNA) can be released and persist for a long period in the environment, particularly their DNA, which is more stable than RNA (up to several years for the DNA and several days for the RNA). Some species of bacteria can perform a genetic transformation, meaning that they can directly take up and integrate free exogenous genetic material from their environment into the host genome through the cell membrane(s) [108–110]. Specifically, DNA may be involved in horizontal transfers of antibiotic resistance genes (ARGs), thus promoting the spread of antimicrobial resistance [111]. These transfers of ARGs were found to be significantly mediated by virulent or/and temperate bacteriophages through transduction mechanisms [112]. The transfer occurs either by the introduction of the bacteriophage DNA into the host genomes (temperate bacteriophages) or by pseudo-lysogeny, where the bacteriophage genome is integrated as a plasmid into the cytoplasm of the host bacteria (virulent bacteriophages) [113]. Mainly isolated from wastewater, the dissemination of these ARGs was also encountered in diverse environments, such as freshwater or urban rivers [114–116].

5.2.2. The Interest in the Natural Populations of Bacteriophages

The natural population of bacteriophages is large and extremely diverse in all environments. In soil, their concentration reputedly varies from 10^3 particles per gram of dry soil in cold deserts to 10^9 particles per gram of dry soil in grasslands and forests [117–119]. In aquatic environments, their concentration varies between 10^3 and 10^7 particles per millilitre of water depending on the type of water (i.e., groundwater, wastewater, marine and freshwater) [120,121]. Given this high diversity, natural populations of bacteriophages could be interesting potential candidates for hydrological tracing to overcome the limitations of the injection of exogenous bacteriophages, as has already been the case for diatoms [9,26,122]. Nowadays, advances in sequencing technologies and bioinformatics tools allow for the easier characterization of the viral diversity for a given habitat [123–125]. This characterization will allow for the determination of the most abundant species and/or genera that can subsequently be selected as potential hydrological tracers [9,126]. Similarly, by detecting and tracing specific sequences extracted from the genetic material of bacteriophages, the speed of the water as well as the preferential water pathways could be determined, especially in terrestrial compartments where bacteriophages are abundant [127].

Finally, bacteriophages introduced at the beginning of tracing experiments may be inactivated as soon as they enter the environment, whereas natural populations of bacteriophages have the advantage of already being adapted to the environment under study. However, for both natural and injected bacteriophages, their fate becomes uncertain once they have left the compartment of origin, and therefore the consideration of the environment of interest is also ideally required.

5.3. The Properties of the Catchment of Interest

5.3.1. Flaws in Considering the Surrounding Environments

Prior to any tracer injection, the characteristics of the ecosystems of interest and the environmental conditions have to be taken into account, as these aspects may be responsible for the loss of the bacteriophage signal during the experiment and its failure. Viral

inactivation is the main phenomenon that can occur when bacteriophages are released into the environment, especially when the latter is not in its natural environment. Inactivation is defined as the loss of the ability of bacteriophages to infect host bacteria. It is then no longer possible to detect them in water samples using the plaque assay. Viral inactivation is highly dependent on environmental factors, such as temperatures, UV radiations and pH [128,129]. Temperature is the main factor in viral inactivation in the environment. In particular, high temperatures are responsible for the loss of the ability of bacteriophages to bind to their host cell through heat-induced changes in protein structure, as demonstrated for the MS2 bacteriophage [130]. These transformations can cause inactivation by disrupting the specific structures required for recognition and binding to host cells. Damage to the viral genome was described as negligible following heat treatment [131]. However, DNA bacteriophages, particularly φX174 and PRD1, were revealed as being highly resistant to a wide range of temperatures (>50 °C) and in various environmental matrices (e.g., surface water, seawater and wastewater) [132]. The optimal temperature for the survival of bacteriophages in the aquatic environment ranges between 4 and 10 °C [30,54,132,133]. A study led by Hodgson et al. (2002) highlighted differences in the bacteriophage travel time and recovery rate according to the season (i.e., winter season vs. summer season). During the cold season, the low light and the high river discharge allowed for a higher recovery rate of the bacteriophage MS2 (98%) than during the summer season (23%) [75]. The negative impact of temperature on MS2 bacteriophage detection was also reported by Gitis et al. (2011) and Cheng et al. (2006) for temperatures between 25 °C and 40 °C [134,135]. In the same vein, UV radiation also increases the inactivation rate of bacteriophages during warm seasons, especially in surface waters and wastewater [32,38]. UV radiation can cause substantial damage to viral nucleic acids, while modest protein damage occurred following a UV treatment on the MS2 bacteriophage [131,136–139]. Among solar radiations, UVB (i.e., $320 < \lambda < 400$ nm) is the most inactivating radiation for bacteriophages [140]. In water loaded with particles such as river or seawater, water turbidity plays an indirect role, since, in turbid waters, radiation penetrates the water to a lesser extent, which limits the inactivation of the bacteriophage [35]. In both aquatic and terrestrial compartments, pH has a key role in the stability of bacteriophages [30,54,141,142]. In alkaline and acidic pH, bacteriophage inactivation was shown to be caused by the deformation, removal or denaturation of the protein capsid [54]. Regarding the bacteriophage T7, a continuous reduction in its concentration was noticed in porous aquifers when a simultaneous increase in the ionic strength and decrease in pH occurred, while the bacteriophage MS2 was found to be intact at a pH = 6.9 in quartz sandy soil columns [45].

In addition to inactivation, the signal of bacteriophages can also be lost during the tracing experiment due to adsorption phenomena. This is defined as the attachment of bacteriophages to other molecules, driven either by van der Waals electrostatic forces or hydrophobic interactions [143,144]. Their adsorption through electrostatic forces is influenced by the pH of the surrounding environment and by charges present on the surface of the molecule [145]. These interaction forces are reversible and mainly occur on mineral surfaces, such as saprolite [80,146] or ferric oxides [147,148]. Hydrophobic interactions are the aggregation of hydrophobic molecules in a hydrophilic media [143,149]. Bacteriophages display different degrees of hydrophobicity, where, for example, the bacteriophage Qb was found to be more hydrophobic than MS2, and, thus, bacteriophages will interact differently depending on their hydrophobicity [150]. This process was studied by Pieper et al. (1997) through the investigation of the effect of organic matter (OM) content on the adsorption of PRD1 during its transportation through sand and gravel aquifers contaminated by sewage. In contaminated aquifers, i.e., those with a high proportion of OM, the adsorption rate in terms of PRD1 on the aquifer particles was reduced, as organic matter was preferentially attached instead of PRD1. This hydrophobic adsorption is reversible and was described as a rapid attachment and slow detachment process [43,80,151]. The possible remobilization of PRD1 bacteriophages after adsorption on aquifer particles has been described. A high pH pulse caused a release of PRD1 into the water body during an experiment through the

detachment of PRD1 from the aquifer surface [43,152]. Bacteriophages adsorb on surfaces when their respective electrical charges oppose each other. However, a change in the pH can cause charge repulsion, resulting in the detachment of bacteriophages. in 2013, Sadeghi et al. studied the effect of calcium ions (Ca^{2+}) on PRD1 transport.

Finally, the loss of the bacteriophage signal can also be related to the intrinsic properties of bacteriophages (e.g., their size, structure and shape), especially in porous media aquifers (i.e., gravel and sand). Bacteriophages were shown to be more dispersed into the fracture or fissure zones through infiltration or percolation processes [45,153,154]. Smaller bacteriophages (<77 nm) are better transported due to their low collision efficiency [82,155]. For instance, H40/1, a small bacteriophage (45 nm diameter), was well distributed through an aquifer [45,148]. However, it was not only its size but also its shape that impacted on its transportation during tracing experiments. Indeed, short-tailed and spherical bacteriophages are adsorbed onto particles to a lesser extent than non-contractile-tailed and rod-shaped bacteriophages, due to their lower contact surface [82]. Not only does the morphology of bacteriophages have an impact on their transportation by causing their loss by adsorption onto particles but it can also be responsible for their inactivation. For instance, physical stresses and interactions with surfaces or particles can break certain structural parts of the viral particle, such as the tail of tailed bacteriophages, leading to their inactivation. In addition, bacteriophages with non-contractile tails are more susceptible to inactivation than those with flexible tails since the rigidity of the non-contractile tail makes it more susceptible to breaking [82,153,155].

5.3.2. Awareness of the Interactions between Bacteriophages and the Surrounding Environment

Whether the bacteriophage tracers used are injected or naturally present in an environment, a consideration of the properties of the environment in which they are used is essential to ensure relevant results from hydrological tracing experiments. As discussed above, the results of a hydrological tracing experiment can be strongly affected by the inactivation of the bacteriophages and their disappearance from the studied environment. As a reminder, the main factors to be controlled to limit the rate of inactivation of bacteriophages are temperature and solar radiation, while pH, size and shape will play an important role in the adsorption of bacteriophages onto soil or water particles, causing their avoidance of detection. Temperatures and solar radiation fluctuate with seasonality and are correlated with each other [156,157]. Surface water temperatures range from 0 °C in the coldest regions of the globe to 35 °C in the warmest, with a range of 9 °C to 24 °C in central Europe (data available daily at https://www.seatemperature.org/, accessed on 27 July 2022). The ground temperature was reported to be about 2°C higher than the air temperature, ranging from −19 °C to 37 °C [158,159]. In addition to seasonal variations, soil and water temperatures depend on depth, with temperatures decreasing with increasing depth [160,161]. The combination of cold weather and low solar radiation will therefore provide better environmental conditions for conducting a tracing experiment using bacteriophages. However, water and soil temperatures are not the main obstacles to tracing experiments, as most of the time tracing experiments are short, which limits the impact of these inactivation and disappearance phenomena to a certain extent.

Suspended sediments, colloidal particles and clay were all identified as being responsible for the disappearance of bacteriophages during hydrological tracing experiments as a result of the adsorption of the colloids on the surface of such environmental components [67,144,146]. To make the best use of bacteriophages, their hydrophobicity and electric charge should be determined before the experiment to predict the hydrophobic and electric interactions, respectively. In the case of H40/1, the bacteriophage was defined as being a moderately hydrophobic (contact angle ≈ 53–61°) and negatively charged (zeta potential = −22 to −24 mM) bacteriophage [82]. The bacteriophage H40/1 would appear to adsorb through hydrophobic interactions rather than using electrostatic forces. A study triggering changes in ionic forces, responsible for electrostatic interaction, did not reveal

a reduction in the bacteriophage concentration during its transportation, suggesting that the bacteriophage did not adsorb [83]. PRD1 has a high hydrophobic surface due to its lipid-protein membrane located within the protein capsid [162], favouring hydrophobic interactions with the OM [163,164]. On the contrary, the adsorption of the MS2 bacteriophage on minerals is mostly driven by electrostatic forces. As explained previously, these forces are mainly driven by the environmental pH and the bacteriophage pI, particularly in soil. Each bacteriophage has its isoelectric point (pI). This is defined as the pH at which a virus carries no net electrical charge and is a characteristic parameter of the viral particle in equilibrium with the surrounding water chemistry. Knowing the pI of a virus enables the prediction of the virus surface charge in the environment. Virus pIs are found in a pH range from 1.9 to 8.4, where they are most frequently measured between 3.5 and 7 [165]. However, only a small number of pIs of viruses are known, and among the bacteriophages used as hydrological tracers, only those of MS2, PRD1, T7, PhiX174 and H6/1 have been measured (pI = 3.9, 4.0, 4.85, 6.6 and <4 respectively). Schematically, when the pH of the surrounding environment is inferior to the virus pI, the overall viral particle charge will be positive. At pH values above 7, the net surface charge of most viruses, including bacteriophages, is negative, resulting in attractive forces between the bacteriophages and certain positively charged particles of the environment [165,166]. The bacteriophage PhiX174, with a pI between 6.6 and 7, will therefore have weak positive charges on its surface, or no charges at all, at a pH of around 7 (roughly the pH of water), whereas the other bacteriophages, with pIs around 4, will be strongly positively charged, leading to a stronger attraction for negative charges. This process allows for the prevention of potential attachments to environmental matrices or molecules [129]. At a pH of above 4, Kvitsand et al. (2015) and Mayotte et al. (2017) demonstrated the adsorption of MS2 (negatively charged; pI = 3.9) on ferric-type surfaces with a net positive charge [60,147]. MS2 cannot only bind to free mineral and metal ions (i.e., Ca^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}) in the medium but also those present on the clay surface [167–170]. According to Rossi and Aragno (1999), attapulgite caused 20% of T7 adsorption within 10 min, reaching 79% at the end of the experiment (total duration of experiments: 180 min). In another experiment, with a pI being almost equal to the pH of the environment, the bacteriophage T7 was reported to recognise both minerals (positive charges) and clay surface (negative charges), as it had both positive and negative charges on its surface [38,67]. Therefore, clay, particularly montmorillonite and attapulgite, induced the inactivation and concentration attenuation of the bacteriophage T7 sediment columns or colloidal particle batch [67,153]. However, in seawater and lake solutions seeded with Φ11M15, T2 and T7 in laboratory experiments, the inactivation of these three bacteriophages was lower for bacteriophages adsorbed on clay surfaces (specifically kaolinite) than for free bacteriophages [171–173]. Moreover, the bacteriophage T7 was still infectious after its adsorption on clay particles, which convinced the authors to assume a protective property of the clay against viral inactivation [67].

In the case of a study involving the injection of bacteriophages, it is important to consider the pI in the selection process of the candidate. If bacteriophages are inappropriately selected, the results of the tracing study may be inconclusive and unusable due to the possible loss of the viral tracer by adsorption to the environmental matrices. It is interesting to mention that during their transportation in the environment, bacteriophages can pass through various ecosystems with different pH values. These changes in environmental conditions during the journeys of bacteriophages should be investigated in advance of the tracing experiment to prevent the possible inactivation of both natural and artificial bacteriophages. It was reported that natural viral communities were less sensitive to UV radiations than lab strains [136]. However, as mentioned previously, it should be noted that the pI is not the only selection criterion regarding adsorption and that the shape or even the size of the virus also matters. As already explained, the tail makes bacteriophages sensitive to inactivation, especially when the tail is not contractile. Therefore, it would be interesting to consider using non-tailed bacteriophages, such as the bacteriophages MS2 or PhiX174, or a tail-head bacteriophage with a contractile tail, such as bacteriophage species belonging

to the families *Siphoviridae* or *Autographiviridae* (e.g., H40/1 and T7, respectively) over non-contractile-tailed bacteriophages, such as species belonging to the family *Myoviridae*.

6. Conclusions and Perspectives

Although bacteriophages have been studied and used as hydrological tracers for almost sixty years, their real potential for this application remains poorly understood. They have been widely used in groundwater studies, but their relevance remains underexplored for hydrological investigations. Due to their small size, they successfully contributed to improving our understanding of subsurface water movements in karstic aquifers and in fractured saprolite [45,80,174], and they have finally emerged as good candidates for exploring hydrological processes. While their potential as hydrological tracers remains to be fully characterised, the combined use of bacteriophage and conventional tracers, such as isotopes, may lead to a better understanding of eco-hydro systems [38,42].

As each bacteriophage has its own behaviour, the transportation of each bacteriophage will be different depending on its intrinsic characteristics and the influence of external factors, which makes it impossible to find a tracer model to use in a specific type of ecosystem [45]. A bacteriophage's size and shape serve as primary criteria. For fractured and fissured media, small bacteriophages will tend to move further in pores, which will reduce the collision efficiency. Adsorption is a common process and is most often encountered during bacteriophage transportation. Taking the hydrophobicity and the electric charge of bacteriophages into account will prevent a high rate of removal. To conclude, the choice of the right bacteriophage for a tracing experiment must be driven by an accurate pre-analysis of both the characteristics of the media and the intrinsic characteristics of bacteriophages.

The different advantages highlighted in the various articles in this review included the sensitivity of the detection method, the possibility to produce a high concentration of bacteriophages for a low volume and their specificity for their hosts. Indeed, bacteriophages were detected before conventional tracers, and they remained detected despite the dilution effect of the experiments [48,82]. Furthermore, several of them were used at the same time without interfering with the detection [35,81,153]. However, the initial high concentration of bacteriophages can be seen as a new type of pollution, as the introduction of an exogenous entity can generate a modification in the natural processes of a habitat. Moreover, the detection method is sensitive but limited to infectious bacteriophages. Complementary molecular techniques need to be considered to detect the damaged bacteriophages still occurring in tracing experiments. Finally, their transportation is influenced by different factors and, more specifically, their adsorption on the surrounding matrices, which has caused the attenuation of bacteriophages during tracing experiments. In addition, other environmental characteristics, such as pH, temperature or radiation, can affect their fate through the inactivation of bacteriophages [29,43,51]. Therefore, the local environment is important to consider when choosing the most appropriate bacteriophage [89].

It is important to note that the design of a tracing experiment using either one or multiple tracers does not differ from a traditional tracing experiment using conventional tracers nor from previous tracing experiments using bacteriophages. The recommendations therefore concern the selection of an appropriate candidate prior to the launch of any experiments in the case of using an artificial tracer. When using natural populations of bacteriophages as a more eco-friendly alternative, no injections are required and only the development of detection methods are needed. In addition, it is recommended to better evaluate the surrounding environment to prevent the loss of the signal of the tracer and to use complementary methods to optimize the obtained results.

Author Contributions: Conceptualization, P.F., L.O. and H.-M.C.; methodology, P.F. and L.O.; validation, P.F., L.O. and H.-M.C.; writing—original draft preparation, P.F.; writing—review and editing, P.F., L.O. and H.-M.C.; visualization, P.F.; supervision, L.O. and H.-M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Luxembourg National Research Fund (FNR) for granting and supporting the PRIDE program containing the Doctoral Training Unit Hydro-CSI project (PRIDE15/10623093—HYDRO-CSI).

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to acknowledge the Luxembourg National Research Fund (FNR) for its financial support of the Doctoral Training Unit Hydro-CSI (ref: PRIDE15/10623093—HYDRO-CSI). Moreover, the authors would like to thank Laurent Pfister (coordinator of the Doctoral Training Unit Hydro-CSI at the Luxembourg Institute of Science and Technology) for his comments on successive iterations of the manuscript and his expertise in hydrology.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript or in the decision to publish the results.

References

1. Leibundgut, C.; Seibert, J. Tracer Hydrology. *Treatise Water Sci.* **2010**, *2*, 215–236. [CrossRef]
2. Mcguire, K.J.; Mcdonnell, J.J. Tracer Advances in Catchment Hydrology. *Hydrol. Process.* **2015**, *29*, 5135–5138. [CrossRef]
3. McGuire, K.J.; McDonnell, J.J. A Review and Evaluation of Catchment Transit Time Modeling. *J. Hydrol.* **2006**, *330*, 543–563. [CrossRef]
4. Flury, M.; Wai, N.N. Dyes as Tracers for Vadose Zone Hydrology. *Rev. Geophys.* **2003**, *41*, 1–37. [CrossRef]
5. Harvey, R.W. Microorganisms as Tracers in Groundwater Injection and Recovery Experiments: A Review. *FEMS Microbiol. Rev.* **1997**, *20*, 461–472. [CrossRef]
6. Behrens, H.; Beims, U.; Dieter, H.; Dietze, G.; Eikmann, T.; Grummt, T.; Hanisch, H.; Henseling, H.; Käss, W.; Kerndorff, H.; et al. Toxicological and Ecotoxicological Assessment of Water Tracers. *Hydrogeol. J.* **2001**, *9*, 321–325. [CrossRef]
7. Schudel, B.; Biaggi, D.; Dervey, T.; Kozel, R.; Müller, I.; Ross, J.H.; Schindler, U. *Utilisation Des Traceurs Artificiels En Hydrogéologie—Guide Pratique*; Série Géologique; Office fédéral des eaux et de la géologie: Washington, DC, USA, 2002; p. 77.
8. Berryman, C.J. *Tracer Tests for Investigating Flow and Transport in the Hyporheic Zone*; Environment Agency: Bristol, UK, 2005; ISBN 9781844326983.
9. Coles, A.E.; Wetzel, C.E.; Martínez-Carreras, N.; Ector, L.; Mcdonnell, J.J.; Frentress, J.; Klaus, J.; Hoffmann, L.; Pfister, L. Diatoms as a Tracer of Hydrological Connectivity: Are They Supply Limited? *Ecohydrology* **2016**, *9*, 631–645. [CrossRef]
10. Kirchner, J.W.; Tetzlaff, D.; Soulsby, C. Comparing Chloride and Water Isotopes as Hydrological Tracers in Two Scottish Catchments. *Hydrol. Process.* **2010**, *24*, 1631–1645. [CrossRef]
11. Klaus, J.; McDonnell, J.J. Hydrograph Separation Using Stable Isotopes: Review and Evaluation. *J. Hydrol.* **2013**, *505*, 47–64. [CrossRef]
12. Burns, D.A. Stormflow-Hydrograph Separation Based on Isotopes: The Thrill Is Gone-What's Next? *Hydrol. Process.* **2002**, *16*, 1515–1517. [CrossRef]
13. Cartwright, I.; Cendón, D.; Currell, M.; Meredith, K. A Review of Radioactive Isotopes and Other Residence Time Tracers in Understanding Groundwater Recharge: Possibilities, Challenges, and Limitations. *J. Hydrol.* **2017**, *555*, 797–811. [CrossRef]
14. Skilton, H.; Wheeler, D. Bacteriophage Tracer Experiments in Groundwater. *J. Appl. Bacteriol.* **1988**, *65*, 387–395. [CrossRef] [PubMed]
15. Martin, C. The Application of Bacteriophage Tracer Techniques in South West Water. *Water Environ. J.* **1988**, *2*, 638–642. [CrossRef]
16. Sklash, M.G.; Farvolden, R.N.; Fritz, P. A Conceptual Model of Watershed Response to Rainfall, Developed through the Use of Oxygen-18 as a Natural Tracer. *Can. J. Earth Sci.* **1976**, *13*, 271–283. [CrossRef]
17. McGlynn, B.; McDonnell, J.; Stewart, M.; Seibert, J. On the Relationships between Catchment Scale and Streamwater Mean Residence Time. *Hydrol. Process.* **2003**, *17*, 175–181. [CrossRef]
18. Burt, T.P.; McDonnell, J.J. Whither Field Hydrology? The Need for Discovery Science and Outrageous Hydrological Hypotheses. *Water Resour. Res.* **2015**, *51*, 5919–5928. [CrossRef]
19. Vitvar, T.; Aggarwal, P.K.; McDonnell, J.J. A Review of Isotope Applications in Catchment Hydrology. In *Isotopes in the Water Cycle: Past, Present and Future of a Developing Science*; Springer: Dordrecht, The Netherlands, 2005; pp. 151–169. ISBN 140203010X.
20. Gibson, J.J.; Aggarwal, P.; Hogan, J.; Kendall, C.; Martinelli, L.A.; Stichler, W.; Rank, D.; Goni, I.; Choudhry, M.; Gat, J.; et al. Isotope Studies in Large River Basins: A New Global Research Focus. *Eos Trans. Am. Geophys. Union* **2002**, *83*, 613–620. [CrossRef]
21. Selker, J.S.; Thévenaz, L.; Huwald, H.; Mallet, A.; Luxemburg, W.; Van De Giesen, N.; Stejskal, M.; Zeman, J.; Westhoff, M.; Parlange, M.B. Distributed Fiber-Optic Temperature Sensing for Hydrologic Systems. *Water Resour. Res.* **2006**, *42*, 1–8. [CrossRef]
22. Westhoff, M.C.; Savenjie, H.H.G.; Luxemburg, W.M.J.; Stelling, G.S.; Van De Giesen, N.C.; Selker, J.S.; Pfister, L.; Uhlenbrook, S. A Distributed Stream Temperature Model Using High Resolution Temperature Observations. *Hydrol. Earth Syst. Sci.* **2007**, *11*, 1469–1480. [CrossRef]
23. Pfister, L.; McDonnell, J.J.; Hissler, C.; Hoffmann, L. Ground-Based Thermal Imagery as a Simple, Practical Tool for Mapping Saturated Area Connectivity and Dynamics. *Hydrol. Process.* **2010**, *24*, 3123–3132. [CrossRef]

24. Glaser, B.; Antonelli, M.; Chini, M.; Pfister, L.; Klaus, J. Technical Note: Mapping Surface-Saturation Dynamics with Thermal Infrared Imagery. *Hydrol. Earth Syst. Sci.* **2018**, *22*, 5987–6003. [CrossRef]
25. Pfister, L.; McDonnell, J.J.; Wrede, S.; Hlúbiková, D.; Matgen, P.; Fenicia, F.; Ector, L.; Hoffmann, L. The Rivers Are Alive: On the Potential for Diatoms as a Tracer of Water Source and Hydrological Connectivity. *Hydrol. Process.* **2009**, *23*, 2841–2845. [CrossRef]
26. Klaus, J.; Wetzel, C.E.; Martínez-Carreras, N.; Ector, L.; Pfister, L. A Tracer to Bridge the Scales: On the Value of Diatoms for Tracing Fast Flow Path Connectivity from Headwaters to Meso-Scale Catchments. *Hydrol. Process.* **2015**, *29*, 5275–5289. [CrossRef]
27. Foppen, J.W.; Orup, C.; Adell, R.; Poulalion, V.; Uhlenbrook, S. Using Multiple Artificial DNA Tracers in Hydrology. *Hydrol. Process.* **2011**, *25*, 3101–3106. [CrossRef]
28. Tang, Y.; Foppen, J.W.; Bogaard, T.A. Transport of Silica Encapsulated DNA Microparticles in Controlled Instantaneous Injection Open Channel Experiments. *J. Contam. Hydrol.* **2021**, *242*, 103880. [CrossRef]
29. Harvey, R.W.; Ryan, J.N. Use of PRD1 Bacteriophage in Groundwater Viral Transport, Inactivation, and Attachment Studies. *FEMS Microbiol. Ecol.* **2004**, *49*, 3–16. [CrossRef]
30. Jończyk, E.; Kłak, M.; Miedzybrodzki, R.; Górski, A. The Influence of External Factors on Bacteriophages-Review. *Folia Microbiol.* **2011**, *56*, 191–200. [CrossRef]
31. Wommack, K.E.; Hill, R.T.; Kessel, M.; Russek-Cohen, E.; Colwell, R.R. Distribution of Viruses in the Chesapeake Bay. *Appl. Environ. Microbiol.* **1992**, *58*, 2965–2970. [CrossRef]
32. Sinton, L.W.; Ching, S.B. An Evaluation of Two Bacteriophages as Sewage Tracers. *Water Air Soil Pollut.* **1987**, *35*, 347–356. [CrossRef]
33. Harden, H.S.; Chanton, J.P.; Rose, J.B.; John, D.E.; Hooks, M.E. Comparison of Sulfur Hexafluoride, Fluorescein and Rhodamine Dyes and the Bacteriophage PRD-1 in Tracing Subsurface Flow. *J. Hydrol.* **2003**, *277*, 100–115. [CrossRef]
34. Hodgson, C.J.; Perkins, J.; Labadz, J.C. Evaluation of Biotracers to Monitor Effluent Retention Time in Constructed Wetlands. *Lett. Appl. Microbiol.* **2003**, *36*, 362–371. [CrossRef]
35. Drury, D.F.; Wheeler, D.C. Applications of a Serratia Marcescens Bacteriophage as a New Microbial Tracer of Aqueous Environments. *J. Appl. Bacteriol.* **1982**, *53*, 137–142. [CrossRef]
36. Rossi, P.; de Carvalho-Dill, A.; Müller, I.; Aragno, M. Comparative Tracing Experiments in a Porous Aquifer Using Bacteriophages and Fluorescent Dye on a Test Field Located at Wilerwald (Switzerland) and Simultaneously Surveyed in Detail on a Local Scale by Radio-Magneto-Tellury (12–240 KHz). *Environ. Geol.* **1994**, *23*, 192–200. [CrossRef]
37. Rstudio Team. *RStudio: Integrated Development for R*; RStudio, PBC.: Boston, MA, USA, 2020.
38. Kinnunen, K. *Tracing Water Movement by Means of Eschirichia Coli Bacteriophages*; The water research institute: Helsinki, Finland, 1978; ISBN 951463344X.
39. Wimpenny, J.W.T.; Cotton, N.; Statham, M. Microbes as Tracers of Water Movement. *Water Res.* **1972**, *6*, 731–739. [CrossRef]
40. Schaub, S.A.; Sorber, C.A. Virus and Bacteria Removal from Wastewater by Rapid Infiltration through Soil. *Appl. Environ. Microbiol.* **1977**, *33*, 609–619. [CrossRef] [PubMed]
41. Martin, R.; Thomas, A. An Example of the Use of Bacteriophage as a Groundwater Tracer. *J. Hydrol.* **1974**, *23*, 73–78. [CrossRef]
42. Drew, D.; Doerfliger, N.; Formentin, K. The Use of Bacteriophages for Multi-Tracing in a Lowland Karst Aquifer in Western Ireland. *Tracer Hydrol.* **1997**, *97*, 33–37.
43. Bales, R.C.; Li, S.; Yeh, T.J.; Lenczewski, M.E.; Gerba, P. Bacteriophage and Microsphere Transport in Saturated Porous Media: Forced-Gradient Experiment at Borden, Ontario. *Water Resour. Res.* **1997**, *33*, 639–648. [CrossRef]
44. Paul, J.H.; Rose, J.B.; Jiang, S.C.; Zhou, X.; Cochran, P.; Kellogg, C.; Kang, J.B.; Griffin, D.; Farrah, S.; Lukasik, J. Evidence for Groundwater and Surface Marine Water Contamination by Waste Disposal Wells in the Florida Keys. *Water Res.* **1997**, *31*, 1448–1454. [CrossRef]
45. Rossi, P.; Doerfliger, N.; Kennedy, K.; Müller, I.; Aragno, M. Bacteriophages as Surface and Ground Water Tracers. *Hydrol. Earth Syst. Sci.* **1998**, *2*, 101–110. [CrossRef]
46. Antonelli, M.; Balasubramanian, M.N.; Ogorzaly, L.; Pfister, L. QPCR (Quantitative Polymerase Chain Reaction) for the Quantification of Bacteriophages in Stream Water Samples to Investigate Hydrological Processes: A Proof-of-Concept Study in the Huewelerbach Experimental Catchment (Luxembourg). In Proceedings of the EGU General assembly, Vienna, Austria, 17–22 April 2016; Volume 18, p. 1.
47. Maurice, L.; Atkinson, T.C.; Williams, A.T.; Barker, J.A.; Farrant, A.R. Catchment Scale Tracer Testing from Karstic Features in a Porous Limestone. *J. Hydrol.* **2010**, *389*, 31–41. [CrossRef]
48. McKay, L.D.; Cherry, J.A.; Bales, R.C.; Yahya, M.T.; Gerba, C.P. A Field Example of Bacteriophage as Tracers of Fracture Flow. *Environ. Sci. Technol.* **1993**, *27*, 1075–1079. [CrossRef]
49. Bricelj, M. Microbial Tracers in Groundwater Research. *RMZ—Mater. Geoenviron.* **2003**, *1*, 67–70.
50. Curk, B.C.; Stichler, W. Deuterium Tracer Experiment in the Unsaturated Zone of Fractured Karst Aquifers. In Proceedings of the International Symposium On Advances In Isotope Hydrology, Vienna, Austria, 21–25 May 2007; Volume 1, pp. 597–604.
51. Paul, J.H.; McLaughlin, M.R.; Griffin, D.W.; Lipp, E.K.; Stokes, R.; Rose, J.B. Rapid Movement of Wastewater from On-Site Disposal Systems into Surface Waters in the Lower Florida Keys. *Estuaries Coasts* **2000**, *23*, 662–668. [CrossRef]
52. Horan, N.J.; Parr, J.; Naylor, P.J. Evaluation of Tracers for the Determination of the Mixing Characteristics of Activated Sludge Reactors. *Environ. Technol.* **1991**, *12*, 603–608. [CrossRef]

53. Langlet, J.; Gaboriaud, F.; Gantzer, C. Effects of PH on Plaque Forming Unit Counts and Aggregation of MS2 Bacteriophage. *J. Appl. Microbiol.* **2007**, *103*, 1632–1638. [CrossRef] [PubMed]
54. Feng, Y.Y.; Ong, S.L.; Hu, J.Y.; Tan, X.L.; Ng, W.J. Effects of PH and Temperature on the Survival of Coliphages MS2 and Qβ. *J. Ind. Microbiol. Biotechnol.* **2003**, *30*, 549–552. [CrossRef]
55. Elhadidy, A.M.; Peldszus, S.; van Dyke, M.I. An Evaluation of Virus Removal Mechanisms by Ultrafiltration Membranes Using MS2 and ΦX174 Bacteriophage. *Sep. Purif. Technol.* **2013**, *120*, 215–223. [CrossRef]
56. Ogorzaly, L.; Tissier, A.; Bertrand, I.; Maul, A.; Gantzer, C. Relationship between F-Specific RNA Phage Genogroups, Faecal Pollution Indicators and Human Adenoviruses in River Water. *Water Res.* **2009**, *43*, 1257–1264. [CrossRef]
57. Ogorzaly, L.; Bertrand, I.; Paris, M.; Maul, A.; Gantzer, C. Occurrence, Survival, and Persistence of Human Adenoviruses and F-Specific RNA Phages in Raw Groundwater. *Appl. Environ. Microbiol.* **2010**, *76*, 8019–8025. [CrossRef]
58. Ogorzaly, L.; Gantzer, C. Development of Real-Time RT-PCR Methods for Specific Detection of F-Specific RNA Bacteriophage Genogroups: Application to Urban Raw Wastewater. *J. Virol. Methods* **2006**, *138*, 131–139. [CrossRef]
59. Jain, R.; Srivastava, R. Metabolic Investigation of Host/Pathogen Interaction Using MS2-Infected Escherichia Coli. *BMC Syst. Biol.* **2009**, *3*, 121. [CrossRef] [PubMed]
60. Mayotte, J.M.; Hölting, L.; Bishop, K. Reduced Removal of Bacteriophage MS2 in during Basin Infiltration Managed Aquifer Recharge as Basin Sand Is Exposed to Infiltration Water. *Hydrol. Process.* **2017**, *31*, 1690–1701. [CrossRef]
61. Bockoven, R.; Gutierrez, J.; Newkirk, H.; Liu, M.; Cahill, J.; Ramsey, J. Complete Genome Sequence of Serratia Marcescens Podophage Parlo. *Microbiol. Resour. Announc.* **2019**, *8*, e00569-19. [CrossRef] [PubMed]
62. Denyes, J.M.; Krell, P.J.; Manderville, R.A.; Ackermann, H.W.; She, Y.M.; Kropinski, A.M. The Genome and Proteome of Serratia Bacteriophage η Which Forms Unstable Lysogens. *Virol. J.* **2014**, *11*, 6. [CrossRef]
63. Bujak, K.; Decewicz, P.; Kaminski, J.; Radlinska, M. Identification, Characterization, and Genomic Analysis of Novel Serratia Temperate Phages from a Gold Mine. *Int. J. Mol. Sci.* **2020**, *21*, 6709. [CrossRef]
64. Tian, C.; Zhao, J.; Zhang, Z.; Chen, X.; Wei, X.; Li, H.; Lin, W.; Ke, Y.; Hu, L.; Jiang, A.; et al. Identification and Molecular Characterization of Serratia Marcescens Phages VB_SmaA_2050H1 and VB_SmaM_2050HW. *Arch. Virol.* **2019**, *164*, 1085–1094. [CrossRef]
65. Chan, L.Y.; Kosuri, S.; Endy, D. Refactoring Bacteriophage T7. *Mol. Syst. Biol.* **2005**, *1*, 1–10. [CrossRef]
66. Huss, P.; Meger, A.; Leander, M.; Nishikawa, K.; Raman, S. Mapping the Functional Landscape of the Receptor Binding Domain of T7 Bacteriophage by Deep Mutational Scanning. *Elife* **2021**, *10*, e63775. [CrossRef]
67. Rossi, P.; Aragno, M. Analysis of Bacteriophage Inactivation and Its Attenuation by Adsorption onto Colloidal Particles by Batch Agitation Techniques. *Can. J. Microbiol.* **1999**, *45*, 9–17. [CrossRef]
68. Kallies, R.; Kiesel, B.; Zopfi, J.; Wick, L.Y.; Chatzinotas, A. Complete Genome Sequence of Alteromonas Virus VB_AspP-H4/4. *Genome Announc.* **2017**, *5*, e00914-17. [CrossRef] [PubMed]
69. Dedeo, M.T.; Finley, D.T.; Francis, M.B. *Viral Capsids as Self-Assembling Templates for New Materials*, 1st ed.; Elsevier Inc.: Amsterdam, The Netherlands, 2011; Volume 103, ISBN 9780124159068.
70. Carr, E.L.; Wilson, M.E.; Adams, S.T.; Arens, D.K.; Ayala, M.; Ayers, H.; Barker, A.; Beecroft, V.; Bishop, E.; Brundage, B.; et al. Genome Sequences of 14 Siphophages That Infect Serratia Marcescens. *Microbiol. Resour. Announc.* **2022**, *11*, e0121221. [CrossRef] [PubMed]
71. Jiang, S.C.; Kellogg, C.A.; Paul, J.H. Characterization of Marine Temperate Phage-Host Systems Isolated from Mamala Bay, Oahu, Hawaii. *Appl. Environ. Microbiol.* **1998**, *64*, 535–542. [CrossRef] [PubMed]
72. Kallies, R.; Kiesel, B.; Schmidt, M.; Ghanem, N.; Zopfi, J.; Hackermüller, J.; Harms, H.; Wick, L.Y.; Chatzinotas, A. Complete Genome Sequence of Pseudoalteromonas Virus VB_PspP-H6/1 That Infects Pseudoalteromonas Sp. Strain H6. *Mar. Genom.* **2019**, *47*, 100667. [CrossRef]
73. Olsen, R.H.; Siak, J.-S.; Gray, R.H. Characteristics of PRD1, a Plasmid-Dependent Broad Host Range DNA Bacteriophage. *J. Virol* **1974**, *14*, 689–699. [CrossRef] [PubMed]
74. Ziedaite, G.; Daugelavic, R.; Bamford, J.K.H.; Bamford, D.H. The Holin Protein of Bacteriophage PRD1 Forms a Pore for Small-Molecule and Endolysin Translocation. *J. Bacteriol.* **2005**, *187*, 5397–5405. [CrossRef]
75. Hodgson, C.J.; Perkins, J.; Labadz, J.C. The Use of Microbial Tracers to Monitor Seasonal Variations in Effluent Retention in a Constructed Wetland. *Water Res.* **2004**, *38*, 3833–3844. [CrossRef]
76. Morozova, V.; Jdeed, G.; Kozlova, Y.; Babkin, I.; Tikunov, A.; Tikunova, N. A New Enterobacter Cloacae Bacteriophage Ec151 Encodes the Deazaguanine Dna Modification Pathway and Represents a New Genus within the Siphoviridae Family. *Viruses* **2021**, *13*, 1372. [CrossRef]
77. McKenna, R.; Xia, D.; Willingmann, P.; Ilag, L.L.; Rossmann, M.G. Structure Determination of the Bacteriophage PhiX174. *Acta Cryst. B* **1992**, *48*, 499–511. [CrossRef]
78. Schnegg, P.-A.; Flynn, R. Online Field Fluorometers for Hydrogeological Tracer Tests. *Isot. Und Tracer Der Wasserforsch.* **2002**, *1879*, 29–36.
79. Goldscheider, N.; Haller, L.; Poté, J.; Wildi, W.; Zopfi, J. Characterizing Water Circulation and Contaminant Transport in Lake Geneva Using Bacteriophage Tracer Experiments and Limnological Methods. *Environ. Sci. Technol.* **2007**, *41*, 5252–5258. [CrossRef] [PubMed]

80. McKay, L.D.; Sanford, W.E.; Strong, J.M. Field-Scale Migration of Colloidal Tracers in a Fractured Shale Saprolite. *Ground Water* **2000**, *38*, 139–147. [CrossRef]
81. Flynn, R.; Hacini, Y.; Schnegg, P.-A.; Costa, R.; Diomande, K.A. Use of Tracer Tests and Geophysical Logging to Understand Solute and Micro-Organism Tracer Responses in Monitoring Wells with Long Screen Intervals in a Gravel Aquifer. *Beiträge Zur. Hydrogeol.* **2006**, *55*, 5–20.
82. Ghanem, N.; Kiesel, B.; Kallies, R.; Harms, H.; Chatzinotas, A.; Wick, L.Y. Marine Phages as Tracers: Effects of Size, Morphology, and Physico− Chemical Surface Properties on Transport in a Porous Medium. *Environ. Sci. Technol.* **2016**, *50*, 12816–12824. [CrossRef] [PubMed]
83. Flynn, R.M.; Sinreich, M. Characterisation of Virus Transport and Attenuation in Epikarst Using Short Pulse and Prolonged Injection Multi-Tracer Testing. *Water Res.* **2010**, *44*, 1138–1149. [CrossRef] [PubMed]
84. Cooper, P.D. The Plaque Assay of Animal Viruses. *Adv. Virus Res.* **1961**, *8*, 319–378. [CrossRef]
85. Dulbecco, R.; Vogt, M. Some Problems of Animal Virology as Studied by the Plaque Technique. *Cold Spring Harb. Symp. Quant. Biol.* **1953**, *18*, 273–279. [CrossRef]
86. Roux, M. On an Invisible Microbe Antagonistic to Dysentery Bacilli. Note by M. F. d'Herelle, Presented by M. Roux. *Comptes Rendus Acad. Des Sci.* **1917**, *165*, 373–375, Erratum in *Bacteriophage* **2011**, *1*, 3–5.. [CrossRef]
87. Shen, C.; Phanikumar, M.S.; Fong, T.T.; Aslam, I.; Mcelmurry, S.P.; Molloy, S.L.; Rose, J.B. Evaluating Bacteriophage P22 as a Tracer in a Complex Surface Water System: The Grand River, Michigan. *Environ. Sci. Technol.* **2008**, *42*, 2426–2431. [CrossRef]
88. Wyer, M.D.; Kay, D.; Watkins, J.; Davies, C.; Kay, C.; Thomas, R.; Porter, J.; Stapleton, C.M.; Moore, H. Evaluating Short-Term Changes in Recreational Water Quality during a Hydrograph Event Using a Combination of Microbial Tracers, Environmental Microbiology, Microbial Source Tracking and Hydrological Techniques: A Case Study in Southwest Wales, UK. *Water Res.* **2010**, *44*, 4783–4795. [CrossRef]
89. Hunt, R.J.; Borchardt, M.A.; Bradbury, K.R. Viruses as Groundwater Tracers: Using Ecohydrology to Characterize Short Travel Times in Aquifers. *Groundwater* **2014**, *52*, 187–193. [CrossRef]
90. Ács, N.; Gambino, M.; Brøndsted, L. Bacteriophage Enumeration and Detection Methods. *Front. Microbiol.* **2020**, *11*, 594868. [CrossRef]
91. Watzinger, F.; Suda, M.; Preuner, S.; Baumgartinger, R.; Ebner, K.; Baskova, L.; Niesters, H.G.M.; Lawitschka, A.; Lion, T. Real-Time Quantitative PCR Assays for Detection and Monitoring of Pathogenic Human Viruses in Immunosuppressed Pediatric Patients. *J. Clin. Microbiol.* **2004**, *42*, 5189. [CrossRef]
92. Toussaint, J.F.; Sailleau, C.; Breard, E.; Zientara, S.; De Clercq, K. Bluetongue Virus Detection by Two Real-Time RT-QPCRs Targeting Two Different Genomic Segments. *J. Virol. Methods* **2007**, *140*, 115–123. [CrossRef]
93. Vester, D.; Lagoda, A.; Hoffmann, D.; Seitz, C.; Heldt, S.; Bettenbrock, K.; Genzel, Y.; Reichl, U. Real-Time RT-QPCR Assay for the Analysis of Human Influenza A Virus Transcription and Replication Dynamics. *J. Virol. Methods* **2010**, *168*, 63–71. [CrossRef]
94. Arya, M.; Shergill, I.S.; Williamson, M.; Gommersall, L.; Arya, N.; Patel, H.R.H. Basic Principles of Real-Time Quantitative PCR. *Expert Rev. Mol. Diagn.* **2005**, *5*, 209–219. [CrossRef]
95. Klymus, K.E.; Ruiz Ramos, D.V.; Thompson, N.L.; Richter, C.A.; Ramos, R.; Development, C. Development and Testing of Species-Specific Quantitative PCR Assays for Environmental DNA Applications. *J. Vis. Exp.* **2020**, *165*, e61825. [CrossRef]
96. Knudsen, S.W.; Ebert, R.B.; Hesselsøe, M.; Kuntke, F.; Hassingboe, J.; Mortensen, P.B.; Thomsen, P.F.; Sigsgaard, E.E.; Hansen, B.K.; Nielsen, E.E.; et al. Species-Specific Detection and Quantification of Environmental DNA from Marine Fishes in the Baltic Sea. *J. Exp. Mar. Biol. Ecol.* **2019**, *510*, 31–45. [CrossRef]
97. Bofill-Mas, S.; Rusiñol, M. Recent Trends on Methods for the Concentration of Viruses from Water Samples. *Curr. Opin. Environ. Sci. Health* **2020**, *16*, 7–13. [CrossRef]
98. Forés, E.; Rusiñol, M.; Itarte, M.; Martínez-Puchol, S.; Calvo, M.; Bofill-Mas, S. Evaluation of a Virus Concentration Method Based on Ultrafiltration and Wet Foam Elution for Studying Viruses from Large-Volume Water Samples. *Sci. Total Environ.* **2022**, *829*, 154431. [CrossRef]
99. Cashdollar, J.L.; Wymer, L. Methods for Primary Concentration of Viruses from Water Samples: A Review and Meta-Analysis of Recent Studies. *J. Appl. Microbiol.* **2013**, *115*, 1–11. [CrossRef] [PubMed]
100. Chevallereau, A.; Pons, B.J.; van Houte, S.; Westra, E.R. Interactions between Bacterial and Phage Communities in Natural Environments. *Nat. Rev. Microbiol.* **2022**, *20*, 49–62. [CrossRef] [PubMed]
101. Maslov, S.; Sneppen, K. Population Cycles and Species Diversity in Dynamic Kill-the-Winner Model of Microbial Ecosystems. *Sci. Rep.* **2017**, *7*, 39642. [CrossRef]
102. Oechslin, F. Resistance Development to Bacteriophages Occurring during Bacteriophage Therapy. *Viruses* **2018**, *10*, 351. [CrossRef] [PubMed]
103. Koskella, B.; Vos, M. Adaptation in Natural Microbial Populations. *Annu Rev. Ecol. Evol. Syst.* **2015**, *46*, 503–522. [CrossRef]
104. Weitz, J.S.; Wilhelm, S.W. Ocean Viruses and Their Effects on Microbial Communities and Biogeochemical Cycles. *F1000 Biol. Rep.* **2012**, *4*, 2–9. [CrossRef]
105. Clokie, M.R.J.; Millard, A.D.; Letarov, A.V.; Heaphy, S. Phages in Nature. *Bacteriophage* **2011**, *1*, 31–45. [CrossRef]
106. Braga, L.P.P.; Spor, A.; Kot, W.; Breuil, M.C.; Hansen, L.H.; Setubal, J.C.; Philippot, L. Impact of Phages on Soil Bacterial Communities and Nitrogen Availability under Different Assembly Scenarios. *Microbiome* **2020**, *8*, 52. [CrossRef]

107. Roux, S.; Brum, J.R.; Dutilh, B.E.; Sunagawa, S.; Duhaime, M.B.; Loy, A.; Poulos, B.T.; Solonenko, N.; Lara, E.; Poulain, J.; et al. Ecogenomics and Potential Biogeochemical Impacts of Globally Abundant Ocean Viruses. *Nature* **2016**, *537*, 689–693. [CrossRef]
108. Chen, I.; Dubnau, D. DNA Uptake during Bacterial Transformation. *Nat. Rev. Microbiol.* **2004**, *2*, 241–249. [CrossRef]
109. Johnston, C.; Martin, B.; Fichant, G.; Polard, P.; Claverys, J.P. Bacterial Transformation: Distribution, Shared Mechanisms and Divergent Control. *Nat. Rev. Microbiol.* **2014**, *12*, 181–196. [CrossRef] [PubMed]
110. Ye, M.; Zhang, Z.; Sun, M.; Shi, Y. Dynamics, Gene Transfer, and Ecological Function of Intracellular and Extracellular DNA in Environmental Microbiome. *iMeta* **2022**, *1*, e34. [CrossRef]
111. Woegerbauer, M.; Bellanger, X.; Merlin, C. Cell-Free DNA: An Underestimated Source of Antibiotic Resistance Gene Dissemination at the Interface Between Human Activities and Downstream Environments in the Context of Wastewater Reuse. *Front. Microbiol.* **2020**, *11*, 671. [CrossRef] [PubMed]
112. Muniesa, M.; Colomer-Lluch, M.; Jofre, J. Could Bacteriophages Transfer Antibiotic Resistance Genes from Environmental Bacteria to Human-Body Associated Bacterial Populations? *Mob. Genet. Elem.* **2013**, *3*, 739–751. [CrossRef]
113. Colavecchio, A.; Cadieux, B.; Lo, A.; Goodridge, L.D. Bacteriophages Contribute to the Spread of Antibiotic Resistance Genes among Foodborne Pathogens of the Enterobacteriaceae Family—A Review. *Front. Microbiol.* **2017**, *8*, 1108. [CrossRef]
114. Sala-Comorera, L.; Nolan, T.M.; Reynolds, L.J.; Venkatesh, A.; Cheung, L.; Martin, N.A.; Stephens, J.H.; Gitto, A.; O'Hare, G.M.P.; O'Sullivan, J.J.; et al. Bacterial and Bacteriophage Antibiotic Resistance in Marine Bathing Waters in Relation to Rivers and Urban Streams. *Front. Microbiol.* **2021**, *12*, 2017. [CrossRef]
115. Zare, S.; Derakhshandeh, A.; Mohammadi, A.; Noshadi, M. Abundance of Antibiotic Resistance Genes in Bacteria and Bacteriophages Isolated from Wastewater in Shiraz. *Mol. Biol. Res. Commun.* **2021**, *10*, 73–83. [CrossRef]
116. Moon, K.; Jeon, J.H.; Kang, I.; Park, K.S.; Lee, K.; Cha, C.J.; Lee, S.H.; Cho, J.C. Freshwater Viral Metagenome Reveals Novel and Functional Phage-Borne Antibiotic Resistance Genes. *Microbiome* **2020**, *8*, 75. [CrossRef]
117. Sinke, L. Viruses in Soils: What Do We Know and Where Do We Go from Here ? Available online: https://www.labroots.com/webinar/viruses-soils-know-here (accessed on 24 June 2019).
118. Williamson, K.E.; Fuhrmann, J.J.; Wommack, K.E.; Radosevich, M. Viruses in Soil Ecosystems: An Unknown Quantity Within an Unexplored Territory. *Annu. Rev. Virol.* **2017**, *4*, 201–219. [CrossRef]
119. Florent, P.; Cauchie, H.-M.; Herold, M.; Jacquet, S.; Ogorzaly, L. Soil PH, Calcium Content and Bacteria as Major Factors Responsible for the Distribution of the Known Fraction of the DNA Bacteriophage Populations in Soils of Luxembourg. *Microorganisms* **2022**, *10*, 1458. [CrossRef]
120. Børsheim, K.Y. Native Marine Bacteriophages. *FEMS Microbiol. Ecol.* **1993**, *102*, 141–159. [CrossRef]
121. Bergh, O.; Børsheim, K.Y.; Bratbak, G.; Heldal, M. High Abundance of Viruses Found in Aquatic Environments. *Nature* **1989**, *340*, 467–468. [CrossRef] [PubMed]
122. Martínez-Carreras, N.; Wetzel, C.E.; Frentress, J.; Ector, L.; McDonnell, J.J.; Hoffmann, L.; Pfister, L. Hydrological Connectivity Inferred from Diatom Transport through the Riparian-Stream System. *Hydrol. Earth Syst. Sci.* **2015**, *19*, 3133–3151. [CrossRef]
123. Kieft, K.; Zhou, Z.; Anantharaman, K. VIBRANT: Automated Recovery, Annotation and Curation of Microbial Viruses, and Evaluation of Viral Community Function from Genomic Sequences. *Microbiome* **2020**, *8*, 90. [CrossRef] [PubMed]
124. Roux, S.; Enault, F.; Hurwitz, B.L.; Sullivan, M.B. VirSorter: Mining Viral Signal from Microbial Genomic Data. *PeerJ* **2015**, *2015*, e985. [CrossRef]
125. Trubl, G.; Hyman, P.; Roux, S.; Abedon, S.T. Coming-of-Age Characterization of Soil Viruses: A User's Guide to Virus Isolation, Detection within Metagenomes, and Viromics. *Soil Syst.* **2020**, *4*, 23. [CrossRef]
126. Mächler, E.; Salyani, A.; Walser, J.-C.; Larsen, A.; Schaefli, B.; Altermatt, F.; Ceperley, N. Environmental DNA Simultaneously Informs Hydrological and Biodiversity Characterization of an Alpine Catchment. *Hydrol. Earth Syst. Sci.* **2021**, *25*, 735–753. [CrossRef]
127. Peters, J. *Fate and Transport of Synthetic DNA in Surface Water*; University of Waterloo: Waterloo, ON, Canada, 2021.
128. Middelboe, M.; Jacquet, S.; Weinbauer, M. Viruses in Freshwater Ecosystems: An Introduction to the Exploration of Viruses in New Aquatic Habitats. *Freshw. Biol.* **2008**, *53*, 1069–1075. [CrossRef]
129. Hurst, C.J.; Gerba, C.P.; Cech, I. Effects of Environmental Variables and Soil Characteristics on Virus Survival in Soil. *Appl. Environ. Microbiol.* **1980**, *40*, 1067–1079. [CrossRef]
130. Drulis-Kawa, Z.; Majkowska-Skrobek, G.; Maciejewska, B. Bacteriophages and Phage-Derived Proteins—Application Approaches. *Curr. Med. Chem.* **2015**, *22*, 1757. [CrossRef]
131. Wigginton, K.R.; Pecson, B.M.; Sigstam, T.; Bosshard, F.; Kohn, T. Virus Inactivation Mechanisms: Impact of Disinfectants on Virus Function and Structural Integrity. *Environ. Sci. Technol.* **2012**, *46*, 12069–12078. [CrossRef] [PubMed]
132. Bertrand, I.; Schijven, J.F.; Sánchez, G.; Wyn-Jones, P.; Ottoson, J.; Morin, T.; Muscillo, M.; Verani, M.; Nasser, A.; de Roda Husman, A.M.; et al. The Impact of Temperature on the Inactivation of Enteric Viruses in Food and Water: A Review. *J. Appl. Microbiol.* **2012**, *112*, 1059–1074. [CrossRef] [PubMed]
133. Fauvel, B.; Ogorzaly, L.; Cauchie, H.M.; Gantzer, C. Interactions of Infectious F-Specific RNA Bacteriophages with Suspended Matter and Sediment: Towards an Understanding of FRNAPH Distribution in a River Water System. *Sci. Total Environ.* **2017**, *574*, 960–968. [CrossRef]
134. Gitis, V.; Dlugy, C.; Gun, J.; Lev, O. Studies of Inactivation, Retardation and Accumulation of Viruses in Porous Media by a Combination of Dye Labeled and Native Bacteriophage Probes. *J. Contam. Hydrol.* **2011**, *124*, 43–49. [CrossRef] [PubMed]

135. Cheng, L.; Chetochine, A.S.; Pepper, I.L.; Brusseau, M.L. Influence of DOC on MS-2 Bacteriophage Transport in a Sandy Soil. *Water Air Soil Pollut.* **2007**, *178*, 315–322. [CrossRef]
136. Weinbauer, M.G.; Wilhelm, S.W.; Suttle, C.A.; Pledger, R.J.; Mitchell, D.L. Sunlight-Induced DNA Damage and Resistance in Natural Viral Communities. *Aquat. Microb. Ecol.* **1999**, *17*, 111–120. [CrossRef]
137. Beck, S.E.; Rodriguez, R.A.; Hawkins, M.A.; Hargy, T.M.; Larason, T.C.; Linden, K.G. Comparison of UV-Induced Inactivation and RNA Damage in MS2 Phage across the Germicidal UV Spectrum. *Appl. Environ. Microbiol.* **2016**, *82*, 1468. [CrossRef]
138. Kim, S.I.; Jin, S.G.; Pfeifer, G.P. Formation of Cyclobutane Pyrimidine Dimers at Dipyrimidines Containing 5-Hydroxymethylcytosine. *Photochem. Photobiol. Sci.* **2013**, *12*, 1409. [CrossRef]
139. Rochette, P.J.; Therrien, J.P.; Drouin, R.; Perdiz, D.; Bastien, N.; Drobetsky, E.A.; Sage, E. UVA-Induced Cyclobutane Pyrimidine Dimers Form Predominantly at Thymine–Thymine Dipyrimidines and Correlate with the Mutation Spectrum in Rodent Cells. *Nucleic Acids. Res.* **2003**, *31*, 2786. [CrossRef]
140. Silverman, A.I.; Peterson, B.M.; Boehm, A.B.; McNeill, K.; Nelson, K.L. Sunlight Inactivation of Human Viruses and Bacteriophages in Coastal Waters Containing Natural Photosensitizers. *Environ. Sci. Technol.* **2013**, *47*, 1870–1878. [CrossRef]
141. Taj, M.K.; Ling, J.X.; Bing, L.L.; Qi, Z.; Taj, I.; Hassani, T.M.; Samreen, Z.; Yunlin, W. Effect of Dilution, Temperature and PH on the Lysis Activity of T4 Phage against E. Coli BL21. *J. Anim. Plant. Sci.* **2014**, *24*, 1252–1255.
142. Jurczak-Kurek, A.; Gasior, T.; Nejman-Faleńczyk, B.; Bloch, S.; Dydecka, A.; Topka, G.; Necel, A.; Jakubowska-Deredas, M.; Narajczyk, M.; Richert, M.; et al. Biodiversity of Bacteriophages: Morphological and Biological Properties of a Large Group of Phages Isolated from Urban Sewage. *Sci. Rep.* **2016**, *6*, 34338. [CrossRef] [PubMed]
143. Zemb, O.; Manefield, M.; Thomas, F.; Jacquet, S. Phage Adsorption to Bacteria in the Light of the Electrostatics: A Case Study Using E. Coli, T2 and Flow Cytometry. *J. Virol. Methods* **2013**, *189*, 283–289. [CrossRef]
144. Schijven, J.F.; Hassanizadeh, S.M. Removal of Viruses by Soil Passage: Overview of Modeling, Processes, and Parameters. *Crit Rev. Environ. Sci. Technol.* **2000**, *30*, 49–127. [CrossRef]
145. Germann, P.F.; Alaoui, A.; Riesen, D. Drag Force Approach to the Transport of Colloids in Unsaturated Soils. *Water Resour. Res.* **2002**, *38*, 18-1–18-15. [CrossRef]
146. Flynn, R.; Hunkeler, D.; Guerin, C.; Burn, C.; Rossi, P.; Aragno, M. Geochemical Influences on H40/1 Bacteriophage Inactivation in Glaciofluvial Sands. *Environ. Geol.* **2004**, *45*, 504–517. [CrossRef]
147. Kvitsand, H.M.L.; Ilyas, A.; Østerhus, S.W. Rapid Bacteriophage MS2 Transport in an Oxic Sandy Aquifer in Cold Climate: Field Experiments and Modeling. *Water Resour. Res.* **2015**, *51*, 9725–9745. [CrossRef]
148. Flynn, R.M.; Mallèn, G.; Engel, M.; Ahmed, A.; Rossi, P. Characterizing Aquifer Heterogeneity Using Bacterial and Bacteriophage Tracers. *J. Environ. Qual.* **2015**, *44*, 1448–1458. [CrossRef]
149. Chattopadhyay, S.; Puls, R.W. Forces Dictating Colloidal Interactions between Viruses and Soil. *Chemosphere* **2000**, *41*, 1279–1286. [CrossRef]
150. Sautrey, G.; Brié, A.; Gantzer, C.; Walcarius, A. MS2 and Qβ Bacteriophages Reveal the Contribution of Surface Hydrophobicity on the Mobility of Non-Enveloped Icosahedral Viruses in SDS-Based Capillary Zone Electrophoresis. *Electrophoresis* **2018**, *39*, 377–385. [CrossRef] [PubMed]
151. Schijven, J.F.; Hoogenboezem, W.; Hassanizadeh, S.M.; Peters, J.H. Modeling Removal of Bacteriophages MS2 and PRD1 by Dune Recharge at Castricum, Netherlands. *Water Resour. Res.* **1999**, *35*, 1101–1111. [CrossRef]
152. Bales, R.C.; Li, S.; Maguire, K.M.; Yahya, M.T.; Gerba, C.P.; Harvey, R.W. Virus and Bacteria Transport in a Sandy Aquifer, Cape Cod, MA. *Groundwater* **1995**, *33*, 653–661. [CrossRef]
153. Flynn, R.; Cornaton, F.; Hunkeler, D.; Rossi, P. Bacteriophage Transport through a Fining-Upwards Sedimentary Sequence: Laboratory Experiments and Simulation. *J. Contam. Hydrol.* **2004**, *74*, 231–252. [CrossRef] [PubMed]
154. Sinton, L.W.; Noonan, M.J.; Finlay, R.K.; Pang, L.; Close, M.E. Transport and Attenuation of Bacteria and Bacteriophages in an Alluvial Gravel Aquifer. *N. Z. J. Mar. Freshw. Res.* **2000**, *34*, 175–186. [CrossRef]
155. Ghanem, N.; Trost, M.; Sánchez Fontanet, L.; Harms, H.; Chatzinotas, A.; Wick, L.Y. Changes of the Specific Infectivity of Tracer Phages during Transport in Porous Media. *Environ. Sci. Technol.* **2018**, *52*, 3486–3492. [CrossRef] [PubMed]
156. Lisovski, S.; Ramenofsky, M.; Wingfield, J.C. Defining the Degree of Seasonality and Its Significance for Future Research. *Integr. Comp. Biol.* **2017**, *57*, 934–942. [CrossRef]
157. Kwiecien, O.; Braun, T.; Brunello, C.F.; Faulkner, P.; Hausmann, N.; Helle, G.; Hoggarth, J.A.; Ionita, M.; Jazwa, C.S.; Kelmelis, S.; et al. What We Talk about When We Talk about Seasonality—A Transdisciplinary Review. *Earth Sci. Rev.* **2022**, *225*, 103843. [CrossRef]
158. Lembrechts, J.J.; van den Hoogen, J.; Aalto, J.; Ashcroft, M.B.; De Frenne, P.; Kemppinen, J.; Kopecký, M.; Luoto, M.; Maclean, I.M.D.; Crowther, T.W.; et al. Global Maps of Soil Temperature. *Glob. Chang. Biol.* **2022**, *28*, 3110–3144. [CrossRef]
159. Zhan, M.J.; Xia, L.; Zhan, L.; Wang, Y. Recognition of Changes in Air and Soil Temperatures at a Station Typical of China's Subtropical Monsoon Region (1961–2018). *Adv. Meteorol.* **2019**, *9*, 6927045. [CrossRef]
160. Tiwari, G.N. Madhuri Effect of Water Depth on Daily Yield of the Still. *Desalination* **1987**, *61*, 67–75. [CrossRef]
161. Caldarescu, D.E.; Brey, T.; Abele, D.; Beierlein, L.; Lohmann, G.; Ionita, M. The Influence of Depth-Dependent Seasonal Temperature Variability on Growth Signal in Arctica Islandica. *Front. Mar. Sci.* **2021**, *8*, 823. [CrossRef]
162. Caldentey, J.; Bamford, J.K.H.; Bamford, D.H. Structure and Assembly of Bacteriophage PRD1, an Escherichia Coli Virus with a Membrane. *J. Struct. Biol.* **1990**, *104*, 44–51. [CrossRef] [PubMed]

163. Kinoshita, T.; Bales, R.C.; Maguire, K.M.; Gerba, C.P. Effect of PH on Bacteriophage Transport through Sandy Soils. *J. Contam. Hydrol.* **1993**, *14*, 55–70. [CrossRef]
164. Schijven, J.F.; Hassanizadeh, S.M.; De Bruin, R.H.A.M. Two-Site Kinetic Modeling of Bacteriophages Transport through Columns of Saturated Dune Sand. *J. Contam. Hydrol.* **2002**, *57*, 259–279. [CrossRef] [PubMed]
165. Michen, B.; Graule, T. Isoelectric Points of Viruses. *J. Appl. Microbiol.* **2010**, *109*, 388–397. [CrossRef]
166. Rossi, P.; Käss, W. Phagen. In *Geohydrologische Markierungstechnik*; Gebruüder Borntraeger: Berlin, Stuttgart, 1998; pp. 209–232.
167. Hijnen, W.A.M.; Brouwer-Hanzens, A.J.; Charles, K.J.; Medema, G.J. Transport of MS2 Phage, Escherichia Coli, Clostridium Perfringens, Cryptosporidium Parvum and Giardia Intestinalis in a Gravel and a Sandy Soil. *Environ. Sci. Technol.* **2005**, *39*, 7860–7868. [CrossRef]
168. Cao, H.; Tsai, F.T.C.; Rusch, K.A. Salinity and Soluble Organic Matter on Virus Sorption in Sand and Soil Columns. *Ground Water* **2010**, *48*, 42–52. [CrossRef]
169. Chu, Y.; Jin, Y.; Flury, M.; Yates, M.V. Mechanisms of Virus Removal during Transport in Unsaturated Porous Media. *Water Resour. Res.* **2001**, *37*, 253–263. [CrossRef]
170. Chu, Y.; Jin, Y.; Baumann, T.; Yates, M.V. Effect of Soil Properties on Saturated and Unsaturated Virus Transport through Columns. *Adsorption* **2003**, *32*, 2017–2025. [CrossRef]
171. Bitton, G. Adsorption of Viruses Onto Surfaces Soil and Water. *Water Res.* **1975**, *9*, 473–484. [CrossRef]
172. Bitton, G.; Mitchell, R. Effect of Colloids on the Survival of Bacteriophages in Seawater. *Water Res.* **1974**, *8*, 227–229. [CrossRef]
173. Babich, H.; Stotzky, G. Reductions in Inactivation Rates of Bacteriophages by Clay Minerals in Lake Water. *Water Res.* **1980**, *14*, 185–187. [CrossRef]
174. Auckenthaler, A.; Raso, G.; Huggenberger, P. Particle Transport in a Karst Aquifer: Natural and Artificial Tracer Experiments with Bacteria, Bacteriophages and Microspheres. *Water Sci. Technol.* **2002**, *46*, 131–138. [CrossRef] [PubMed]

This review provided an overview of the application of the bacteriophages in hydrological processes as tracers, and the results obtained among the various experiment. Despite their numerous benefits, their limitations were specifically raised, but above all solutions were proposed to circumvent these issues and thus optimise their use as hydrological tracers. The main points of this review concerning bacteriophages as artificial tracers are important to consider in the framework of this thesis. Indeed, beyond finding solutions, the review will be guiding us in the selection of new hydrological tracers. The PhD project has been built around the potential pollution that the injection of exogenous bacteriophages could cause to the environments studied. Indeed, the soils are tremendously diverse in bacteriophages and therefore these populations could be imagined to be used as environmental tracers. Additional points to investigate prior to conducting any tracing experiments were addressed in the review, including the complementarity of several detection methods and the consideration of the characteristics of the catchment of interest, which are very important to implement also in the process of finding a new hydrological tracer. To conclude, this review not only helps to improve the application of bacteriophages as artificial tracers but also opens doors to new perspectives using bacteriophages as environmental tracers too.

Chapter 2:

OBJECTIVES AND METHODOLOGIES

Chapter 2:

OBJECTIVES AND METHODOLOGIES

2.1 Research aims and objectives

Before the advent of hydrological tracers, early studies of water sources and flow paths relied primarily on hydrometric measurements (e.g., soil moisture content, groundwater levels, discharge, precipitations) (McDonnell et al., 1999; Tetzlaff et al., 2015). Since the first tracing experiment in the 1860s, our understanding of the basic hydrological functions of catchments has been greatly improved using isotopes and geochemical tracers. A wide variety of hydrological tracers, the most common being fluorescent dyes and hydrogen/oxygen isotopes, have been used to track water flow paths and identify their sources. In the 1960s, Finnish scientists Kari Kinnunen and Seppo Niemelä introduced bacteriophages as hydrological tracers to study the water path from a sewage treatment plant after its discharge into a river. Since then, bacteriophages have won over hydrologists mainly because of their detection sensitivity, their specificity for their respective bacterial hosts and most importantly, their harmlessness to humans and ecosystems. However, this safety for ecosystems can be questioned today. The setup of tracing experiments requires the injection of high volume and concentration of bacteriophages specifically selected not to be naturally present in the environmental compartments of interest. Not only can the injected bacteriophages interact with and disrupt the presence of naturally occurring bacteria in the environment of interest, but they can also remain in the surrounding environment for a period of up to weeks or months, either as intact bacteriophages or as free genetic materials. To date, the consequences of the injection of bacteriophages and the presence of their remains in the environment are still poorly understood. Within an era where the protection of the environment is central, it is crucial to consider the potential impact of injecting artificial tracers like bacteriophages. Since 2009, some countries have elaborated regulations and guidelines for using artificial tracers by implementing protection surveys or permits (Environment Agency, 2021; Wolkersdorfer and LeBlanc, 2012). Using viral particles as artificial tracers presented thus a major limitation to ecological awareness and led us to investigate and explore the potential of the naturally occurring bacteriophages in the soil.

This thesis project proposes the innovative aspect of the meeting between the scientific fields of hydrology and virology, by introducing bacteriophages into the toolbox of hydrological tracers. The pedosphere harbours an incredible diversity of bacteriophages, which allows considering the naturally occurring populations in soils to be used as tracers. Although the use of bacteriophages as hydrological tracers is not a novel concept, using the natural population of bacteriophage present in soils has never been done before. This would open new sustainable perspectives for the exploration of hydrological systems while providing new knowledge on bacteriophage communities in soils. Despite the large number of bacteriophages encountered in the terrestrial compartment, knowledge of their fate and survival remains poorly studied.

In this context, **the objective of this proof-of-concept study is to assess the potential of natural soil bacteriophages to serve as environmental tracers of hydrological processes, with the ambition of identifying the bacteriophage communities and proposing relevant and sustainable environmental tracer candidates**. To achieve the goal, several scientific questions, gaps and methodological challenges need to be addressed. Firstly, the natural populations of bacteriophages in soils need to be characterised, and the role of biotic and abiotic factors on the occurrence of the identified bacteriophages living in soils needs to be understood. In this way, not only can some bacteriophages be selected as tracers, but knowledge of their interactions with the surrounding ecosystems can be improved, making it possible to control their persistence in the environment during tracing experiments. Secondly, the ability of natural bacteriophages to be transported remains unknown and needs to be investigated to ensure their reliability as hydrological tracers. For this purpose, the identification of bacteriophages capable of being mobilised from the soil matrix is necessary, as well as their potential detection in neighbouring compartments. Thus, two complementary work packages (WPs) formed the backbone of this PhD project (Figure 3). The first WP aims to study DNA-bacteriophage communities in several types of soils in Luxembourg (WP1), while the second WP focuses on the mobilisation of DNA-bacteriophages from the soil to the soil water, potentially triggered by precipitations (WP2). The spatial and temporal aspects of the first and second WPs will provide the most complete picture possible of the bacteriophage dynamics in the terrestrial compartment, with the elements available at present. Moreover, the complementary results obtained by these two WPs would afterwards guide the selection of certain bacteriophages as potential candidates for hydrological tracers

based on the criteria that define an ideal tracer and predict at best their fate according to their relationship with environmental properties and climatic conditions.

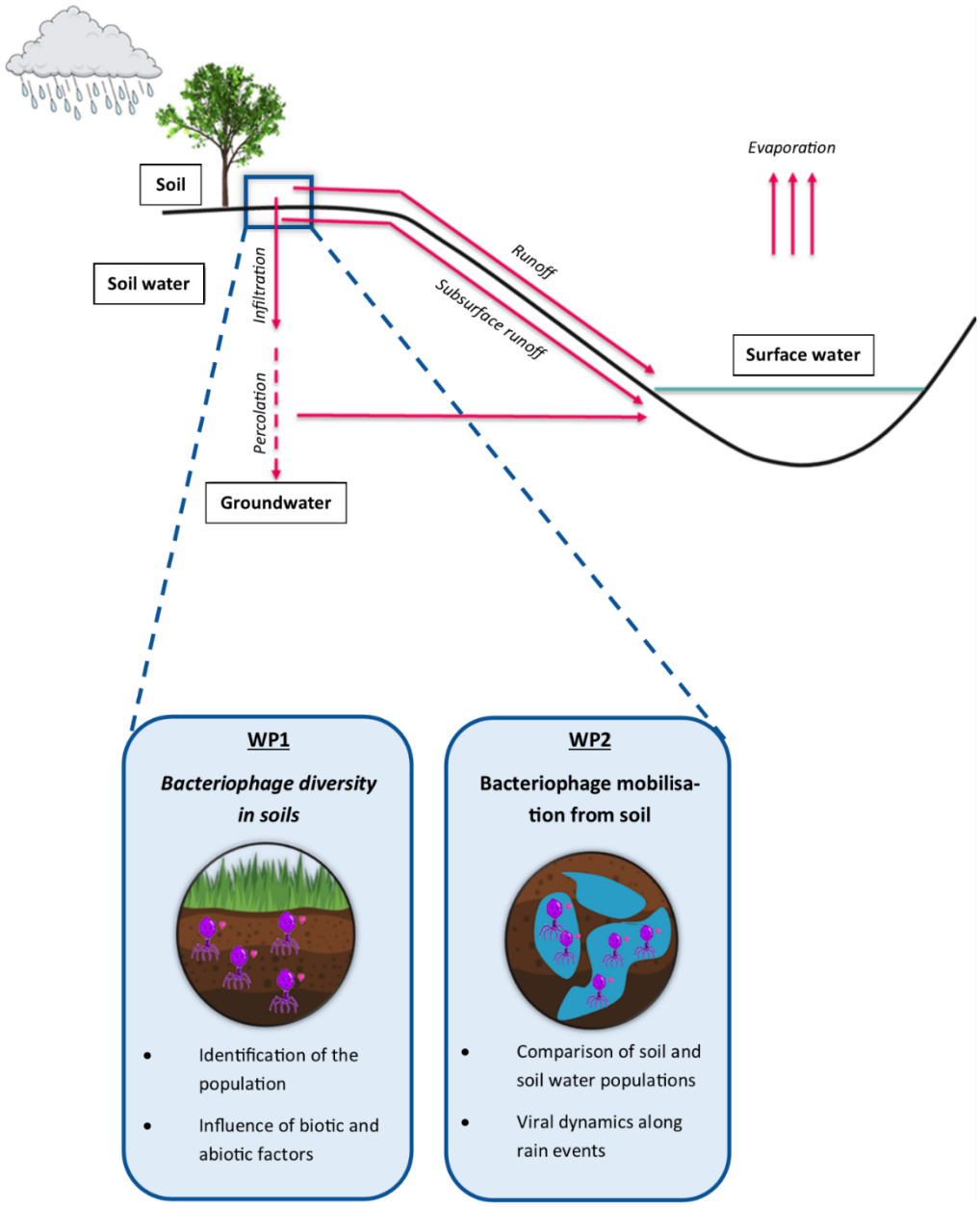


Figure 3: General scheme of the research aim and objectives of the PhD project.

2.2 Methodology

In the frame of identifying the naturally occurring populations of bacteriophages in soil, an innovative workflow has been designed (Figure 4). This section will therefore be devoted to explaining the methodological strategies employed to achieve the objectives of the PhD project, as well as the methodologies associated with them. Firstly, a detailed description of

the catchment area will be provided regarding its geology, soil characteristics and hydrology to help understand why this catchment was suitable for this project. Secondly, the entire methodology workflow designed for characterising and identifying bacteriophage communities will be described. It should be noted that the methodological and technical aspects extensively described and explained in scientific articles present in the result sections of this manuscript will only be mentioned hereafter.

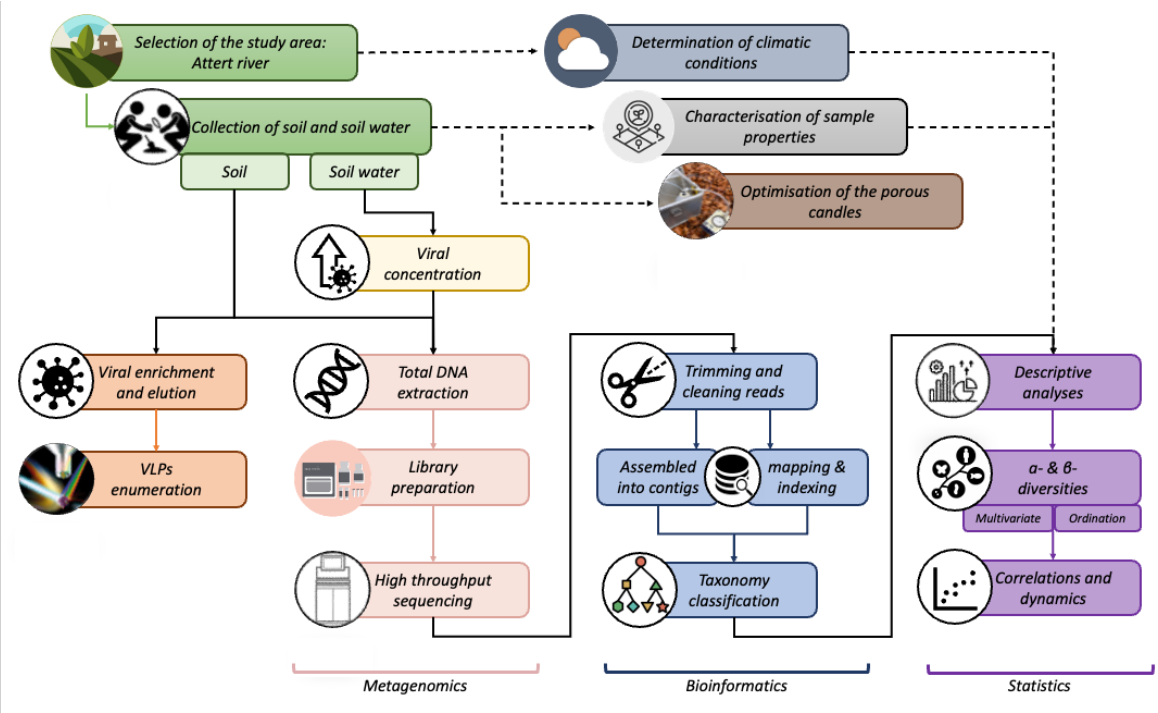


Figure 4: Workflow of the methodology designed during the PhD project to identify naturally occurring bacteriophage populations.

2.2.1 Studied areas

The research project was carried out in the Attert River basin, located northwest of Luxembourg. This small basin (310 km^2) is of great scientific interest as it presents a diversity not only in soil and subsoil types but also in land use. In addition, the basin lies on the two geological regions of Luxembourg, the Oesling and the Gutland, thus giving a good representation of Luxembourg's soils at a smaller scale. To achieve the objectives of the WP1, eight different sites were selected within this basin, including three forest sites: Weierbach, Retgenbusch and Daerent, as well as five grassland sites: Hueschterbach, Mollbach, Koulbich, Pall1 and Pall 2 (Figure 5). All the sites are located on a tributary of the Attert river. The selection of the sites was made according to their proximity to a

watercourse but, above all, to cover the great diversity of the basin by choosing sites with different types of vegetation, diverse soil textures and located in the two geological regions.

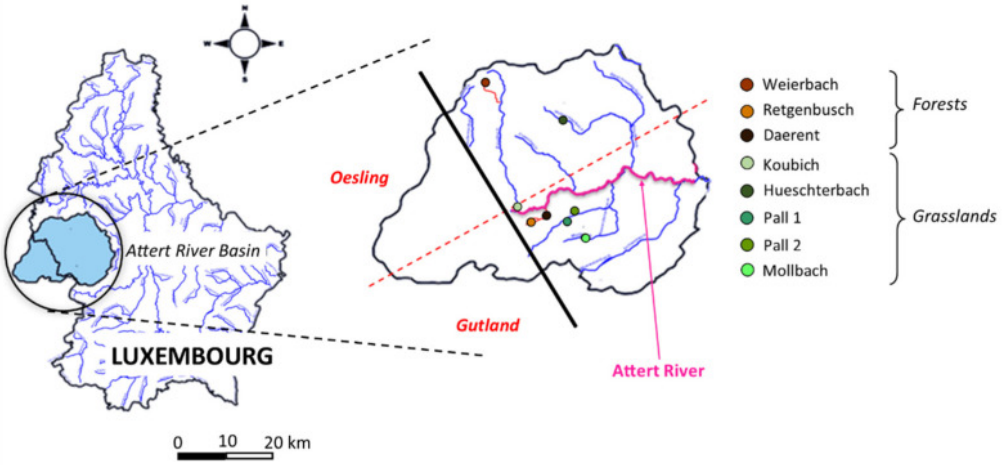


Figure 5: Map of the study site, the Attert River basin, with the location of the eight selected sampling sites (modified from Geoportail.lu). The plain black line represents roughly the border between the Luxembourg and Belgium sides of the basin, and the dashed red line is the limit between the two Luxembourg regions: Oesling and Gutland.

Regarding the WP2, the focus was solely made on the Weierbach site, a catchment area well-studied by the LIST hydrology team (Antonelli et al., 2016; Hissler et al., 2021; Martínez-Carreras et al., 2015), for time-scale experiments where bacteriophage dynamics will be studied over time during precipitation events. This site is well-equipped so that subsoil properties and local climatic conditions can be easily obtained. Additionally, the Weierbach has been well studied at LIST which has given us access to a large amount of existing data. Further details regarding the Attert river basin can be found in the "Material and Method" section of the articles in Chapter 3.

2.2.2 Sample collection and selected data

For the WP1, a field campaign was conducted in September 2019 to collect soil samples from all the above-mentioned sites. To do so, soil samples were collected *in situ* at 20 cm depth using augers on a delimited surface (25 m^2). Five to seven replicates per site were carried out and pooled together to remove any local spatial variations. Regarding the WP2,

two campaigns were conducted, the first in December 2020 and the second in March 2021, for 1 month each to collect soil and soil water samples while targeting precipitation events. The soil was collected similarly as during the campaign of September 2019, and the soil water was collected along the first 20 cm of the soil using porous candles. For the porous candles, laboratory experiments were necessary and thus performed before their installation since little was known about the passage of viruses through the pores of the device. The results successfully confirmed the device's efficiency and are described in Section 3.2.1. Finally, all soil samples were subjected to soil characterisation, carried out by the Administration des Services Techniques de l'Agriculture (ASTA) of Luxembourg, to determine the physicochemical properties of the soils, such as pH, organic matter, nitrogen content, granulometry, cation exchange capacity and nutrient contents. In addition, climatic data (I,e, precipitations, air and soil temperatures) and soil moisture were obtained, when available, from the hydro-climatological observation and sensing technology team at LIST. Besides, the pH of the soil water was measured after each sampling, and the antecedent precipitation indices (APIs) were calculated from 1 day to 5 days to analyses. All the physicochemical properties and climactic data were considered so that their influence on the populations of bacteriophages could be investigated. Supplementary details regarding the exact methods used to determine all physicochemical properties and sources for all climatic data can be found in the "Material and Method" sections of the articles in Chapter 3.

2.2.3 Viral identification and diversity analyses

Among the large diversity of bacteriophages, the majority have a DNA genome. Of more than thirty bacteriophage families, only six have an RNA genome, four of which belong to the order *Norzivirales* and two to the order *Timlovirales*. These two orders were discovered by metagenomics and recently added to the viral taxonomy. Therefore, despite the numerous studies conducted on the members of the family *Fiersviridae* (e.g. MS2, *Norzivirales*), not only is little known about the new RNA-bacteriophages but they also represent a minority of the viruses available in the viral database to date. For these reasons, viral identification and diversity analyses were carried out with a focus on DNA-bacteriophages.

In the context of identifying the natural DNA-bacteriophage population from the soil, the first step was to collect viruses from the soil matrix. For this purpose, a viral enrichment and elution protocol were optimised using eluent buffers and ultracentrifugation. From the

concentrated solutions, the enumeration of virus-like particles (or VLPs) using flow cytometry (FCM) was performed to determine the concentrations of VLPs in the soils of each site from the WP1. More information about the exact protocol and results can be found in Chapter 3.1.2.

The aquatic compartment contains a lower concentration of viruses than soil (from 10^3 to 10^7 viral particles per millilitre, depending on the aquatic compartment of interest). Therefore, a viral concentration step was performed using ultrafiltration on the soil water samples to improve the DNA yield as much as possible and thus ensure successful results for subsequent analyses.

Since the PhD project focuses on using the natural populations of DNA-bacteriophages instead of injecting laboratory strains, culture-based techniques or microscopy methods are unsuitable for population exploration from complex ecosystems, while metagenomics analysis is. Therefore, total DNA extraction was first conducted on the bulk soils from the collected samples of WP1 and WP2, as well as on the soil water concentrates. Then, library preparation and high throughput sequencing were carried out on the DNA extracts to obtain the metagenomes. All information regarding the devices and kit references can be found in the sections in the Chapter "Results" of this manuscript. To get the taxonomy identification of the metagenomes, bioinformatics analyses are required by first trimming and cleaning the raw reads, then assembling them into contigs and mapping them against databases. The contigs and the mapped reads are then merged to obtain the taxonomy classification for each identified microbial species (Figure 6, pipeline 3). An optimisation of this pipeline was performed and is explained in detail in the section below. Additionally, all tool versions used in the pipeline, as well as the specific parameters employed, are described in the Chapter "Results" of this manuscript.

The entire pipeline was used to identify total microbial populations, from which bacteria and viruses were extracted for further analyses. Finally, relative abundances of viruses and bacteria were calculated from the bioinformatics results and a description of the viral population, including bacteriophages, was explored at several taxonomical levels. Then, alpha and beta diversities were determined using multivariate data and ordination analyses (RDA, CoCA, PERMANOVA). Ultimately, these statistical analyses allowed the exploration of the relationship between bacteriophage populations and the physicochemical

properties of soils and the associated bacterial populations. Furthermore, correlations and linear regressions were performed to analyse the temporal dynamics of some bacteriophages of interest.

2.2.4 Optimisation of the bioinformatics pipeline

The above-mentioned bioinformatics analysis was obtained after optimisation testing of three different pipelines (Figure 6). For all pipelines, the quality of the raw sequencing reads was checked, and Illumina adapters were removed using Trimmomatic v0.39. From the quality-filtered reads, the first pipeline consisted of taxonomically classifying the reads and estimating the total microbial abundance using Kraken 2 and Bracken, respectively. Then, the microbial population results were visualised via a sunburst chart, using Krona. The second pipeline involved the assembly of the quality-filtered reads into contigs performed using Spades, followed by a taxonomic classification of the assembled contigs against the complete non-redundant (nr) microbial protein sequence database (released version of 2019) from the national centre for biotechnology information (NCBI) with Blastx and the DIAMOND sequence aligner. For both pipelines, a robustness check was performed using two different publicly available datasets from the NCBI database. These datasets were chosen based on the similarity of the samples (i.e., environmental samples) and the pre-bioinformatic analyses (i.e., DNA extraction, library, and sequencing), and then a comparison of the proportions of total microbial reads identified was performed. However, it was noticed that the second pipeline gives presence/absence results since it does not allow determining the abundance for each identified species due to the lack of a step. This was then overcome by the implementation of the third and final pipeline. As with the previous pipeline, contig assemblies were made separately for each sample using SPAdes v3.15.4. The raw reads were mapped back to the contigs with minimap2 v2.24, sorted and indexed using SAMtool v1.15.1. Finally, the taxonomic classification of the mapped contigs was carried out against the complete non-redundant (nr) microbial protein sequence database (released version of April 2022) from NCBI using Mmseqs2 v13.45111.

This pipeline only includes non-virus-specific bioinformatics tools allowing us to obtain the full microbial populations (i.e. bacterial, fungi, mammals). Workflows to specifically detect viruses, like iVirus (Bolduc et al., 2017) or FastViromeExplorer (Tithi et al., 2017), as well as virus-specific tools such as VirSorter (Roux et al., 2015a), VirFinder (Ren et al., 2017),

Vibrant (Kieft et al., 2020) are nowadays well developed. All the above-mentioned workflows and tools were tested using several databases (nr, viral RefSeq and IMG/VR), however, none of them provided successful results with very few viruses detected lower than at the family level.

In addition, it is important to note that the selected pipeline is reference-based, and the use of the database-independent pipeline using the binning process to assign the unknown fraction of reads was intentionally not retained. The complementarity of the two techniques would provide a complete picture of the viral community present in the terrestrial compartment. However, as the aim of the thesis was to select bacteriophage candidates and use them as tracers, it was essential to focus on the known part of the population.

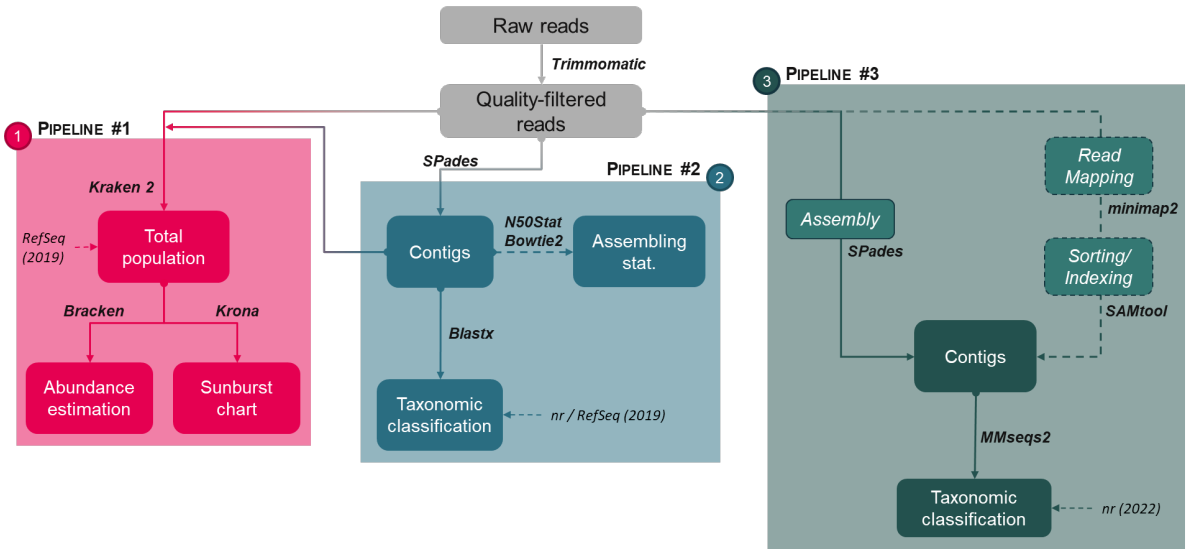


Figure 6: Workflows for the optimisation of the bioinformatics pipeline to obtain the microbial taxonomic classification.

Chapter 3:

RESULTS

Chapter 3:

RESULTS

3.1 WP1: Bacteriophage diversity in the soils of Luxembourg

As described in the previous chapter, two complementary work packages (WPs) were built to achieve the objective of this PhD work. The WP1 aimed at investigating DNA-bacteriophage communities in several types of soils in Luxembourg and exploring the potential influence of some abiotic and biotic factors on their distribution. The hypotheses presented for this WP1 were:

1. Abiotic factors (i.e., pH, cation content and so on), well known to affect bacteriophage survival, may also have an impact on their diversity and distribution in terrestrial environments
2. The bacterial community present in the soil may influence the relationship between bacteriophages and soil properties

To best answer the research questions of this WP1, optimisation methods for the recovery of viruses from soil samples and the improvement of DNA yield and pre-sequencing protocols had to be carried out before getting into the heart of the abundance analyses and virus population identification.

Thus, in this section, the steps leading to a final virus collection and elution protocol followed by those leading to the selection of kits for DNA extraction and for library preparation will be described and discussed. Next, the description of DNA-bacteriophage communities in Luxembourg soils will be presented as well as the relationship between these viral communities and certain biotic and abiotic factors. The latter results will be presented in the format of a scientific article, as this one has been published. Each part will be accompanied by introductory and concluding paragraphs which explain in detail the context of each part.

3.1.1 Optimisation methodology 1: Elution and concentration of viruses and improvement of DNA yield and library construction from soil samples

The study of soil viruses generally involves the collection of viruses from the soil itself before any analysis. As described earlier in this manuscript, several studies have shown the high adsorption potential of bacteriophages on soil particles and the influence of certain parameters on this adsorption process (e.g. pH, virus pI, soil type). This adsorption can thus complicate the application of subsequent analyses, such as enumeration or identification since the adsorbed viruses will then be recovered very little or not at all. However, this adsorption process is known to be reversible, giving the viruses the ability to detach from the soil matrix under certain chemical and mechanical conditions. In this way, by playing on these conditions in the laboratory, which in this case are the pH, the elution solutions and the mechanical forces, we can improve the virus collection and elution from soil samples. To complete this optimisation protocol, a concentration step is then carried out using ultrafiltration with different cut-off points, allowing better results to be obtained when quantifying and identifying the recovered viruses.

After the recovery of the viruses from the soil, the extraction of their DNA and the preparation of the libraries were carried out to allow the implementation of the sequencing methods necessary to identify the viral populations. Here again, the optimisation of these two methods was applied by testing, firstly, different types of DNA extraction kits to obtain the best final DNA yield, and secondly, different types of library preparation kits, for better sequencing performance.

It should be noted that among the many papers using these methods of elution and concentration as well as pre-sequencing analyses, several variations of these methods have been conducted without using a standard protocol applicable to soil samples. Thus, optimisation of these protocols was necessary and was based on already existing protocols. Finally, this work was published as **supplementary material** in the following scientific article: "Florent, P.; Cauchie, H.-M.; Herold, M.; Jacquet, S.; Ogorzaly, L. Soil pH, Calcium Content and Bacteria as Major Factors Responsible for the Distribution of the Known Fraction of the DNA Bacteriophage Populations in Soils of Luxembourg. Microorganisms 2022, 10, 1458. https://doi.org/10.3390/microorganisms10071458".

microorganisms

MDPI

Supplementary protocol

Section S1: Viral elution and concentration protocol

Materials and method

To optimise and validate our isolation method of viruses from other possible soil fractions (e.g. bacteria, eukaryotes, dust, soil particles…), an elution and concentration protocol was optimised on the soil of the Weierbach site, using bacteriophage ΦX174 (*Microviridae*) as surrogate [97–104]. Feed solutions of ΦX174 were produced with quantities comprised between 1.45 and 2.35 x 10^9 PFU (Plaque-Forming Unit) and spiked into the tested soil samples. The experiment was conducted in triplicate for each soil. The optimisation included the experimentation of three different parts of the protocol: mechanical detachment, chemical detachment, cell debris cleaning and concentration process. Regarding the detachment of the viral particles from the soil matrices, a combination of mechanical and chemical actions was assessed to increase the viral recovery rate. The mechanical detachment was explored through three options: (i) sonication during 3 minutes at 4°C (Sonicator S300 Elmasonic, 37 kHz, 300 W), (ii) bead-beating for 3 minutes at room temperature, 15 Hz (100-240V/60Hz, Retsch, Germany), and (iii) the absence of any mechanical process. In parallel, a chemical detachment was carried out investigating six eluent solutions, selected according to the literature [97,99,101]: (i) beef extract 3% mixed with glycine 0.05M (BG) at pH = 9.5, (ii) beef extract 10% (B10%) at pH = 9, (iii) glycine 0.25M (Gly) at pH = 8, (iv) potassium citrate (PC) at pH = 7, (v) sodium pyrophosphate (SP) at pH = 7, (vi) phosphate-buffered saline (PBS) at pH = 7. Regarding the viral concentration, an ultracentrifugal filtration was performed using an Amicon Ultra-15 (UFC905024, Merck Millipore Ltd.) with two tested cut-off points (30 kD and 50 kD). The feed solutions of ΦX174, as well as the recovered solutions, at every step were enumerated using the double agar layer assay according to the standard procedures ISO 10705-2:2001, and the Log Reduction Value (LRV) was estimated for each experimental condition as follow:

$$LRV = \log\left(\frac{Qr}{Qf}\right)$$

where Q_f and Q_r are the quantities of ΦX174 in the feed and recovered solutions, respectively. The protocol was then applied to the seven remaining sites for which the LRV was calculated as described above in the validation process of the optimisation.

Results

Viral mechanical detachment

The results regarding the optimisation of the viral mechanical detachment step are reported in Table S3. The sonication as well as the bead-beater methods revealed a loss in ΦX174 quantity of 0.7 log while the absence of these two methods in the protocol induced a loss of 0.8 log. According to the non-parametric test Kruskal-Wallis One Way ANOVA on rank, no significant difference was detected between all three methods ($p = 0.07$, $\alpha = 0.05$). As the two mechanical methods did not significantly allow improving the recovery of ΦX174 particles during the viral detachment, none of them was kept. In addition, the sonication method is known to be non-homogeneous in the spread of the sonic waves.

Table S3: Recovery of bacteriophage ΦX174 comparing three methods for mechanical viral detachment from the Weierbach soil. A Kruskal-Wallis one-way ANOVA on ranks, followed by a Dunn test, was performed on the results of the three methods to analyse their effectiveness on the elution of ΦX174 compared to the spiking solution.

Solutions	ΦX174 quantity Mean ± sd (PFU)	LRV Mean ± sd	p-value
Spiking	$2.05 \times 10^9 \pm 5.5 \times 10^8$	-	-
Sonication	$4.41 \times 10^8 \pm 1.62 \times 10^8$	- 0.70 ± 0.19	NS
Bead-beater	$4.16 \times 10^8 \pm 5.59 \times 10^7$	- 0.70 ± 0.06	NS
Nothing	$3.26 \times 10^8 \pm 4.89 \times 10^7$	- 0.80 ± 0.07	NS

LRV = Log Reduction Value; NS = Not significant

Viral chemical detachment

The second optimised method was viral chemical detachment for which the results are reported in Table S4. Overall, a reduction in ΦX174 quantity of more than 1 log from the spiking was observed for glycine, PBS, PC and SP solutions. In contrary, BG and B10%, showed a reduction below 1 log, with a better recovery for the mix beef extract and glycine than for the beef extract alone (LRV = -0.47 log vs. LRV = -0.72 log, respectively). A non-parametric test (Kruskal-Wallis one way ANOVA on rank) confirmed significant differences ($p = 0.01082$, $\alpha = 0.05$) between the different eluent solutions. More precisely, a significant difference was noticed for PBS, PC, SP, and Gly compared to the feed solution (p-value = 0.0057, 0.0211, 0.0350 and 0.0005, respectively). In contrary, B10% and BG did not show significant reduction in ΦX174 quantity compared to the feed solution (p-value = 0.2357 and 0.5532, respectively). In addition to the 1.5-fold decrease in the viral recovery of BG compared to B10%, carrying out the molecular technique after using BG is preferred from B10% since the amount of beef extract in much lower in the BG (3%) than in the B10% (10%). Indeed, the beef extract present in the eluent solution can bring DNA contamination during the metagenomics analyses. The eluent solution beef extract (3%) and glycine (0.05M) was thus selected.

Table S4: Recovery of bacteriophage ΦX174 from the Weierbach soil comparing six different eluent solutions. B10% = Beef extract 10% (pH = 9); BG = Beef extract 3% + Glycine 0.05M (pH = 9.5); Gly = Glycine 0.25M (pH = 8); PBS = Phosphate-buffered saline (pH = 7); PC = Potassium citrate (pH = 7); SP = Sodium pyrophosphate (pH = 7). A Kruskal-Wallis one-way ANOVA on ranks, followed by a Dunn test, was performed on the results of each eluent solution to analyse their impact on the recovery of ΦX174 compared to the spiking solution.

Solutions	ΦX174 quantity Mean ± sd (PFU	LRV Mean ± sd	p-value
Spiking	$2.35 \times 10^9 \pm 2.12 \times 10^8$	-	-
B10%	$4.51 \times 10^8 \pm 1.03 \times 10^8$	- 0.72 ± 0.11	0.2357
BG	$7.97 \times 10^8 \pm 1.00 \times 10^8$	- 0.47 ± 0.06	0.5532
GLY	$5.34 \times 10^7 \pm 2.32 \times 10^7$	- 1.67 ± 0.19	0.0005***
PBS	$9.43 \times 10^7 \pm 2.73 \times 10^7$	- 1.41 ± 0.12	0.0057**
PC	$1.16 \times 10^8 \pm 1.43 \times 10^7$	- 1.31 ± 0.05	0.0211*
SP	$1.23 \times 10^8 \pm 1.80 \times 10^7$	- 1.28 ± 0.06	0.0350*

Significance: ***p<0.001; ** p<0.01; * p<0.05. sd = standard deviation; LRV = Log Reduction Value; PFU = Plaque forming unit.

Concentration of viral particles

The average quantities of ΦX174 recovered after concentration were quantified for both concentrates and eluate (Amicons 50 kDa & 30 kDa) and are reported in Table S5.

After centrifugation, a reduction of 0.47 log was observed. After concentration by ultrafiltration, the log reductions of both Amicons 50 kDa & 30 kDa were similar with -0.89 log and -0.81 log, respectively and no significant difference with the feed solutions (p-value = 1.000 and 0.955, respectively). In contrast, the eluates showed different patterns between the two tested Amicons. The quantification of ΦX174 in the eluate allowed defining the number of lost bacteriophages. The eluate of the Amicon 50kDa was revealed to have a significant difference (p = 0.006) with a reduction of 8.3 log from the feed solution. However, no significant loss was observed in the eluate of the Amicon 30 kDa (LRV = -4.79 log, p-value = 0.055). Indeed, the quantity of bacteriophages in the eluate of the Amicon 30 kDa was found 4-log higher than in the Amicon 50 kDa, meaning that a fraction of ΦX174 was not properly recovered in the concentrate of the former.

Table S5: Recovery of bacteriophage ΦX174 from the Weierbach soil after concentration step, comparing Amicon with two different cut-off points (i.e., 30 kDa and 50 kDa). A Kruskal-Wallis one-way ANOVA on ranks, followed by a Dunn test, was performed on the results of each eluent solution to analyse their impact on the recovery of ΦX174 compared to the feed solution.

Solutions	ΦX174 quantity Mean ± sd (PFU)	LRV Mean ± sd	p-value
Spiking	$2.35 \times 10^9 \pm 2.12 \times 10^8$	-	-
Centrifuge	$7.97 \times 10^8 \pm 8.18 \times 10^7$	- 0.47 ± 0.04	1.000
Concentrate 50 kDa	$3.56 \times 10^8 \pm 1.66 \times 10^8$	- 0.89 ± 0.27	1.000
Eluate 50 kDa	$9.00 \times 10^0 \pm 8.29 \times 10^0$	- 8.3 ± 0.23	0.006**
Concentrate 30 kDa	$4.50 \times 10^8 \pm 3.29 \times 10^8$	- 0.81 ± 0.27	0.955
Eluate 30 kDa	$8.39 \times 10^4 \pm 1.82 \times 10^4$	- 4.79 ± 0.51	0.055

Significance: ** p<0.01; sd = standard deviation; LRV = Log Reduction Value; PFU = Plaque forming unit.

It is noteworthy that a clean-up treatment with chloroform (1:20 v/v) was performed to remove any remaining cellular debris from the final viral concentrates. After vortexing for 5 min, the solutions were centrifuged at 10,000 g for 5 min. As a result, contamination was observed during the plaque assays and therefore did not allow successful viral recovery. In general, bacteriophages show good stability in chloroform, provided that the sensitivity of the bacteriophage is known [102]. Indeed, in some cases it has been found that chloroform can potentially cause inactivation of some bacteriophages [103,104]. For these reasons, this clean-up treatment was not retained for the final protocol.

Comparison for all sites: protocol validation

Once the optimisation for the viral elution and concentration was successfully carried out on the soil of the Weierbach, the protocol was performed on the samples of the remaining soils (i.e., Daerent, Retgenbusch, Pall 1, Pall 2, Hueschterbach, Koulbich and Mollbach). The results obtained were then reported on the Table S6, as the average quantity of ΦX174 recovered after each step (i.e., chemical elution and concentration). The LRVs were found to be comprised between 0.47 and 1 log after the elution step where no significant difference was detected from the feed solution (p-value = 0.172). After the concentration step, all forest sites showed reductions in ΦX174 between 0.68 and 0.89 log, while ΦX174 spiked in grassland soils were reduced by between 1.07 and 1.34 log. Overall, the LRVs obtained after concentration step were found significant different from the feed solution (p-value = 0.02). Hueschterbach, Pall 1 and Pall 2 showed high LRVs after the concentration step, however, as the final quantity of ΦX174 remained satisfactory for these

three sites and the methodology was desired to be applied similarly to all sites, the results were considered satisfactory.

Table S6: Recovery of bacteriophage ΦX174 at each step of the elution and concentration protocol for each study site. Kruskal-Wallis one-way ANOVA on ranks was performed on the raw data at the level of confidence of 95%.

	Feed solution	Elution step		Concentration step	
Sites	ΦX174 quantity Mean ± sd (PFU)	ΦX174 quantity Mean ± sd (PFU)	LRV Mean ± sd	ΦX174 quantity Mean ± sd (PFU)	LRV Mean ± sd
Daerent	$2.04 \times 10^9 \pm 2.83 \times 10^7$	$3.95 \times 10^8 \pm 1.92 \times 10^8$	- 0.76 ± 0.27	$5.73 \times 10^8 \pm 4.03 \times 10^8$	- 0.68 ± 0.47
Hueschterbach	$1.45 \times 10^9 \pm 3.54 \times 10^7$	$1.29 \times 10^8 \pm 4.04 \times 10^6$	- 1.05 ± 0.01	$6.65 \times 10^7 \pm 1.06 \times 10^7$	- 1.34 ± 0.07
Koulbich	$1.84 \times 10^9 \pm 1.41 \times 10^7$	$2.26 \times 10^8 \pm 1.44 \times 10^7$	- 0.91 ± 0.03	$1.59 \times 10^8 \pm 2.84 \times 10^7$	- 1.07 ± 0.07
Mollbach	$1.84 \times 10^9 \pm 1.41 \times 10^7$	$1.70 \times 10^8 \pm 7.55 \times 10^6$	- 1.03 ± 0.02	$1.59 \times 10^8 \pm 2.60 \times 10^7$	- 1.07 ± 0.07
Pall 1	$1.72 \times 10^9 \pm 7.07 \times 10^6$	$1.91 \times 10^8 \pm 3.31 \times 10^7$	- 0.96 ± 0.08	$9.79 \times 10^7 \pm 6.06 \times 10^6$	- 1.24 ± 0.03
Pall 2	$1.72 \times 10^9 \pm 7.07 \times 10^6$	$2.06 \times 10^8 \pm 3.20 \times 10^7$	- 0.92 ± 0.06	$1.23 \times 10^8 \pm 3.66 \times 10^7$	- 1.14 ± 0.13
Retgenbusch	$2.04 \times 10^9 \pm 2.83 \times 10^7$	$4.07 \times 10^8 \pm 1.33 \times 10^8$	- 0.72 ± 0.15	$3.35 \times 10^8 \pm 2.35 \times 10^7$	- 0.76 ± 0.03
Weierbach	$2.35 \times 10^9 \pm 2.12 \times 10^8$	$7.97 \times 10^8 \pm 1.00 \times 10^8$	- 0.47 ± 0.06	$3.56 \times 10^8 \pm 2.03 \times 10^8$	- 0.89 ± 0.33

sd = standard deviation; LRV = Log reduction value; PFU = Plaque forming unit

Conclusion

The protocol for viral elution and concentration was optimised on three steps: mechanical detachment, chemical detachment and virus concentration. As a result, the mechanical detachment did not allow increasing the detachment rate of bacteriophage ΦX174. Therefore, only the chemical detachment using a solution mixing beef extract (3%) with glycine (0.05M) at pH 9.5 was performed followed by the concentration step using an Amicon 50 kDa. Then, all final solutions underwent a VLPs counting (See manuscript content) as well as DNA extraction (See supplementary material part B).

Section S2: Viral and total DNA extraction optimisation

Materials and method

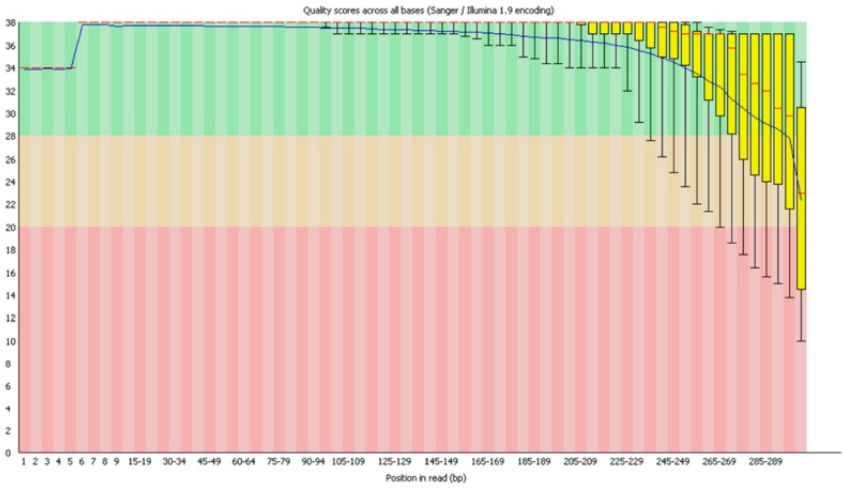
As for the optimisation of the viral elution and concentration, the DNA extraction and library preparation were first conducted for the Weierbach, on both viral concentrate and soil sample, all in triplicate. Regarding the viral concentrates, a DNase treatment using Invitrogen™ kit TURBO DNA-free™ (AM1907, Thermo Fisher Scientific) was tested prior to DNA extraction to remove free DNA. Then, three different extraction kits were selected: the DNeasy® PowerSoil® kit (Qiagen, Hilden, Germany), QIAmp DNA mini kit (51304, Qiagen) and AllPrep PowerViral DNA/RNA kit (28000-50, Qiagen). The obtained DNA solutions were quantified using Invitrogen™ Qubit™ 2.0 fluorometer (ThermoFischer). Regarding the protocol on soil samples, only DNeasy® PowerSoil® kit and PowerViral kit were used as they were specifically conceived for soil sample extraction. On the extracted DNA from all extractions, library preparations were performed using Nextera XT DNA kit (FC-131-1096, Illumina) and the amplified DNA were quantified using qPCR (Applied biosystems ViiA 7, ThermoFisher). It is noteworthy that when the DNA quantity was detected below the limit detection of the device, the quantity was taken as recommended by the manufacturer of the library preparation protocol. Finally, paired-end sequence reads were generated using the Illumina MiSeq (2x350 bp). The quality control on the raw sequences was assessed using FastQC 0.11.8.

Results

The results obtained on the viral concentrates and soil samples are reported in Table S7. The viral concentrates showed better DNA recovery with the QiAmp kit than with the PowerSoil or PowerViral kits, for which the quantities were detected below the detection limit. However, the library preparations were not successful on the DNA extracted by QiAmp. Instead, despite the inability to quantify, libraries could be constructed on DNA extracts from PowerSoil and PowerViral kits. Nevertheless, the quantities of libraries were found to be lower than required ($< 10^4$ pM), making sequencing impossible. The DNA extracted with the PowerSoil kit on the soil samples were, in contrary, sufficient to successfully construct the libraries ($> 10^4$ pM), which was not observed for the extraction performed with PowerViral. The libraries from PowerSoil kit were

sequenced using MiSeq sequencer, but the reads produced was revealed to have very poor quality (Figure S2).

A



B

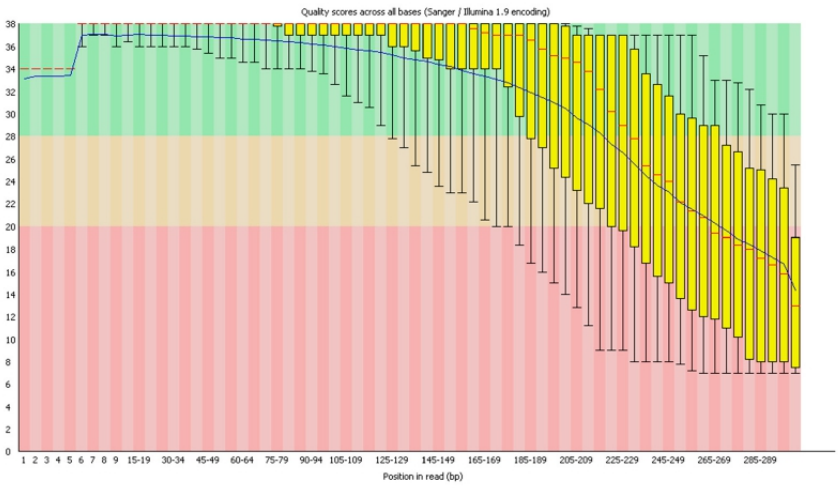


Figure S2: Per base sequence quality from FastQC for read forward 1 (A) and read reverse 2 (B) of the sequencing ot the Weierbach soil.

Table S7: Summary of each step from the DNase treatment to the library preparation from viral concentrates and soil sample of the Weierbach site.

	Viral concentrate					Soil sample	
Initial volume/mass	200 µL	200 µL	200 µL	200 µL	200 µL	250 µg	250 µg
DNase treatment	Yes	No	Yes	No	Yes	No	No
DNA extraction kit	PowerSoil	PowerSoil	QIAmp	QIAmp	PowerViral	PowerSoil	PowerViral
DNA quantification (mean)	< 0.05 ng/µL*	< 0.05 ng/µL*	0.575 ng/µL [1]	0.551 ng/µL [1]	< 0.05 ng/µL*	51 ng/µL	25.5 ng/µL
Libraries quantification (mean)	0.8 pM	2 pM	< DL	< DL	25.5 pM	2.74×10^4 pM	2.74×10^3 pM

* Below the detection limit of the Qubit 2.0; DL = Detection Limit.

[1] It is noteworthy that a cleaning step using DNeasy PowerClean CleanUp kit (12877-50, Qiagen) was tested on the extracted DNA obtained through the QIAmp kit to increase the DNA concentrations. However, the obtained concentrations were detected below the detection limit of the Qubit 2.0, thus the remaining steps were not carried out.

Conclusion

The DNA extractions followed by the library preparations were not successfully addressed to run the sequencing on the viral concentrates. On the contrary, the libraries obtained from the soil samples were sequenced but revealed very poor quality. Therefore, future analyses were explored, particularly the use of Nextera Flex kit followed by NovaSeq 6000 sequencer, which resulted in raw sequences from the soil samples, as described in the main manuscript.

References

97. Chen, L.; Xun, W.; Sun, L.; Zhang, N.; Shen, Q.; Zhang, R. Effect of Different Long-Term Fertilization Regimes on the Viral Community in an Agricultural Soil of Southern China. *Eur. J. Soil Biol.* **2014**, *62*, 121–126, doi:10.1016/j.ejsobi.2014.03.006.
98. Doan, T.T.; Bouvier, C.; Bettarel, Y.; Bouvier, T.; Henry-des-Tureaux, T.; Janeau, J.L.; Lamballe, P.; Nguyen, B. Van; Jouquet, P. Influence of Buffalo Manure, Compost, Vermicompost and Biochar Amendments on Bacterial and Viral Communities in Soil and Adjacent Aquatic Systems. *Appl. Soil Ecol.* **2014**, *73*, 78–86, doi:10.1016/j.apsoil.2013.08.016.
99. Williamson, K.E.; Corzo, K.A.; Drissi, C.L.; Buckingham, J.M.; Thompson, C.P.; Helton, R.R. Estimates of Viral Abundance in Soils Are Strongly Influenced by Extraction and Enumeration Methods. *Biol. Fertil. Soils* **2013**, *49*, 857–869, doi:10.1007/s00374-013-0780-z.
100. Williamson, K.E.; Wommack, K.E.; Radosevich, M. Sampling Natural Viral Communities from Soil for Culture-Independent Analyses. *Appl. Environ. Microbiol.* **2003**, *69*, 6628–6633, doi:10.1128/AEM.69.11.6628-6633.2003.
101. Swanson, M.M.; Fraser, G.; Daniell, T.J.; Torrance, L.; Gregory, P.J.; Taliansky, M. Viruses in Soils: Morphological Diversity and Abundance in the Rhizosphere. *Ann. Appl. Biol.* **2009**, *155*, 51–60, doi:10.1111/j.1744-7348.2009.00319.x.
102. Ateba, C.N.; Akindolire, M.A. Isolation and Characterisation of Bacteriophages with Lytic Activity Against Virulent Escherichia Coli O157:H7: Potential Bio-Control Agents. **2019**, doi:10.20944/PREPRINTS201901.0132.V1.
103. Gupta, V.; Saxena, H.M. Isolation and Characterization of BpL1, a Broad Acting Lytic Bacteriophage against Brucella. *Int. J. Curr. Microbiol. Appl. Sci.* **2017**, *6*, 2486–2496, doi:10.20546/IJCMAS.2017.611.292.
104. Hyman, P. Phages for Phage Therapy: Isolation, Characterization, and Host Range Breadth. *Pharmaceuticals* **2019**, *12*, doi:10.3390/PH12010035.

The optimisation of the method of viral elution and concentration allowed the successful recovery of the viruses from the soil samples collected from the eight selected sites, using an eluent solution based on beef extract and ultrafiltration using Amicon device with a cut-off at 50 kDa. However, when quantifying viral DNA after the elution and concentration protocol, the yield of recovered viral DNA was found to be relatively low, preventing the sequencing methods from being applied. Indeed, the library preparations carried out on the Weierbach soil concentrates did not allow sequencing to be carried out on these samples. In contrast, sequencing could be performed on DNA extracts from raw soil samples without prior elution and concentration steps. However, this resulted in very low-quality sequences, making it difficult to perform bioinformatics analyses for viral identification.

Thus, for the continuation of the study concerning the diversity of viral populations in Luxembourg soils, the viral concentrates obtained were used for the quantification of the virus-like-particles (VLPs), while the DNA extracts coming from raw soil samples from each selected site were subjected to sequencing and bioinformatics analyses for the taxonomic assignment of viruses and bacteria. The results and discussions of these analyses can be found in the following section.

3.1.2 Soil pH, calcium content and bacteria as major factors responsible for the distribution of the known fraction of the DNA bacteriophage populations in soils of Luxembourg

Following the optimisation of methods concerning the recovery of viruses from soils, a shotgun metagenomics protocol followed by bioinformatics analyses was implemented. Then, the data relating to the soil properties and bacterial abundances were put in parallel with the taxonomic data obtained and the viral abundances to determine the diversity in DNA-bacteriophages for each selected soil (eight in total including five grasslands and three forests). Thus, the purpose of this part is to tackle the lack of knowledge that remains regarding the diversity of bacteriophages in soils and to study their distribution according to different criteria. This section is the heart of the first part of this PhD project allowing us to conclude on the DNA-bacteriophage population naturally occurring in soil that could serve as hydrological tracers as part of the objective and the hypotheses proposed within the framework of WP1, presented previously in the introduction of this part.

It should be noted that this work has been published in the journal *Microorganisms* and is presented as such in the following section. The paper includes a description of the methodology used, exploration and discussion of the results on the alpha and beta diversity of the bacteriophage communities and the effects of the soil physicochemical properties and the bacterial community on the distribution of the virus community.

Scientific article available at:

Florent, P.; Cauchie, H.-M.; Herold, M.; Jacquet, S.; Ogorzaly, L. Soil pH, Calcium Content and Bacteria as Major Factors Responsible for the Distribution of the Known Fraction of the DNA Bacteriophage Populations in Soils of Luxembourg. Microorganisms 2022, 10, 1458. https://doi.org/10.3390/microorganisms10071458

microorganisms

MDPI

Article

Soil pH, Calcium Content and Bacteria as Major Factors Responsible for the Distribution of the Known Fraction of the DNA Bacteriophage Populations in Soils of Luxembourg

Perrine Florent [1,2], Henry-Michel Cauchie [1], Malte Herold [1], Stéphan Jacquet [3] and Leslie Ogorzaly [1,*]

[1] Environmental Research and Innovation Department (ERIN), Luxembourg Institute of Science and Technology (LIST), 4422 Belvaux, Luxembourg; perrine.florent@list.lu (P.F.); henry-michel.cauchie@list.lu (H.-M.C.); malte.herold@list.lu (M.H.)
[2] Faculté des Sciences, de la Technologie et de la Communication (FSTC), Doctoral School in Science and Engineering (DSSE), University of Luxembourg, 4365 Esch-sur-Alzette, Luxembourg
[3] INRAE, UMR CARRTEL, Université Savoie Mont Blanc, 74200 Thonon-les-Bains, France; stephan.jacquet@inrae.fr
* Correspondence: leslie.ogorzaly@list.lu; Tel.: +352-2758885-5069

Abstract: Bacteriophages participate in soil life by influencing bacterial community structure and function, biogeochemical cycling and horizontal gene transfer. Despite their great abundance, diversity, and importance in microbial processes, they remain little explored in environmental studies. The influence of abiotic factors on the persistence of bacteriophages is now recognized; however, it has been mainly studied under experimental conditions. This study aimed to determine whether the abiotic factors well-known to influence bacteriophage persistence also control the natural distribution of the known DNA bacteriophage populations. To this end, soil from eight study sites including forests and grasslands located in the Attert River basin (Grand Duchy of Luxembourg) were sampled, covering different soil and land cover characteristics. Shotgun metagenomics, reference-based bioinformatics and statistical analyses allowed characterising the diversity of known DNA bacteriophage and bacterial communities. After combining soil properties with the identified DNA bacteriophage populations, our *in-situ* study highlighted the influence of pH and calcium cations on the diversity of the known fraction of the soil DNA bacteriophages. More interestingly, significant relationships were established between bacteriophage and bacterial populations. This study provides new insights into the importance of abiotic and biotic factors in the distribution of DNA bacteriophages and the natural ecology of terrestrial bacteriophages.

Keywords: DNA bacteriophages; soil; metagenomics; bacteria; soil properties

check for updates

Citation: Florent, P.; Cauchie, H.-M.; Herold, M.; Jacquet, S.; Ogorzaly, L. Soil pH, Calcium Content and Bacteria as Major Factors Responsible for the Distribution of the Known Fraction of the DNA Bacteriophage Populations in Soils of Luxembourg. *Microorganisms* **2022**, *10*, 1458. https://doi.org/10.3390/microorganisms10071458

Academic Editors: Huixin Li, Li Xu and Hui Cao

Received: 23 June 2022
Accepted: 16 July 2022
Published: 19 July 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Soil is a complex compartment where biodiversity and interactions between microbes, animals and plants contribute to its life and optimal functioning. Generally, plants provide organic matter as well as nutrients to soil (e.g., nitrogen, carbon, phosphorous) and are responsible for its structure through the development of the rhizosphere [1,2]. Soil-dwelling animals help to degrade and mix organic matter throughout the soil and participate in the recycling of nutrients from dead biomass [3,4]. Microbes, including fungi, archaea and bacteria, play a key role in organic matter decomposition, element cycling and nutrient availability to plants [5–7]. This microbial activity, particularly due to the quantity, diversity and functional roles of prokaryotes, is considered central to soil processes [8–10]. Viruses are also biological entities fully part of soil biodiversity with essential but relatively unexplored functions [11].

Since the beginning of research on viral diversity in soils in the 1970s [11,12], knowledge of soil viruses has been expanded well, especially for bacteriophages (bacteria-infecting viruses), whose abundance can reach up to 10^{10} particles per gram of soil [13,14].

Bacteriophages are ubiquitous in the environment and can be found in agricultural soils, forest soils (approximately 10^9 bacteriophages per gram of dry soil), wetlands, or even cold deserts (approximately 10^3 bacteriophages per gram of dry soil) [13]. Bacteriophages seem to be more abundant in wetlands than in forests due to high soil water content, but less abundant in agricultural soils than in forests where the soil temperature is lower [13]. In fact, many abiotic factors may be involved in the distribution, abundance and diversity of bacteriophages in soils. However, most of them have only been explored to investigate their effect on bacteriophage persistence. Temperature is recognised as an important, if not the main, factor in viral inactivation [15]. The ideal temperature range to prevent viral inactivation is considered to be 4 to 10 °C, where bacteriophages are generally stable at pH values between 5 and 9, but can be degraded under strongly alkaline (pH > 11) or acidic (pH < 2) conditions [16–18]. These extreme pH conditions can inactivate virus particles through the denaturation of their proteins or nucleic acids [18–21]. In addition, soil texture, especially clay type, alters the detection of bacteriophage as it strongly influences adsorption and retention phenomena in the soil. Indeed, clay soils present a very dense structure with a low permeability and a large specific surface area, which is auspicious for higher adsorption–desorption reactions with bacteriophages [22]. In addition, due to hydrophobicity interactions and the presence of electrical charges, bacteriophages tend to adsorb more onto clay surfaces than onto sandy or silty soil particles [23]. This process also helps to protect bacteriophages from other inactivating factors [11,24].

However, knowledge of the influence of these abiotic factors on bacteriophage persistence has been mainly gained under experimental conditions using bacteriophage models (e.g., ΦX174, MS2), which may not be transferable when studying environmental samples [19,21,25,26]. Indeed, the natural bacteriophage diversity and the impact of such abiotic factors remain largely underexplored in terrestrial compared to aquatic environments [27–29]. Regarding the studies exploring the diversity of bacteriophages on in situ terrestrial ecosystems, virus-like particles (VLPs) or viral operational taxonomic units (vOTUs) have dominantly been studied [30–33]. While the former does not allow the identification of viruses, the latter is rather a proxy for viral species. Therefore, the use of metagenomics in determining the taxonomical classification of viruses and their respective diversity would not only allow completing the knowledge about the influence of abiotic factors on the bacteriophage species communities but also explore the relationship with the bacterial communities.

Coevolution between bacteriophages and their bacterial hosts is widely recognised, with reciprocal influences [11,34]. Bacteriophages are unable to self-replicate and therefore must infect one or more bacterial hosts to replicate and spread. As agents of bacterial mortality, bacteriophages are likely to have a significant influence on bacterial community dynamics, structure and function, as well as biogeochemical cycling, horizontal gene transfer processes and the pre-decomposition of the organic matter [35,36]. However, viral-mediated mortality induces interest at the expense of understanding the influence of bacteria on bacteriophage occurrence in soils [37–39].

With the advent of viral metagenomics, databases increasingly include new viral sequences, which participate in the rapid expansion of our understanding of the virosphere. Based on the viruses already identified, the influence of selected abiotic factors (i.e., soil properties, vegetation and land use) on the distribution of the known DNA bacteriophages can be assessed in different types of soil. Therefore, in this study, we used a combination of approaches and methods (i.e., physicochemical analysis, metagenomics, reference-based bioinformatics, direct counting and statistics) to obtain an original picture of the natural viral and bacterial diversities and their ecological interactions with soil properties in terrestrial environments of Luxembourg. The aim of the study was to determine whether the abiotic factors well known to influence bacteriophage persistence also control the natural distribution of the known DNA bacteriophage populations in the studied soil samples, and to analyse the role of bacterial populations in this phenomenon. Two main hypotheses were tested: (i) abiotic factors (i.e., pH, cations) well known to affect bacteriophage survival also

have an impact on their diversity and distribution in terrestrial environments, and (ii) the bacterial community present in the soil influences the relationship between bacteriophages and soil properties.

2. Materials and Methods

2.1. Studied Area

The Attert River basin area reaches 310 km^2 and covers a wide range of bedrock geologies and land use [40], ultimately being representative of Luxembourg's main physiographic characteristics. Its northern boundary lies on schist bedrock (belonging to the Ardennes massif; Oesling region), while its southern part exhibits alternating sedimentary layers of marls and sandstone (representative of the eastern limit of the Paris basin; Gutland region). The Attert River originates near Nobressart (Belgium) and extends over 38 km from west to east, joining the Alzette River basin in Bissen, Luxembourg. The study is focused on the Luxembourg side of the Attert River basin (Figure 1, Table 1) where 8 sub-basins have been selected compiling 3 forested sites and 5 grassland sites. All of them are located near one of the tributaries of the Attert River.

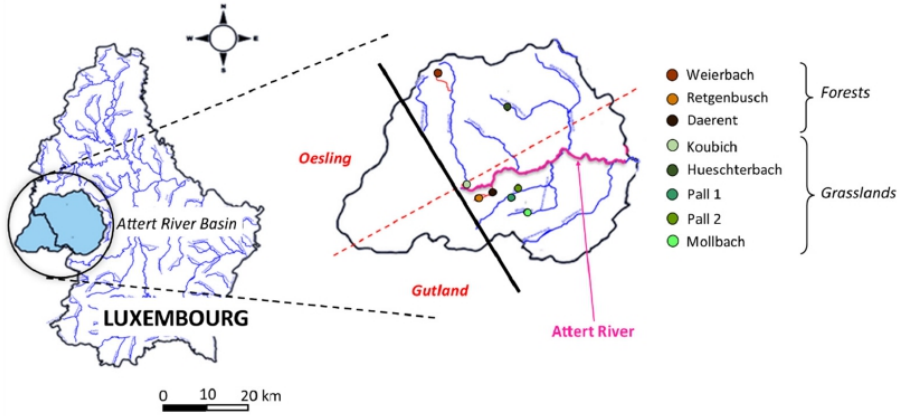


Figure 1. Map of the Attert River basin with the location of the eight sampling sites (modified from Geoportail.lu). Brown colours: forest sites; green colours: grasslands. With an oversimplified scheme, the plain black line represents the border between the Luxembourg and Belgian sides of the basin while the dashed red line is the limit between Oesling and Gutland regions. The Attert River is highlighted in pink, the affluents of the Attert River are in blue and the affluents of affluents, in orange.

Table 1. List of the samples with their GPS coordinates.

Sample Sites	Sample ID	Vegetation	GPS Coordinates
Weierbach	WPF12092019	Forest	(N) 49.831937 (E) 5.80341
Retgenbusch	RPF12092019	Forest	(N) 49.743436 (E) 5.834341
Daerent	DPF12092019	Forest	(N) 49.748226 (E) 5.856064
Koulbich	KPF12092019	Grassland	(N) 49.751942 (E) 5.826879
Hueschterbach	HPF12092019	Grassland	(N) 49.809914 (E) 5.863031
Pall 1	P1PF12092019	Grassland	(N) 49.74617 (E) 5.871577
Pall 2	P2PF12092019	Grassland	(N) 49.748935 (E) 5.881184
Mollbach	MPF12092019	Grassland	(N) 49.7347405 (E) 5.888961

The first forest site is the Weierbach sub-basin located in the northwest of the Attert River basin, in the Oesling region. The two other forest sub-basins are Retgenbusch and Daerent, located on the same tributary of the Attert River basin in the southwestern part of the river (Gutland region). All forest sites have distinct vegetation types; Weierbach is a beech forest, while Daerent is an oak forest and Retgenbusch is a spruce forest.

The first grassland site, Hueschterbach, is the only sub-basin located in the Oesling region and is characterised by schist bedrock. The four other grassland sites are in the Gutland region: Mollbach a sub-basin located in the southwest of the Attert River basin; Pall located in the north of the Mollbach sub-basin, for which two distinct sites (Pall 1 and Pall 2) were chosen on the tributary of the same name; the sub-basin Koulbich located in the northwest of the Attert River basin is known for pasture activities of barley and wheat cultures.

2.2. Sample Collection

The field campaign was conducted on 12 September 2019. The topsoil was collected for all selected sites cited above. Five to seven replicates were randomly sampled within a delimited surface of 25 m^2, with a soil auger (3 cm in diameter) on the first 20 cm of depth. To exclude the intra-site spatial variability and to ease laborious shotgun metagenomic and bioinformatic analyses, 5 to 7 soil replicates were pooled altogether to set up one sample per site, representing around 300 g per site [41–43]. All soil samples were split into two parts, either for physicochemical analyses or stored at −20 °C for molecular analyses.

2.3. Soil Characterisation

The characterisation of the soil properties includes first the pH measurements made on 10 g of soil according to the ISO 10,390 [44] for pH_{H2O} and pH_{KCl} and to VDLUFA A5.1.1 [45] for pH_{CaCl2}. Then, the nutrients dosing of phosphorus (P_2O_5) and potassium (K_2O) was performed according to the VDLUFA A6.2.1.1 [46] using 5 g of soil with 100 mL of Ca-acetate-lactate at pH = 4.1. On the other hand, the dosing of magnesium (Mg) and sodium (Na) was conducted following the VDLUFA A6.2.1.7 [47], using 10 g of soil with 100 mL of $CaCl_2$ (0.01 M). In addition, the carbon-to-nitrogen ratio (C/N) was calculated after quantifying the total organic carbon (C_{org}) and the total nitrogen (N_{tot}) both on 300 g of soil, according to ISO 10,694 [48] and ISO 13,878 [49], respectively. The cation exchangeable capacity (CEC) and cation saturation rate (S/T) were analysed according to the ISO 23,470 [50] on 2.5 g of soil. From the extract CoHex, calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^+) and sodium (Na^+) CoHex were analysed by ICP-OES (inductively coupled plasma–optical emission spectrometry). Finally, the soil granulometry was determined by a sieving method on 10 g of soil mixed with 100 mL of H_2O_2 and 10 mL HCl [51].

2.4. Viral Quantification

2.4.1. Viral Elution and Concentration

After the optimisation of the elution and concentration protocol (Supplementary Materials, Section S1), 5 g of soil, previously stored at −20 °C, was placed at room temperature and then mixed with 15 mL of the eluent solution, i.e., 3% beef extract and 0.05 M glycine (at pH = 9.5) and shaken vigorously using an orbital shaker for 30 min at 250 rpm (rotation per minute) at 4 °C. The samples were then centrifuged (Eppendorf 5801R) at 5000× *g* for 15 min on a fixed rotor at 4 °C. The supernatant was collected and underwent a 0.22 µm Millex® sterile filter unit with a Millipore Express® PES membrane (SLGP033RS, Merck Millipore Ltd., Tullagreen, Ireland), followed by ultracentrifugal filtration using an Amicon Ultra-15 50 kD (UFC905024, Merck, Darmstadt, Germany), upon the recommendations of the manufacturer. After centrifugation at 5000× *g* for 15 min on a fixed rotor at 4 °C, the concentrate solutions, with a volume varying from 0.3 to 3 mL depending on the site, were finally transferred into 2 mL cryotubes and stored at 4 °C.

2.4.2. Viral-like Particles (VLPs) Counting

From the viral concentrates obtained after the viral elution and concentration steps, the abundances of VLPs were determined by flow cytometry (i.e., a benchtop FACSCalibur), after the samples were divided into 3 technical replicates. Briefly, after homogenisation by centrifugation, the viral sample was fixed using glutaraldehyde (0.25% final concentration) and mixed with autoclaved and <0.02 µm filtered TE buffer (0.1 mM Tris and 1 mM EDTA,

pH 8), SYBR© Green I (at a final concentration of 5×10^{-5}, Molecular Probes, Eugene, OR, USA). The samples underwent a 10 min heat treatment at 70 °C before the Flow CytoMetry (FCM) analysis [52].

2.5. Identification of the Microbial Populations

2.5.1. Total DNA Extraction and Shotgun Metagenomics Sequencing

After a comparison of different protocols (Supplementary Materials, Section S2), DNA extraction was performed using the DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany) from 0.25 g of frozen soil (−20 °C) for each site, according to the manufacturer's recommendations The DNA elution step was performed in 60 µL instead of 100 µL to increase the DNA concentration. The final DNA solutions were stored at −20 °C until further analyses.

From the total extracted DNA, library preparations were completed using the Nextera DNA Flex Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Paired-end sequence reads were generated using the Illumina NovaSeq 600 (2×150 bp).

2.5.2. Reference-Based Bioinformatics

The raw sequencing reads were quality controlled and Illumina adapters were trimmed with Trimmomatic 0.39 [53]. Assemblies were performed separately for each sample using SPAdes v3.15.4 [54] with the "meta" option and default parameters Taxonomic classification of contigs was carried out against the complete non-redundant (nr) microbial protein sequence database (released version of April 2022) from NCBI using MMseqs2 v13.45111 [55] and the easy-taxonomy workflow that determines ancestors by searching translated ORFs (translation Table 11) [56]. Quality-filtered reads from each metagenome were mapped back to the assembled contigs using minimap2 v2.24 with the "sr" preset [57] and sorted and indexed with SAMtools v1.15.1 [58]. Finally, metagenomic profiles were exported as comma-separated value (CSV) files. Metagenomic abundances were calculated based on the number of reads mapping to each contig. Mapped reads were divided by contig length and the total number of mapped reads per sample [59,60].

2.6. Statistical Analyses

Statistical analyses were performed with RStudio, version 1.4.1106, and R 4.0.4 [61]. Figures were generated using the package 'ggplot2'. Regarding the ecological diversity, both α and β-diversities of bacteria and DNA bacteriophages were studied. The α-diversity is the average number of distinct species groups at a local scale, considering the richness of a population [62]. As popular α-diversity indexes, the Shannon index (H) and the species richness (S) were calculated for each site, using the R package 'vegan' [63]. Then, the evenness (E), which defines the distribution of the abundance of the species in a community, was calculated as follows [64,65]:

$$E = \frac{H}{\log(S)}$$

The β-diversity is all changes in the species composition of a population between several habitats in a region. This diversity considers both the richness and evenness of populations [66]. Using Bray–Curtis dissimilarities, a multivariate method, the RDA (redundancy analysis), was performed to compare the different sites as a function of the bacteriophage populations at the genus level, through the analysis of their variation from one another. To build the RDA, a selection of the 10 most abundant genera was first performed, as rare genera might generate noise in the analysis). Then, abiotic factors (i.e., sand, clay, silt content, soil pH, magnesium concentration, carbon-to-nitrogen ratio and Ca^{2+} concentration) were selected by removing the variables for which strong correlations (>r = 0.80) were found to avoid co-variations, using the function 'pairs'. In addition, all abiotic factors that significantly constructed the RDA were kept (i.e., pH and Ca^{2+}). The determination of the explanatory variables for the RDA was conducted using 'vegan' and

correlations of the abiotic factors with each axis were defined using the package 'psych'. Permutational multivariate analysis of variance (PERMANOVA) was conducted to establish the impact of some abiotic factors on the distribution of the DNA bacteriophages. From the calculation of the Bray–Curtis dissimilarities to the PERMANOVA and RDA, the package 'vegan' was used to conduct these analyses. Finally, a co-correspondence analysis (CoCA) was performed to assess the co-variation of the distribution of the total bacterial and bacteriophage genera obtained from the metagenomic analyses, among the different sites. This analysis is based on contingency table data and highlights co-similarities between two groups of variables (here, bacterial species data and DNA bacteriophage genera data). CoCA was performed using the package 'coca', combined with a Mantel test using Pearson correlations.

3. Results

3.1. Soil Description

The granulometric analysis of the soil revealed similar textures between the sites (Table 2). Indeed, selected sites are mainly silty, especially Mollbach and Retgenbusch, for which the silt content is around 52–58%. However, Weierbach and Pall 1 are clay loam with approximately 42% silt, while Daerent and Huechterbach are sandy loam with 53% sand. Concerning Pall 2, the proportion for the different textures are rather equal (28% clay, 38% silt, 34% sand). Koulbich is a loam soil, with a similar proportion of silt and sand (≈40%) but little clay (19%). The gravimetric water content (GWC) was the highest for Weierbach (24.6%) and Pall 1 (20.9%) and the lowest for Koulbich (10.8%).

The physical-chemical properties of soils are reported in Table 2. Within the forest sites, the soils of Weierbach and Retgenbusch are highly acidic (respectively, pH = 4.1 and 4.5) and their respective cation exchange capacity and calcium concentration are quite low (CEC of Retgenbusch = 2.6 milliequivalent per 100 g (meq per 100 g) and Ca^{2+} = 1.6 meq per 100 g, CEC of Weierbach = 8.5 meq per 100 g and Ca^{2+} = 2.1 meq per 100 g). Their nutrient content was also relatively poor, especially for phosphorus (P_2O_5) and potassium (K_2O), suggesting that these soils are not very fertile. In addition, the cation saturation rate (S/T) of Retgenbusch is superior to 85% (S/T = 101%), which can explain the high calcium content in the soil, while the soil of Weierbach shows a regular calcium intake (20% $\leq$ S/T = 42% $\leq$ 85%). The soil of Weierbach is distinguished by a slow organic matter decomposition (carbon-to-nitrogen ratio or C/N = 18 and high C_{org} content), displayed by a need for nitrogen to ensure an efficient organic decomposition. The soil of Daerent is fertile (CEC = 17.1 meq per 100 g, pH = 6.3, K_2O = 21 mg per 100 g of dry soil) and has a maximum decomposition rate (C/N = 10). Regarding the grassland sites, Pall 1, Pall 2 and Mollbach soils are all fertile (CEC = 23.7, 17.2, 14.3 meq per 100 g, respectively) with a high concentration of calcium (Ca^{2+} = 16.6, 15.1 and 13.1 meq per 100 g), while Koulbich and Hueschterbach are not quite (CEC = 7.4 and 8.5 meq/100 g, respectively) with lower calcium concentrations (Ca^{2+} = 5.8 and 8.7 meq per 100 g). However, all of them are saturated in calcium, with a cation saturation rate superior to 100%. Finally, the grassland soils have a rapid decomposition (C/N ≈ 8.5) with a maximum organic decomposition for Pall 1 (C/N = 9.8) and have a favourable pH (pH ≈ 6–6.5) and mineral nutrient for plant growth.

Table 2. Physical-chemical properties of the eight studied soils: GWC: gravimetric water content, C_{org}: organic carbon, N_{tot}: total nitrogen, CEC: cation exchangeable capacity, S/T: cation saturation rate, Ca^{2+}: calcium concentration.

Site Names	GWC	pH	C/N	C_{org}	N_{tot}	Mg	Na	P_2O_5	K_2O	CEC	Ca^{2+}	S/T	Textural Class	Clay	Silt	Sand
	%	-	-	%	%	mg per 100 g ds	mg per 100 g ds	mg per 100 g ds	mg per 100 g ds	Meq kg^{-1}	Meq kg^{-1}	%		%	%	%
Weierbach	24.6	4.1	18	9.7	0.54	3	2	5	13	85	21	42	CL	23.9	42.2	33.9
Retgenbusch	13.7	4.5	8.6	1.9	0.22	3	1	3	9	26	16	101	A	27.2	58.5	14.3
Daerent	15.8	6.3	10	3.9	0.39	46	2	4	21	171	126	107	SL	53.9	28.0	18.1
Koulbich	10.8	5.7	8.3	1.9	0.23	12	3	9	11	74	58	105	L	37.7	43.6	18.6
Hueschterbach	14.7	6.4	8.8	2.2	0.25	11	3	20	16	85	87	126	L	52.0	29.6	18.4
Pall 1	20.9	6.8	9.8	5.1	0.52	17	2	6	17	237	166	100	CL	24.2	42.6	33.2
Pall 2	16.3	6.2	8.8	3.6	0.41	31	3	7	15	172	151	115	CL	33.9	38.1	28.1
Mollbach	18.3	6.0	8.3	3.1	0.23	52	5	13	21	143	131	112	SL	28.1	52.5	19.4

ds = dry soil, meq kg: milliequivalent per kilogram (quantity of positive charges per kilogram); textural class: CL = clay loam; L = silt loam; SL = sandy loam; L = loam [67].

3.2. Description of the DNA-Bacteriophage Populations

3.2.1. VLPs Enumeration

The average concentration of VLPs found in every soil was about 10^{10} VLPs per gram of dry soil (Figure 2). The highest concentrations were recorded for the soils of Retgenbusch, Hueschterbach, Daerent and Pall 2 with approximately 1.5×10^{10} VLPs per gram of dry soil. The lowest concentration was for the soil of Weierbach with 3.3×10^{9} VLPs per gram of dry soil. and displayed a 0.48 log difference with the lowest VLPs concentration (after the Weierbach), Koulbich and 0.67 log with Hueschterbach, the highest VLPs concentration. Finally, Koulbich showed on average a lower concentration of 0.18 log compared to all the other sites.

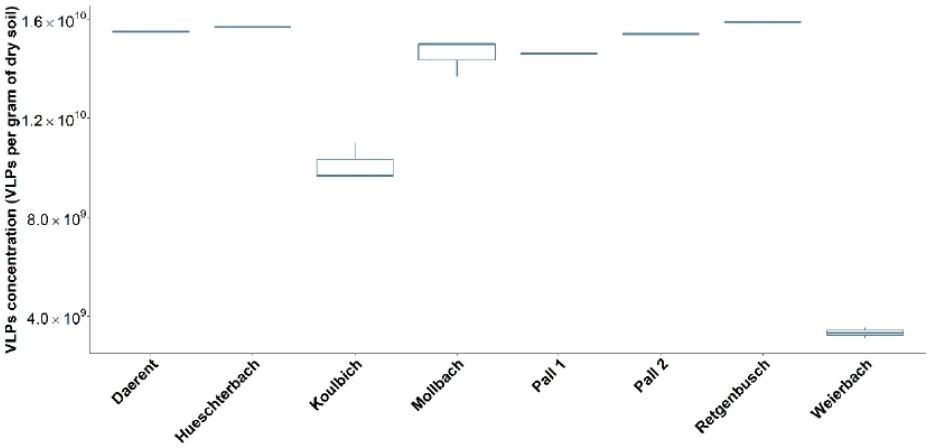


Figure 2. Boxplot of the virus-like particles (VLPs) concentration per gram of dry soil for all studied sites.

3.2.2. Taxonomic Classification

The statistics of the shotgun metagenomic sequencing are reported in Table 3.

The shotgun sequencing produced between 40 to 57 million paired reads per sample generating an average of 6.2 million contigs after assembly. From 75 to 99% of the total raw reads mapped back to the contigs. Of these mapped reads, between 0.01% and 0.04% were virus-affiliated reads (Supplementary Table S1), corresponding to a total of 37 known viral families for all sites included. Among these families, 13 DNA bacteriophage families were identified and represented between 89% and 96% of all virus-affiliated reads (Figure 3A). The DNA bacteriophage composition was strongly characterised by the *Caudovirales* order with between 75% and 93% of the total metagenomics identification. This high proportion was mainly due to the high abundance of *Siphoviridae* (13.9% to 49%), *Myoviridae* (5% to 12%) and *Podoviridae* (4% to 10%). *Caudovirales* gathers all the identified DNA bacteriophage families, except for Autolykiviridae (unclassified order) in Daerent and Mollbach soils, *Microviridae* (Petitvirales) and *Tectiviridae* (order Kalamavirales) both present in every site. By investigating each site, *Siphoviridae* was found to always be the most identified family, especially in the soils of Pall 1, Hueschterbach, Mollbach, Koulbich and Pall 2 (with, respectively, 49.58%, 44.83%, 35.15% and 34.80%) while its abundance was the lowest for Retgenbusch (13.9%). Regarding *Myoviridae* and *Podoviridae*, the former was highly abundant in Koulbich (10.65%), Retgenbusch (10.9%) and Weierbach (12.3%) while the latter was rather highly present in the soils of Weierbach (10.2%) and Mollbach (10.3%). It is noteworthy that most of the assigned DNA bacteriophages from the libraries belonged to the unclassified *Caudovirales* (between 29% and 51%). Of the two ssDNA bacteriophage families, i.e., *Inoviridae* and *Microviridae*, both were detected by the metagenomics with a low abundance in all sites (between 0.09% and 1.6% for the former and between 0.06% and 1% for the latter), while all the previously mentioned families are dsDNA bacteriophages.

Table 3. Statistics of the metagenomic sequencing: raw data, assembly and mapping on the eight studied soils.

Sites	Number of Read Pairs	Yield in Mbp	Average Quality (Q-Score)	Number of Total Mapped Read Pairs	Percentage of Mapped Read Pairs	Number of Total Contigs	GC Content	N50
Daerent	45,506,199	12,929	35.6	36,248,481	79.66%	5,469,310	63.70%	271
Hueschterbach	42,222,600	13,046	35.88	36,987,700	87.60%	5,727,441	63.95%	269
Koulbich	64,930,676	18,587	35.53	49,069,306	75.57%	7,792,136	63.18%	268
Mollbach	50,983,302	14,522	35.63	35,064,164	68.77%	6,306,081	63.40%	264
Pall 1	46,905,743	13,397	35.62	40,072,879	85.43%	5,980,767	64.72%	273
Pall 2	56,510,613	16,136	35.6	48,535,078	85.89%	6,883,923	63.40%	272
Retgenbusch	49,316,386	13,878	35.59	46,692,284	94.68%	5,677,103	60.01%	295
Weierbach	57,936,000	16,650	35.22	57,518,714	99.28%	5,898,678	60.95%	323

Mbp = millions of base pairs; GC = guanine cytosine; N50 = the sequence length of the shortest contig at 50% of the total genome length; total references to total microbial metagenomes (i.e., eukaryote, archaea, bacteria and viruses).

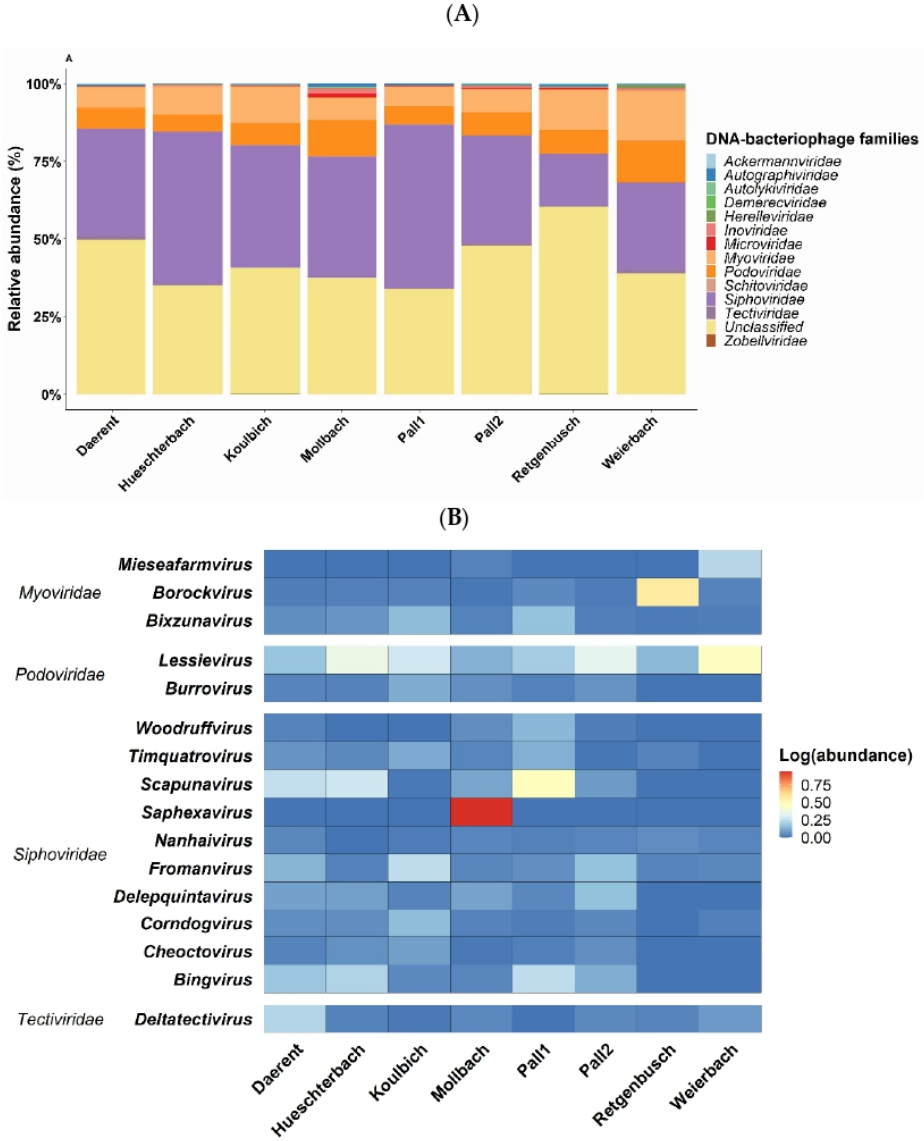


Figure 3. (**A**) Bar charts of the relative abundance (in %) of DNA bacteriophage families from the known fraction of all studied sites. (**B**) Heatmap of the most occurrent DNA bacteriophage genera identified (selection of genera with a log-abundance above 0.24) as a function of the sites. The gradient represents the log-transformed abundance, where red colour represents the most abundant genera and blue, the least ones.

To visualise the composition of the DNA bacteriophage population, a heatmap on the log-transformation abundances, at the genus level, was performed on the 10 most abundant genera, all sites included (Figure 3B). Genus *Lessievirus* (*Podoviridae*) was detected in every site, and more specifically in the soil of Weierbach, for which *Mieseafarmvirus* (*Myoviridae*) was also present. The soil of Mollbach was specifically represented by *Saphexavirus* (*Siphoviridae*), while in Retgenbusch, *Borockvirus* (*Myoviridae*) was the most abundant genus. Pall 1 was found to be mainly represented by *Scapunavirus* (*Siphoviridae*) followed by *Bingvirus* (*Siphoviridae*). *Scapunavirus* was also found in a slightly higher abundance in Daer-

ent and Hueschterbach. Koulbich and Pall 2 did not display high abundances for specific genera, but still showed the presence of *Fromanvirus* (*Siphoviridae*) besides *Lessievirus*.

3.3. Impact of the Soil Properties on Known DNA-Bacteriophage Distribution

3.3.1. α-Diversity: Known DNA-Bacteriophage Diversity within Sites

The DNA bacteriophage α-diversity of the known microbial fraction of each soil was investigated using the Shannon index, species evenness and species richness (Table 4). All sites displayed similar diversity (around H = 2) where Retgenbusch was the least diverse soil (H = 2.31) and Weierbach the most one (H = 2.83). Indeed, the soil of Weierbach presented the lowest number of DNA bacteriophage species (S = 91) and the highest evenness (E = 0.63). A one-way analysis of variance (one-way ANOVA) was performed on the Shannon index to analyse the hypothetical impact of the selected soil properties on the DNA bacteriophage diversity within the sites. As a result, none of the soil properties showed a significant effect on the DNA bacteriophage alpha-diversity.

Table 4. Shannon diversity index (H), evenness (E) and richness (S) on genus level of DNA bacteriophage from the known fraction of the population from the soil of the studied sites.

Sites	Shannon Index (H)	Evenness (E)	Species Richness (S)
Daerent	2.39	0.48	147
Hueschterbach	2.54	0.51	140
Koulbich	2.58	0.51	149
Mollbach	2.79	0.55	155
Pall 1	2.65	0.52	160
Pall 2	2.62	0.51	174
Retgenbusch	2.31	0.49	117
Weierbach	2.83	0.63	91

3.3.2. β-Diversity: Known DNA-Bacteriophage Diversity between Sites

The β-diversity analysis allowed comparing the DNA bacteriophage subset populations from one site to another. The PERMANOVA test (Table 5) performed on the DNA bacteriophage genera abundancy did not reveal a significant effect of the vegetation (i.e., forest and grasslands) on the distribution of the DNA bacteriophages (p-value = 0.075, $\alpha = 0.05$).

Table 5. PERMANOVA tests to analyse the influence of the selected abiotic factors on the distribution of the known DNA bacteriophage genera ($\alpha = 0.05$).

Environmental Variables	*p*-Value
Vegetation	0.075
Ca^{2+}	0.008 **
Carbon-to-nitrogen ratio	0.349
pH	0.003 **
Silt content	0.125
Sand content	0.764
Clay content	0.901
Mg content	0.083
GWC	0.846

Significance: ** $p < 0.01$.

However, soil pH and calcium concentration were associated with the differences in the DNA bacteriophage compositions between the sites (p-value = 0.003 and 0.008, respectively, $\alpha = 0.05$). A redundancy analysis (afterwards called RDA; Figure 4) was carried out to analyse the variations of bacteriophage genera depending on the soil properties of the sites. The RDA was built over the two first dimensions, covering together 67.5% of the fitted variation. Among all the abiotic factors, soil pH was the explanatory variable that had a

significant weight on the construction of the RDA (p-value = 0.008, α = 0.05). On the one hand, the axis RDA1 was significantly correlated at 88.4% with soil pH (p-value < 0.01, α = 0.05) and at 79.2% with calcium cation (p-value < 0.01, α = 0.05). The RDA2, on the other hand, was not significantly correlated with any of the tested soil properties.

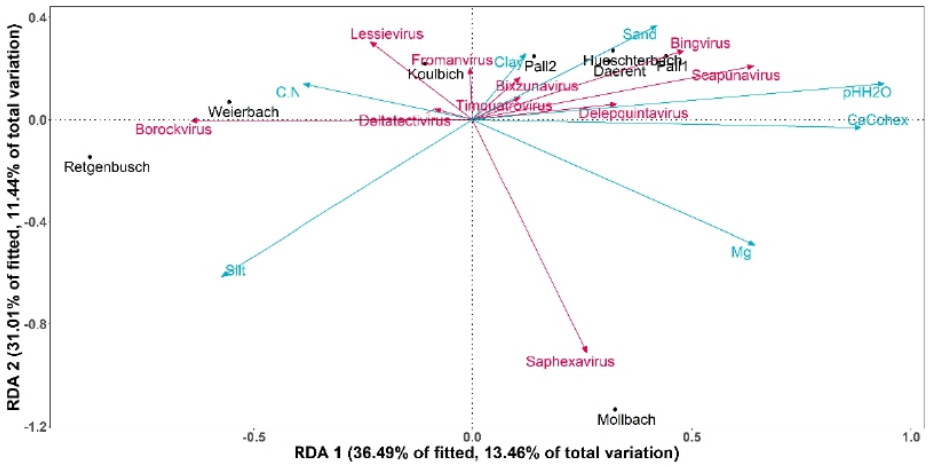


Figure 4. Biplot of redundancy analysis (RDA) on the 10 most abundant genera of DNA bacteriophage in relation to the soil properties of each site. The pink vectors represent the DNA bacteriophage genera with a Hellinger transformation, the blue vectors display the selected soil properties and the sites are shown in black. The RDA was built over the two first dimensions, with 36.49% of the fitted variations explained by RDA1 and 31.01% by RDA2. (C.N = carbon to nitrogen ratio; Mg = magnesium concentration; pHH_2O = soil pH measured in water, CaCohex = calcium cation available).

Along the RDA1 axis, both soil pH and calcium concentration explained the sites distributions. Indeed, Weierbach and Retgenbusch were displayed apart from the other sites, as they were the most acidic soils (pH = 4.1 and 4.5, respectively) and had the lowest concentration in calcium (Ca^{2+} = 21 and 16 positive charges per kg of soil, respectively). The RDA showed a particular correlation between the sites of Weierbach and Retgenbusch and the genus *Borockvirus*. On the contrary, Pall 1, Huescherbach and Daerent presented the highest soil pH (pH = 6.8, 6.4 and 6.3, respectively) with relatively high concentrations of calcium (Ca^{2+} = 166, 126 and 87 positive charges per kg of soil, respectively). In terms of DNA bacteriophage genera, these soil properties (i.e., pH and Ca^{2+}) showed a negative correlation with *Bingvirus* and *Scapunavirus*. Finally, Mollbach showed a particular distribution, related to the magnesium content, as indeed its soil presented the highest concentration of magnesium (Mg = 52 mg/100 g of soil). Additionally, *Saphexavirus* displayed a positive correlation specifically to the magnesium concentration.

3.4. Known Bacterial Populations and Connections with the Known DNA-Bacteriophage Communities

As reported in Supplementary Table S1, bacterial sequences represented 74 to 77% of the total microbial sequences identified in the libraries. First, bacterial populations of all sites were mainly composed of the following phyla, *Acidobacteria*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (see Supplementary Figure S1). As for the DNA bacteriophages, the taxonomical composition of the bacterial populations was then analysed at the genus level by selecting the most abundant bacteria (abundance above 1000 mapped reads per species, all sites included). Retgenbusch and Weierbach showed similar patterns in bacterial genera with the lowest soil bacterial diversity (H = 2.78 and 2.89, respectively) (see Supplementary Table S2) and similar bacterial composition as is visible in Figure 5.

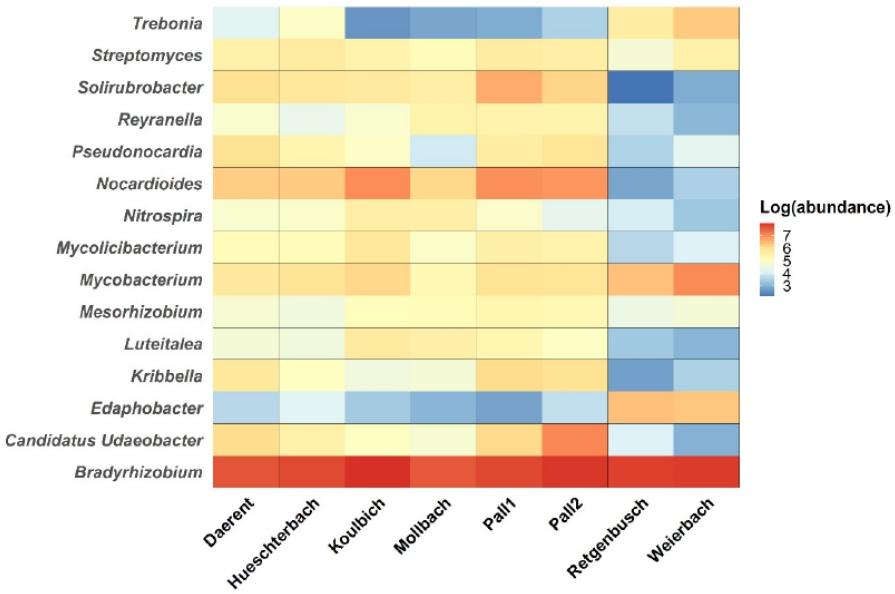


Figure 5. Heatmap of the most occurrent bacterial genera identified (selection with a log-abundance above 3) as a function of the sites. The gradient represents the log-transformed abundance of the genera, where red colour represents the most abundant genera and blue, the least ones.

In addition, these two sites showed differences in bacterial compositions from the other sites. Indeed, Regtenbusch and Weierbach showed higher abundance for the genera *Trebonia*, *Edaphobacter* and *Mycobacterium*, for which the relative abundances were lower in the other soils. Regarding the other genera, with the exception of *Streptomyces* and *Bradyrhizobium*, all of them showed high abundance for all sites except for Regtenbusch and Weierbach. Finally, *Streptomyces* and *Bradyrhizobium*, were detected in every soil with the highest abundance reported for the latter. The differences in bacterial community composition between the sites were studied through a PERMANOVA test, performed on the bacteria genera abundance. As noticed for the DNA bacteriophages, the vegetation (i.e., forest and grasslands) did not significantly impact the bacteria distribution (p-value = 0.121, α = 0.05). In addition, soil pH and calcium concentration also displayed significant effect on the bacterial compositions between the sites (p-value = 0.006 and 0.002, respectively, α = 0.05). Contrary to the observations on the DNA bacteriophages, magnesium concentration played a significant role on the bacterial composition (p-value = 0.027, α = 0.05). As for the DNA bacteriophage investigation, RDA was carried out to analyse the variations of bacterial genera depending on the same selected soil properties of the sites (Figure 6). The RDA was built over the two first dimensions, covering together 93.77% of the fitted variation. Among all the abiotic factors, soil pH was the sole explanatory variable that had a significant weight on the construction of the RDA (p-value = 0.01, α = 0.05). On one hand, the axis RDA1 was significantly correlated at 92.16% with soil pH (p-value < 0.01, α = 0.05) and at 79.21% with calcium (p-value < 0.01, α = 0.05). On the other hand, the RDA2 was not significantly correlated with any of the tested soil properties. Weierbach and Retgenbusch were found apart from all the other sites, and both related to only *Edaphobacter* and *Mycobacterium*.

As DNA bacteriophages and bacteria are known for being exclusively linked to one another, the covariation of these two communities was analysed using a symmetric CoCA. This ordination method was built over the two first dimensions that covered 67.4% of the total covariation (Figure 7). It allows exploring the distribution of the whole known bacterial and DNA bacteriophage subset populations at the genera level by analysing both simultaneously. The patterns for both bacterial and DNA bacteriophage CoCA were similar and confirmed the distinct group formed by Weierbach and Retgenbusch. The

latter was displayed offset from the other sites while Daerent, Pall 1, Pall 2, Mollbach and Hueschterbach were similar to each other on both bacterial and bacteriophage CoCA. A Mantel test using Pearson correlation carried on DNA bacteriophage and bacterial genera confirmed a strong positive correlation (R-value = 0.75, significance = 0.001).

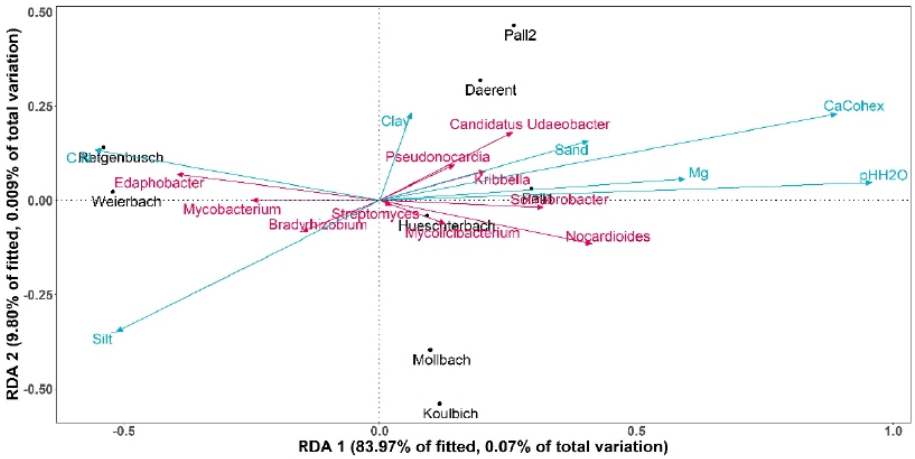


Figure 6. Biplot of redundancy analysis (RDA) on the 10 most abundant genera of bacteria in relation to the soil properties of each site. The pink vectors represent the bacterial genera, the blue vectors display the selected soil properties and the sites are shown in black. The RDA was built over the two first dimensions, with 83.97% of the fitted variations explained by RDA1 and 9.80% by RDA2. (C.N = carbon to nitrogen ratio; Mg = magnesium content; pHH_2O = soil pH measured in water, CaCohex = calcium cation available).

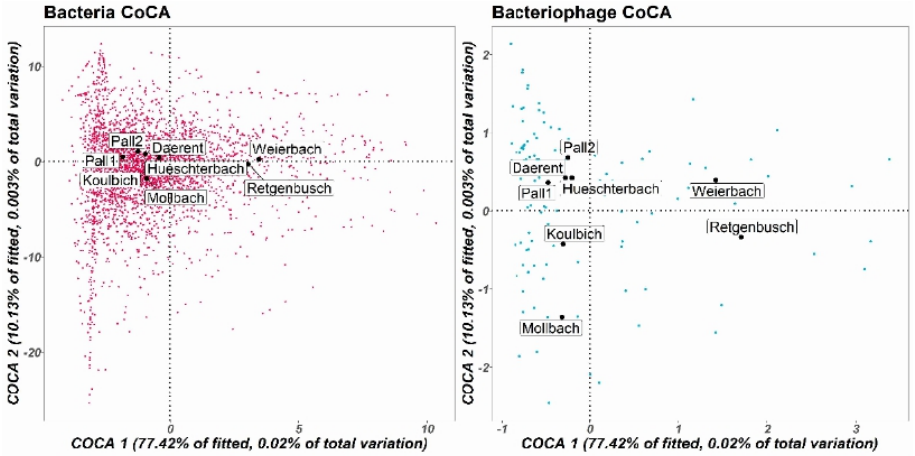


Figure 7. Symmetric co-correspondence analysis (Sym-CoCA) of both total bacteria and DNA bacteriophage community compositions. The bacterial genera are represented in pink and bacteriophage genera, in blue. Here, they were built over the two first dimensions, where 77.42% of the fitted covariation between the two matrices was explained by COCA1 and 10.13% by COCA2. The sites are displayed in black.

4. Discussion

In our study, a viral concentration of approximately 10^9 to 10^{10} VLPs per gram of dry soil was found, which is consistent with previous works [14,68,69]. Although VLP counts are not always considered to be a good representative of virus concentrations, the counts performed here were carried out in the same manner as previously on freshwater

sediments using the FACSCalibur FCM table. This method was previously validated and provided similar VLP counts to those obtained by EFM [70]. A good correlation was found, highlighting the high sensitivity of the device and thus, allowing obtaining unambiguous signatures for VLPs by discriminating noise from VLPs (virus-like particles) to PLPs (prokaryote-like particles). It is noteworthy, however, that flow cytometry analysis does not allow identifying viruses or discriminating bacteriophages from the rest of the VLPS [71]. To draw a complete picture of the viral community, a taxonomic identification of the known DNA bacteriophage populations was complementarily performed through shotgun metagenomics and reference-based bioinformatics. The taxonomy was assigned using the nr database released in April 2022; however, it is noteworthy that the viral taxonomy built by the International Committee on Taxonomy of Viruses (ICTV) is nowadays updated according to the genetic similarities between bacteriophages, including reclassification of the families observed in the current study, such as *Siphoviridae*, *Myoviridae* and *Podoviridae*.

Our study showed *Caudovirales* as the most frequently identified order in all sites, while *Siphoviridae*, *Podoviridae* and *Myoviridae* were found as the most abundant families observed within the identified DNA bacteriophages, which is consistent with most of the literature that studied viral soil diversity [13,26,29]. However, the high proportion of these three families is rather the reflection of the taxonomy made available on the databases up to now, where most of the bacteriophage species are classified into these families [72]. For this reason, it is important to consider low taxonomic levels in diversity studies. In the current study, the viral genera obtained from the libraries did not display specific patterns related to the ecosystems (i.e., forests vs. grasslands). The three selected forest sites presented different vegetal covers (i.e., beech, spruce and oak) with diverse types of soil textures. The forest sites might not be sufficiently similar to constitute a common pattern in DNA bacteriophage diversity. However, several patterns in the known DNA bacteriophage distribution are distinguishable by considering the specific soil parameters. The soil pH as well as the calcium concentration significantly drove the ecological distribution of the known DNA bacteriophage communities among the different studied soils. More interestingly, the distribution of both DNA bacteriophage and bacterial communities in diverse types of soil were found to be strongly linked to one another and their distributions were explained by the same abiotic factors, i.e., soil pH and calcium concentration. Additionally, a complementary effect of magnesium concentration solely on the bacterial distribution was detected. The differences in microbial diversity were highlighted especially for the soils of Weierbach and Retgenbusch. Indeed, these two sites did not only display an acidic pH (around 4) with low calcium availability (<5 meq per 100 g of soil), but also low abundance and diversity of the known microbial populations at the genus level. While studying the known DNA bacteriophage populations, these same three soils were represented by the significant presence of one specific DNA bacteriophage genus. Indeed, *Saphexavirus*, *Borockvirus* and *Lessievirus* were highly abundant in the soil of Mollbach, Retgenbusch and Weierbach, respectively, compared to the other studied soils. Some studies reported a dominance of small ssDNA viruses rather than dsDNA-tailed bacteriophages in the soils [73–75]. In the present study, ssDNA bacteriophages (*Microviridae* and *Inoviridae*) were also detected, but in low frequency in comparison to dsDNA bacteriophages. Most ssDNA bacteriophages detected from environmental metagenomes belonged to the *Microviridae* family, for which all members infect a narrow range of hosts [76]. However, most ssDNA bacteriophages remain scarcely characterised within the taxonomy as *Inoviridae* represents 11% and *Microviridae* 2% of the ICTV database [77], reflecting a potential bias in the determination of their abundance in the soil samples.

Calcium is an important component participating in soil life. It plays a role in the soil structure, firstly as a binding agent in the aggregation of soil particles, secondly as a macronutrient in plant nutrition and thirdly as a factor facilitating soil permeability [78]. Then, calcium has a biological role in promoting an optimal environment to enhance soil microorganism activities [79]. Finally, calcium is highly related to soil pH. Indeed, soils with low pH generally display low calcium availability. As high concentrations of hydrogen ions

displace calcium concentration from binding sites, it results in the leaching of "free" calcium, particularly in the more porous soils [80,81]. Such soils, such as the ones of Weierbach and Retgenbusch, i.e., low pH and calcium concentration, did display a low microbial diversity among the bacterial and DNA bacteriophage populations characterised in this study. On the contrary, Pall 1, Pall 2 and Daerent with much higher calcium concentrations (between 10 and 20 meq per 100 g of soil) and an ideal range of soil pH (between 6 and 7) showed higher microbial diversity. In laboratory experiments, divalent cations (such as calcium and magnesium) were found to interact with bacteriophage MS2 by increasing its attachment to the surface of river natural organic matter [82]. In soil, adsorption onto surface particles is the most important process implied in the bacteriophages-soil interactions [11]. This interaction is highly dependent upon the type of cations found in the environment. Calcium cation (positively charged) is highly involved in the adsorption of clay–humus complex (CHC), negatively charged on its surface, through electrostatic forces [83,84]. Bacteriophages can then adsorb onto the CHC by the presence of the cations on the surface. Indeed, bacteriophages are charged on their surface; however, this charge is highly dependent on pH. At neutral pH (7), most bacteriophages are negatively charged and can interact with the available cations [85]. Reducing the pH will induce some bacteriophage surfaces to turn into positive charges, according to their isoelectric point (pI). Once positive, bacteriophages can compete with cations to bind with the clay charges of the CHC. Indeed, bacteriophages tend to be more retained in soils rich in clay. The latter has a lower specific surface, due to large particles and a greater ion-exchange capacity, which highly adsorb bacteriophages [86]. Therefore, the combination between calcium cation and pH can play a role in the bacteriophage dynamic in soils. However, not only did these factors show a significant effect on the DNA bacteriophage distribution, but they also did on the bacterial distribution. Additionally, our results showed a strong positive correlation between both bacterial and DNA bacteriophages distribution among the sites, suggesting a strong interaction. Therefore, soil pH seems like the most significant factor in leading the known DNA bacteriophage distributions, directly or/and indirectly through the shaping of bacterial communities. Calcium cation availability might rather play a role in the dynamic of DNA bacteriophages, through their adsorption with the soil particles.

The advances in metagenomics and bioinformatics have enabled deeper investigations for the identification of viruses [87,88]; however, some technical issues remain in the analysis of soil viruses [89]. As viruses tend to adsorb on soil particles, virus elution and concentration steps are often required before performing metagenomics. Nevertheless, this step generally ends in low recovery efficiencies [90,91], as noticed in our results during the optimisation of the elution and concentration protocol. Indeed, even though the viral recovery was satisfactory to conduct a viral quantification using flow cytometry, it resulted in low DNA yield after nucleic acid extraction. Therefore, it affected the good quality of viral sequences obtained after conducting the shotgun metagenomics, leading us to carry out the DNA extraction directly from the soil samples. As the analysis is solely relying on virus-affiliated reads that can be obtained from total DNA metagenomes, few viral contigs were obtained with N50 comprising between 200 and 300 per library. This can limit the interpretation of relationships between viruses and other microbes within the same samples.

Much remains to be understood about the diversity, dynamics and roles of bacteriophages in soils and metagenomics approaches have only recently enabled increasing our knowledge, particularly under in situ conditions. In laboratory experiments, some physicochemical properties, especially pH and ion strength, influenced bacteriophage inactivation [92,93]. Our results seemed to show the effect of these similar factors on bacteriophage diversity in soils; however, it is noteworthy that the study was focused on the known fraction of the microbial populations in the diverse selected soils. The OTU-based approach is less dependent on reference databases but works by clustering sequences according to similarity thresholds (often set at a sequence similarity level above 97%) [94–96]. This approach does not identify the taxonomic species but is rather a proxy for them and

has shown to be phylogenetically incoherent [94–96]. Therefore, further analyses using complementary molecular and bioinformatics methods, including untargeted approaches, could be applied to obtain a more complete picture of bacteriophage dynamics. Additionally, the investigation of the influence of certain abiotic factors on bacteriophage diversity based on what has already been characterised could open perspectives for the use of these species as, for instance, biotechnologies or in the development of new culture models for laboratory experiments.

5. Conclusions

In this study, the natural diversity and distribution of DNA bacteriophage populations in soils were explored as well as their relationship with the soil-dwelling bacterial populations. Through the complementary use of metagenomics, reference-based bioinformatics and multidimensional analyses, we identified the known fraction of DNA bacteriophage communities in forest and grassland soils and deciphered the influence of some soil properties and microbial communities on the DNA bacteriophage occurrence. Both soil pH and calcium content were observed to influence the distribution of DNA bacteriophage and bacterial populations. A strong positive correlation between DNA bacteriophage and bacterial communities was confirmed, suggesting in addition to soil pH and calcium content, bacteria are also involved. Therefore, our results add to the growing body of evidence that bacteriophages are likely to play key roles in soil ecology.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10071458/s1, Figure S1: Bar charts of the relative abundance (in %) of bacterial phylum (selected genera with an abundance above 100 occurrences) from the known fraction of all studied sites; Figure S2: Per base sequence quality from FastQC for read forward 1 (A) and read reverse 2 (B) of the sequencing ot the Weierbach soil; Table S1: Percentages of relative abundances identified for each taxonomic domain; Table S2: Shannon diversity index (H), evenness (E) and species richness (S) of bacteria populations in the soil of the studied sites; Table S3: Recovery of bacteriophage ΦX174 comparing three methods for mechanical viral detachment from the Weierbach soil; Table S4: Recovery of bacteriophage ΦX174 from the Weierbach soil comparing six different eluent solutions; Table S5: Recovery of bacteriophage ΦX174 from the Weierbach soil after concentration step, comparing Amicon with two different cut-off points (i.e., 30 kDa and 50 kDa); Table S6: Recovery of bacteriophage ΦX174 at each step of the elution and concentration protocol for each study site; Table S7: Summary of each step from the DNase treatment to the library preparation from viral concentrates and soil sample of the Weierbach site. References [97–102] are cited in the supplementary materials.

Author Contributions: Conceptualization, P.F., L.O. and H.-M.C.; methodology, P.F., L.O., M.H., S.J. and H.-M.C.; bioinformatics, M.H.; flow cytometry: S.J.; validation, P.F., L.O., M.H., S.J. and H.-M.C.; writing—original draft preparation, P.F.; writing—review and editing, P.F., L.O., M.H., S.J. and H.-M.C.; visualization, P.F.; supervision, L.O. and H.-M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Luxembourg National Research Fund (FNR) for granting and supporting the PRIDE program containing the Doctoral Training Unit Hydro-CSI project (PRIDE15/10623093—HYDRO-CSI).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw sequence data generated and analysed within this study are available in the National Centre for Biotechnology Information Databases (Sequence Read Archive) under the BioProject number PRJNA771214.

Acknowledgments: The authors would like to acknowledge the Luxembourg National Research Fund (FNR) for granting and supporting the PRIDE program containing the Doctoral Training Unit Hydro-CSI project (PRIDE15/10623093—HYDRO-CSI). The authors would also like to thank Thomas Mion and Magdalena Calusinska for their valuable assistance in the bioinformatics pipeline by providing us with their expertise in this field. The soil analyses could not have been done without the cooperation of Lionel Leydet (Administration des services techniques de l'agriculture, ASTA). We would finally thank our colleagues, Christophe Hissler and Nathalie Goeders for discussing with us regarding, respectively, the soil data and statistics analyses.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Lange, M.; Eisenhauer, N.; Sierra, C.A.; Bessler, H.; Engels, C.; Griffiths, R.I.; Mellado-Vázquez, P.G.; Malik, A.A.; Roy, J.; Scheu, S.; et al. Plant Diversity Increases Soil Microbial Activity and Soil Carbon Storage. *Nat. Commun.* **2015**, *6*, 6707. [CrossRef]
2. Vezzani, F.M.; Anderson, C.; Meenken, E.; Gillespie, R.; Peterson, M.; Beare, M.H. The Importance of Plants to Development and Maintenance of Soil Structure, Microbial Communities and Ecosystem Functions. *Soil Tillage Res.* **2018**, *175*, 139–149. [CrossRef]
3. Eldridge, D.; James, A. Soil-disturbance by Native Animals Plays a Critical Role in Maintaining Healthy Australian Landscapes. *Ecol. Manag. Restor.* **2009**, *10*, S27–S34. [CrossRef]
4. Lou, X.; Zhao, J.; Lou, X.; Xia, X.; Feng, Y.; Li, H. The Biodegradation of Soil Organic Matter in Soil-Dwelling Humivorous Fauna. *Front. Bioeng. Biotechnol.* **2021**, *9*, 1406. [CrossRef] [PubMed]
5. Odelade, K.A.; Babalola, O.O. Bacteria, Fungi and Archaea Domains in Rhizospheric Soil and Their Effects in Enhancing Agricultural Productivity. *Int. J. Environ. Res. Public Health* **2019**, *16*, 3873. [CrossRef]
6. Rashid, M.I.; Mujawar, L.H.; Shahzad, T.; Almeelbi, T.; Ismail, I.M.I.; Oves, M. Bacteria and Fungi Can Contribute to Nutrients Bioavailability and Aggregate Formation in Degraded Soils. *Microbiol. Res.* **2016**, *183*, 26–41. [CrossRef]
7. Buerkert, A.; Joergensen, R.G.; Ludwig, B.; Schlecht, E. Chapter 18—Nutrient and Carbon Fluxes in Terrestrial Agro-Ecosystems. In *Marschner's Mineral Nutrition of Higher Plants*, 3rd ed.; Academic Press: San Diego, CA, USA, 2012; pp. 473–482. ISBN 978-0-12-384905-2.
8. Ortiz, A.; Sansinenea, E. The Role of Beneficial Microorganisms in Soil Quality and Plant Health. *Sustainability* **2022**, *14*, 5358. [CrossRef]
9. Beule, L.; Vaupel, A.; Moran-Rodas, V.E. Abundance, Diversity, and Function of Soil Microorganisms in Temperate Alley-Cropping Agroforestry Systems: A Review. *Microorganisms* **2022**, *10*, 616. [CrossRef]
10. Armon, R. Soil Bacteria and Bacteriophages. In *Biocommunication in Soil Microorganisms*; Witzany, G., Ed.; Springer: Heidelberg, Germany, 2011; Volume 23, pp. 67–112. ISBN 978-3-642-14511-7.
11. Kimura, M.; Jia, Z.J.; Nakayama, N.; Asakawa, S. Ecology of Viruses in Soils: Past, Present and Future Perspectives. *Soil Sci. Plant Nutr.* **2008**, *54*, 1–32. [CrossRef]
12. Coyne, M.S. A Cartoon History of Soil Microbiology. *J. Nat. Resour. Life Sci. Educ.* **1996**, *25*, 30–36. [CrossRef]
13. Williamson, K.E.; Fuhrmann, J.J.; Wommack, K.E.; Radosevich, M. Viruses in Soil Ecosystems: An Unknown Quantity Within an Unexplored Territory. *Annu. Rev. Virol.* **2017**, *4*, 201–219. [CrossRef] [PubMed]
14. Williamson, K.E.; Corzo, K.A.; Drissi, C.L.; Buckingham, J.M.; Thompson, C.P.; Helton, R.R. Estimates of Viral Abundance in Soils Are Strongly Influenced by Extraction and Enumeration Methods. *Biol. Fertil. Soils* **2013**, *49*, 857–869. [CrossRef]
15. Bertrand, I.; Schijven, J.F.; Sánchez, G.; Wyn-Jones, P.; Ottoson, J.; Morin, T.; Muscillo, M.; Verani, M.; Nasser, A.; de Roda Husman, A.M.; et al. The Impact of Temperature on the Inactivation of Enteric Viruses in Food and Water: A Review. *J. Appl. Microbiol.* **2012**, *112*, 1059–1074. [CrossRef] [PubMed]
16. Corapcioglu, M.Y.; Vogel, J.R.; Munster, C.L.; Pillai, S.D.; Dowd, S.; Wang, S. Virus Transport Experiments in a Sandy Aquifer. *Water Air Soil Pollut.* **2006**, *169*, 47–65. [CrossRef]
17. Fauvel, B.; Cauchie, H.M.; Gantzer, C.; Ogorzaly, L. Influence of Physico-Chemical Characteristics of Sediment on the in Situ Spatial Distribution of F-Specific RNA Phages in the Riverbed. *FEMS Microbiol. Ecol.* **2019**, *95*, fiy240. [CrossRef]
18. Jurczak-Kurek, A.; Gasior, T.; Nejman-Faleńczyk, B.; Bloch, S.; Dydecka, A.; Topka, G.; Necel, A.; Jakubowska-Deredas, M.; Narajczyk, M.; Richert, M.; et al. Biodiversity of Bacteriophages: Morphological and Biological Properties of a Large Group of Phages Isolated from Urban Sewage. *Sci. Rep.* **2016**, *6*, 34338. [CrossRef]
19. Feng, Y.Y.; Ong, S.L.; Hu, J.Y.; Tan, X.L.; Ng, W.J. Effects of PH and Temperature on the Survival of Coliphages MS2 and Qβ. *J. Ind. Microbiol. Biotechnol.* **2003**, *30*, 549–552. [CrossRef]
20. Nishide, M.; Tsujimoto, K.; Uozaki, M.; Ikeda, K.; Yamasaki, H.; Koyama, A.H.; Arakawa, T. Effects of Electrolytes on Virus Inactivation by Acidic Solutions. *Int. J. Mol. Med.* **2011**, *27*, 803–809. [CrossRef]
21. Taj, M.K.; Ling, J.X.; Bing, L.L.; Qi, Z.; Taj, I.; Hassani, T.M.; Samreen, Z.; Yunlin, W. Effect of Dilution, Temperature and PH on the Lysis Activity of T4 Phage against E. Coli BL21. *J. Anim. plant Sci.* **2014**, *24*, 1252–1255.
22. Cuadros, J. Clay Minerals Interaction with Microorganisms: A Review. *Clay Miner.* **2017**, *52*, 235–261. [CrossRef]
23. Park, J.A.; Kang, J.K.; Kim, J.H.; Kim, S.B.; Yu, S.; Kim, T.H. Bacteriophage Removal in Various Clay Minerals and Clay-Amended Soils. *Environ. Eng. Res.* **2015**, *20*, 133–140. [CrossRef]

24. Harvey, R.W.; Ryan, J.N. Use of PRD1 Bacteriophage in Groundwater Viral Transport, Inactivation, and Attachment Studies. *FEMS Microbiol. Ecol.* **2004**, *49*, 3–16. [CrossRef] [PubMed]
25. Williamson, K.E.; Radosevich, M.; Wommack, K.E. Abundance and Diversity of Viruses in Six Delaware Soils. *Appl. Environ. Microbiol.* **2005**, *71*, 3119–3125. [CrossRef] [PubMed]
26. Leroy, M.; Prigent, M.; Dutertre, M.; Confalonieri, F.; Dubow, M. Bacteriophage Morphotype and Genome Diversity in Seine River Sediment. *Freshw. Biol.* **2008**, *53*, 1176–1185. [CrossRef]
27. Parmar, K.; Dafale, N.; Pal, R.; Tikariha, H.; Purohit, H. An Insight into Phage Diversity at Environmental Habitats Using Comparative Metagenomics Approach. *Curr. Microbiol.* **2018**, *75*, 132–141. [CrossRef] [PubMed]
28. Mohiuddin, M.; Schellhorn, H.E. Spatial and Temporal Dynamics of Virus Occurrence in Two Freshwater Lakes Captured through Metagenomic Analysis. *Front. Microbiol.* **2015**, *6*, 960. [CrossRef] [PubMed]
29. Gong, Z.; Liang, Y.; Wang, M.; Jiang, Y.; Yang, Q.; Xia, J.; Zhou, X.; You, S.; Gao, C.; Wang, J.; et al. Viral Diversity and Its Relationship With Environmental Factors at the Surface and Deep Sea of Prydz Bay, Antarctica. *Front. Microbiol.* **2018**, *9*, 1–17. [CrossRef]
30. Narr, A.; Nawaz, A.; Wick, L.Y.; Harms, H.; Chatzinotas, A. Soil Viral Communities Vary Temporally and along a Land Use Transect as Revealed by Virus-like Particle Counting and a Modified Community Fingerprinting Approach (FRAPD). *Front. Microbiol.* **2017**, *8*, 1975. [CrossRef]
31. Trubl, G.; Jang, H.B.; Roux, S.; Emerson, J.B.; Solonenko, N.; Vik, D.R.; Solden, L.; Ellenbogen, J.; Runyon, A.T.; Bolduc, B.; et al. Soil Viruses Are Underexplored Players in Ecosystem Carbon Processing. *mSystems* **2018**, *3*, e00076-18. [CrossRef]
32. Cao, M.-M.; Liu, S.-Y.; Bi, L.; Chen, S.-J.; Wu, H.-Y.; Ge, Y.; Han, B.; Zhang, L.-M.; He, J.-Z.; Han, L.-L. Distribution Characteristics of Soil Viruses Under Different Precipitation Gradients on the Qinghai-Tibet Plateau. *Front. Microbiol.* **2022**, *13*, 848305. [CrossRef]
33. Han, L.L.; Yu, D.T.; Bi, L.; Du, S.; Silveira, C.; Cobián Güemes, A.G.; Zhang, L.M.; He, J.Z.; Rohwer, F. Distribution of Soil Viruses across China and Their Potential Role in Phosphorous Metabolism. *Environ. Microbiome* **2022**, *17*, 6. [CrossRef]
34. Koskella, B.; Brockhurst, M.A. Bacteria–Phage Coevolution as a Driver of Ecological and Evolutionary Processes in Microbial Communities. *FEMS Microbiol. Rev.* **2014**, *38*, 916–931. [CrossRef]
35. Fazzino, L.; Anisman, J.; Chacón, J.M.; Heineman, R.H.; Harcombe, W.R. Lytic Bacteriophage Have Diverse Indirect Effects in a Synthetic Cross-Feeding Community. *ISME J.* **2020**, *14*, 123–134. [CrossRef] [PubMed]
36. Sullivan, M.B.; Weitz, J.S.; Wilhelm, S. Viral Ecology Comes of Age. *Environ. Microbiol. Rep.* **2017**, *9*, 33–35. [CrossRef] [PubMed]
37. Klimenko, A.I.; Matushkin, Y.G.; Kolchanov, N.A.; Lashin, S.A. Bacteriophages Affect Evolution of Bacterial Communities in Spatially Distributed Habitats: A Simulation Study. *BMC Microbiol.* **2016**, *16*, S10. [CrossRef]
38. Ashelford, K.E.; Day, M.J.; Fry, J.C. Elevated Abundance of Bacteriophage Infecting Bacteria in Soil. *Appl. Environ. Microbiol.* **2003**, *69*, 285–289. [CrossRef] [PubMed]
39. Braga, L.P.P.; Spor, A.; Kot, W.; Breuil, M.C.; Hansen, L.H.; Setubal, J.C.; Philippot, L. Impact of Phages on Soil Bacterial Communities and Nitrogen Availability under Different Assembly Scenarios. *Microbiome* **2020**, *8*, 52. [CrossRef]
40. Marx, S.; Flammang, F. *La Cartographie Des Sols Au Grand-Duché de Luxembourg Version Provisoire_V4*; Ministère de L'agriculture, de la Viticulture et du Développement Rural: Luxembourg, 2015.
41. Heffernan, A.L.; Aylward, L.L.; Toms, L.M.L.; Sly, P.D.; Macleod, M.; Mueller, J.F. Pooled Biological Specimens for Human Biomonitoring of Environmental Chemicals: Opportunities and Limitations. *J. Expo. Sci. Environ. Epidemiol.* **2014**, *24*, 225–232. [CrossRef]
42. Caudill, S.P. Characterizing Populations of Individuals Using Pooled Samples. *J. Expo. Sci. Environ. Epidemiol.* **2010**, *20*, 29–37. [CrossRef]
43. Roy, K.; Ghosh, D.; DeBruyn, J.M.; Dasgupta, T.; Wommack, K.E.; Liang, X.; Wagner, R.E.; Radosevich, M. Temporal Dynamics of Soil Virus and Bacterial Populations in Agricultural and Early Plant Successional Soils. *Front. Microbiol.* **2020**, *11*, 1494. [CrossRef]
44. AFNOR. *ISO 10390: Sols, Biodéchets Traités et Boues—Détermination Du PH*, 3rd ed.; Association Française de Normalisation: Geneva, Switzerland, 2021.
45. VDLUFA. *Methodenbuch A5.1.1: Bestimmung des PH-Wertes*; Verlag: Darmstadt, Germany, 2016.
46. VDLUFA. *Methodenbuch A6.2.1.1: Bestimmung von Phosphor und Kalium im Calcium-Acetat-Lactat-Auszug*; Verlag: Darmstadt Germany, 2012.
47. VDLUFA. *Methodenbuch A6.2.1.7: Bestimmung von Pflanzenverfügbarem Kalium und Natrium im Calciumchloridauszug auf Gewichtsbasis*; Verlag: Darmstadt, Germany, 1997.
48. AFNOR. *ISO 10694: Qualité du Sol–Dosage du Carbone Organique et du Carbon Total Après Combustion Sèche (Analyse Élémentaire)*, 1st ed.; Association Française de Normalisation: Geneva, Switzerland, 1995.
49. AFNOR. *ISO 13878: Qualité du Sol–Détermination de la Teneur Totale en Azote par Combustion Sèche ("Analyse Élémentaire")*, 1st ed.; Association Française de Normalisation: Geneva, Switzerland, 1998.
50. AFNOR. *ISO 23470: Qualité Du Sol–Détermination de La Capacité D'échange Cationique (CEC) Effective et des Cations Échangeables à L'aide d'une Solution de Trichlorure de Cobaltihexammine*, 1st ed.; Association Française de Normalisation: Geneva, Switzerland, 2007.
51. Marx, S.; Flammang, F. *Cartographie des Sols au Grand-Duché de Luxembourg*; Ministère de L'agriculture, de la Viticulture et du Développement Rural: Luxembourg, 2018.
52. Jacquet, S.; Dorigo, U.; Personnic, S. A Few Tests Prior to Flow Cytometry and Epifluorescence Analyses of Freshwater Bacterioand Virioplankton Communities. *Flow Cytom. Princ. Methodol. Appl.* **2013**, *1*, 1–30.

53. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A Flexible Trimmer for Illumina Sequence Data. *Bioinformatics* **2014**, *30*, 2114–2120. [CrossRef] [PubMed]
54. Nurk, S.; Meleshko, D.; Korobeynikov, A.; Pevzner, P.A. MetaSPAdes: A New Versatile Metagenomic Assembler. *Genome Res.* **2017**, *27*, 824–834. [CrossRef] [PubMed]
55. Steinegger, M.; Söding, J. MMseqs2 Enables Sensitive Protein Sequence Searching for the Analysis of Massive Data Sets. *Nat. Biotechnol.* **2017**, *35*, 1026–1028. [CrossRef]
56. Mirdita, M.; Steinegger, M.; Breitwieser, F.; Sö Ding, J.; Levy Karin, E. Fast and Sensitive Taxonomic Assignment to Metagenomic Contigs. *Bioinformatics* **2021**, *37*, 3029–3031. [CrossRef]
57. Li, H. Minimap2: Pairwise Alignment for Nucleotide Sequences. *Bioinformatics* **2018**, *34*, 3094–3100. [CrossRef]
58. Danecek, P.; Bonfield, J.K.; Liddle, J.; Marshall, J.; Ohan, V.; Pollard, M.O.; Whitwham, A.; Keane, T.; McCarthy, S.A.; Davies, R.M.; et al. Twelve Years of SAMtools and BCFtools. *Gigascience* **2021**, *10*, giab008. [CrossRef]
59. Palermo, C.N.; Fulthorpe, R.R.; Saati, R.; Short, S.M. Metagenomic Analysis of Virus Diversity and Relative Abundance in a Eutrophic Freshwater Harbour. *Viruses* **2019**, *11*, 792. [CrossRef]
60. Zhao, Y.; Li, M.C.; Konaté, M.M.; Chen, L.; Das, B.; Karlovich, C.; Williams, P.M.; Evrard, Y.A.; Doroshow, J.H.; McShane, L.M. TPM, FPKM, or Normalized Counts? A Comparative Study of Quantification Measures for the Analysis of RNA-Seq Data from the NCI Patient-Derived Models Repository. *J. Transl. Med.* **2021**, *19*, 269. [CrossRef]
61. RStudio Team. *RStudio: Integrated Development for R. RStudio*; PBC: Boston, MA, USA, 2020; Available online: http://www.rstudio.com/ (accessed on 11 July 2022).
62. Thukral, A.K. A Review on Measurement of Alpha Diversity in Biology. *Agric. Res. J.* **2017**, *54*, 10. [CrossRef]
63. Oksanen, J.; Blanchet, F.G.; Friendly, M.; Kindt, R.; Legendre, P.; McGlinn, D.; Minchin, P.R.; O'Hara, R.B.; Simpson, G.L.; Solymos, P.; et al. vegan: Community Ecology Package. R Package Version 2.5-7. 2020. Available online: https://CRAN.R-project.org/package=vegan (accessed on 11 July 2022).
64. Mulder, C.P.H.; Bazeley-White, E.; Dimitrakopoulos, P.G.; Hector, A.; Scherer-Lorenzen, M.; Schmid, B. Species Evenness and Productivity in Experimental Plant Communities. *Oikos* **2004**, *107*, 50–63. [CrossRef]
65. Moore, J.C. Diversity, Taxonomic versus Functional. In *Encyclopedia of Biodiversity*, 2nd ed.; Academic Press: Cambridge, MA, USA, 2013; pp. 648–656. ISBN 9780123847195.
66. Socolar, J.B.; Gilroy, J.J.; Kunin, W.E.; Edwards, D.P. How Should Beta-Diversity Inform Biodiversity Conservation? *Trends Ecol. Evol.* **2016**, *31*, 67–80. [CrossRef] [PubMed]
67. USDA (United States Department of Agriculture, Natural resources conservation service, Soil Texture Calculator. Available online: https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/survey/?cid=nrcs142p2_054167 (accessed on 18 July 2022).
68. Pratama, A.A.; van Elsas, J.D. The 'Neglected' Soil Virome—Potential Role and Impact. *Trends Microbiol.* **2018**, *26*, 649–662. [CrossRef]
69. Williamson, K.E.; Wommack, K.E.; Radosevich, M. Sampling Natural Viral Communities from Soil for Culture-Independent Analyses. *Appl. Environ. Microbiol.* **2003**, *69*, 6628–6633. [CrossRef] [PubMed]
70. Duhamel, S.; Jacquet, S. Flow Cytometric Analysis of Bacteria- and Virus-like Particles in Lake Sediments. *J. Microbiol. Methods* **2006**, *64*, 316–332. [CrossRef]
71. Colombet, J.; Billard, H.; Viguès, B.; Balor, S.; Boulé, C.; Geay, L.; Benzerara, K.; Menguy, N.; Ilango, G.; Fuster, M.; et al. Discovery of High Abundances of Aster-Like Nanoparticles in Pelagic Environments: Characterization and Dynamics. *Front. Microbiol.* **2019**, *10*, 2376. [CrossRef] [PubMed]
72. Turner, D.; Kropinski, A.M.; Adriaenssens, E.M. A Roadmap for Genome-Based Phage Taxonomy. *Viruses* **2021**, *13*, 506. [CrossRef]
73. Kim, K.H.; Chang, H.W.; Nam, Y.D.; Roh, S.W.; Kim, M.S.; Sung, Y.; Jeon, C.O.; Oh, H.M.; Bae, J.W. Amplification of Uncultured Single-Stranded DNA Viruses from Rice Paddy Soil. *Appl. Environ. Microbiol.* **2008**, *74*, 5975–5985. [CrossRef]
74. Reavy, B.; Swanson, M.M.; Cock, P.J.A.; Dawson, L.; Freitag, T.E.; Singh, B.K.; Torrance, L.; Mushegian, A.R.; Taliansky, M. Distinct Circular Single-Stranded DNA Viruses Exist in Different Soil Types. *Appl. Environ. Microbiol.* **2015**, *81*, 3934–3945. [CrossRef]
75. Grujcic, V.; Nuy, J.K.; Salcher, M.M.; Shabarova, T.; Kasalicky, V.; Boenigk, J.; Jensen, M.; Simek, K. Cryptophyta as Major Bacterivores in Freshwater Summer Plankton. *ISME J.* **2018**, *12*, 1668–1681. [CrossRef]
76. Cherwa, J.E.; Fane, B.A. Microviridae: Microviruses and Gokushoviruses. *eLS* **2011**, *5*, 1–10. [CrossRef]
77. Székely, A.J.; Breitbart, M. Single-Stranded DNA Phages: From Early Molecular Biology Tools to Recent Revolutions in Environmental Microbiology. *FEMS Microbiol. Lett.* **2016**, *363*, 27. [CrossRef]
78. Wuddivira, M.N.; Camps-Roach, G. Effects of Organic Matter and Calcium on Soil Structural Stability. *Eur. J. Soil Sci.* **2007**, *58*, 722–727. [CrossRef]
79. Zavarzin, G.A. Microbial Geochemical Calcium Cycle. *Microbiology* **2002**, *71*, 1–17. [CrossRef]
80. Whittinghill, K.A.; Hobbie, S.E. Effects of PH and Calcium on Soil Organic Matter Dynamics in Alaskan Tundra. *Biogeochemistry* **2011**, *111*, 569–581. [CrossRef]
81. Ng, J.F.; Ahmed, O.H.; Jalloh, M.B.; Omar, L.; Kwan, Y.M.; Musah, A.A.; Poong, K.H. Soil Nutrient Retention and PH Buffering Capacity Are Enhanced by Calciprill and Sodium Silicate. *Agronomy* **2022**, *12*, 219. [CrossRef]
82. Pham, M.; Mintz, E.A.; Nguyen, T.H. Deposition Kinetics of Bacteriophage MS2 to Natural Organic Matter: Role of Divalent Cations. *J. Colloid Interface Sci.* **2009**, *338*, 1–9. [CrossRef]

83. Brydon, J.E.; Sowden, F.J. A Study o the Clay-Humus Complexes of a Chernozemic and a Podzol Soil. *J. Soil Sci.* **2011**, *39*, 136–143. [CrossRef]
84. Xu, X.; Li, Y.; Hu, X.; Xie, G.; Xu, H.; Gao, M.; Zhang, X.; Zhang, R.; Tang, C.; Hu, X. Effect of Humic Acid on the Adsorption/Desorption Behaviors of Trivalent Chromium on Calcium Modified Montmorillonite and Kaolinite. *ChemistrySelect* **2022**, *7*, e202104302. [CrossRef]
85. Sadeghi, G.; Behrends, T.; Schijven, J.F.; Hassanizadeh, S.M. Effect of Dissolved Calcium on the Removal of Bacteriophage PRD1 during Soil Passage: The Role of Double-Layer Interactions. *J. Contam. Hydrol.* **2013**, *144*, 78–87. [CrossRef]
86. McLeod, M.; Aislabie, J.; Smith, J.; Fraser, R.; Roberts, A.; Taylor, M. Viral and Chemical Tracer Movement through Contrasting Soils. *J. Environ. Qual.* **2001**, *30*, 2134–2140. [CrossRef] [PubMed]
87. Fernandez-Cassi, X.; Rusinol, M.; Martinez-Puchol, S. Viral Concentration and Amplification from Human Serum Samples Prior to Application of Next-Generation Sequencing Analysis. In *The Human Virome: Methods and Protocols*; Springer: New York, NY, USA, 2018; Volume 1838, pp. 173–188. ISBN 9781493986828.
88. Zhang, Y.Z.; Shi, M.; Holmes, E.C. Using Metagenomics to Characterize an Expanding Virosphere. *Cell* **2018**, *172*, 1168–1172. [CrossRef]
89. Trubl, G.; Hyman, P.; Roux, S.; Abedon, S.T. Coming-of-Age Characterization of Soil Viruses: A User's Guide to Virus Isolation, Detection within Metagenomes, and Viromics. *Soil Syst.* **2020**, *4*, 23. [CrossRef]
90. Baer, A.; Kehn-Hall, K. Viral Concentration Determination Through Plaque Assays: Using Traditional and Novel Overlay Systems. *J. Vis. Exp.* **2014**, *93*, e52065. [CrossRef] [PubMed]
91. Van Twest, R.; Kropinski, A.M. *Bacteriophages Enrichment from Water and Soil*; Clokie, M.R.J., Kropinski, A.M., Eds.; Humana Press: Totowa, NJ, USA, 2009; Volume 501, ISBN 1402037902.
92. Walshe, G.E.; Pang, L.; Flury, M.; Close, M.E.; Flintoft, M. Effects of PH, Ionic Strength, Dissolved Organic Matter, and Flow Rate on the Co-Transport of MS2 Bacteriophages with Kaolinite in Gravel Aquifer Media. *Water Res.* **2010**, *44*, 1255–1269. [CrossRef]
93. Furiga, A.; Pierre, G.; Glories, M.; Aimar, P.; Roques, C.; Causserand, C.; Berge, M. Effects of Ionic Strength on Bacteriophage MS2 Behavior and Their Implications for the Assessment of Virus Retention by Ultrafiltration Membranes. *Appl. Environ. Microbiol.* **2011**, *77*, 229–236. [CrossRef] [PubMed]
94. Fierer, N.; Breitbart, M.; Nulton, J.; Salamon, P.; Lozupone, C.; Jones, R.; Robeson, M.; Edwards, R.A.; Felts, B.; Rayhawk, S.; et al. Metagenomic and Small-Subunit RRNA Analyses Reveal the Genetic Diversity of Bacteria, Archaea, Fungi, and Viruses in Soil. *Appl. Environ. Microbiol.* **2007**, *73*, 7059–7066. [CrossRef]
95. Nguyen, N.P.; Warnow, T.; Pop, M.; White, B. A Perspective on 16S RRNA Operational Taxonomic Unit Clustering Using Sequence Similarity. *NPJ Biofilms Microbiomes* **2016**, *2*, 16004. [CrossRef]
96. Schloss, P.D.; Westcott, S.L. Assessing and Improving Methods Used in Operational Taxonomic Unit-Based Approaches for 16S RRNA Gene Sequence Analysis. *Appl. Environ. Microbiol.* **2011**, *77*, 3219–3226. [CrossRef]
97. Chen, L.; Xun, W.; Sun, L.; Zhang, N.; Shen, Q.; Zhang, R. Effect of Different Long-Term Fertilization Regimes on the Viral Community in an Agricultural Soil of Southern China. *Eur. J. Soil Biol.* **2014**, *62*, 121–126. [CrossRef]
98. Doan, T.T.; Bouvier, C.; Bettarel, Y.; Bouvier, T.; Henry-des-Tureaux, T.; Janeau, J.L.; Lamballe, P.; Van Nguyen, B.; Jouquet, P. Influence of Buffalo Manure, Compost, Vermicompost and Biochar Amendments on Bacterial and Viral Communities in Soil and Adjacent Aquatic Systems. *Appl. Soil Ecol.* **2014**, *73*, 78–86. [CrossRef]
99. Swanson, M.M.; Fraser, G.; Daniell, T.J.; Torrance, L.; Gregory, P.J.; Taliansky, M. Viruses in Soils: Morphological Diversity and Abundance in the Rhizosphere. *Ann. Appl. Biol.* **2009**, *155*, 51–60. [CrossRef]
100. Ateba, C.N.; Akindolire, M.A. Isolation and Characterisation of Bacteriophages with Lytic Activity Against Virulent Escherichia Coli O157:H7: Potential Bio-Control Agents. *Preprints* **2019**, 2019010132. [CrossRef]
101. Gupta, V.; Saxena, H.M. Isolation and Characterization of BpL1, a Broad Acting Lytic Bacteriophage against Brucella. *Int. J. Curr. Microbiol. Appl. Sci.* **2017**, *6*, 2486–2496. [CrossRef]
102. Hyman, P. Phages for Phage Therapy: Isolation, Characterization, and Host Range Breadth. *Pharmaceuticals* **2019**, *12*, 35. [CrossRef]

The identification of DNA-bacteriophage populations of soils was successfully achieved through the implementation of a methodology combining metagenomics, bioinformatics and biostatistics from the extracted viral DNA. Thus, among the most abundant families, *Siphoviridae*, *Myoviridae* and *Podoviridae* are those found in all the sites studied. However, on further analysis of their distribution within sites according to soil properties, little or no variation was visible, which necessitated the identification of bacteriophages at a higher taxonomic level. Variations could be demonstrated according to the pH and calcium content. Most importantly, the Weierbach soil, the model site for the DTU in which this PhD work is involved, was found to be very different from the other sites with particularly acidic soil, high organic matter content and low virus richness and concentration.

However, although identification is a necessary first step in the use of new bacteriophages as environmental tracers, it is far from sufficient on its own. Indeed, if the identified bacteriophages are not capable of being mobilised from their initial point of detection, then it will be impossible to use them in tracing experiments. This mobilisation is therefore crucial to consider, especially as it can depend on many environmental factors, and this topic is discussed and explored in detail in the next section of the manuscript.

3.2 WP2: Mobilisation of the bacteriophages from the soil matrix to the soil water

Once the DNA-bacteriophage community was characterised and the main drivers of their distribution were identified, we needed to understand whether these DNA-bacteriophage species were to be mobilised from their natural habitat, the soil. Indeed, to potentially be used as hydrological tracers, not only is their abundance of high importance but their ability to be transported has also to be considered. The second WP (WP2) aimed at studying the mobilisation of DNA-bacteriophages from soil to soil water triggered by precipitation events, where two hypotheses were:

1. Gradual changes in bacteriophage's community composition within and between the soil and soil water compartments may be triggered by precipitation events
2. The dynamics of certain DNA-bacteriophages may be observed by the loss of some DNA-bacteriophages in soils and the appearance of the same ones or others in soil water.

In contrast to the first work package, which dealt with different soil types, this second work package focuses specifically on the Weierbach site. While soil sampling is relatively straightforward, soil water sampling is somewhat more difficult. Indeed, the use of porous candles is often put into practice to collect soil water in the case of chemical water analyses. However, porous candles have never been used to recover bacteriophages potentially present in soil water. Therefore, in this section, we have first looked at the application of these porous candles for the recovery of bacteriophages both in the laboratory and directly on site. These results are presented in the format of a published scientific article. Subsequently, viral populations, particularly DNA-bacteriophage species, have been studied from both soil water and soil samples. Finally, their dynamics as a function of climatic data, notably rainfall, have been explored to highlight potential variations in their abundance on a temporal scale. The latter results are presented in the format of a scientific article, as this one is in the process of being submitted for publication.

As for the WP1, each part will be accompanied by introductory and concluding paragraphs which explain in detail the context of each part.

3.2.1 Optimisation methodology 2: Investigation of the passage of bacteriophages through the porous candles

To answer the question addressed in the second WP, both soil and soil water sampling is necessary. Indeed, not only the characterisation of the bacteriophage population in the soil is important but also that of the soil water in order to be able to compare these two communities and conclude on a potential mobilisation from the soil to the soil water. However, while soil sampling is fairly simple in its design and implementation, soil water sampling is more resource intensive. Although the technology for extracting water from soil was already known and applied for many chemical analyses, its use for virus sampling was not yet known. Viruses are known to adsorb onto different surfaces, especially those with high clay content. The porous candles used for soil water collection are covered with a porous ceramic layer. This layer is mainly composed of clay, which could interact with the bacteriophages, hindering their correct collection. For this reason, scientific experiments were carried out to check whether bacteriophages could be successfully collected without being retained by this ceramic layer.

A scientific paper has been published in which these results are presented, described and discussed. This paper is presented as such in the next section.

Scientific article available at:

Florent, P., Cauchie, H.-M., Herold, M., & Ogorzaly, L. (2022). Bacteriophages pass through candle-shaped porous ceramic filters: Application for the collection of viruses in soil water. *MicrobiologyOpen*, 11, e1314. https://doi.org/10.1002/mbo3.1314

Check for updates

Received: 18 April 2022 | Accepted: 26 August 2022

DOI: 10.1002/mbo3.1314

ORIGINAL ARTICLE

MicrobiologyOpen Open Access WILEY

Bacteriophages pass through candle-shaped porous ceramic filters: Application for the collection of viruses in soil water

Perrine Florent[1,2] | **Henry-Michel Cauchie**[1] | **Malte Herold**[1] | **Leslie Ogorzaly**[1]

[1]Environmental Research and Innovation Department (ERIN), Luxembourg Institute of Science and Technology (LIST), Belvaux, Luxembourg

[2]Faculté des Sciences, de la Technologie et de la Communication (FSTC), Doctoral School in Science and Engineering (DSSE), University of Luxembourg, Esch-sur-Alzette, Luxembourg

Correspondence

Leslie Ogorzaly, Environmental Research and Innovation Department (ERIN), Luxembourg Institute of Science and Technology (LIST), 41 Rue du Brill, Belvaux L-4422, Luxembourg.
Email: leslie.ogorzaly@list.lu

Funding information

Fonds National de la Recherche Luxembourg, Grant/Award Number: 10623093

Abstract

Despite the ubiquity of viruses in soils, their diversity in soil water has not been explored, mainly due to the difficulty of collecting them. In hydrology, soil water is usually collected using porous candles. This study proposes using these porous candles as a new tool for sampling viruses in soil water to analyze their passage through the ceramic part of the candles. The recovery of the viruses was determined after filtration under laboratory conditions using three model bacteriophages (MS2, ΦX174, and Φ6) and *Escherichia coli*, at neutral and acidic pH. Then, a field experiment was carried out where soil water filtration and viral identification by metagenomic shotgun were performed. At neutral pH, all bacteriophages tested successfully passed through the porous candles during the filtration process, with reductions of 0.02 log, 0.16 log, and 0.55 log for MS2 ΦX174 and Φ6, respectively. At pH 4.4, the passage of MS2 was not affected while ΦX174 underwent a slight reduction in recovery, probably caused by adsorption onto the filter material. Regarding the application of the porous candles in the field, the results obtained allowed the successful recovery of viruses, exposing porous candles as a new method suitable for the collection of viruses from soil water in the context of the study of viral communities.

KEYWORDS

bacteriophage, metagenomics, microbial ecology, transport

1 | INTRODUCTION

Soil is a complex ecosystem composed of organic matter, minerals, air, and water. Although soil water accounts for about 20%–30% of the total soil composition, to date, it remains the least studied fraction (Kalev & Toor, 2018). Within soil environments, water freely circulates through soil pores, consisting of macro (>0.08 mm) and micropores (<0.08 mm), allowing the transport of water either between soil aggregates in the macropores or within aggregates in the micropores (Easton & Bock, 2016; Gardner, 1962). As water moves through the soil, it has the potential to carry most particles smaller than the soil pores with it, including viruses. The soil is very diverse and is the most abundant compartment of the biosphere for viruses (Kimura et al., 2008), whose abundance can reach up to 10^{10} particles per gram of soil (Ashelford et al., 2003; Kimura et al., 2008; Williamson et al., 2013). Viruses form an essential part of the microbial activities in soil and can be found in every type of soil, such as agricultural soils, forest soils, wetlands, or even cold deserts (Florent et al., 2022; Williamson et al., 2017). Bacteriophages are reported as being the most abundant viruses in soils, and thus,

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2022 The Authors. *MicrobiologyOpen* published by John Wiley & Sons Ltd.

knowledge of soil viral diversity was gained by investigating bacteriophage diversity (Ashelford et al., 2003; Swanson et al., 2009; Williamson et al., 2017). Viruses are more stable in cool, and wet environments, resulting in higher viral abundance reported in wetlands than in forests, but lower in agricultural soils than in forests (Hurst et al., 1980; Schijven & Hassanizadeh, 2000; Williamson et al., 2017). Many abiotic factors may be involved in explaining the distribution, abundance, and diversity of viruses in soils, such as soil pH, cation availability, or organic matter (Cao et al., 2016; C. Wang et al., 2019). The role of viruses is now recognized, particularly in terms of the control of bacterial population growth or in the interaction within biogeochemical cycles (Braga et al., 2020; Trubl et al., 2018). Due to the electrical charges present on the surface of viruses, most of them can interact with soil particles, through the adsorption process. This process is defined as the attachment of viral particles to the surface of any type of molecule or material (Schijven & Hassanizadeh, 2000; Zemb et al., 2013). Adsorption by electrostatic forces is affected by the environmental pH and the surface charges of the molecules on which the viruses can adsorb (Flynn & Sinreich, 2010; Germann et al., 2002). The adsorption of viruses was reported to occur on mineral surfaces, such as saprolite, colloidal particles or even clay (Flynn et al., 2004; McKay et al., 2000). This has led investigations to focus preferentially on the soil matrix, rather than soil water (Braga et al., 2020; McLeod et al., 2004).

Soil water sampling is usually carried out by ceramic filtration using porous candles with the general aim of determining the chemical composition of the soil water (i.e., pesticide, trace metal, nitrogen contents) (Paul et al., 2003; Rawles & Brakensiek, 1982; Riekerk & Morris, 1983; Rosenqvist, 1959; Yang et al., 2013). This method is based on a suction mechanism, allowing water to flow through the pores of the candles and excluding any particles larger than the pore size (typically 2 µm). The pore size is an important parameter in water treatment applications. These ceramic filters proved effective in removing microbial contaminants, such as some bacteria, protozoa, and microbial cysts, but could not entirely remove viruses, due to them being much smaller than the pore size (Lelago Bulta et al., 2019; Nigay et al., 2020; Zereffa & Bekalo, 2017). Ceramic suction samplers were used in sewage-contaminated soils (Wang et al., 1980) and the study of pathogenic virus transport in wastewaters (Lance & Gerba, 1984). In these studies, the ceramic samplers proved their efficiency in recovering viruses from tap and sewage waters moving through soils, under either saturated or unsaturated flows. However, soil water collection was carried out on soil columns constructed for laboratory experiments and no studies have sampled soil water in the field to recover natural populations of bacteriophages.

In this study, we propose using porous ceramic candles as a novel sampling method to collect viruses in soil water. However, the passage of viruses through the ceramic part of the candle is still not fully understood. Bacteriophages were reported to adsorb onto the positively charged materials added to the ceramic part of the candle (Brown & Sobsey, 2009; Michen et al., 2013). Therefore, this study aimed to determine whether viruses can pass through porous ceramic candles without this causing their loss. We assumed that the viruses were small enough to pass through the porous candle but that adsorption onto the ceramic part could interfere with their filtration. To evaluate the filtration process of viruses through porous candles, two complementary methodological approaches were set up. First, three bacteriophage models and one bacterium were selected based on their size, isoelectric point (pI), hydrophobicity, and shape to perform a series of in vitro experiments under controlled conditions. Subsequently, to verify the potential of porous candles to collect viruses directly from soil water, an *in situ* experiment was conducted with the collection of soil water samples from a forest in Luxembourg, called Weierbach. The samples were then subjected to shotgun metagenomic analysis to detect the presence of viral DNA and thus confirm the effectiveness of the candles in collecting viruses.

2 | EXPERIMENTAL PROCEDURES

2.1 | Laboratory experiments

2.1.1 | Bacteriophage strains, stock production, and quantification

To conduct the first set of experiments on porous candles, three bacteriophages were selected (MS2, ΦX174, and Φ6) depending on their size, isoelectric point (pI), hydrophobicity, and shape. *Emesvirus zinderi* (former MS2) is a small (28 nm), icosahedral, and highly hydrophobic ssRNA-bacteriophage with a pI of 3.5 (Dedeo et al., 2011; McKay et al., 1993). *Sinsheimervirus ΦX174* (ssDNA bacteriophage) has almost the same morphology as MS2, with an icosahedron of 27 nm (Elhadidy et al., 2013). With a pI ranging from 6.6 to 6.8, ΦX174 is one of the least electrostatic and hydrophobic bacteriophages known (Mi, 2020). Finally, *Cytovirus* Φ6 is a hydrophobic lipidic-enveloped ssRNA-bacteriophage, with a spherical shape of approximately 85 nm with a pI = 6.94 (Bamford et al., 1987; Fedorenko et al., 2020; Kozlowski, 2016).

The three bacteriophages were obtained from culture collections: *E. zinderi* (former *Escherichia coli* bacteriophage MS2, ATCC 15597-B1, LGC), *Sinsheimervirus ΦX174* (ATCC 13706-B1, LGC), and *Cytovirus Φ6* (DSM 21518, Deutsche Sammlung von Mikroorganismen und Zellkulturen). Also, MS2 was replicated using *E. coli* Hfr K12 strain (CIP 104130, Collection Institut Pasteur) as the bacterial host according to the ISO method 10705-1:2001. Stocks of ΦX174 were produced with *E. coli* WG5 strain (CIP 107680, Collection Institut Pasteur), as described in the ISO method 10705-2:2001. The replication of Φ6 was conducted using the bacterium *Pseudomonas* sp. (DSM 21482, Deutsche Sammlung von Mikroorganismen und Zellkulturen). The bacterial host was grown in 50 mL of TSB (Tryptone Soy Broth) for 18 h at 26°C, under agitation (110 rpm). A volume of 500 µL from the overnight culture was mixed with a fresh 50 mL of TSB for 5 to 6 h at 26°C, under agitation (110 rpm). Then, 1 mL of the stock suspension of bacteriophage Φ6 (>10^4 PFU mL^{-1})

was added to the bacterial vial and incubated at 26°C under agitation (110 rpm) until the lysis process was observed (overnight) (Pinheiro et al., 2020).

Following viral replication, bacteriophage suspensions were centrifuged at ×3000*g* for 20 min to remove the cell debris. The supernatant was then decontaminated by filtration using 0.22 µm membrane filters (MillexGP Millipore Express PES membrane, Millipore). Finally, the different bacteriophage stocks were stored at 4°C.

The bacteriophage stocks were enumerated using the double agar layer procedure according to the standard procedures ISO 10705-1:2001 and ISO 10705-2:2001 for MS2 and ΦX174, respectively. The plaque assay for Φ6 was conducted with the same protocol as for MS2 and ΦX174, using the *Pseudomonas* bacterial sp. (DSM 21482). Briefly, the host bacterium was grown on TSB for 18 h at 26°C, under agitation (110 rpm). Then, 500 µL of the previous culture was mixed with 50 mL of TSB, for 4 to 5 h (DO ≈ 0.1) and then incubated at 26°C, under agitation (110 rpm). So, 2.5 mL of ssTSA (semi-solid Tryptone Soy Agar with 0.75% agar), 1 mL of *Pseudomonas*, and 1 mL of diluted Φ6 solution were mixed and poured onto the first layer of TSA (Tryptone Soy Agar with 1.5% agar). The Petri dishes were then placed into incubation at 26°C for 24 h. The concentrations of the bacteriophage stocks were 5.00×10^9, 5.43×10^9, and 3.90×10^6 PFU mL^{-1} for MS2, ΦX174, and Φ6, respectively.

2.1.2 | *Escherichia coli* K12 strain, stock production, and quantification

In addition to the previous bacteriophages, *E. coli* K12 bacterial strain was selected to verify the effective exclusion of bacteria due to pore size. It is a Gram-negative bacterium that belongs to the *Escherichia* genus. It has a rod-shaped morphology and is about 2.0 µm long with a 0.25 to 1.0 µm diameter (Neidhardt & Kushner, 2017; Rogers & Kadner, 2020). The *E. coli* K12 strain was produced as described in the ISO method 10705-1:2001, used to produce the bacterial host of the MS2 bacteriophages. The enumeration of *E. coli* K12 strain followed the culture-based method described in the standard method ISO 10705-1:2001. Then, the bacterial solutions were quantified and expressed in CFU mL^{-1} (colony forming units per mL). The concentration of the stock solution was estimated at 1.00×10^8 CFU mL^{-1}.

2.1.3 | Porous ceramic filtration: Experimental design

The water was filtered using candle-shaped filters (2440110, SDEC, Reignac sur Indre), with a diameter of 21 mm and a length of 95 mm (Figure 1a). The candles are made of polytetrafluoroethylene (or PTFE) covered with a quartz ceramic (silicon dioxide, SiO_2), with a 2-micron pore size. This porous ceramic candle has a porous area of 33 cm^2 and hydraulic conductivity of 3.31×10^{-7} cm s^{-1}. This type of candle was chosen as it does not retain any elements or chemical compounds from samples. Moreover, the chemical elements of the candles (e.g., aluminum, sodium, iron, zinc…) were quantified as being below the detection limits, so that they do not hinder the quality of the water samples (SDEC, 2002).

The porous candle was placed in a 100-mL microbial solution (i.e., solution of MS2, Φ6, ΦX174, or *E. coli*) in a glass test tube and connected to a collection bottle via a plastic extension on a polyacetal (polyoxymethylene or POM) screw cap. The collection bottle was also connected to a P2000 electric vacuum pump with an automatic drive (1800000, SDEC, Reignac sur Indre). The pump was set to −0.8 bars to depressurize the collection bottle, thus generating the suction of the microbial solution through the porous candle by capillarity (Figure 1b).

Two experimental conditions were investigated to assess the pH effect on the filtration process through the porous candle: pH = 7.0 and pH = 4.4. For each experiment, 500 mL of microbial spike solutions (i.e., MS2, ΦX174, Φ6, and *E. coli* K12) were prepared with peptone saline water (pH 7.0 and pH 4.4) to reach a feed concentration of 10^6 PFU mL^{-1} for MS2 and ΦX174, 10^3 PFU mL^{-1} for Φ6 and 10^7 CFU mL^{-1} for *E. coli* K12. Each experiment was performed in quadruplicate using four replicate porous ceramic candles. For the experiment at pH 4.4, only the bacteriophages MS2 and ΦX174 were tested. A pH of 4.4 was chosen because it is the lowest pH measured at the Weierbach site. At this pH, the net surface charges of ΦX174 became positive (pH below the pI of the bacteriophage), whereas MS2 remained negatively charged. The bacteriophage solutions were acidified using hydrochloric acid (HCl, pH = 3.1). The microbial feed solutions, as well as the permeate solutions, were enumerated by the procedures previously described (see Sections 2.2 and 2.3), and the Log Reduction Value (LRV) was estimated for each experimental condition as follows:

$$\mathrm{LRV} = \log\left(\frac{C_p}{C_f}\right)$$

where C_f and C_p are the concentration of microorganisms in the feed solution and permeate, respectively.

2.2 | Field experiments

2.2.1 | Study site and soil water collection

The field campaign was carried out on the Weierbach plateau (latitude: 49°83'46"55"'N, longitude: 5°79'64"27"'E) and took place in November 2020. The Weierbach is a subbasin located northwest of the Attert River basin, in the Grand Duchy of Luxembourg. The subbasin has an area of 0.45 km² and belongs to the Ardennes massif in the Oesling region (Martínez-Carreras et al., 2012). The five porous candles used in the previously mentioned lab experiments were randomly installed at 20-cm soil depth in a delimited area of 25 m^2 on the Weierbach plateau. The candles were then connected to both a collection bottle and to a P2000 electric vacuum pump, as with the lab experiments (Figure 1c). The pump was set to −0.8 bars to trigger

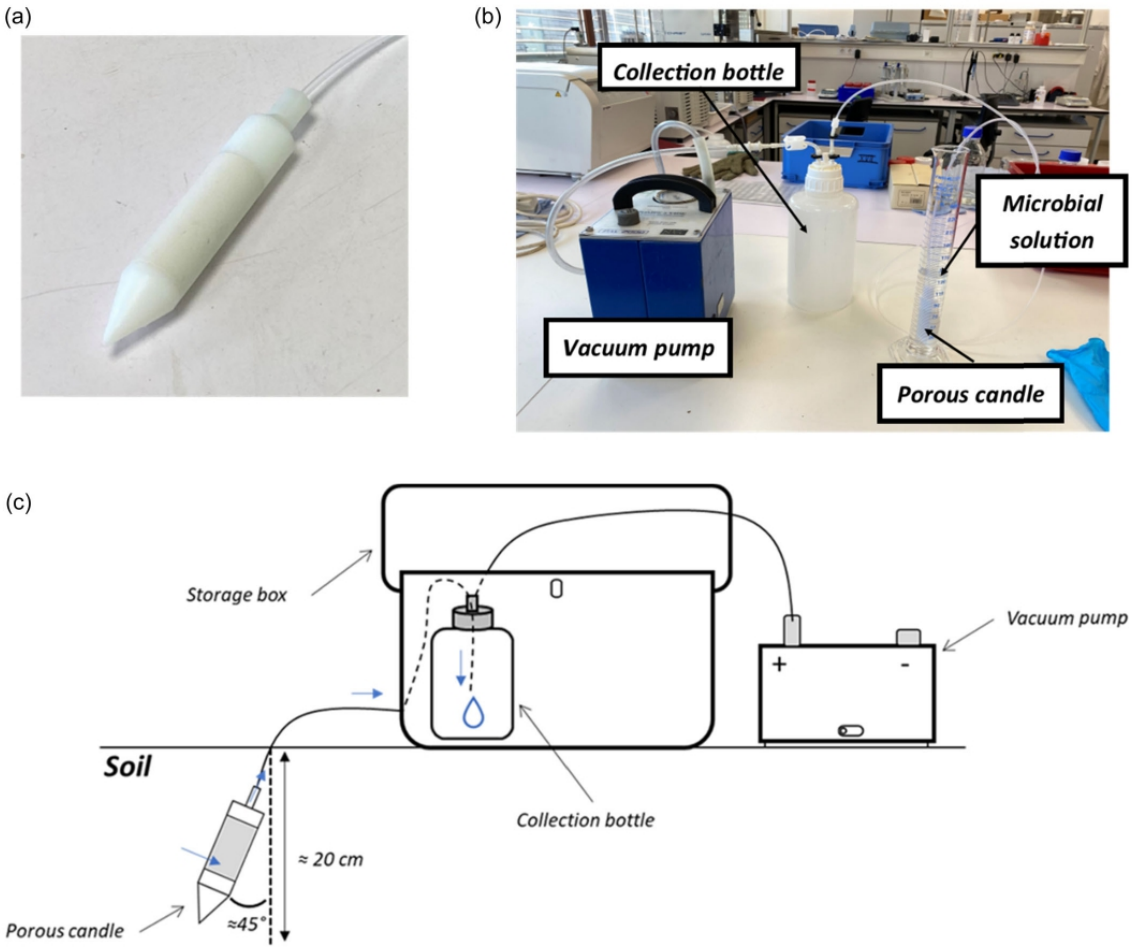


FIGURE 1 (a) Picture of a porous ceramic candle, (b) scheme of the experimental filtration design, and (c) scheme of the porous candle installed in soils, where the blue arrows show the transport of soil water from soil suction to sampling in the collection bottle.

the collection of soil water. Since the aspiration process took approximately 24 h, soil water samples were recovered from the collection bottle on the next sampling day. In addition, the volume of water collected using the porous candles in the field is often uncertain, thus, the soil water recovered in the five collection bottles was pooled to increase the final volume. Ultimately, a total of six pooled replicates were collected with final volumes ranging from 43 to 165 mL. The pH of each soil water sample was measured, and the samples were then stored at 4°C before further analyses.

2.2.2 | The concentration of soil water samples

This step aimed to increase the concentration of viral particles from the soil water samples collected directly in the field using a laboratory enrichment protocol. For this purpose, a primary concentration step using a Centricon® plus-70 centrifugal ultrafilter with a cut-off point of 10 kD (UFC710008, Millipore) was performed at ×3200*g* for 20 min, at 4°C on volume samples ranging from 43 to 165 mL, according to the manufacturer's recommendations. This step resulted in a final volume of approximately 4 mL for all samples. A secondary concentration step was carried out using an Amicon Ultra-4 10 kD (UFC801024, Millipore) at ×4000*g* for 20 min, at 4°C, according to the recommendations of the manufacturer, to reach this final volume (200 µL) for all samples.

2.2.3 | Total DNA extraction and shotgun metagenomics sequencing

Total DNA extractions were performed on the 200 µL of soil water concentrates, using the DNeasy® PowerSoil® kit (Qiagen). The

protocol followed the manufacturer's recommendations: cell lysis, followed by DNA fixation and elution. However, the recommended volume of 100 µL for the elution step was reduced to 60 µL to concentrate the DNA. Final DNA concentrations were quantified using the Invitrogen™ Qubit™ 2.0 fluorometer (Thermo Fischer) and ranged from 0.37 to 1.0 ng μL^{-1}. The extracted DNA was then stored at −20°C until further analyses. The viral communities were determined using shotgun metagenomics sequencing. From the total extracted DNA of soil water samples, library preparations were completed using the Nextera DNA Flex kit (Illumina). Paired-end sequence reads were generated using the Illumina NovaSeq. 600 (2 × 150 bp).

2.2.4 | Reference-based bioinformatics

The raw sequencing reads were quality-controlled (sliding window of 4 and minimum quality score of 20, minimum read length of 100 bps) and Illumina adapters (NexteraPE-PE. fa) were trimmed with Trimmomatic v0.39 (Bolger et al., 2014). Assemblies were performed separately for each sample using SPAdes v3.15.4 (Nurk et al., 2017) with the "meta" option and default parameters. Taxonomy classification of contigs was carried out against the complete nonredundant (nr) microbial protein sequences database (released version of April 2022, downloaded in May 2022) from NCBI using MMseqs. 2 v13.45111 (Steinegger & Söding, 2017) and the easy-taxonomy workflow that determines ancestors by searching translated ORFs (translation table 11) (Mirdita et al., 2021). Quality-filtered reads from each metagenome were mapped back to the assembled contigs using minimap2 v2.24 with the "sr" preset (Li, 2018), then sorted and indexed with SAMtools v1.15.1 (Danecek et al., 2021). Finally, metagenomic profiles were exported as comma-separated value (csv) files. Relative abundances were calculated based on the number of reads mapping to each contig. Mapped reads were divided by contig length and the total number of mapped reads per sample (Palermo et al., 2019; Zhao et al., 2021).

A mathematical transformation of the relative abundances of the microbial species was performed to normalize the replicates since the volumes recovered were different for each candle. The transformation was performed as followed:

$$At = \frac{A \times r}{V_i}$$

where At is the transformed relative abundance in 1 mL of sample, A is the relative abundance, r is the ratio of the volume (µL) extracted after DNA extraction to the volume (µL) analyzed during metagenomic sequencing, and V_i is the initial volume (ml) collected for each soil water sample.

2.3 | Statistical analyses

Statistical analyses were performed with RStudio, version 1.4.1106, and R version 4.0.4 (Rstudio Team, 2020). The figure was generated from the percentage of the transformed relative abundance using the package "ggplot2," "ggpubr," and "cowplot." Nonparametric tests (Kruskal–Wallis One Way ANOVA on rank) were used followed by Dunn's test (multiple pairwise comparisons) with Holm's correction to compare the microbial concentrations before and after filtration through the porous candles. These statistical tests were performed separately for the experiments at pH = 7 and pH = 4.4 (N = 32 and N = 16, respectively).

3 | RESULTS

3.1 | Laboratory experiments

The results regarding the filtration of all microorganisms at pH 7.0 are reported in Table 1. For bacteriophages MS2, ΦX174, and Φ6, 100 mL solutions with the respective feed concentrations of 3.65×10^6, 1.55×10^6, and 2.44×10^3 PFU mL^{-1} were brought into contact with the porous candles. After filtration through the candles, the permeate concentrations were 3.48×10^6, 1.07×10^6, and 6.88×10^2 PFU mL^{-1} for MS2, ΦX174, and Φ6, respectively. For all bacteriophage solutions, no statistical difference was observed between the permeates and the feed solutions (Kruskal–Wallis test: $p = 0.7622$ for MS2, $p = 0.8175$ for ΦX174, and $p = 1$ for Φ6, at $\alpha = 0.05$). The filtration process induced an LRV of 0.02 log, 0.16 log, and 0.55 log for MS2, ΦX174, and Φ6, respectively.

For *E. coli* K12 bacterial strain, the median permeate concentration (1.50×10^4 CFU mL^{-1}) was significantly different from that of the feed solution (7.07×10^7 CFU mL^{-1}) after filtration (Kruskal–Wallis test: $p = 0.0113$, $\alpha = 0.05$). Furthermore, of the four candles used, the LRV of *E. coli* K12 ranged from 2 log to 6 log (−3.8 log median), showing variability in the retention of *E. coli* K12 in the filter.

For the second set of experiments, bacteriophages MS2 and ΦX174 solutions were prepared at pH = 4.4 and underwent passage through four different porous candles as previously described. The initial concentrations of the MS2 and the ΦX174 solutions were 1.63×10^6 and 3.60×10^5 PFU mL^{-1}, respectively. After passing through the porous candles, the concentrations were 1.25×10^6 and 1.01×10^4 PFU mL^{-1} for MS2 and ΦX174, respectively (Table 1). No statistical difference between the concentrations before and after filtration was observed (Kruskal–Wallis test: $p = 0.3412$ for MS2 and $p = 0.1137$ for ΦX174, $\alpha = 0.05$).

At pH 7.0, MS2 and ΦX174 bacteriophages showed a LRV of 0.02 and 0.16, respectively. At pH 4.4, the LRV was 0.11 for MS2 bacteriophage, while the LRV of ΦX174 was 1.57. The results for MS2 and ΦX174 did not reveal a significant reduction after the acidification of the bacteriophage solutions (Kruskal–Wallis test: $p = 0.2045$ and 0.1363, $\alpha = 0.05$, respectively).

3.2 | Total endogenous DNA viral populations recovered from collected soil water

The statistics of the metagenomic shotgun sequencing are reported in Table 2. Shotgun sequencing produced approximately 50 million

TABLE 1 Estimated concentrations of microorganisms in feed solutions and permeates in porous candle experiments conducted at pH 7.0 and pH 4.4, and their associated LRVs

Microorganisms	pH 7.0 Feed solution Concentration + CI (PFU ml^{-1}) or (CFU ml^{-1})	Permeate ($n = 4$) Median concentration + IQR (PFU ml^{-1}) or (CFU ml^{-1})	LRV Median + IQR	pH 4.4 Feed solution Concentration + CI (PFU ml^{-1}) or (CFU ml^{-1})	Permeate ($n = 4$) Median concentration + IQR (PFU ml^{-1}) or (CFU ml^{-1})	LRV Median + IQR
MS2	$3.65 \times 10^6 \pm 3.50 \times 10^5$	$3.48 \times 10^6 \pm 8.38 \times 10^5$	−0.02 ± 0.06	$1.63 \times 10^6 \pm 4.50 \times 10^4$	$1.25 \times 10^6 \pm 2.24 \times 10^4$	−0.11 ± 0.01
ΦX174	$1.55 \times 10^6 \pm 2.50 \times 10^5$	$1.07 \times 10^6 \pm 2.39 \times 10^5$	−0.16 ± 0.09	$3.60 \times 10^5 \pm 1.00 \times 10^4$	$1.01 \times 10^4 \pm 9.05 \times 10^3$	−1.57 ± 0.42
Φ6	$2.44 \times 10^3 \pm 1.50 \times 10^1$	$6.88 \times 10^2 \pm 1.89 \times 10^2$	−0.55 ± 0.12	ND	ND	ND
Escherichia coli K12	$7.07 \times 10^7 \pm 6.00 \times 10^5$	$1.50 \times 10^4 \pm 1.13 \times 10^5$[a]	−3.80 ± 2.72	ND	ND	ND

Abbreviations: CFU ml^{-1}, colony forming unit per milliliter; CI, confidence interval; IQR, interquartile range; LRVs, Log Reduction Values; ND, not determined; PFU ml^{-1}, plaque forming unit per milliliter.

[a]Significatively different from the feed solution for $0.01 < p < 0.05$.

TABLE 2 Statistics of the metagenomic sequencing: raw data, assembly, and mapping on the soil water samples

Soil water replicates	Number of read-pairs	Yield in Mbp	Average quality (Q-score)	Number of total contigs	GC-content	N50	Identified total sequences[a]
SW1	52,568,931	14,966	35.70	748,555	57.47%	547	37.04%
SW2	50,275,130	14,523	35.62	437,122	56.15%	358	41.22%
SW3	53,588,349	15,350	35.56	675,363	56.33%	535	44.66%
SW4	48,195,690	13,687	35.81	1,675,417	57.18%	496	38.58%
SW5	48,213,814	13,625	35.59	1,813,938	55.99%	476	37.42%
SW6	51,119,776	14,665	35.47	1,837,051	56.95%	463	33.99%

Note: Total references to the total microbial metagenome (i.e., eukaryote, archaea, bacteria, and viruses).

Abbreviations: GC, guanine cytosine; Mbp, millions of base pairs; N50, the sequence length of the shortest contig at 50% of the total genome length.

[a]Calculations based on the number of total contigs.

paired reads and of these, 300,000 to 1.5 million contigs were created after assembly. Using the nr (non redundant) database and after *e*-value filtering, approximately 30%–45% of the contigs were successfully identified. Of the identified fraction, on average, 95.5% of the sequences were identified as bacterial genomes, and the second most identified microbes were viruses with an average of 2.04% viral genomes (Figure 2a). These viral genomes were mostly represented by DNA-bacteriophages (85.83% of the total viral sequences), while 1.05% represented giant viruses (all of them were nucleocytoplasmic large DNA virus, NCLDV), 0.29% eukaryotic viruses, 0.12% virophages and the remainder of the sequences (12.45%) were unclassified viruses (Figure 2b). While exploring viral composition in detail, a total of 38 viral families were identified, of which 18 were bacteriophage families (i.e., 95.05% of the viral sequences) and 13 belonged to the *Caudovirales* order. The high proportion of this order was mainly the result of a large abundance of unclassified *Caudovirales* (59.86%), *Siphoviridae* (11.72%), *Myoviridae* (8.10%), and *Podoviridae* (3.26%). In addition, five other families were identified: *Sphaerolipoviridae* (0.003%, *Halopanivirales* order), *Tectiviridae* (0.74%, *Kalamavirales* order), *Microviridae* (0.014%, *Petitvirales* order), *Inoviridae* (0.048%, *Tubulavirales* order), and *Corticoviridae* (0.017%, *Vinavirales* order) (Figure 2c).

4 | DISCUSSION

In the investigation of the passage of viruses through porous candles to be collected from soil water, the model bacteriophages selected in this study succeeded in demonstrating this process. In the study of Van der Laan et al., bacteriophages showed lower retention than *E. coli* bacteria at a neutral pH (≈−0.6 log for MS2 vs. ≈−1 log for *E. coli* on average), which is consistent with the results obtained in our study (−0.03 log for MS2 and −4 log on average for *E. coli*) (Van der Laan et al., 2014). Indeed, all tested bacteriophages successfully passed through the porous candles, mainly due to their size (27 nm for MS2 and ΦX174 and 75 nm for Φ6), which is considerably smaller than 2-µm pores. In contrast, *E. coli* K12 was significantly retained during filtration, especially due to its length (2 µm), thus proving the

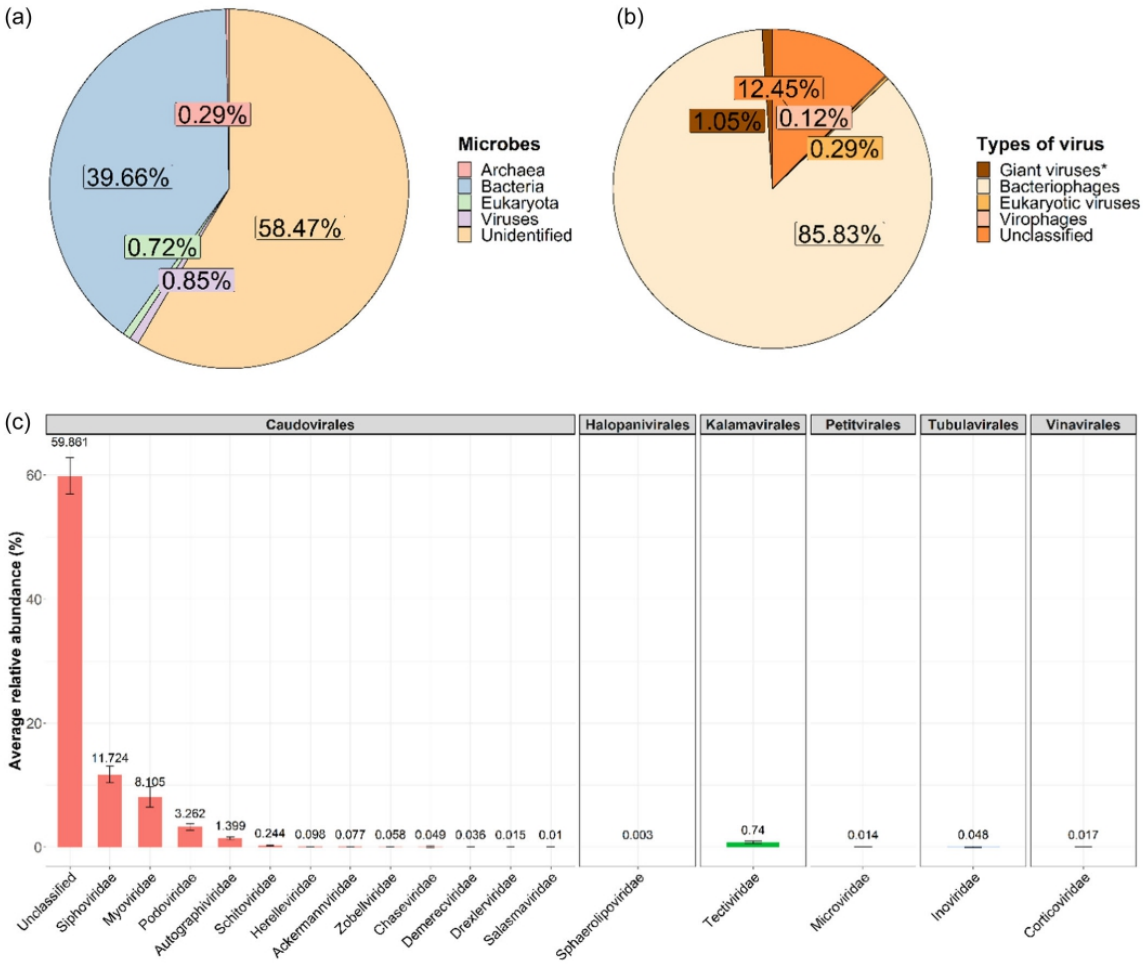


FIGURE 2 Pie charts of the relative abundances of (a) the total microbial populations and (b) the total viral populations recovered from soil water samples ($n = 6$) collected by the porous candles. (c) Bar chart representing the relative abundance of each DNA-bacteriophage family comprising the soil water samples ($n = 6$).

effective exclusion of particles larger than 2 µm. Until now, the largest viruses reported, also known as giant viruses, belong to the *Mimivirus* genus and are known to infect protists. They are non-tail-shaped particles with a capsid of approximately 500 nm and a genome size of 1300 kb (Drulis-Kawa et al., 2014; Serwer et al., 2007), which are still smaller than the pores of the porous candles. Theoretically, and based on the initial results, viruses should not be affected by size exclusion when passing through porous candles. However, other phenomena may be responsible for virus loss during the filtration process through candles. Viruses have electrical charges on their surface, inducing interactions with other molecules, such as minerals. These electrical charges depend strongly on the pH of the surrounding solutions, which is linked to the pI of viruses. The first in vitro experiment was performed at a neutral pH (pH = 7.0) so that all the bacteriophages studied had a negative electrical charge on their surface. To study whether pH could cause adsorption phenomena between the bacteriophage and the candle coating, a second experiment consisted of reducing the pH of bacteriophage solutions to 4.4 so that the bacteriophages had different surface charges. At this pH value, MS2 remained negatively charged while ΦX174 was positively charged. As expected, the concentration of MS2 did not significantly decrease after candle filtration, regardless of the change in pH. In contrast, the changes in surface charges of ΦX174 triggered a slight reduction (−1.57 log), however, this was not significantly different from the feed solution.

Two main mechanisms can be considered in the reduction of ΦX174: its inactivation or adsorption. An acidic pH (≈2) tends to inactivate bacteriophages due to the denaturation of proteins or nucleic acids (Feng et al., 2003; Jurczak-Kurek et al., 2016; Nishide et al., 2011). Furthermore, an acidic pH can also be responsible for the aggregation

process. Indeed, bacteriophages tend to aggregate with each other when the pH goes below the pI (Jończyk et al., 2011; Langlet et al., 2007). This process increases the apparent size of the particles to a few micrometers, inducing potential obstacles during their passage through the filter pores. This phenomenon can be observed in a plaque assay since the aggregation of bacteriophages results in a reduction in the number of plaques (Langlet et al., 2007, 2008). However, our results (Appendix) did not reveal that a pH of 4.4 had a significant effect on the inactivation of ΦX174, supporting the adsorption hypothesis. A study on viral adsorption during filtration processes highlighted the stability of MS2 at different pH values compared to ΦX174 (Elhadidy et al., 2013). More precisely, the repulsive forces between ΦX174 and the ceramic surface were reduced as the pH decreased. The adsorption process occurs either through electrostatic forces or hydrophobic interactions (Chattopadhyay & Puls, 2000; Flynn et al., 2004; Käss, 2004; Zemb et al., 2013). The ceramic part of the candles used in this study is composed of quartz, also called silicon dioxide, formed of silicon (Si^{+4}) and oxygen (O^{-2}), a hydrophobic molecule. Note, MS2 is highly hydrophobic, while ΦX174 has low hydrophobicity (Farkas et al., 2015; Kreißel et al., 2012; Oudega et al., 2021). Therefore, ΦX174 will preferentially interact with the hydrophobic surface of the candle, compared to MS2. However, a slight reduction of ΦX174 was observed when the pH was changed while the hydrophobic interactions were not impacted by pH change, reinforcing potential adsorption by electrostatic interactions. The silicon (cation) is in the central position of the quartz surrounded by four oxygen atoms (anion), which makes the quartz inert and incapable of electrostatic interaction with ΦX174. However, the ceramic part is made of clay, a natural soil material composed of silica, alumina, and water, and presents a permanent negative charge (Kumari & Mohan, 2021; Mohammed et al., 2021). Since bacteriophage ΦX174 is positively charged after the change in pH, electrostatic interactions with the negative charges of the ceramic could have caused the observed reduction in ΦX174 concentration. Such interactions are well known and have particularly been documented between bacteriophages and mineral surfaces, such as saprolite (Flynn et al., 2004; McKay et al., 2000) or metal oxides (i.e., 2–4 log of loss) (Flynn et al., 2015; Kvitsand et al., 2015). Despite the slight difference observed in the reduction of ΦX174, it is important to note that this reduction was not significantly different from that of the feed solution, allowing us to assume the effectiveness of the porous candles in collecting bacteriophages, and thus, viruses smaller than 2 µm.

Soil viral communities are predominantly composed of bacteriophages, and more particularly of DNA-bacteriophages (Emerson, 2019; Trubl et al., 2020). This study therefore focused the aim on the collection of DNA viruses. However, it should be noticed that RNA viruses are also an important part of the soil viral community, and they could be collected and detected using library preparation adapted for RNA viruses (i.e., including a retro-transcription step). As the analysis of the Weierbach soils showed, DNA viruses were successfully collected from the soil water with the porous candles and were the second most identified population after bacteria. In particular, DNA-bacteriophages were the most frequently found viruses with identified contigs belonging mainly to the most representative families of viral taxonomy: *Siphoviridae*, *Myoviridae*, and *Podoviridae*. The taxonomy was assigned using the nr database released in April 2022, however, it is noticeable that the viral taxonomy built by the International Committee on Taxonomy of Viruses (ICTV) is nowadays updated according to the genetic similarities between bacteriophages, including a reclassification of the families observed in the current study, such as *Siphoviridae*, *Myoviridae*, and *Podoviridae*. However, it is noteworthy that the bacterial contigs from our samples represented 90% of the total identified contigs. Among the limitations of viral metagenomics, the small size of viral genomes (i.e., up to 500 kbp) compared to bacterial genomes (i.e., up to 8 Mbp) can make viral genome reconstruction challenging during *de novo* assembly (Hatfull & Hendrix, 2011; Koduru, 2019; Rose et al., 2016). Indeed, although viruses are more abundant in environments than their hosts, the viral genomes are less represented than those of their hosts within the bulk metagenomes, which can lead to an over-representation of bacterial contigs after shotgun metagenomics compared to that of the viruses. To mitigate this, enrichment and amplification approaches are required before extraction to increase the DNA concentration (Gaafar & Ziebell, 2020; Hall et al., 2014; Lien et al., 2007). Additionally, despite the growing number of viruses being discovered, viral genomes in the general databases (e.g., RefSeq, nr, Genbank) remain underrepresented compared to bacterial genomes (Rose et al., 2016). These databases are also dependent on research topics as they are built based on repository submission, and thus, require extensive alignment methods (Phadke et al., 2021). In this study, bioinformatics tools not specifically targeting viruses were used to identify all microbial populations (i.e., virus, bacteria, archaea…), as the detection of other microorganisms than viruses was also of interest in the study of porous candle efficiency. However, when studying viral diversity, this bioinformatics pipeline may under-detect viruses and virus-specific bioinformatics tools would be preferable for further analysis (Roux et al., 2015). Nevertheless, our bioinformatics pipeline did successfully detect viral populations from the collected soil water and allowed us to prove that viruses passed through the porous candles.

Most publications report on the use of ceramic filtration in microbiology, which aims to remove microbes, including viruses, during water treatment processes while their passage through the ceramic has been poorly studied (Van der Laan et al., 2014; Michen et al., 2012; Nigay et al., 2020). When using porous ceramic filters to clean water of biological pathogens, such as bacteria, parasites, or even fungi and suspended particles (e.g., turbidity, soil aggregates), viral removal has always been a challenge (Hammel et al., 2014; Lelago Bulta et al., 2019; Zereffa & Bekalo, 2017). The generally small size of viruses prevents them from being retained outside of the filter (Michen et al., 2012). This ability of viruses to pass through the pores may be beneficial for collecting them from soil water. Indeed, this method has already been used to collect trace metals, pesticides, or nitrogen in the soil (SDEC, 2002), and it may be applicable for viruses. Soil is a complex compartment with many organisms that can be detected by molecular techniques. When collecting soil water only particles smaller than 2 µm can pass through the porous candles. This can be an interesting advantage when collecting soil water for viral analyses, especially to reduce the sampling quantity of larger bacteria. Additionally, porous

candles are easy to install in the soil and can be reused in new sites after rinsing, without significantly disturbing the soil structure (Curley et al., 2011). This soil water sampler works in all types of soil (i.e., clay, sandy, silty) and allows analyses at any depth at any time (Grobler et al., 2003). However, it is noteworthy that the efficiency of the device will be decreased in drier soils, and the collected volume will depend on the soil water content. Based on the results obtained in the laboratory experiments, the variability between porous candles in the recovery of bacteriophage was found to be low. However, the laboratory experiments do not take into account the complexity of the soil. First, viruses can attach to soil particulates through either electrostatic forces or hydrophobic bindings, which may interfere with the collection of viruses from soil water (Bitton, 1975; Davis et al., 2006). The attachment process in the soil is highly complex since it depends on many factors, such as the soil pH and texture, or the organic matter content, and thus, is very difficult to investigate. Then, variability in nutrient concentrations after collection with ceramic samplers has been observed (Hansen & Harris, 1975). In addition, the presence of macropores and micropores in soils results in preferential pathways for water, which are impossible to predict during collection (Curley et al., 2011). This can lead to difficulties in anticipating the volume of soil water collected, which is highly dependent on soil properties and climate conditions (Holder et al., 1991). After the collection of soil viruses, metagenomics analysis has identified viruses that were able to pass through the porous candles, but it does not provide information on their infectivity or integrity. Indeed, these viruses may be either intact or in the state of free DNA or RNA. Culture-based tests would be needed to verify this, however, this application is difficult to implement for soil viral populations, since first, most viruses are still poorly known or not known at all, and second, most host strains are not available for detection and replication in the laboratory.

The porous candles showed very promising results in the collection of viruses from soil water since no significant loss after passage through the ceramic was observed under laboratory conditions, and natural viruses including bacteriophages were successfully detected after in situ samplings. Therefore, this new virological sampling device could open new perspectives in the study of terrestrial viral diversity and its ecological dynamics.

5 | CONCLUSIONS

Porous ceramic candles have proven to be useful for collecting bacteriophages from liquid media in a laboratory experiment and for collecting viruses from soil water in the field. Indeed, their small size prevented the size-exclusion of the viruses through the candles. As expected, the acidification of the solution did not affect the passage of MS2. More surprisingly, ΦX174 was also not significantly impacted, despite the change in its surface charges. However, a slight reduction in the concentration of ΦX174 was observed upon acidification of the ΦX174 solution, mostly due to adsorption by electrostatic forces between bacteriophages on the ceramic part of the candle. Ultimately, the successful collection of the viruses during the field experiment allowed us to conclude the ability and the interest of the porous candles to collect such small microorganisms from soil water.

AUTHOR CONTRIBUTIONS

Perrine Florent: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Writing–original draft (lead); Writing–review & editing (equal). **Henry-Michel Cauchie**: Formal analysis (supporting); Funding acquisition (supporting); Investigation (supporting); Methodology (supporting); Supervision (supporting); Writing–original draft (supporting); Writing–review & editing (equal). **Malte Herold**: Investigation (supporting); Methodology (supporting); Software (lead). **Leslie Ogorzaly**: Conceptualization (supporting); Formal analysis (supporting); Funding acquisition (supporting); Investigation (supporting); Methodology (supporting); Supervision (lead); Validation (supporting); Writing–original draft (supporting); Writing–review & editing (equal).

ACKNOWLEDGMENTS

The authors would like to acknowledge the Luxembourg National Research Fund (FNR) for granting and supporting the PRIDE program containing the Doctoral Training Unit Hydro-CSI project (PRIDE15/10623093—HYDRO-CSI). The authors would also like to thank Jean-François Iffly and Jérôme Juilleret for their valuable assistance in the set-up of the experiment and their expertise in porous candles.

CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

The raw sequence data generated and analyzed in the current study are available in the National Centre for Biotechnology Information Databases (Sequence Read Archive) repository, under accession number PRJNA823538: http://www.ncbi.nlm.nih.gov/bioproject/823538.

ETHICS STATEMENT

None required.

ORCID

Malte Herold http://orcid.org/0000-0003-2627-0159
Leslie Ogorzaly http://orcid.org/0000-0001-8393-4818

REFERENCES

Ashelford, K. E., Day, M. J., & Fry, J. C. (2003). Elevated abundance of bacteriophage infecting bacteria in soil. *Applied and Environmental Microbiology*, *69*, 285–289.

Bamford, D. H., Romantschuk, M., & Somerharjul, P. J. (1987). Membrane fusion in prokaryotes: bacteriophage Φ6 membrane fuses with the pseudomonas syringae outer membrane. *EMBO Journal*, *6*, 1467–1473.

Bitton, G. (1975). Adsorption of viruses onto surfaces soil and water. *Water Research*, *9*, 473–484.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for illumina sequence data. *Bioinformatics*, *30*, 2114–2120.

Braga, L. P. P., Spor, A., Kot, W., Breuil, M. C., Hansen, L. H., Setubal, J. C., & Philippot, L. (2020). Impact of phages on soil bacterial

communities and nitrogen availability under different assembly scenarios. *Microbiome*, *8*, 1–14.

Brown, J., & Sobsey, M. D. (2009). Ceramic media amended with metal oxide for the capture of viruses in drinking water. *Environmental Technology*, *30*, 379–391.

Cao, H., Chen, R., Wang, L., Jiang, L., Yang, F., Zheng, S., Wang, G., & Lin, X. (2016). Soil ph, total phosphorus, climate and distance are the major factors influencing microbial activity at a regional spatial scale. *Scientific Reports*, *6*, 25815.

Chattopadhyay, S., & Puls, R. W. (2000). Forces dictating colloidal interactions between viruses and soil. *Chemosphere*, *41*(8), 1279–1286. https://doi.org/10.1016/s0045-6535(99)00519-6

Curley, E. M., O'Flynn, M. G., & McDonnell, K. P. (2011). The use of porous ceramic cups for sampling soil pore water from the unsaturated zone. *International Journal of Soil Science*, *6*, 1–11.

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., Whitwham, A., Keane, T., McCarthy, S. A., Davies, R. M., & Li, H. (2021). Twelve years of SAMtools and BCFtools. *GigaScience*, *10*, 1–4.

Davis, J. A., Farrah, S. R., & Wilkie, A. C. (2006). Adsorption of viruses to soil: impact of anaerobic treatment. *Water Science and Technology*, *54*, 161–167.

Dedeo, M. T., Finley, D. T., & Francis, M. B. (2011). *Viral capsids as self-assembling templates for new materials* (1st ed.). Elsevier Inc.

Drulis-Kawa, Z., Olszak, T., Danis, K., Majkowska-Skrobek, G., & Ackermann, H. W. (2014). A giant pseudomonas phage from Poland. *Archives of Virology*, *159*, 567–572.

Easton, Z. M., & Bock, E. (2016). *Soil and soil water relationships* (pp. 1–9). Virginia Cooperative Extension.

Elhadidy, A. M., Peldszus, S., & Van Dyke, M. I. (2013). An evaluation of virus removal mechanisms by ultrafiltration membranes using MS2 and φX174 bacteriophage. *Separation and Purification Technology*, *120*, 215–223.

Emerson, J. B. (2019). Soil viruses: A new hope. *mSystems*, *4*, 1–4.

Farkas, K., Varsani, A., & Pang, L. (2015). Adsorption of rotavirus, MS2 bacteriophage and surface-modified silica nanoparticles to hydrophobic matter. *Food and Environmental Virology*, *7*, 261–268.

Fedorenko, A., Grinberg, M., Orevi, T., & Kashtan, N. (2020). Survival of the enveloped bacteriophage Phi6 (a surrogate for SARS-CoV-2) in evaporated saliva microdroplets deposited on glass surfaces. *Nature*, *10*, 1–10.

Feng, Y. Y., Ong, S. L., Hu, J. Y., Tan, X. L., & Ng, W. J. (2003). Effects of ph and temperature on the survival of coliphages MS2 and Qβ. *Journal of Industrial Microbiology and Biotechnology*, *30*, 549–552.

Florent, P., Cauchie, H.-M., Herold, M., Jacquet, S., & Ogorzaly, L. (2022). Soil ph, calcium content and bacteria as major factors responsible for the distribution of the known fraction of the DNA bacteriophage populations in soils of Luxembourg. *Microorganisms*, *10*, 1458.

Flynn, R. M., Hunkeler, D., Guerin, C., Burn, C., Rossi, P., & Aragno, M. (2004). Geochemical influences on H40/1 bacteriophage inactivation in glaciofluvial sands. *Environmental Geology*, *45*, 504–517.

Flynn, R. M., Mallèn, G., Engel, M., Ahmed, A., & Rossi, P. (2015). Characterizing aquifer heterogeneity using bacterial and bacteriophage tracers. *Journal of Environmental Quality*, *44*, 1448–1458.

Flynn, R. M., & Sinreich, M. (2010). Characterisation of virus transport and attenuation in epikarst using short pulse and prolonged injection multi-tracer testing. *Water Research*, *44*, 1138–1149.

Gaafar, Y. Z. A., & Ziebell, H. (2020). Comparative study on three viral enrichment approaches based on RNA extraction for plant virus/viroid detection using high-throughput sequencing. *PLoS One*, *15*, 1–17.

Gardner, W. H. (1962). *How water moves in the soil*. Crops and soils magazine.

Germann, P. F., Alaoui, A., & Riesen, D. (2002). Drag force approach to the transport of colloids in unsaturated soils. *Water Resources Research*, *38*, 18-1–18-15.

Grobler, L., Claassens, A. S., & Annandale, J. G. (2003). Ceramic suction samplers: A reliable method for extracting soil solutions for analysis. *South African Journal of Plant and Soil*, *20*, 161–164.

Hall, R. J., Wang, J., Todd, A. K., Bissielo, A. B., Yen, S., Strydom, H., Moore, N. E., Ren, X., Huang, Q. S., Carter, P. E., & Peacey, M. (2014). Evaluation of rapid and simple techniques for the enrichment of viruses prior to metagenomic virus discovery. *Journal of Virological Methods*, *195*, 194–204.

Hammel, E. C., Ighodaro, O. L. R., & Okoli, O. I. (2014). Processing and properties of advanced porous ceramics: An application based review. *Ceramics International*, *40*, 15351–15370.

Hansen, E. A., & Harris, A. R. (1975). Validity of soil-water samples collected with porous ceramic cups. *Soil Science Society of America Journal*, *39*, 528–536.

Hatfull, G. F., & Hendrix, R. W. (2011). Bacteriophages and their genomes. *Current Opinion in Virology*, *1*, 298–303.

Holder, M., Brown, K. W., Thomas, J. C., Zabcik, D., & Murray, H. E. (1991). Capillary-wick unsaturated zone soil pore water sampler. *Soil Science Society of America Journal*, *55*, 1195–1202.

Hurst, C. J., Gerba, C. P., & Cech, I. (1980). Effects of environmental variables and soil characteristics on virus survival in soil. *Applied and Environmental Microbiology*, *40*, 1067–1079.

Jończyk, E., Kłak, M., Miedzybrodzki, R., & Górski, A. (2011). The influence of external factors on bacteriophages-review. *Folia Microbiologica (Praha)*, *56*, 191–200.

Jurczak-Kurek, A., Gąsior, T., Nejman-Faleńczyk, B., Bloch, S., Dydecka, A., Topka, G., Necel, A., Jakubowska-Deredas, M., Narajczyk, M., Richert, M., Mieszkowska, A., Wróbel, B., Węgrzyn, G., & Węgrzyn, A. (2016). Biodiversity of bacteriophages: Morphological and biological properties of a large group of phages isolated from urban sewage. *Scientific Reports*, *6*, 1–17.

Kimura, M., Jia, Z. J., Nakayama, N., & Asakawa, S. (2008). Ecology of viruses in soils: Past, present and future perspectives. *Soil Science and Plant Nutrition*, *54*, 1–32.

Kalev, S. D., & Toor, G. S. (2018). The composition of soils and sediments. In B. Török & T. Dransfield (Eds.), *Green Chemistry* (pp. 339–357). Elsevier.

Käss, W. (2004). Phagen. In *Geohydrologische Markierungstechnik* (pp. 209–232). Schweizerbart Science Publishers, Lehrbuch der Hydrogeologie.

Koduru, S. K. (2019). The impact of bioinformatics tools in the development of antimicrobial drugs and other agents. In V. Buddola (Ed.), *Recent developments in applied microbiology and biochemistry* (pp. 335–347). Academic Press.

Kozlowski, L. P. (2016). Proteome-pI: Proteome isoelectric point database. *Nucleic Acids Research*, *45*, 1–5.

Kreißel, K., Bösl, M., Lipp, P., Franzreb, M., & Hambsch, B. (2012). Study on the removal efficiency of UF membranes using bacteriophages in bench-scale and semi-technical scale. *Water Science and Technology*, *66*, 1195–1202.

Kumari, N., & Mohan, C. (2021). Basics of clay minerals and their characteristic properties. In G. Morari & D. Nascimento (Eds.), *Clay and clay minerals* (pp. 1–30). IntechOpen.

Kvitsand, H. M. L., Ilyas, A., & Østerhus, S. W. (2015). Rapid bacteriophage MS2 transport in an oxic sandy aquifer in cold climate: Field experiments and modeling. *Water Resources Research*, *51*, 9725–9745.

Van der Laan, H., van Halem, D., Smeets, P. W. M. H., Soppe, A. I. A., Kroesbergen, J., Wubbels, G., Nederstigt, J., Gensburger, I., & Heijman, S. G. J. (2014). Bacteria and virus removal effectiveness of ceramic pot filters with different silver applications in a long term experiment. *Water Research*, *51*, 47–54.

Lance, J. C., & Gerba, C. P. (1984). Virus movement in soil during saturated and unsaturated flow. *Applied and Environmental Microbiology*, *47*, 335–337.
Langlet, J., Gaboriaud, F., Duval, J. F. L., & Gantzer, C. (2008). Aggregation and surface properties of f-specific RNA phages: Implication for membrane filtration processes. *Water Research*, *42*, 2769–2777.
Langlet, J., Gaboriaud, F., & Gantzer, C. (2007). Effects of ph on plaque forming unit counts and aggregation of MS2 bacteriophage. *Journal of Applied Microbiology*, *103*, 1632–1638.
Lelago Bulta, A., Arega, G., & Micheal, W. (2019). Evaluation of the efficiency of ceramic filters for water treatment in kambata tabaro zone, Southern Ethiopia. *Environmental Systems Research*, *8*, 1–15.
Li, H. (2018). Minimap2: Pairwise alignment for nucleotide sequences. *Bioinformatics*, *34*, 3094–3100.
Lien, K. Y., Lin, J. L., Liu, C. Y., Lei, H. Y., & Lee, G. B. (2007). Purification and enrichment of virus samples utilizing magnetic beads on a microfluidic system. *Lab on a Chip*, *7*, 868–875.
Martínez-Carreras, N., Krein, A., Gallart, F., Iffly, J. F., Hissler, C., Pfister, L., Hoffmann, L., & Owens, P. N. (2012). The influence of sediment sources and hydrologic events on the nutrient and metal content of fine-grained sediments (attert river basin, Luxembourg). *Water, Air, and Soil Pollution*, *223*, 5685–5705.
McKay, L. D., Cherry, J. A., Bales, R. C., Yahya, M. T., & Gerba, C. P. (1993). A field example of bacteriophage as tracers of fracture flow. *Environmental Science and Technology*, *27*, 1075–1079.
McKay, L. D., Sanford, W. E., & Strong, J. M. (2000). Field-scale migration of colloidal tracers in a fractured shale saprolite. *Ground Water*, *38*, 139–147.
McLeod, M., Aislabie, J., Ryburn, J., & McGill, A. (2004). Microbial and chemical tracer movement through granular, ultic, and recent soils. *New Zealand Journal of Agricultural Research*, *47*, 557–563.
Mi, X. (2020). Detection and thermal stabilization of virus based on surface properties (PhD thesis). Michigan Technologica University.
Michen, B., Fritsch, J., Aneziris, C., & Graule, T. (2013). Improved virus removal in ceramic depth filters modified with MgO. *Environmental Science and Technology*, *47*, 1526–1533.
Michen, B., Meder, F., Rust, A., Fritsch, J., Aneziris, C., & Graule, T. (2012). Virus removal in ceramic depth filters based on diatomaceous earth. *Environmental Science and Technology*, *46*, 1170–1177.
Mirdita, M., Steinegger, M., Breitwieser, F., Sö Ding, J., & Levy Karin, E. (2021). Fast and sensitive taxonomic assignment to metagenomic contigs. *Bioinformatics*, *37*, 3029–3031.
Mohammed, I., Al Shehri, D., Mahmoud, M., Kamal, M. S., & Alade, O. S. (2021). A surface charge approach to investigating the influence of oil contacting clay minerals on wettability alteration. *ACS Omega*, *6*, 12841–12852.
Neidhardt, F. C., & Kushner, S. (2017). *Escherichia coli*. In S. Maloy & K. Hughes (Eds.), *Reference module in life sciences* (pp. 522–527), from a previous edition of Brenner's Encyclopedia of Genetics. Elsevier.
Nigay, P.-M., Salifu, A. A., Obayemi, J. D., White, C. E., Nzihou, A., & Soboyejo, W. O. (2020). Assessment of ceramic water filters for the removal of bacterial, chemical, and viral contaminants. *Journal of Environmental Engineering (Reston, VA, United States)*, *146*, 1–8.
Nishide, M., Tsujimoto, K., Uozaki, M., Ikeda, K., Yamasaki, H., Koyama, A. H., & Arakawa, T. (2011). Effects of electrolytes on virus inactivation by acidic solutions. *International Journal of Molecular Medicine*, *27*, 803–809.
Nurk, S., Meleshko, D., Korobeynikov, A., & Pevzner, P. A. (2017). MetaSPAdes: A new versatile metagenomic assembler. *Genome Research*, *27*, 824–834.
Oudega, T. J., Lindner, G., Derx, J., Farnleitner, A. H., Sommer, R., Blaschke, A. P., & Stevenson, M. E. (2021). Upscaling transport of bacillus subtilis endospores and coliphage phiX174 in heterogeneous porous media from the column to the field scale. *Environmental Science and Technology*, *55*, 11060–11069.
Palermo, C. N., Fulthorpe, R. R., Saati, R., & Short, S. M. (2019). Metagenomic analysis of virus diversity and relative abundance in a eutrophic freshwater harbour. *Viruses*, *11*(9), 792. https://doi.org/10.3390/v11090792
Paul, K. I., Polglase, P. J., O'Connell, A. M., Carlyle, J. C., Smethurst, P. J., & Khanna, P. K. (2003). Defining the relation between soil water content and net nitrogen mineralization. *European Journal of Soil Science*, *54*, 39–48.
Phadke, S., Macherla, S., & Scheuermann, R. H. (2021). Database and analytical resources for viral research community. *Encyclopedia of Virology*, 141–152. https://doi.org/10.1016/B978-0-12-809633-8.20995-3
Pinheiro, L. A. M., Pereira, C., Barreal, M. E., Gallego, P. P., Balcão, V. M., & Almeida, A. (2020). Use of phage ϕ6 to inactivate pseudomonas syringae pv. actinidiae in kiwifruit plants: In vitro and ex vivo experiments. *Applied Microbiology and Biotechnology*, *104*, 1319–1330.
Rawles, W. J., & Brakensiek, D. L. (1982). Estimating soil water retention from soil properties. *Journal of the Irrigation and Drainage Division*, *108*, 166–171.
Riekerk, H., & Morris, L. A. (1983). A constant-potential soil water sampler. *Soil Science Society of America Journal*, *47*, 606–608.
Rogers, K., & Kadner, R. J. (2020). *Bacteria*. Encyclopedia Britannica.
Rose, R., Constantinides, B., Tapinos, A., Robertson, D. L., & Prosperi, M. (2016). Challenges in the analysis of viral metagenomes. *Virus Evolution*, *2*, 1–11.
Rosenqvist, I. T. (1959). Physico-chemical properties of soils: Soil-water systems. *Journal of the Soil Mechanics and Foundations Division*, *85*, 31–53.
Roux, S., Enault, F., Hurwitz, B. L., & Sullivan, M. B. (2015). VirSorter: mining viral signal from microbial genomic data. *PeerJ*, *2015*, e985.
RStudio Team. (2020). RStudio: Integrated development for R. RStudio; PBC. Accessed July 11, 2022. http://www.rstudio.com/
Schijven, J. F., & Hassanizadeh, S. M. (2000). Removal of viruses by soil passage: Overview of modeling, processes, and parameters. *Critical Reviews Environmental Science Technology*, *30*, 49–127.
SDEC. (2002). Lysimètres en PTFE/quartz pour ETM éléments métalliques en trace. Accessed September 6, 2022. https://www.sdec-france.com/lysimetres-ptfe-quartz-pour-solution-du-sol.html
Serwer, P., Hayes, S. J., Thomas, J. A., & Hardies, S. C. (2007). Propagating the missing bacteriophages: A large bacteriophage in a new class. *Virology Journal*, *4*, 1–5.
Steinegger, M., & Söding, J. (2017). MMseqs. 2 enables sensitive protein sequence searching for the analysis of massive data sets. *Nature Biotechnology*, *35*, 1026–1028.
Swanson, M. M., Fraser, G., Daniell, T. J., Torrance, L., Gregory, P. J., & Taliansky, M. (2009). Viruses in soils: Morphological diversity and abundance in the rhizosphere. *Annals of Applied Biology*, *155*, 51–60.
Trubl, G., Hyman, P., Roux, S., & Abedon, S. T. (2020). Coming-of-age characterization of soil viruses: A user's guide to virus isolation, detection within metagenomes, and viromics. *Soil System*, *23*(4), 23.
Trubl, G., Jang, H. B., Roux, S., Emerson, J. B., Solonenko, N., Vik, D. R., Solden, L., Ellenbogen, J., Runyon, A. T., Bolduc, B., Woodcroft, B. J., Saleska, S. R., Tyson, G. W., Wrighton, K. C., Sullivan, M. B., & Rich, V. I. (2018). Soil viruses are underexplored players in ecosystem carbon processing. *mSystem*, *3*, 1–59.
Wang, C., Zhou, X., Guo, D., Zhao, J., Yan, L., Feng, G., Gao, Q., Yu, H., & Zhao, L. (2019). Soil ph is the primary factor driving the distribution and function of microorganisms in farmland soils in northeastern China. *Annals of Microbiology*, *69*, 1461–1473.
Wang, D. S., Lance, J. C., & Gerba, C. P. (1980). Evaluation of various soil water samplers for virological sampling. *Applied and Environmental Microbiology*, *39*, 662–664.
Williamson, K. E., Corzo, K. A., Drissi, C. L., Buckingham, J. M., Thompson, C. P., & Helton, R. R. (2013). Estimates of viral

abundance in soils are strongly influenced by extraction and enumeration methods. *Biology and Fertility of Soils*, *49*, 857–869.
Williamson, K. E., Fuhrmann, J. J., Wommack, K. E., & Radosevich, M. (2017). Viruses in soil ecosystems: An unknown quantity within an unexplored territory. *Annual Review of Virology*, *4*, 201–219.
Yang, D., Qi, S., Zhang, J., Wu, C., & Xing, X. (2013). Organochlorine pesticides in soil, water and sediment along the jinjiang river mainstream to quanzhou bay, southeast China. *Ecotoxicology and Environmental Safety*, *89*, 59–65.
Zemb, O., Manefield, M., Thomas, F., & Jacquet, S. (2013). Phage adsorption to bacteria in the light of the electrostatics: A case study using *E. coli*, T2 and flow cytometry. *Journal of Virological Methods*, *189*, 283–289.
Zereffa, E. A., & Bekalo, T. B. (2017). Clay ceramic filter for water treatment. *Materials Science and Applied Chemistry*, *34*, 69–74.
Zhao, Y., Li, M.-C., Konaté, M. M., Chen, L., Das, B., Karlovich, C., Williams, P. M., Evrard, Y. A., Doroshow, J. H., & McShane, L. M. (2021). TPM, FPKM, or normalized counts? A comparative study of quantification measures for the analysis of RNA-seq data from the NCI patient-derived models repository. *Journal of Translational* Medicine, *19*(1). https://doi.org/10.1186/s12967-021-02936-w

How to cite this article: Florent, P., Cauchie, H.-M., Herold, M., & Ogorzaly, L. (2022). Bacteriophages pass through candle-shaped porous ceramic filters: Application for the collection of viruses in soil water. *MicrobiologyOpen*, 11, e1314. https://doi.org/10.1002/mbo3.1314

APPENDIX A:

Effect of acidic pH on ΦX174 inactivation

Material and methods

Acidic pH (here, 4.4) could induce an indirect reduction in the concentration of ΦX174 through the adsorption process, however, pH is also known to directly affect the inactivation of ΦX174, leading to concentration reductions (Feng et al., 2003; Nishide et al., 2011; Jurczak-Kurek et al., 2016). Therefore, to test whether pH 4.4 could cause a reduction in ΦX174 concentration, a solution of ΦX174 was prepared with peptone saline water and acidified to pH 4.4 using hydrochloric acid (HCl, pH = 3.1) to reach a concentration of 1.60×105 PFU mL^{-1}. The concentrations of ΦX174 were determined every 2 h for a total duration of 8 h, thus covering the time of the laboratory experiment for ΦX174, using the double agar layer assay (according to the procedures of ISO 10705-2:2001). The effect of pH on ΦX174 inactivation was tested using a nonparametric test (Kruskal–Wallis One Way ANOVA on rank) for small comparisons ($n = 2$ replicates).

Results

The analysis performed on the effect of pH 4.4 on ΦX174 inactivation revealed a constant concentration of ΦX174 for 8 h (Figure A1). The initial concentration of ΦX174 of 1.60×105 PFU mL^{-1} was reduced to 1.22×105 PFU ml^{-1} after 4 h and to about 1.12×105 PFU mL^{-1} at the end of the experiment (after 8 h). No significant difference in ΦX174 concentration was found all along the experiment ($p = 0.1204$, $\alpha = 0.05$).

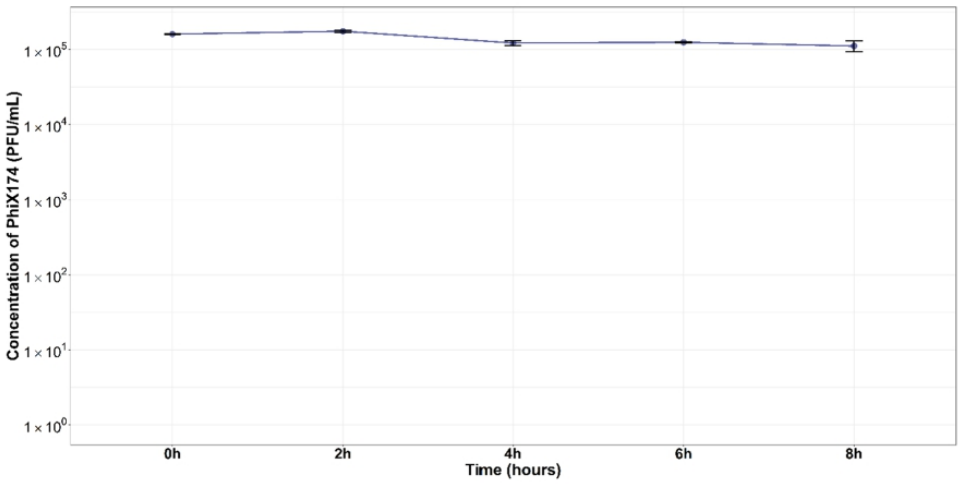


FIGURE A1 Effect of acidic pH (4.4) on ΦX174 concentration over 8 h experiment. A nonparametric test (Kruskal–Wallis One Way ANOVA on rank) for small comparisons ($n = 2$) was performed on the ΦX174 concentrations.

The experiments conducted demonstrated the effectiveness of porous candles for the recovery of bacteriophages during the collection of soil water. Indeed, under several pH conditions and using bacteriophages with different surface charges as well as *E. coli* bacterium, no significant adsorption was noticed at the soil pH corresponding to that of the Weierbach. Furthermore, when taxonomically identifying the total population recovered from the sampled soil water, the presence of bacteriophages was confirmed with approximately 85% of the viruses identified as bacteriophages. Thus, it was concluded that the porous candle collection device was very suitable for the recovery of viruses and more specifically, bacteriophages from soil water.

Thus, the validation of this new sampling method allowed the implementation of field experiments on the Weierbach to better understand the potentially existing variations in bacteriophage abundances between soil and soil water. These variations between these two soil components, the soil matrix and the soil water, may provide information on the onset of bacteriophage mobilisation, and those triggered by rainfall. This topic is the focus of the following section and is the core of the WP2 analyses presented below.

3.2.2 Mobilisation of the bacteriophages from the soil matrix to the soil water

Soil and soil water sampling was carried out during two field campaigns specifically targeting precipitation events. These precipitation events are thought to trigger potential colloid mobilisations, including naturally occurring bacteriophages in the soil. Thus, water flows within the soil could carry these bacteriophages with them. This hypothesis is central to the second WP, for which the main objective is to demonstrate the mobilisation of bacteriophages from soil to soil water. In this section, the identification of bacteriophage populations was done through shotgun metagenomics followed by a bioinformatics pipeline. Statistical analyses were then carried out to study not only the alpha and beta diversity of the bacteriophage communities but also their variation according to available climatic data such as precipitation, antecedent precipitation indices, and soil and air temperatures. These variations are expected to correlate with precipitation intensities, with the hypothesis that bacteriophage abundance would decrease in soil samples but increase in soil water samples. Furthermore, in order to compare the two types of samples (soil and soil water), the abundances of bacteriophage species were also studied as a function of certain soil properties such as pH, which has already proven in the first WP to play an important role in the distribution of bacteriophages.

In the context of this PhD work, this second WP will give us more information on the dynamics of bacteriophages, but above all, we hope to highlight patterns in the variation of certain bacteriophage families or even species. Thus, this would allow us, together with the complementary study of WP1, to have the first picture of bacteriophages potentially capable of being used as hydrological tracers.

These analyses will be the subject of a scientific article to be submitted for publication shortly. Being in a state of progress worthy of a final version, the results are presented in the form of the scientific article that it will be.

Temporal variability of natural bacteriophage populations from the terrestrial compartment in response to precipitation events.

FLORENT Perrine[1,2], CAUCHIE Henry-Michel[1], HEROLD Malte[1], OGORZALY Leslie[1]

[1] Environmental Research and Innovation Department (ERIN), Luxembourg Institute of Science and Technology (LIST), 41 rue du Brill L-4422 Belvaux, LUXEMBOURG

[2] University of Luxembourg (FSTC-DSSE), 2 avenue de l'Université L-4365 Esch-sur-Alzette, Luxembourg

Corresponding authors: leslie.ogorzaly@list.lu

1. Introduction

In the late 1980s, the investigation of bacteriophage diversity in marine waters triggered scientific interest in their role in the ecological functions of their hosts' (Ackermann and DuBow, 1987a, 1987b; Bergh et al., 1989). Subsequently, with the development of molecular techniques, the discovery of a large number of bacteriophages raised the awareness that bacteriophages are not only predators of bacteria but also entities in their own right. Particularly, with an increasing level of characterisation, bacteriophages have been implicated in important roles in numerous environmental processes such as. biogeochemical cycles, horizontal gene transfer, or variation in bacterial biomass (Hall et al., 2020; Weitz and Wilhelm, 2012). More recently, the fate and dynamics of bacteriophage populations have been the focus of ecological studies, however predominantly for the aquatic

compartment and to a lesser extent in the terrestrial compartment (Cai et al., 2022; Mohiuddin and Schellhorn, 2015; Sime-Ngando, 2014). The characterisation of soil bacteriophage populations has revealed different distributions according to soil properties (Florent et al., 2022a; Williamson et al., 2005), but little is known about their temporal variability in their natural terrestrial habitat and their potential mobilisation to other compartments.

The water cycle links all compartments (i.e. atmosphere, hydrosphere and pedosphere). This complex system is the continuous movement of water between different reservoirs and in different forms (i.e. liquid, vapour, solid). Water can enter the terrestrial compartment by infiltration and then move either vertically, percolate into deeper layers and thus reach the groundwater, or horizontally, to create subsurface flows and potentially reach surface water (Seiler and Gat, 2007). The infiltration rate is highly dependent on precipitation events. The more precipitation there is, the more infiltration there will be until the soil is saturated, and a runoff process occurs. Water flows through the soil and carries particles and colloids, including bacteriophages. The transport of bacteriophages in soil has been studied in tracing experiments with bacteriophages as hydrological tracers to investigate water flow paths, water contamination or microbial source tracking (Antonelli et al., 2016; Kinnunen, 1978; Rossi et al., 1998). In addition, numerous articles have studied the fate of bacteriophages in various environments, examining the potential effects of abiotic factors (i.e. temperature, pH, radiation) on their survival (Clokie et al., 2011; Jończyk et al., 2011; Kimura et al., 2008). However, all experiments (field or laboratory) were conducted using laboratory strains of bacteriophages that are cultivable. The natural population of bacteriophages in ecosystems is mainly composed of non-cultivable and unidentified species, for which transport and environmental effects remain largely underexplored (Batinovic et al., 2019).

Further analysis considering natural populations is needed to confirm and increase the accuracy of the knowledge gained so far.

Soil is composed of four elements which are mineral (45%), organic matter (5%), water (20-30%) and air (20-30%) (Mandal, 2008). After precipitation, infiltrated water circulating in the soil can trigger the mobilisation of colloids from one soil component to another (Mohanty et al., 2016; Rousseau et al., 2004). This mobilisation is strongly dependent not only on the water flow rate but also and especially on abiotic factors. Bacteriophages, as colloidal particles, are known to adsorb and desorb onto and from soil particles, which strongly influences bacteriophage transport. Bacteriophages have an isoelectric point (pI) which indicates the net electrical charge (i.e. positive or negative) present on their surface and allows adsorption of bacteriophages on the surrounding environment surfaces (Bitton, 1975; Rossi and Aragno, 1999; Wang et al., 2017). Adsorption of bacteriophages in the soil can also take place through hydrophobic interactions, particularly with organic matter (Leelamaanie and Karube, 2010; Sautrey et al., 2018). This adsorption-desorption process can therefore play a role in the mobilisation of bacteriophages since their attachment to soil components can hinder their transport with the water flow.

Before exploring in depth, the influence of adsorption processes on bacteriophage transport, it is first necessary to better understand the capacity of bacteriophages to be mobilised. As part of the terrestrial compartment, studying the connection between two of the terrestrial reservoirs will provide insight into the potential dynamics of bacteriophages. To this end, bacteriophage populations from soil samples will be compared to those present in the soil water fraction so that the temporal variability of bacteriophage species common to both types of samples can be studied. The proportion of soil water is strongly related to precipitation as its volume may increase immediately after rainfall, decrease when infiltrated or stabilise when soil saturation is reached (He et al., 2012). Therefore, the the variability of

bacteriophages abundances will be studied in relation to precipitation events to understand whether bacteriophage dynamics respond to the occurrences of precipitations.

The main objective of this paper is to examine whether natural soil DNA-bacteriophages species can be found in soil water after a precipitation event, indicating a potential mobilisation of DNA-bacteriophages in terrestrial environments. The two main hypotheses are (i) temporal variability of DNA-bacteriophage communities is significantly different in the soil and soil water compartments in both campaigns; (ii) dynamics of certain DNA-bacteriophage species observed in the two types of samples is triggered by precipitation events.

2. Materials and methods

2.1. Study area

This study was conducted in the Weierbach plateau. The Weierbach is a sub-basin located in the Northwest of the Attert River basin, in the commune of Rambrouch, in the Grand-Duchy of Luxembourg (latitude: 49°83’46’’55’’’N, longitude: 5°79’64’’27’’’E). The sub-basin has an area of 0.45 km² and belongs to the Ardennes massif of the Oesling region. It is underlain by schist bedrock and consists of two distinct zones: plateau landscapes with deep periglacial deposits and high storage capacity and hillslopes with shallow soils and low storage capacity (Martínez-Carreras et al., 2016; Pfister et al., 2017). The schist bedrock exhibits low permeability, explaining the high runoff rate on the Weierbach (about 500 mm and 55.5% of runoff coefficient from 2005 to 2008) (Pfister et al., 2017). The site has an altitude ranging from 458 to 513 metres above sea level. The climate is semi-oceanic with an average annual precipitation of 953 mm (2006–2014) (Hissler et al., 2021; Pfister et al., 2017) and average monthly temperatures of up to 18°C in summer and a minimum of 0°C in winter (Martínez-Carreras et al., 2010). Weierbach is a predominantly forested site,

covered by 76% mixed oak and beech forest and 24% coniferous forest (Martínez-Carreras et al., 2015). The soil is characterised as a dystric (saturation < 50%) endoskeletal cambisol with a thin organic A horizon (over 0-8 cm depth) and a cambic B horizon (over 8-40 cm depth) (Scaini et al., 2018).

2.2. Soil and soil water samples

2.2.1. Collection design

Two separate campaigns were conducted depending on the period of soil water recharge. The first campaign was conducted from 13 November to 14 December 2020, at the start of soil water recharge, while the second campaign was conducted from 2 March to 25 March 2021, once the soil compartment had been recharged with water (Figure S1). During these two campaigns, both soil and soil water were collected by targeting precipitation events. The sampling strategy was based on weather forecasts obtained from AccuWeather (https://www.accuweather.com) at the Rambrouch location. On a delimited area of 25 m² on the Weierbach plateau, a total of 8 and 10 soil samples and 8 and 11 soil water samples were collected during the November and March campaigns, respectively. For each campaign, sampling was distributed so that samples were taken ideally before, during and after a targeted rainfall event. The sampling strategy was carried out at a frequency of approximately 2 to 3 samples per week for a total duration of 1 month per campaign.

Five soil replicates were randomly sampled on the previously mentioned delineated area to a depth of 20 cm using a 3 cm diameter auger. To eliminate spatial variability during collection, the soil replicates were pooled to form one sample per site, representing a final weight of approximately 300-500 g per sample. All soil samples were then stored at -20°C before being divided into two parts, for either metagenomic or physicochemical analyses.

Soil water samples were collected using candle-shaped filters (2440110, SDEC), as previously described (Florent et al., 2022b; SDEC, 2002). Five porous candles were randomly installed on the same delimited surface of the Weierbach plateau. Briefly, the porous candles were placed in the soil and connected to a collection bottle in which the soil water was collected by a depressurisation system using an electric vacuum pump (1800000, SDEC). As the suction process took approximately 24 hours, soil water sampling started one day before the soil sampling and collected from the collection bottle on the next sampling day. As with the soil samples, the soil water replicates were pooled to form one sample per site, representing final volumes of 40 - 150 mL and 60 - 120 mL per collection for the November and March campaigns, respectively. After measuring pH, all soil water samples were stored at 4°C before being processed and analysed.

2.2.2. Soil characterisation

The characterisation of the soil properties includes first the pH measurements made on 10 g of soil according to the ISO 10390 (AFNOR, 2021) for pH_{H2O} and pH_{KCl} and to VDLUFA A5.1.1 (VDLUFA, 2016) for pH_{CaCl2}. Then, the nutrient dosing of phosphorus (P_2O_5) and potassium (K_2O) was performed according to the VDLUFA A6.2.1.1 (VDLUFA, 2012) and that of magnesium (Mg) and sodium (Na) following the VDLUFA A6.2.1.7 (VDLUFA, 1997). In addition, the total organic carbon (C_{org}) and the total nitrogen (N_{tot}) were obtained according to ISO 10694 (AFNOR, 1995) and ISO 13878 (AFNOR, 1998), respectively, allowing the calculation of the carbon-to-nitrogen ratio (C/N). The cation exchangeable capacity (CEC) and cation saturation rate (S/T) were analysed according to the ISO 23470 (AFNOR, 2007). Finally, soil granulometry was determined as well as gravimetric and volumetric water contents (GWC and VWC for short, respectively). All these parameters were determined on the soil of the Weierbach, as described in detail in Florent et al., 2022.

147

148 **2.3.** Environmental parameters

149 For both campaigns, several environmental characteristics were measured: precipitation
150 (mm), air temperature (°C), soil temperature (°C) and soil moisture (**%**). The precipitation
151 data coming from the closest meteorological station (Roodt, 49°79'N, 5°82'O) were
152 provided by the "Administration des Services Techniques de l'Agriculture" (ASTA,
153 http://www.agrimeteo.lu). Soil and air temperatures were measured at 10 cm depth using
154 Vaissala HMP 45 C sensors coupled with a logger Campbell CR 800 at stations available on
155 the Weierbach plateau and shared by the hydro-climatological observation and sensing
156 technology team of the Luxembourg Institute of Science and Technology (LIST).

157

158 **2.4.** Enrichment of soil water samples

159 The purpose of this step is to increase the concentration of viral particles in soil water
160 samples using a laboratory enrichment protocol. For this purpose, a primary concentration
161 step using a Centricon® plus-70 centrifugal ultrafilter with a cut-off point of 10 kD (ref:
162 UFC710008, Merck) was performed, according to the manufacturer's recommendations.
163 This step resulted in a volume of approximately 4 mL for all pooled samples. However, as
164 the volume required to perform the DNA extractions was approximately 200 µL, a secondary
165 concentration was carried out using an Amicon Ultra-4 10 kD (ref: UFC801024, Merck)
166 according to the recommendations of the manufacturer, to reach this final volume (200 µL)
167 for all samples.

168

169 **2.5.** Total DNA extraction

170 Total DNA extractions of the soil samples as well as on the concentrates of the soil water
171 samples were carried out using the DNeasy® PowerSoil® kit (Qiagen, Hilden, Germany)

172 from 0.25 g of soil or 200 µL of concentrated soil water, respectively. The protocol followed
173 the manufacturer's recommendations: cell lysis, followed by DNA fixation and elution.
174 However, the recommended volume of 100 µl for the elution step was reduced to 60 µl to
175 concentrate the DNA. Final DNA concentrations were quantified using the Invitrogen™
176 Qubit™ 2.0 fluorometer (ThermoFisher) and ranged from 30 to 100 ng/µL for all soil
177 samples, and from 0.37 to 1.0 ng/µL for the soil water samples from the November
178 campaign. The DNA concentrations of soil water samples from the March campaign were
179 insufficient for metagenomic analyses. Therefore, an additional DNA concentration step was
180 performed using the CentriVap Centrifugal Vacuum Concentrator (sm 100930709,
181 Avantor™) reducing the extracted DNA volume to 20 µL and increasing the concentrations
182 to around 0.30-1.8 ng/µL. However, some samples did not yield sufficient DNA
183 concentrations and thus, were discarded while the other samples were stored at -20°C until
184 further analysis.
185
186 **2.6.** Shotgun metagenomics sequencing and reference-based bioinformatics
187 The viral communities were determined using a shotgun metagenomics method. From the
188 total extracted DNA from soil and concentrated soil water samples, library preparations were
189 completed using the Nextera DNA Flex kit (Illumina). Paired-end sequence reads were
190 generated using the Illumina NovaSeq 600 (2x150 bp). The raw sequencing reads were
191 quality-controlled (sliding window of 4 and minimum quality score of 20, minimum read
192 length of 100 bps) and Illumina adapters (NexteraPE-PE.fa) were trimmed with
193 Trimmomatic v0.39 (Bolger et al., 2014). Assemblies were performed separately for each
194 sample using SPAdes v3.15.4 (Nurk et al., 2017) with the "meta" option and default
195 parameters. Taxonomy classification of contigs was carried out against the complete non-
196 redundant (nr) microbial protein sequence database (released version of April 2022,

197 downloaded in May 2022) from NCBI using MMseqs2 v13.45111 (Steinegger and Söding,
198 2017) and the easy-taxonomy workflow that determines ancestors by searching translated
199 ORFs (translation table 11) (Mirdita et al., 2021). Quality-filtered reads from each
200 metagenome were mapped back to the assembled contigs using minimap2 v2.24 with the
201 "sr" preset (Li, 2018) and sorted and indexed with SAMtools v1.15.1 (Danecek et al., 2021).
202 Finally, metagenomic profiles were exported as comma-separated value (csv) files. Relative
203 abundances were calculated based on the number of reads mapping to each contig. Mapped
204 reads were divided by contig length and the total number of mapped reads per sample
205 (Palermo et al., 2019; Zhao et al., 2021).
206 A mathematical transformation of the relative abundances of the microbial species in soil
207 water samples was performed to normalise the replicates since the water volumes recovered
208 using porous candles were different from one sample to another. The transformation was
209 performed as followed:

210 $$At = \frac{A * r}{Vi}$$

211 Where *At* is the transformed relative abundance in 1 mL of sample, *A* is the relative
212 abundance, *r* is the ratio of the volume (µL) extracted after DNA extraction to the volume
213 (µL) analysed during metagenomic sequencing, and *Vi* is the initial volume (mL) collected
214 for each soil water sample.
215

216 **2.7.** Biostatistical analyses

217 Statistical analyses were performed with RStudio, version 1.4.1106, and R version 4.0.4
218 (Rstudio Team, 2020). All figures were generated using the package 'ggplot2'. All analyses
219 in this article considered the relative abundances of bacteriophages as described above,
220 however it is important to note that the temporal variations of total abundance of

bacteriophages according to precipitations was performed by considering the absolute abundances of bacteriophages. Regarding the ecological diversity, both α and β-diversity of DNA-bacteriophages were studied and calculated as described in Florent et al., 2022. In addition to all the measured environmental parameters described above, the antecedent precipitation index (API) was calculated using the 'HydroBE' package v0.9 (Simon S., 2019). This index provides information on the moisture stored in the soil and is calculated based on the rainfall of the previous days (Ali et al., 2010). The impact of all environmental factors previously mentioned on the variation of bacteriophage communities was tested using permutational multivariate analysis of variance (PERMANOVA) analysis through the package 'Vegan'. Using Bray–Curtis dissimilarities, a redundancy analysis (RDA for short) was performed to explore the distribution of the bacteriophage composition at the family level as a function of the type of sample (soil and soil water) and the campaigns (November and March), using the package 'Vegan'. For the analyses of the bacteriophage populations at the species level, the 10 most abundant species were extracted from the entire data, since rare species might generate noise in the analysis. Pearson correlations were performed between bacteriophage populations and environmental factors, and then linear regressions were conducted on the species-factor pairs that showed a significant correlation to study variations in bacteriophage abundance as a function of the respective environmental factors.

2.8. Accession number

The raw sequence data generated and analysed during the current study are available in the National Centre for Biotechnology Information Databases (Sequence Read Archive) under the BioProject number PRJNA823538.

3. Results

3.1. Physicochemical characteristics of Weierbach soil and climatic conditions during the two campaigns

The physicochemical properties of the Weierbach soil are characterised by a silty-clay and acidic soil with a pH of around 4, and a moderately acidic to neutral soil water ($4.5 < pH_{SoilWater} < 7$). With a carbon/nitrogen (or C/N) ratio of about 20, microorganisms will tend to immobilise soil nitrogen resulting in nitrogen deficiency for plants. In addition, the nutrient content is relatively low, (magnesium - Mg, sodium - Na, phosphorous - P_2O_5 and potassium - K_2O), implying low soil fertility. When analysing the soil properties separately for the two campaigns, soil pH, C/N ratio and nutrient content showed very little variations (pH between 4 and 4.3, C/N between 18 and 20 and changes in nutrient concentration of a maximum 3 mg per 100 g of soil). In contrast, soil water pH varied during the campaigns with pH values ranging from 4.5 to 6.2 in November and from 5.1 to 7 in March (Supplementary Table S1).

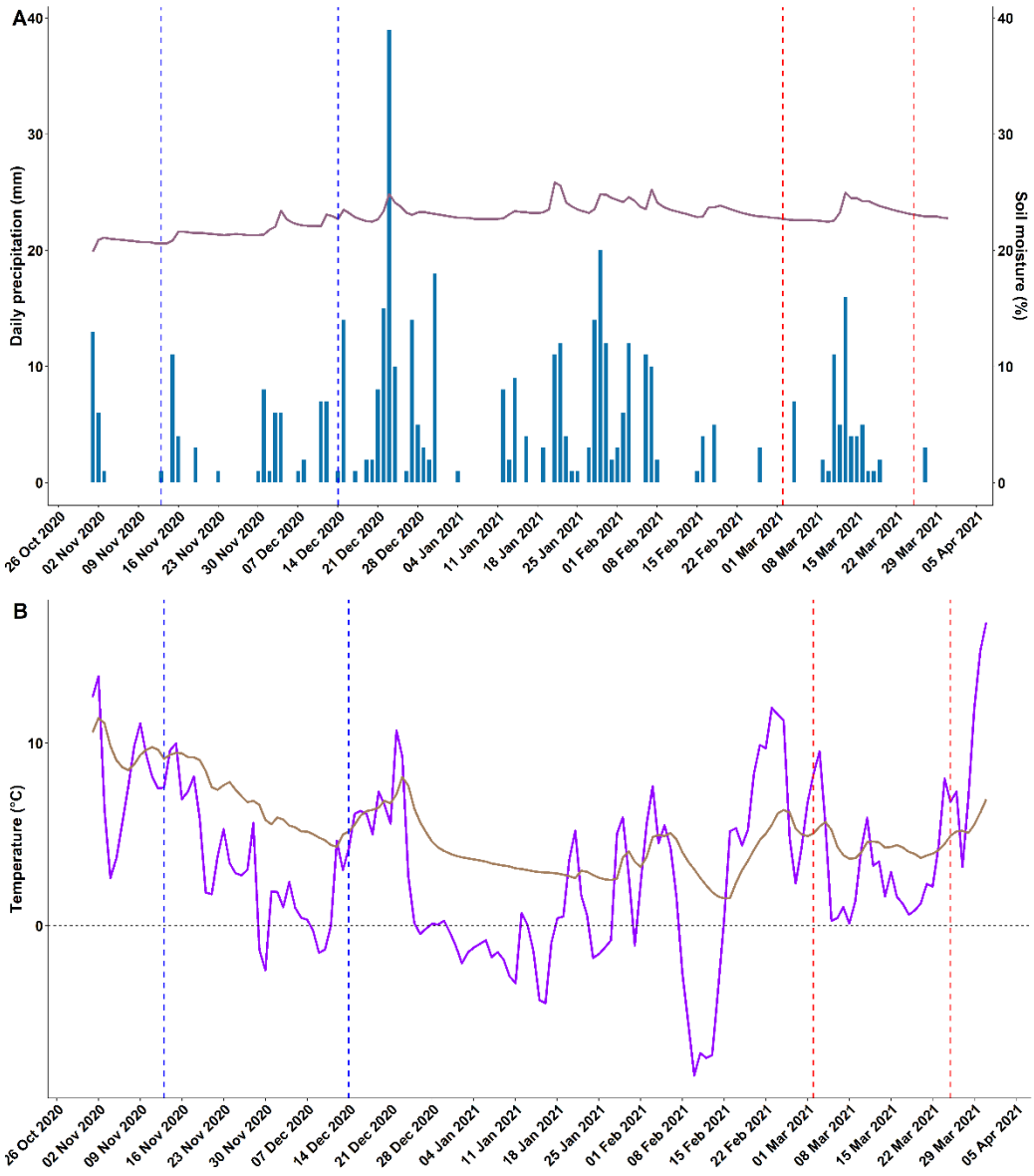


Figure 1: (A) Hydrograph presenting the daily total precipitation volume in millimetres (in blue) combined with the percentage of soil moisture (in dark purple) and (B) Time-temperature graph representing the daily average air temperature (in purple) and soil temperature (in brown) in degrees Celsius (°C) for the Weierbach plateau from the 1st of November 2020 to the 1st of April 2021. The blue dashed lines represent the first field campaign (from November 13, 2020 to December 14, 2020) while the red dashed lines represent the second field campaign (from March 2 to March 25, 2021).

Climatic data were extracted over the period of 1st November 2020 to 1st April 2021 to cover the two sampling campaigns. December and January recorded the highest rainfall (170.3 mm and 107.8 mm respectively) and the coldest monthly mean temperatures (2.7°C in December and -0.3°C in January). November was recorded as the driest month with total monthly precipitation of 41.6 mm and the warmest with a mean air temperature of 6.0°C (Figure 1B). During these five months, three precipitation events were observed, the first around the 20th of December, the second around the 30th of January and the last around the 10th of March (Figure 1A). The ideal climatic conditions for exploring transport processes in the soil during a precipitation event are (i) a high daily precipitation volume with dry periods before and after each event and (ii) high air and soil temperatures to avoid freezing of the soil surfaces, which would make it impossible for rainwater to infiltrate the soil. These criteria were met in particular for the rain events of December and March, chosen for the case of this study.

The soil temperature fluctuated between 1.5 °C and 11.7 °C over the 5 months, with November being the warmest month on record with an average temperature of 8.7 °C and January the coldest with an average of 3.1°C. Despite the small variations in soil temperature, its profile varied in a similar way to the air temperature (Figure 1B). The VWC considers soil bulk density and thus, unlike the GWC, provides information on the relative amount of water in the soil, which represents the soil moisture. For this reason, only the VWC was considered for further analyses. Daily VWC was relatively constant over the 5 months with a minimum of 21% in November and a maximum of 23% in February. However, the daily VWC was increasing after rainfall events. For this reason, only the VWC was considered for further analyses. VWC was relatively constant over the 5 months with the lowest in November at 21% and the highest in February at 23%. However, the daily variations in VWC followed rainfall with increases in VWC when rainfall increased (Figure 1A).

294

295 **3.2.** Composition of bacteriophage communities in soil and soil water over time

296 The statistics of the metagenomic shotgun sequencing are reported in Table 1. Shotgun
297 sequencing produced between 47 and 60 million read pairs in the soil samples and 39 to 95
298 million read pairs in soil water samples. Metagenomic assemblies resulted in over 5 million
299 contigs for each of the soil samples (for both campaigns), while the soil water samples
300 produced between 300,00 and 1.5 million contigs per sample. Using the nr database and after
301 e-value filtering, about 69% to 72% and 70% to 73% of the assembled contigs were
302 successfully taxonomically assigned in the soil during the campaigns of November and
303 March, respectively. Regarding the soil water samples, about 33 to 44% and 35 to 53% of
304 the assembled contigs were taxonomically assigned during the campaigns of November and
305 March, respectively. Of these identified sequences, about 0.05% and 0.8-1.1% were
306 identified as viral sequences in the soil and soil water samples, respectively, in the November
307 campaign, while 0.03% and 0.6-1% were identified during the March campaign.

Table 1: Statistics of the metagenomic sequencing: raw data, assembly and mapping on the soil and soil water for the November campaign (A) and March campaign (B). SW= soil water sample, S= soil sample.

(A)

Sampling dates	Sample ID	Number of read-pairs	Yield in Mbp	Average quality (Q-score)	Number of total contigs	GC-content	N50	Identified total contigs*	Absolute numbers of identified viral sequences	Identified vir sequences**
13.11.2020	SW1	52,568,931	14,966	35.70	748,555	57.47%	547	37.05%	3,801	0.81%
16.11.2020	S1	57,791,172	16,259	35.51	5,588,182	60.66%	306	71.76%	958	0.06%
	SW2	50,275,130	14,523	35.62	437,122	56.15%	358	41.22%	2,363	0.92%
20.11.2020	S2	51,258,401	14,655	35.52	5,801,124	60.48%	306	71.28%	618	0.04%
	SW3	53,588,349	15,350	35.56	675,363	56.33%	535	44.66%	4,040	1.08%
26.11.2020	S3	54,607,186	15,431	35.7	5,140,292	60.68%	325	69.85%	631	0.04%
	SW4	48,195,690	13,687	35.81	1,675,417	57.18%	496	38.58%	9,708	0.94%
01.12.2020	S4	47,680,677	13,567	35.56	5,309,702	60.16%	307	72.24%	685	0.05%
	SW5	-	-	-	-	-	-	-	-	-
04.12.2020	S5	50,061,626	14,346	35.67	5,536,755	60.13%	311	71.35%	719	0.04%
	SW6	48,213,814	13,625	35.59	1,813,938	55.99%	476	37.42%	12,484	1.10%
07.12.2020	S6	57,578,885	16,569	35.58	6,169,623	60.12%	307	70.64%	883	0.05%
	SW7	51,119,776	14,665	35.47	1,837,051	56.95%	463	33.99%	12,344	1.02%
10.12.2020	S7	60,167,261	17,167	35.73	6,472,486	60.30%	302	70.95%	703	0.04%
	SW8	-	-	-	-	-	-	-	-	-
14.12.2020	S8	57,404,889	16,383	35.48	5,445,055	60.29%	335	69.21%	814	0.05%

Mbp = millions of base pairs; GC = Guanine Cytosine; N50 = the sequence length of the shortest contig at 50% of the total genome length; Total references to the total microbial metagenome (i.e., eukaryote, archaea, bacteria and viruses). * Calculations based on the number of total contigs; ** calculations based on the percentage of identified total sequence.

(B)

Sites	Samples	Number of read-pairs	Yield in Mbp	Average quality (Q-score)	Number of total contigs	GC-content	N50	Identified total contigs*	Absolute numbers of identified viral sequences	Identified viral sequences**
02.03.2021	SW1	89,910,054	24,372	35.94	428,914	55.09%	522	52.66%	1,824	0.90%
03.03.2021	S1	50,154,381	13,881	35.63	5,104,903	60.67%	320	71.56%	497	0.03%
	SW2	65,692,938	17,583	35.87	824,486	56.69%	456	47.53%	2,556	0.59%
05.03.2021	S2	40,594,124	11,117	35.55	4,700,052	60.69%	299	72.74%	414	0.03%
	SW3	95,466,873	25,298	36.09	1,204,325	55.75%	497	41.46%	5,327	0.76%
08.03.2021	S3	47,180,484	12,998	35.62	4,591,724	60.36%	328	70.05%	448	0.03%
	SW4	55,007,509	15,239	35.66	460,231	55.18%	703	38.18%	1,943	0.68%
10.03.2021	S4	51,103,588	14,253	35.4	5,691,110	60.57%	306	72.18%	488	0.03%
	SW5	-	-	-	-	-	-	-	-	-
11.03.2021	S5	-	-	-	-	-	-	-	-	-
	SW6	39,906,319	11,206	35.3	432,792	55.09%	616	47.83%	2,215	0.98%
12.03.2021	S6	-	-	-	-	-	-	-	-	-
	SW7	57,350,350	15,558	35.74	1,442,377	55.17%	571	35.09%	8,364	0.97%
15.03.2021	S7	50,795,701	13,785	35.72	5,433,267	60.38%	309	72.02%	471	0.03%
	SW8	-	-	-	-	-	-	-	-	-
18.03.2021	S8	40,087,756	11,027	35.52	4,414,535	60.21%	307	72.27%	412	0.03%
	SW9	86,315,701	23,427	36.0	593,570	56.05%	579	43.08%	2,997	0.89%
22.03.2021	S9	45,459,549	12,660	35.61	5,146,599	60.63%	315	72.05%	472	0.03%
	SW10	79,757,835	19,872	36.16	991,041	56,97%	500	46.43%	5,073	0.96%
24.03.2021	S10	53,268,475	14,910	35.54	5,798,366	60.48%	314	72.56%	522	0.03%
	SW11	91,628,435	24,387	35.99	1,108,817	55.30%	551	39.94%	5,938	0.89%
25.03.2021	S11	53,222,865	14,668	35.36	5,727,624	60.77%	302	72.82%	552	0.03%

Mbp = millions of base pairs; GC = Guanine Cytosine; N50 = the sequence length of the shortest contig at 50% of the total genome length; Total references to the total microbial metagenome (i.e., eukaryote, archaea, bacteria and viruses). * Calculations based on the number of total contigs; ** calculations based on the percentage of identified total sequence

Of the bacteriophage population identified, 42.1% and 66.1% were unclassified at the family level for the November campaign and 41.9% and 65.5% for the March campaign, for soil and soil water samples respectively (Figure 1). In bacteriophage composition, 18 bacteriophage families could be detected, where the most abundant families were found to have similar variation in soil and soil water between the two campaigns, with *Siphoviridae* (≈30% in soil and ≈16% in soil water) being the most abundant followed by *Myoviridae* (≈16% in soil and ≈9% in soil water) and *Podoviridae* (≈9% in soil and ≈7% in soil water). The composition of bacteriophages in the soil water samples appeared to be relatively similar over time, particularly for the November campaign accross all families. In March, *Siphoviridae* showed a higher abundance on 2 and 8 March. More variation was seen in the soil samples, with *Myoviridae* showing slightly increased abundances on 16 November and 1 December and was most abundant on 22 March.

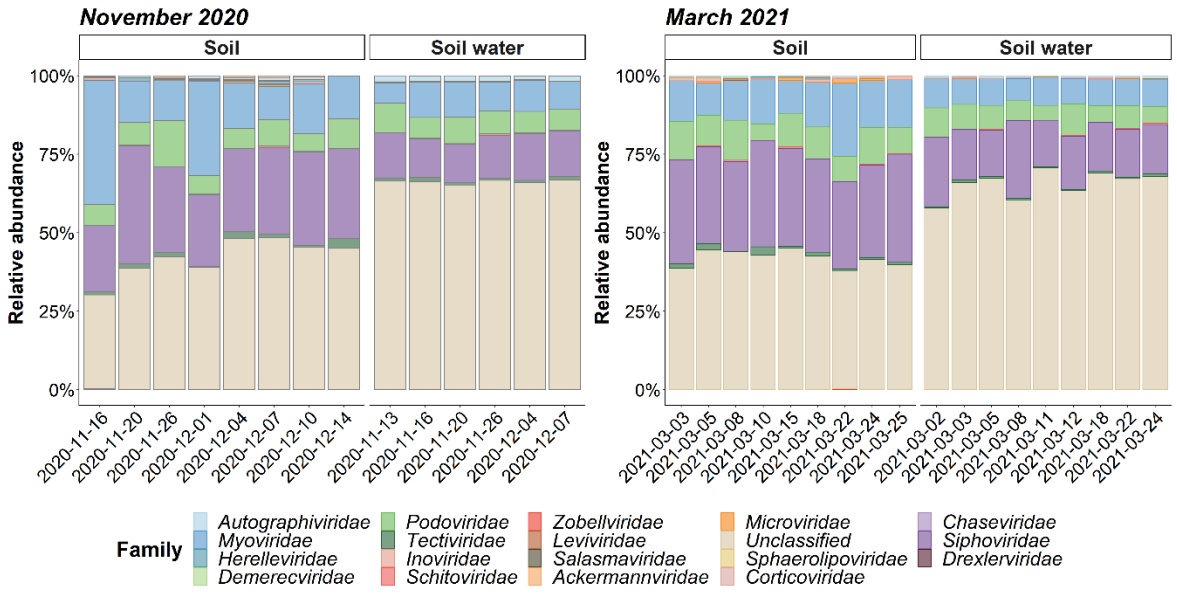


Figure 2: Bar charts representing the relative abundances (%) of bacteriophages at the family level over time during the two field campaigns in November and March in both soil and soil water.

Bacteriophage diversity, calculated using the Shannon index, was found to be 2.81 ± 0.11 and 2.31 ± 0.14 in soil and soil waters respectively during the campaign of November and 2.78 ± 0.12 and 2.25 ± 0.13 respectively, during the campaign of March. The diversity, as well as the evenness of bacteriophages, was significantly higher in soil than in soil water (p-values < 0.001 for the two campaigns), while soil water was significantly richer in bacteriophage species than soil (p-value < 0.001), during both campaigns. In general, fewer species were identified in soil than in soil water, with about 100 species in soil and between 400-800 species and 200-450 species in soil water for the campaigns of November and March, respectively. However, with a low evenness, soil water samples were rather composed of rare species, compared to soil samples ($E < 0.4$ and ≈ 0.6, for soil water and soil samples respectively) (Figure 2).

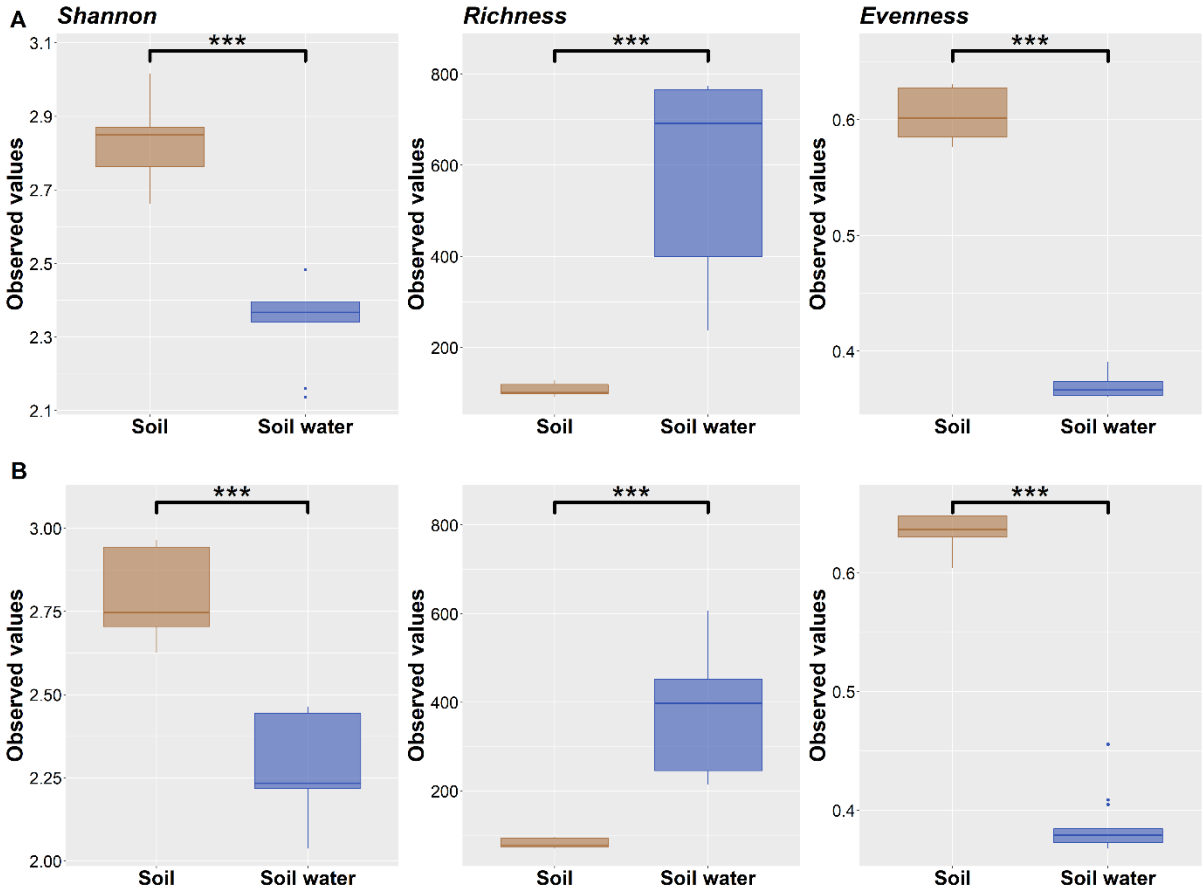


*Figure 3: Boxplots of diversity indices (Shannon, richness and evenness) in soil (in brown) and soil water (in blue) for the campaign of November (n = 18) (A) and the campaign of March (n = 14) (B). The statistical tests were conducted using a Wilcoxon test with a Bonferroni correction (p-value < 0.001: ***).*

The significant differences in bacteriophage community diversity between soil and soil water were subsequently explored through redundancy analysis (afterwards called RDA). This analysis made it possible to relate the bacteriophages identified in the samples at the taxonomic family level to the selected environmental factors, conducted separately for the two campaigns (Figure 3). The two RDA were built over the two first dimensions, covering together 64.25% and 70.78% of the fitted variation for the campaign of November and March, respectively. Among all the environmental factors, pH was the explanatory variable that had significant weight on the construction of the RDA (p-value = 0.002). Regarding the

RDA of the November campaign, the axis RDA1 was positively correlated at 64% with pH (p-value < 0.01) while the axis RDA2 was rather negatively correlated with the precipitations and API at 1 day, both at 28.1% but not significantly (p-value = 0.05). The RDA of the November campaign revealed a strong positive correlation of 84.64% (p-value < 0.01) between the axis RDA1 and pH, while the axis RDA2 did not show a correlation with any of the selected environmental factors. Thus, of the environmental factors tested during the two campaigns in November and March, pH showed influences in the distribution of bacteriophages between the soil and soil water samples. In November, *Autographiviridae* and *Siphoviridae* were significantly correlated to the pH (r = 0.67 and -0.73, respectively), while in March, in addition to these two families previously mentioned (r = 0.53 and -0.85, respectively), *Inoviridae*, *Myoviridae*, and *Podoviridae* were also correlated to the pH (r = -0.56, -0.69 and -0.68, respectively). On the contrary, no correlation was found between bacteriophage families and any other environmental factors tested.

Finally, when testing the impact of these same environmental factors on the variation of bacteriophage communities within each type of sample (soil and soil water) separately, the PERMANOVA analysis did not reveal a significant effect from any factor.

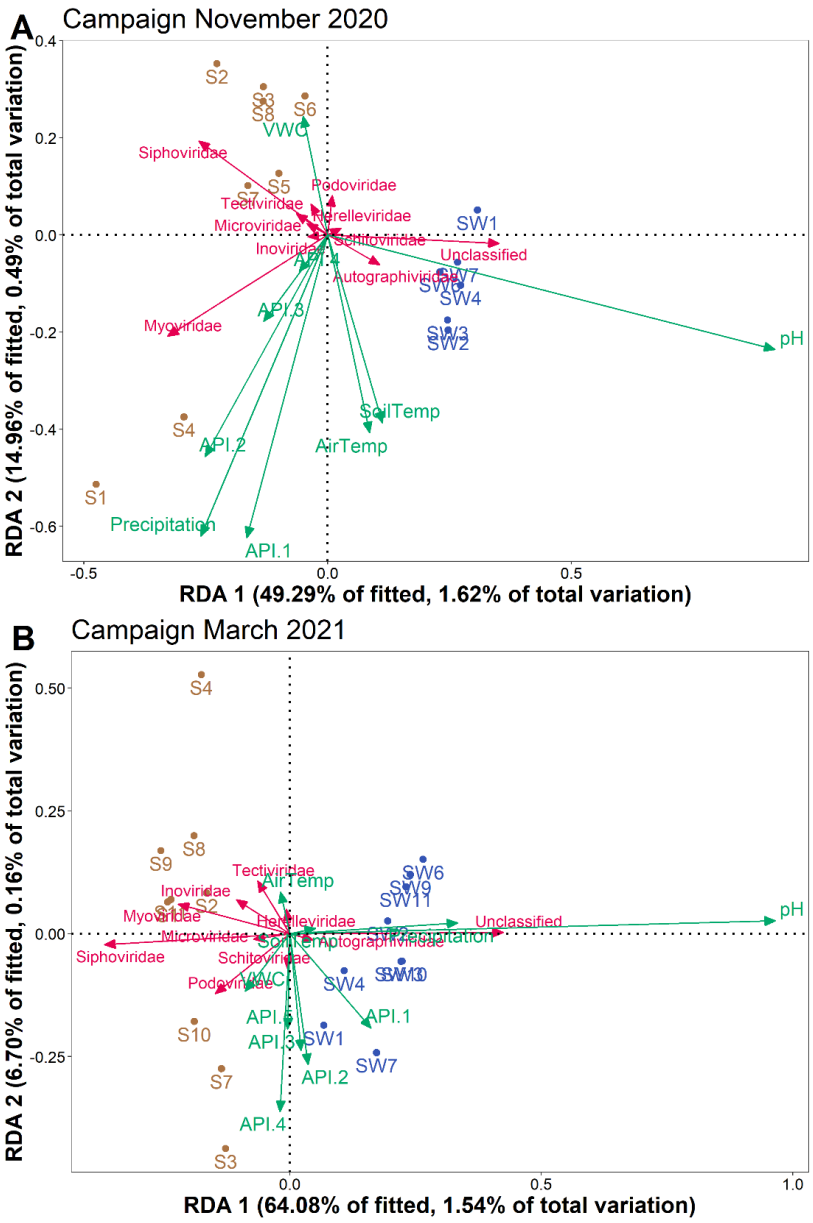


Figure 4: Biplot of redundancy analysis (RDA) of the DNA-bacteriophage families in relation to the environmental factors during (A) the campaign of November 2020 and (B) the campaign of March 2021. The pink vectors represent the bacteriophage families, and the green vectors display the selected environmental factors. The soil samples are represented in brown colour with the letter "S" and the soil water samples in blue with the letters "SW". The RDA was built over the two first dimensions, with 43.87% of the fitted variations explained by RDA1 and 4.11% by RDA2. (API = Antecedent precipitation index

389 *followed by the number of days; SoilTemp = soil temperature; AirTemp = air temperature;*
390 *VWC = volumetric water content).*
391

392 To further analyse the effects of environmental factors, and more particularly rainfall, the
393 change in bacteriophage's community composition at the taxonomic level of the species was
394 explored since it was not noticed at the family level. Given the large number of species
395 identified, this exploration focused on selecting the 10 most abundant species. Across all
396 campaigns and samples, the order *Caudovirales* contained the highest number of
397 bacteriophage species, as uncultured and unidentified bacteriophages. *Siphoviridae sp.* are
398 was very abundant, especially in the soil samples of both campaigns while unidentified
399 bacteriophage species belonging to the families *Myoviridae* and *Podoviridae* were found to
400 be equally abundant in both types of samples for the two field seasons studied. Then,
401 bacteriophage species belonging to specific genera were detected differently depending on
402 the campaign studied. In fact, the presence of unidentified species belonging to the genus
403 *Naesvirus* (*Myoviridae*) was detected in November, with a notable variation in the soil water
404 samples. During the March campaign, this genus was not detected, but unidentified species
405 belonging to the genera *Fromanvirus* (*Siphoviridae*) and *Bridgettevirus* (*Siphoviridae*) were
406 specifically detected in both types of samples. Finally, the samples from the two campaigns
407 of November and March were similarly composed of four DNA-bacteriophage identified at
408 the specie level, *Lessievirus bcepil02* (*Podoviridae*), *Ochrobactrum phage vB_OspP_OH*
409 (*Podoviridae*), *Mieseafarmvirus RSL1* (*Myoviridae*) and *Alcaligenes phage*
410 *vB_Af_QDWS535* (*Podoviridae*). Among these identified species, bacteriophages *bcepil02*
411 and *OH* were the most abundant in the soil of the two campaigns (November and March)
412 but were not detected in the soil water. Bacteriophage *QDWS535* was abundant in the soil
413 water samples compared to the soil samples of the November campaign, with a positive

correlation with the pH (r = 0.70, p-values = 0.005). Soil samples displayed more acidic pH than soil water samples (soil pH around 4 and soil water pH between 5 to 6), meaning that DNA-bacteriophages dominantly abundant in soil samples will be associated with acidic pH (here, inferior to 5). In addition to these common species, each campaign was characterised by specific DNA-bacteriophage species. The samples from the campaign of November were represented by the presence of *phage 71_18* (*Siphoviridae*), *Cinunavirus CN1A* (*Siphoviridae*), *Borockvirus brock* (*Myoviridae*) and *Burkholderia phage FLC9* (*Myoviridae*). Regarding the samples of the campaign of March, four specific DNA-bacteriophage species were detected: *freshwater phage C440* (*Podoviridae*) and *Omegavirus Thibault* (*Siphoviridae*).

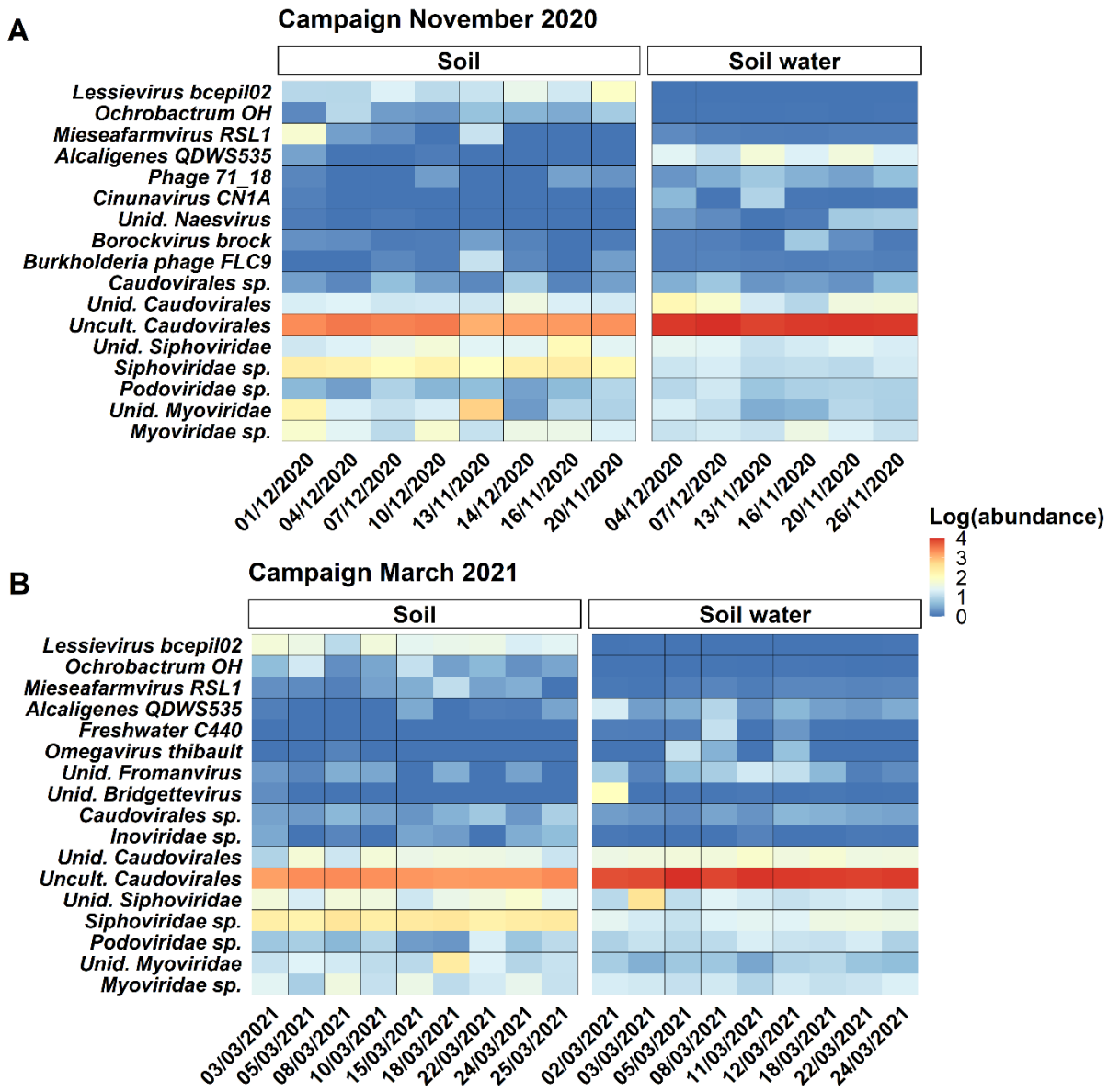


Figure 5: Heatmaps representing the log-transformed relative abundances of DNA-bacteriophage species in soil and soil water over time for the campaign of November (A) and the campaign of March (B), where the redder, the higher the abundance and the bluer, the lower the DNA-bacteriophage abundance.

As for the differences in the family composition of bacteriophages, the difference in the abundance of these species between the soil and soil water was explained by the pH among all environmental factors tested (PERMANOVA tests: p-value = 0.002 in November and p-value

= 0.001 in March) (Supplementary Figure 2). More precisely, bacteriophage *bcepil02* and *OH* were negatively correlated to the pH in the samples of the two campaigns (Pearson correlation: r = -0.67 and -0.84, p-values = 0.008 and < 0.001, respectively for *bcepil02*; r = -0.66 and -0.59, p-values = 0.011 and 0.01, respectively for *OH*). In addition, bacteriophage *RSL1* was also found to be negatively correlated to the pH but only in samples of the March campaign (Pearson correlation: r = -0.48, p-value = 0.042). Significant correlations with the pH were found for bacteriophages 71_18 (Pearson correlation: r = 0.58, p-value = 0.029) and CNA1 (Pearson correlation: r = 0.57, p-value = 0.032) in November, and for bacteriophage *Omegavirus Thibault* (Pearson correlation: r = 0.48, p-value = 0.045) in March.

3.3. Temporal variations in DNA-bacteriophage abundance during the precipitation events

The November precipitation event was quite variable in its intensity, also reflected in the total abundance of bacteriophages in both types of samples. In the soil, the abundance of bacteriophages decreased slightly during the first half of the campaign and was then marked by two increases in abundance during the first rainfall intensity at the end of November/beginning of December and the second intensity in mid-December. The absolute abundance of bacteriophage in soil water, on the other hand, continued to increase slightly throughout the rainfall event. The March precipitation, in contrast to November, showed a bell-shaped variation in intensity with a peak around mid-March. Bacteriophage abundance did not show a similar variation to that of the precipitation. In the soil samples, the bacteriophage abundance showed a slight increase after the first peak of rain intensity in early March. Then a second peak is revealed after the main precipitation event, during the rain-free period at the end of March. Finally, in the soil water, the abundance of bacteriophages was rather constant.

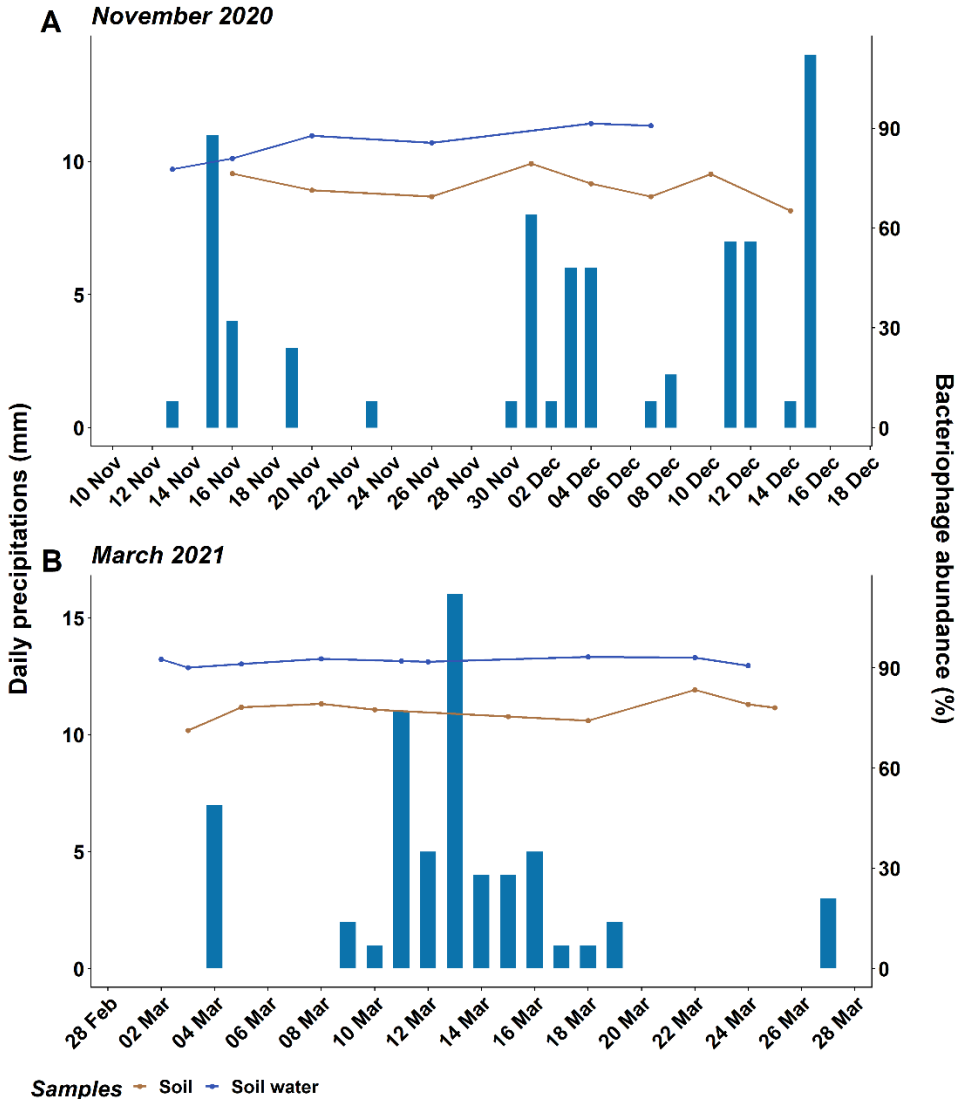


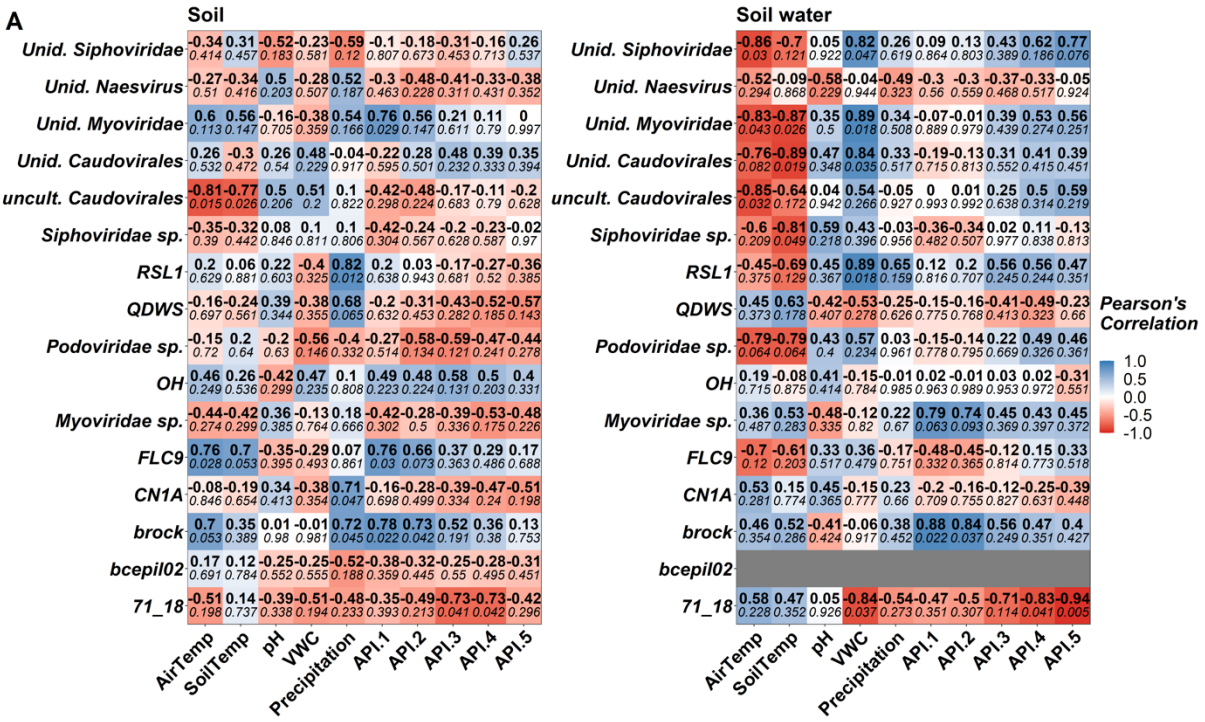
Figure 6: Hydrographs over time for the two field campaigns of November (A) and March (B) representing daily precipitations (in mm) in blue, with parallel (in the second axis) variations in the total abundance (in %) of the identified bacteriophages represented by lines, in brown for variations in soil samples and in blue for those in soil water samples.

To further understand variations in DNA-bacteriophage abundance, the most abundant species detected were related to several environmental factors, including rainfall (Figure 6). Besides the pH previously mentioned, other environmental factors showed effects on these DNA-bacteriophage species. In November, unidentified species belonging to the *Caudovirales* order were negatively correlated with air and soil temperatures in both soil and soil water samples (r

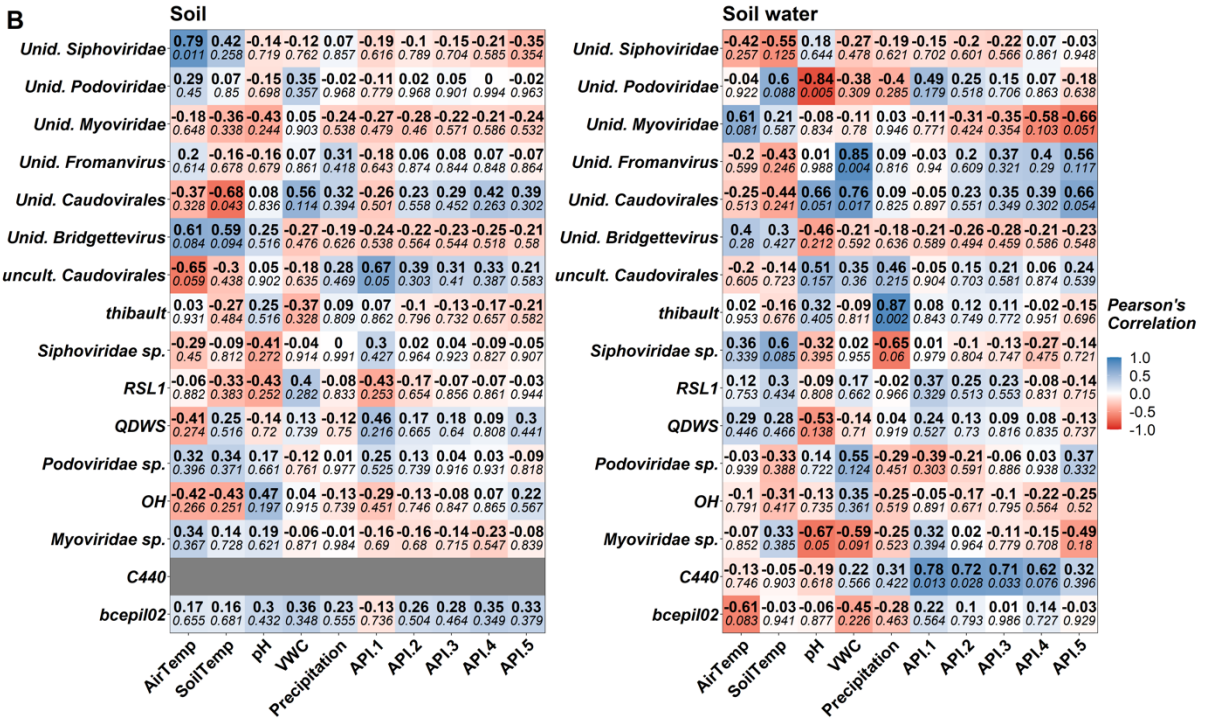
» -0.81, p-value < 0.05) (Figure 6A). Additionally, unidentified species belonging to *Myoviridae* showed a positive correlation with API at 1 day in soil samples (r = 0.76, p-value = 0.029) and with VWC in soil water (0.89, p-value = 0.018), while they were negatively correlated with air and soil temperatures in soil water samples (r = -0.83 and -0.87, p-value = 0.043 and 0.026, respectively). Finally, unidentified species belonging to the *Siphoviridae* were negatively correlated with air and soil temperatures only in soil water samples (r = -0.86 and -0.81, p-value = 0.03 and 0.049, respectively). Regarding the identified species detected in November, bacteriophages RSL1, CN1A and brock showed a positive correlation with precipitations (r = 0.82, 0.71 and 0.72, p-value = 0.012, 0.047 and 0.045, respectively), but only in the soil samples. None of the species was found to correlate with the precipitations in the soil water samples. However, unlike the two other species mentioned above, bacteriophage RSL1 was positively correlated with VWC in the soil water samples (r = 0.89, p-value = 0.018). Bacteriophage 71_18 was the only other species being correlated with VWC in the soil water samples, but in contrast to RSL1, its abundance was increased with decreasing soil moisture (r = -0.84, p-value = 0.037 with API at 3 and 4 days). More interestingly, this bacteriophage 71_18 was strongly correlated with the soil moisture stored in both soil and soil water samples. While its abundance significantly decreased 3 to 4 days behind the precipitation in the soil (r = -0.73, p-value = 0.043), in the soil water samples, the more soil moisture is stored, the less abundant bacteriophage 71_18 became (-0.47 < r < -0.94, 0.351 < p-value < 0.005, API from 1 to 5 days). Bacteriophage brock was, in contrary, positively correlated to the stored soil moisture in both soil and soil water samples, especially calculated at 1 and 2 days (r ≈ 0.75 and 0.86, p-value ≈ 0.03 and 0.03 in the respective samples).

During the March campaign, fewer correlations were noticeable (Figure 6B). None of the species showed a correlation with the precipitation in soil water, while only bacteriophage Thibault was positively correlated to the precipitation in the soil water samples (r = 0.87, p-

494 value = 0.002). The abundance of the unidentified species belonging to the order *Caudovirales*
495 and the genus *Fromanvirus* showed to increase with the VWC in soil water samples (r = 0.76
496 and 0.85, p-value = 0.017 and 0.004). Finally, bacteriophage C440 was not detected in the soil
497 samples, and its abundance was correlated with the soil moisture stored for 1 to 3 days (r =
498 $0.71 < r < 0.71$, $0.013 <$ p-value < 0.033). However, it is important to note that this correlation
499 decreased with increasing soil moisture storage time.
500



501



502

503 *Figure 7: Correlations between the most abundant bacteriophage species and the selected*
504 *environmental variables, during (A) the campaign of November 2020 and (B) the campaign of*
505 *March 2021. Pearson's correlation coefficients r are displayed as text values as well as*
506 *gradient colour (blue representing positive correlations, red as being negative correlations*
507 *and white, no correlations). Unid. = Unidentified; uncult. = uncultured; API = Antecedent*

precipitation index followed by the number of days; SoilTemp = soil temperature; AirTemp = air temperature; VWC = volumetric water content. The grey content represents the absence of correlation due to the absence of the species in their respective samples.

The rainfall events studied showed a significant correlation with two of the most abundant species identified. Their temporal variations during the rainfall events can be seen in Figure 8. In November, the bacteriophage *RSL1* was correlated with precipitation, during which the abundance of the bacteriophage showed two peaks of abundance in agreement with the first two peaks of rain intensity. These abundance peaks occurred only in the soil samples, whereas in soil water, the bacteriophage was detected at very low levels. An increase in abundance could still be noticed at the beginning of December, after the strong increase in abundance in soil. Regarding the field campaign conducted in March, only bacteriophage *Thibault* showed a significant correlation with precipitation. The variation in abundance of this bacteriophage was especially visible in soil water, where a first increase was noticed at the beginning of the most important rain intensity peak but at a time lag with the first peak. The second increase in abundance occurred during the rainless period after the precipitation ended. In the soil, on the other hand, the abundance varied very little with detection mainly visible between the first peak on March 4 and the beginning of the second on March 10. It is interesting to note that for these two bacteriophages, *RSL1* was also detected in March but did not show any correlation with precipitation, whereas *Thibault* was only detected during the November campaign.

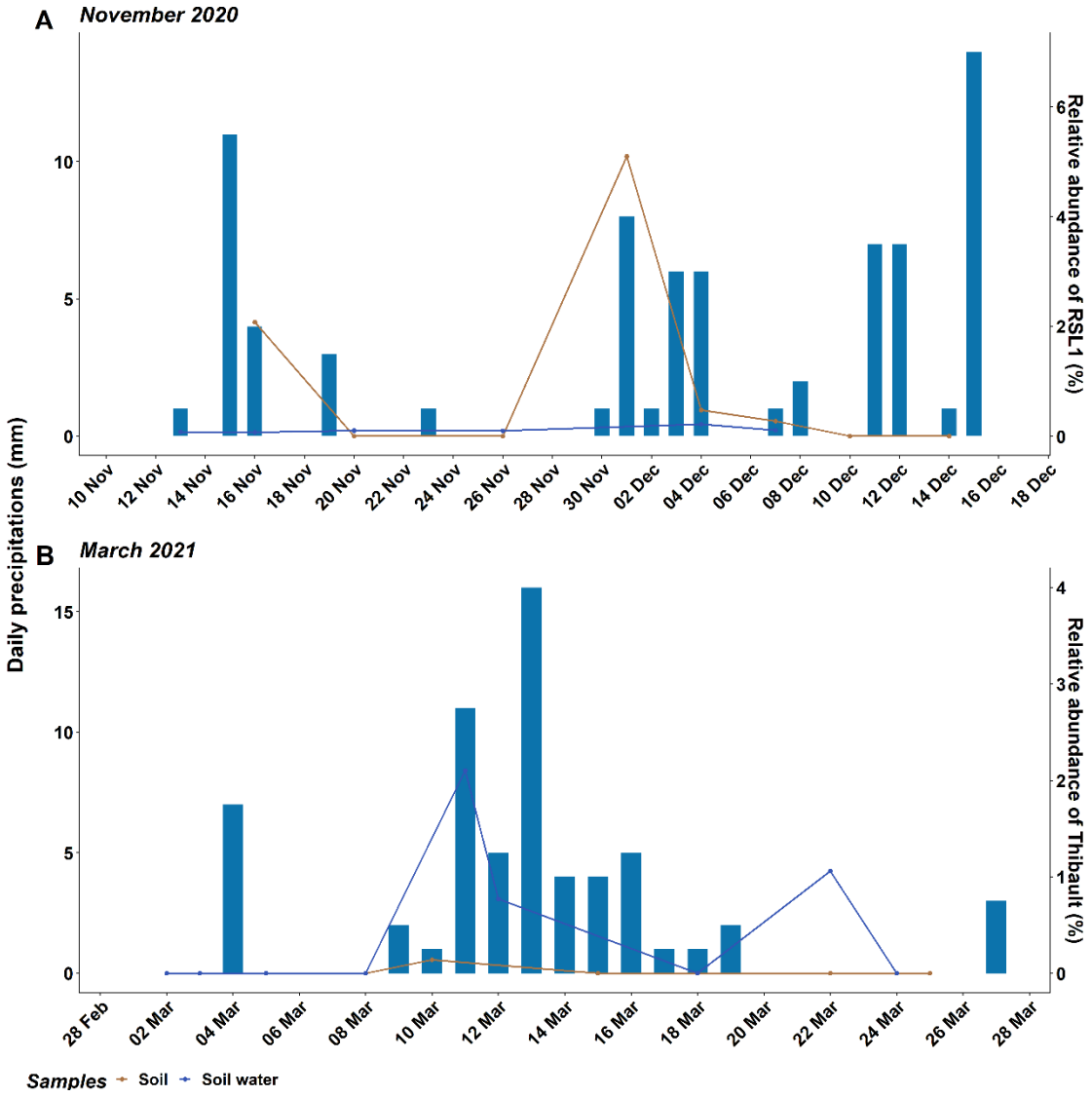


Figure 9: Variations in relative abundance (%) of bacteriophage RSL1 (A) and bacteriophage Thibault (B) represented by the lines (brown for soil samples and in blue for soil water samples) during the daily precipitations (in mm) (blue bar chart) during the field campaigns of November and March, respectively.

4. Discussion

The determination of the natural populations of DNA-bacteriophages in the terrestrial compartment demonstrated clear differences in diversity as well as a taxonomical composition between the soil and the soil water. All samples were dominantly composed of *Siphoviridae*, *Myoviridae* and *Podoviridae* DNA-bacteriophage families. It should be noted that as of March 2022, the *Caudovirales* order was dissolved as well as the three families *Siphoviridae*, *Myoviridae* and *Podoviridae* and most species were then reclassified within the class *Caudoviricetes* (Turner et al., 2021). However, the classification of these three families in terms of morphotypes remains valid, with *Myovirus* as morphotype A (head with a tubular tail, baseplate and collar), *Siphovirus* as morphotype B (icosahedral with noncontractile tail or prolate capsid) and *Podovirus* as morphotype C (icosahedral with short noncontractile tail and stacked disks with fibres) (Turner et al., 2021). Finally, the presence of RNA-bacteriophage families, *Fiersiviridae* et *Corticoviridae*, was noticed but at very low abundance, which was probably due to a flaw in the assembly of reads into contigs since no retro-transcription step was performed to detect RNA viruses (Smits et al., 2014; Sutton et al., 2019). No pattern in the distribution of these morphotypes was found between or within the different samples (i.e. soil and soil water). However, ithe bacteriophage composition between the two campaigns showed some differences with the presence of common bacteriophage species such as bacteriophages bcepIL02 and RSL1, but also by specific species present in either of the campaign. the distribution of the bacteriophage community appeared to be influenced by precipitation, soil and air temperatures, and was characterised by the presence of certain bacteriophages, RSL1, 71_18 and Brock. The variation of the latter was distinguished by their significant correlations with precipitation, VWC and API, respectively.During the March campaign, the bacteriophage distribution seemed to be potentially impacted by the soil moisture, where only *Omegavirus*

562 *Thibault* and bacteriophage C440 revealed a significant correlation with precipitation and API,
563 respectively.
564 For all campaigns studied, the differences in DNA-bacteriophage composition of soil and soil
565 water samples were found to be dominantly driven by the environmental pH, with the soil pH
566 being more acidic than the soil water pH (4 against 5-6). Some species were detected
567 preferentially in one or the other samples, being the case for *Lessievirus bcepIL02* or
568 *Ochrobactrum OH* specifically detected in the soil samples. Conversely, species such as
569 *Alcaligenes QDWS535* and *Freshwater phage C440* were mainly detected in the soil water
570 samples. Thus, bacteriophages bcepIL02 or OH would be more favourable to rather acidic
571 soils, whereas bacteriophages QDWS535 and C440 are associated with pH values close to 7.
572 Among these species, bacteriophage bicepil02 was particularly abundant in the soil of the two
573 campaigns studied (November 2020 and March 2021), which was also noted in the context of
574 previous analyses that took place in September 2019. This bacteriophage would therefore be
575 part of the established soil bacteriophage population of the Weierbach. However, very little is
576 known about the ecology of this bacteriophage. Therefore, information about its bacterial host,
577 *Burkholderia cepacia*, may help to further understand its presence in this terrestrial
578 environment. This bacterium, without which bacteriophage bicepIL02 cannot replicate, is
579 known to be related to soil pH and to specifically live in acidic soils but to also be found in
580 marine environments (Hall et al., 2015; Paul et al., 2021). The specificity of this bacteriophage
581 for soil is also reflected by its absence from the soil water samples. It is most likely that
582 bacteriophage BcepIL02 might not be part of the established populations found in the soil
583 water, if its bacterial host is not present, especially since it was not detected before the rainfall
584 events started.
585 As colloidal particles, the ability for bacteriophages to be transported within and between
586 ecosystems requires the movement of water (Batinovic et al., 2019). Following the water cycle,

the natural populations of bacteriophages in the soil could then be moved away from their natural habitat by precipitations, due to the increased volume of water and the initiation of water flow (Luo et al., 2017). Little is known about the impact of precipitations on the natural bacteriophage dynamics in soil, but our results revealed some patterns. Firstly, the precipitations did trigger potential mobilisation from the soil to the soil water for any of the bacteriophages mentioned above. Regarding the two species specifically detected in soil samples, their absence from the soil water and their constant abundance in the soil sample along the rainfall events potentially indicates that they are most likely adsorbed to the soil particles and incapable of desorption. Unfortunately, no information is available on their surface electric charge, which could have been helpful to corroborate this fact since pH and surface electric charges are primarily influenced by the process of adsorption. Regarding the bacteriophages detected solely in the soil water sample, the hypothesis is that the bacteriophages were brought into the soil from the surface but were not detected in the soil samples due to potential rapid infiltration (Schaub and Sorber, 1977). Secondly, our results reported a precipitation effect on two of the most abundant DNA-bacteriophage species: *Ralstonia phage RSL1* and *Omegavirus Thibault*. Bacteriophage RSL1 is known to infect the bacterium *Ralstonia solanacearum*, a bacterium mainly found in soil as it plays a role in nitrogen-fixation, carbon decomposition and crop pathogenicity (Fujiwara et al., 2011; Gill et al., 2011; Miller et al., 2002; Yamada et al., 2010). Therefore, it is expected that the bacteriophage RSL1 rather occurs in soil, as already shown in the soil of Weierbach in previous analyses (Florent et al., 2022a), whereas in soil water the abundance of the bacteriophage was lower. Concerning the bacteriophage Thibault, apart from the structural information, no study has reported the presence of this bacteriophage among natural populations of bacteriophages in ecosystems, thus influencing the availability of information concerning the ecological distribution of this bacteriophage. However, its bacterial host, *Mycolicibacterium smegmatis MC2 155*, is also better known for its non-

612 pathogenic aspect for which the bacteria is used as a laboratory model strain in studies on
613 pathogenic species of the genus *Mycobacterium*, a dominant soil genus (Ofer et al., 2012). The
614 species *Mycolicibacterium smegmatis MC2 155* is believed to be present in a variety of
615 environments, such as soil, fresh and marine waters, and has shown strong persistence in
616 environments with high salt concentrations (Berney et al., 2014; Yamada et al., 2018).
617 The two field campaigns studied in this research work are very different from each other. At
618 first, the two precipitation events clearly displayed diverse patterns, with bell-shaped
619 precipitation for March 2021 while that of November was more diffuse in the peaks of intensity.
620 Moreover, even if these two campaigns came about during the cold period of 2020/2021, they
621 took place at very strategic times. The targeted precipitation event in November occurred just
622 after the dry period characterized by soil moisture of around 5-10% at the lowest. Soil moisture
623 recorded for November/December was around 20% while for March, soil water recharge was
624 much more established than for November with soil moisture around 23%, even rising to 26%.
625 Water from precipitation will behave differently after hitting the soil surface depending on the
626 state of the soil. Indeed, soil that has experienced dry periods, such as that of Weierbach in
627 November, will be more compact with large cracks or fissures (Masroor et al., 2022). This state
628 will favour a rapid circulation of water through the soil, and the water will therefore have very
629 little contact with most soil surfaces (e.g. horizons O and A) (Schimel, 2018). In contrast, in
630 periods following increased levels of rainfall, such as for the March campaign, the soil is
631 gradually loaded with water, making it more porous but without cracks. This state of the soil
632 presents a higher surface tension favouring capillary water until reaching higher water volumes
633 reducing the pores (Schimel, 2018). This soil dynamics can be transferred to the temporal
634 variations observed for bacteriophage RSL1. On the one hand, bacteriophage Thibault was not
635 detected in November and is therefore not concerned by these dynamics. Bacteriophage RSL1,
636 on the other hand, showed slightly different temporal variations between the two campaigns.

637 The abundance of RSL1 in November soil varied significantly depending on rainfall but it was
638 not detected in soil water, probably due to the fact that the water infiltrating too quickly through
639 the soil did not allow the bacteriophage to be transferred from soil to soil water. The
640 bacteriophage RSL1 in the soil in November was detected more strongly in only two samples,
641 corresponding to the first two peaks of precipitation intensity, whereas in March, the temporal
642 variations in abundance were more constant with bacteriophage detection in almost all the
643 samples. In November, a few days after the precipitation peaks, a decrease in the detection of
644 bacteriophage RSL1 was observed, which can be explained by the infiltration of the
645 bacteriophage by the rains towards the deeper layers of soil. In March, the temporal variations
646 in RSL1 were shown to be more constant, which could be the result of a stable volume of water
647 in the soil. Thus, the transport of the bacteriophage from the soil to the water would be favoured
648 when the soil is more replenished with water, as is the case for the March campaign where
649 bacteriophage RSL1 was a little more abundant in the soil water compared to the November
650 campaign.
651 However, it is important to highlight the limits regarding the results obtained on bacteriophage
652 temporal and spatial variations. We can effectively conclude on a potential effect of
653 precipitations on the variations in abundance of bacteriophage species between soil
654 compartments. Further analyses are nevertheless needed to obtain a more accurate picture of
655 the dynamics, especially in the long term, with sampling at a greater frequency to confirm these
656 first promising results on the possible mobilisation of bacteriophages in the soil.
657

658 **5. Conclusions**

659 The Weierbach soil has a very specific bacteriophage population with a relatively low richness.
660 Two species are particularly found, *Ralstonia phage RSL1* and *Lessievirus bcepIL02*, with a
661 high abundance, especially for the latter. As a matter of fact, the bacteriophage bcepIL02 was

present in all the soil samples of the two campaigns studied, in September and March, whereas it was not detected in the soil water samples. In the context of analysing the dynamics of bacteriophages between soil and soil water potentially triggered by precipitation events, bacteriophage bcepIL02 is most likely not capable of mobilisation from the soil. Among the most abundant bacteriophages, other species however revealed a significant correlation of the temporal variations in their abundance with precipitation events, such as *Ralstonia phage RSL1* in November and *Omegavirus Thibault* in March. While bacteriophage bcepIL02 would probably not be able to be transported due to potential adsorption processes on the soil, bacteriophages RSL1 and Thibault would be rather sensitive to water transport. In addition to the fact that these bacteriophages varied temporally with precipitation, their temporal variation was also dependent on soil water recharge. In soil that has undergone a recent water recharge after a long period of drought (i.e. November campaign), bacteriophages might preferentially infiltrate into the deeper layers of the soil due to the presence of fractures, whereas in less dry soils with a constant volume of water (i.e. March campaign, soil-to-soil water transport of bacteriophages might rather be favoured.

Acknowledgements

The authors would like to acknowledge the Luxembourg National Research Fund (FNR) for granting and supporting the PRIDE program containing the Doctoral Training Unit Hydro-CSI project (PRIDE15/10623093 – HYDRO-CSI). The authors would also like to thank Thomas Mion and Dr Magdalena Calusinska for their valuable assistance in the bioinformatics pipeline by providing us with their expertise in this field. The soil analyses could not have been done without the cooperation of Lionel Leydet (Administration des services techniques de l'agriculture, ASTA). Finally, the authors would also like to thank Jean-François Iffly and

686 Jérôme Juilleret for their valuable assistance in the settlement and monitoring of the porous
687 candles in the Weierbach.

688

689 **References**

690 Ackermann, H.-W., DuBow, M.S., 1987a. Viruses of prokaryotes: General Properties of
691 Bacteriophages (Volume 1), CRC Press. ed. CRC Press, Boca Raton, Florida.

692 Ackermann, H.-W., DuBow, M.S., 1987b. Viruses of prokaryotes: General Properties of
693 Bacteriophages (Volume 2), CRC Press. ed. CRC Press, Boca Raton, Florida.

694 AFNOR, 2021. ISO 10390: Sols, biodéchets traités et boues - détermination du pH.

695 AFNOR, 2007. ISO 23470: Qualité du sol - Détermination de la capacité d'échange cationique
696 (CEC) effective et des cations échangeables à l'aide d'une solution de trichlorure de
697 cobaltihexammine.

698 AFNOR, 1998. ISO 13878: Qualité du sol - Détermination de la teneur totale en azote par
699 combustion sèche ("analyse élémentaire").

700 AFNOR, 1995. ISO 10694: Qualité du sol - Dosage du carbone organique et du carbon total
701 après combustion sèche (analyse élémentaire).

702 Ali, S., Ghosh, N.C., Singh, R., 2010. Rainfall-runoff simulation using a normalized antecedent
703 precipitation index. Hydrological Sciences Journal-Journal Des Sciences Hydrologiques
704 55, 266–274. doi:10.1080/02626660903546175

705 Antonelli, M., Balasubramanian, M.N., Ogorzaly, L., Pfister, L., 2016. qPCR (quantitative
706 polymerase chain reaction) for the quantification of bacteriophages in stream water
707 samples to investigate hydrological processes: a proof-of-concept study in the
708 Huewelerbach experimental catchment (Luxembourg), in: EGU General Assembly. p. 1.

709 Batinovic, S., Wassef, F., Knowler, S.A., Rice, D.T.F., Stanton, C.R., Rose, J., Tucci, J.,
710 Nittami, T., Vinh, A., Drummond, G.R., Sobey, C.G., Chan, H.T., Seviour, R.J., Petrovski,
711 S., Franks, A.E., 2019. Bacteriophages in Natural and Artificial Environments. Pathogens
712 8. doi:10.3390/PATHOGENS8030100

713 Bergh, O., Børsheim, K.Y., Bratbak, G., Heldal, M., 1989. High abundance of viruses found
714 in aquatic environments. Nature 340, 467–468. doi:10.1038/340301a0

715 Berney, M., Greening, C., Conrad, R., Jacobs, W.R., Cook, G.M., 2014. An obligately aerobic
716 soil bacterium activates fermentative hydrogen production to survive reductive stress
717 during hypoxia. Proceedings of the National Academy of Sciences of the United States of
718 America 111, 11479–11484. doi:10.1073/PNAS.1407034111/-
719 /DCSUPPLEMENTAL/PNAS.201407034SI.PDF

720 Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: A flexible trimmer for Illumina
721 sequence data. Bioinformatics 30, 2114–2120. doi:10.1093/bioinformatics/btu170

722 Clokie, M.R.J., Millard, A.D., Letarov, A. v., Heaphy, S., 2011. Phages in nature.
723 Bacteriophage 1, 31–45. doi:10.4161/bact.1.1.14942

724 Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A.,
725 Keane, T., McCarthy, S.A., Davies, R.M., Li, H., 2021. Twelve years of SAMtools and
726 BCFtools. GigaScience 10, 1–4. doi:10.1093/GIGASCIENCE/GIAB008

727 Florent, P., Cauchie, H.-M., Herold, M., Jacquet, S., Ogorzaly, L., 2022a. Soil pH, Calcium
728 Content and Bacteria as Major Factors Responsible for the Distribution of the Known
729 Fraction of the DNA Bacteriophage Populations in Soils of Luxembourg. Microorganisms
730 10, 1458. doi:10.3390/MICROORGANISMS10071458

731 Florent, P., Cauchie, H.-M., Herold, M., Ogorzaly, L., 2022b. Bacteriophages pass through
732 candle-shaped porous ceramic filters: Application for the collection of viruses in soil
733 water. MicrobiologyOpen 11, e1314. doi:10.1002/MBO3.1314

734 Hall, C.M., Busch, J.D., Shippy, K., Allender, C.J., Kaestli, M., Mayo, M., Sahl, J.W., Schupp,
735 J.M., Colman, R.E., Keim, P., Currie, B.J., Wagner, D.M., 2015. Diverse Burkholderia
736 Species Isolated from Soils in the Southern United States with No Evidence of B.
737 pseudomallei. PLoS ONE 10. doi:10.1371/JOURNAL.PONE.0143254

738 Hall, J.P.J., Harrison, E., Pärnänen, K., Virta, M., Brockhurst, M.A., 2020. The Impact of
739 Mercury Selection and Conjugative Genetic Elements on Community Structure and
740 Resistance Gene Transfer. Frontiers in Microbiology 11. doi:10.3389/FMICB.2020.01846

741 He, Z., Zhao, W., Liu, H., Chang, X., 2012. The response of soil moisture to rainfall event size
742 in subalpine grassland and meadows in a semi-arid mountain range: A case study in
743 northwestern China's Qilian Mountains. Journal of Hydrology 420–421, 183–190.
744 doi:10.1016/J.JHYDROL.2011.11.056

745 Hissler, C., Martínez-Carreras, N., Barnich, F., Gourdol, L., Iffly, J.F., Juilleret, J., Klaus, J.,
746 Pfister, L., 2021. The Weierbach experimental catchment in Luxembourg: A decade of
747 critical zone monitoring in a temperate forest - from hydrological investigations to
748 ecohydrological perspectives. Hydrological Processes 35, 1–7. doi:10.1002/HYP.14140

749 Jończyk, E., Kłak, M., Miedzybrodzki, R., Górski, A., 2011. The influence of external factors
750 on bacteriophages-review. Folia Microbiologica 56, 191–200. doi:10.1007/s12223-011-
751 0039-8

752 Kimura, M., Jia, Z.J., Nakayama, N., Asakawa, S., 2008. Ecology of viruses in soils: Past,
753 present and future perspectives. Soil Science and Plant Nutrition 54, 1–32.
754 doi:10.1111/j.1747-0765.2007.00197.x

755 Kinnunen, K., 1978. Tracing water movement by means of Eschirichia coli bacteriophages.
756 The water research institute, Helsinki.

757 Li, H., 2018. Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics 34,
758 3094–3100. doi:10.1093/BIOINFORMATICS/BTY191

759 Luo, E., Aylward, F.O., Mende, D.R., Delong, E.F., 2017. Bacteriophage Distributions and
760 Temporal Variability in the Ocean's Interior. MBio 8, 1–13. doi:10.1128/MBIO.01903-17

761 Mandal, U.K., 2008. Soil Physical and Chemical Properties in Relation to Conservation of
762 Natural Resources, CORE.

763 Martínez-Carreras, N., Hissler, C., Gourdol, L., Klaus, J., Juilleret, J., Iffly, J.F., Pfister, L.,
764 2016. Storage controls on the generation of double peak hydrographs in a forested
765 headwater catchment. Journal of Hydrology 543, 255–269.
766 doi:10.1016/J.JHYDROL.2016.10.004

767 Martínez-Carreras, N., Udelhoven, T., Krein, A., Gallart, F., Iffly, J.F., Ziebel, J., Hoffmann,
768 L., Pfister, L., Walling, D.E., 2010. The use of sediment colour measured by diffuse
769 reflectance spectrometry to determine sediment sources: Application to the Attert River
770 catchment (Luxembourg). Journal of Hydrology 382, 49–63.
771 doi:10.1016/j.jhydrol.2009.12.017

772 Martínez-Carreras, N., Wetzel, C.E., Frentress, J., Ector, L., McDonnell, J.J., Hoffmann, L.,
773 Pfister, L., 2015. Hydrological connectivity inferred from diatom transport through the
774 riparian-stream system. Hydrology and Earth System Sciences 19, 3133–3151.
775 doi:10.5194/hess-19-3133-2015

776 Masroor, M., Sajjad, H., Rehman, S., Singh, R., Hibjur Rahaman, M., Sahana, M., Ahmed, R.,
777 Avtar, R., 2022. Analysing the relationship between drought and soil erosion using

778 vegetation health index and RUSLE models in Godavari middle sub-basin, India.
779 Geoscience Frontiers 13, 101312. doi:10.1016/J.GSF.2021.101312

780 Mirdita, M., Steinegger, M., Breitwieser, F., Sö Ding, J., Levy Karin, E., 2021. Fast and
781 sensitive taxonomic assignment to metagenomic contigs. Bioinformatics 37, 3029–3031.
782 doi:10.1093/BIOINFORMATICS/BTAB184

783 Mohanty, S.K., Saiers, J.E., Ryan, J.N., 2016. Colloid Mobilization in a Fractured Soil: Effect
784 of Pore-Water Exchange between Preferential Flow Paths and Soil Matrix. Environmental
785 Science and Technology 50, 2310–2317. doi:10.1021/ACS.EST.5B04767

786 Nurk, S., Meleshko, D., Korobeynikov, A., Pevzner, P.A., 2017. MetaSPAdes: A new versatile
787 metagenomic assembler. Genome Research 27, 824–834. doi:10.1101/gr.213959.116

788 Ofer, N., Wishkautzan, M., Meijler, M., Wang, Y., Speer, A., Niederweis, M., Gur, E., 2012.
789 Ectoine biosynthesis in Mycobacterium smegmatis. Applied and Environmental
790 Microbiology 78, 7483–7486. doi:10.1128/AEM.01318-
791 12/SUPPL_FILE/ZAM999103756SO1.PDF

792 Paul, S.I., Rahman, M.M., Salam, M.A., Khan, M.A.R., Islam, M.T., 2021. Identification of
793 marine sponge-associated bacteria of the Saint Martin's island of the Bay of Bengal
794 emphasizing on the prevention of motile Aeromonas septicemia in Labeo rohita.
795 Aquaculture 545, 737156. doi:10.1016/J.AQUACULTURE.2021.737156

796 Pfister, L., Martínez-Carreras, N., Hissler, C., Klaus, J., Carrer, G.E., Stewart, M.K.,
797 McDonnell, J.J., 2017. Bedrock geology controls on catchment storage, mixing, and
798 release: A comparative analysis of 16 nested catchments. Hydrological Processes 31,
799 1828–1845. doi:10.1002/HYP.11134

800 Rossi, P., Doerfliger, N., Kennedy, K., Müller, I., Aragno, M., 1998. Bacteriophages as surface
801 and ground water tracers. Hydrology and Earth System Sciences 2, 101–110.

802 Rousseau, M., di Pietro, L., Angulo-Jaramillo, R., Tessier, D., Cabibel, B., 2004. Preferential
803 Transport of Soil Colloidal Particles: Physicochemical Effects on Particle Mobilization.
804 Vadose Zone Journal 3, 247–261. doi:10.2113/3.1.247

805 Rstudio Team, 2020. RStudio: Integrated development for R. RStudio, PBC. Boston, MA.

806 Scaini, A., Hissler, C., Fenicia, F., Juilleret, J., Iffly, J.F., Pfister, L., Beven, K., 2018. Hillslope
807 response to sprinkling and natural rainfall using velocity and celerity estimates in a slate-
808 bedrock catchment. Journal of Hydrology 558, 366–379.
809 doi:10.1016/j.jhydrol.2017.12.011

810 Schaub, S.A., Sorber, C.A., 1977. Virus and bacteria removal from wastewater by rapid
811 infiltration through soil. Applied and Environmental Microbiology 33, 609–619.

812 Schimel, J.P., 2018. Life in Dry Soils: Effects of Drought on Soil Microbial Communities and
813 Processes. Annual Review of Ecology, Evolution, and Systematics Annu. Rev. Ecol. Evol.
814 Syst 12, 409–432. doi:10.1146/annurev-ecolsys-110617

815 SDEC, 2002. Lysimètres en PTFE / quartz pour ETM éléments métalliques en trace [WWW
816 Document]. URL https://www.sdec-france.com/lysimetres-ptfe-quartz-pour-solution-du-
817 sol.html (accessed 10.26.21).

818 Seiler, K.-P., Gat, J.R., 2007. Groundwater Recharge from Run-Off, Infiltration and
819 Percolation. Water Science and Technology Library 55. doi:10.1007/978-1-4020-5306-1

820 Simon S., 2019. getApi: Calculate the antecedent precipitation index [WWW Document].
821 Hydro-Giub/HydroBE: Miscellaneous Functions of the Group of Hydrology Bern. URL
822 https://rdrr.io/github/hydro-giub/hydroBE/man/getApi.html (accessed 10.16.22).

823 Smits, S.L., Bodewes, R., Ruiz-Gonzalez, A., Baumgärtner, W., Koopmans, M.P., Osterhaus,
824 A.D.M.E., Schürch, A.C., 2014. Assembly of viral genomes from metagenomes. Frontiers
825 in Microbiology 5. doi:10.3389/FMICB.2014.00714/ABSTRACT

826 Steinegger, M., Söding, J., 2017. MMseqs2 enables sensitive protein sequence searching for
827 the analysis of massive data sets. Nature Biotechnology 2017 35:11 35, 1026–1028.
828 doi:10.1038/nbt.3988

829 Sutton, T.D.S., Clooney, A.G., Ryan, F.J., Ross, R.P., Hill, C., 2019. Choice of assembly
830 software has a critical impact on virome characterisation. Microbiome 7, 1–15.
831 doi:10.1186/S40168-019-0626-5/FIGURES/4

832 Turner, D., Kropinski, A.M., Adriaenssens, E.M., 2021. A roadmap for genome-based phage
833 taxonomy. Viruses 13, 1–10. doi:10.3390/v13030506

834 VDLUFA, 2016. Methodenbuch A5.1.1: Bestimmung des pH-Wertes.

835 VDLUFA, 2012. Methodenbuch A6.2.1.1: Bestimmung von phosphor und kalium im calcium-
836 acetat-lactat-auszug.

837 VDLUFA, 1997. Methodenbuch A6.2.1.7: Bestimmung von pflanzenverfügbarem kalium und
838 natrium im calciumchloridauszug auf gewichtsbasis.

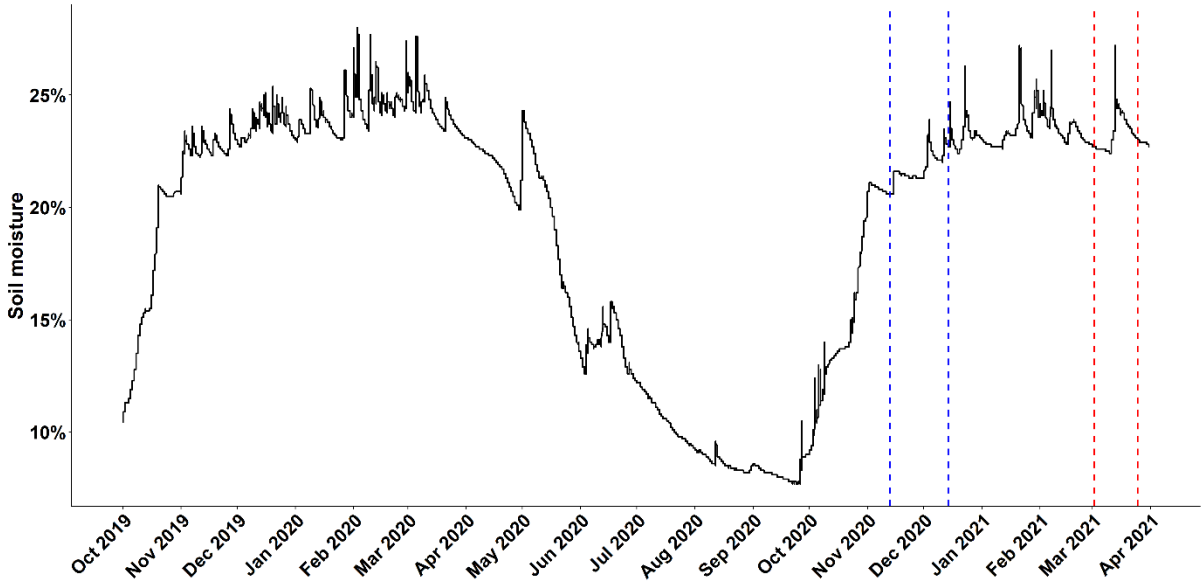
839 Weitz, J.S., Wilhelm, S.W., 2012. Ocean viruses and their effects on microbial communities
840 and biogeochemical cycles. F1000 Biology Reports 4, 2–9. doi:10.3410/B4-17

841 Williamson, K.E., Radosevich, M., Wommack, K.E., 2005. Abundance and diversity of viruses
842 in six Delaware soils. Applied and Environmental Microbiology 71, 3119–3125.
843 doi:10.1128/AEM.71.6.3119

844 Yamada, H., Yamaguchi, M., Igarashi, Y., Chikamatsu, K., Aono, A., Murase, Y., Morishige,
845 Y., Takaki, A., Chibana, H., Mitarai, S., 2018. Mycolicibacterium smegmatis, Basonym
846 Mycobacterium smegmatis, Expresses Morphological Phenotypes Much More Similar to
847 Escherichia coli Than Mycobacterium tuberculosis in Quantitative Structome Analysis
848 and CryoTEM Examination. Frontiers in Microbiology 9, 1992.
849 doi:10.3389/FMICB.2018.01992/BIBTEX

850 **Supplementary figures**

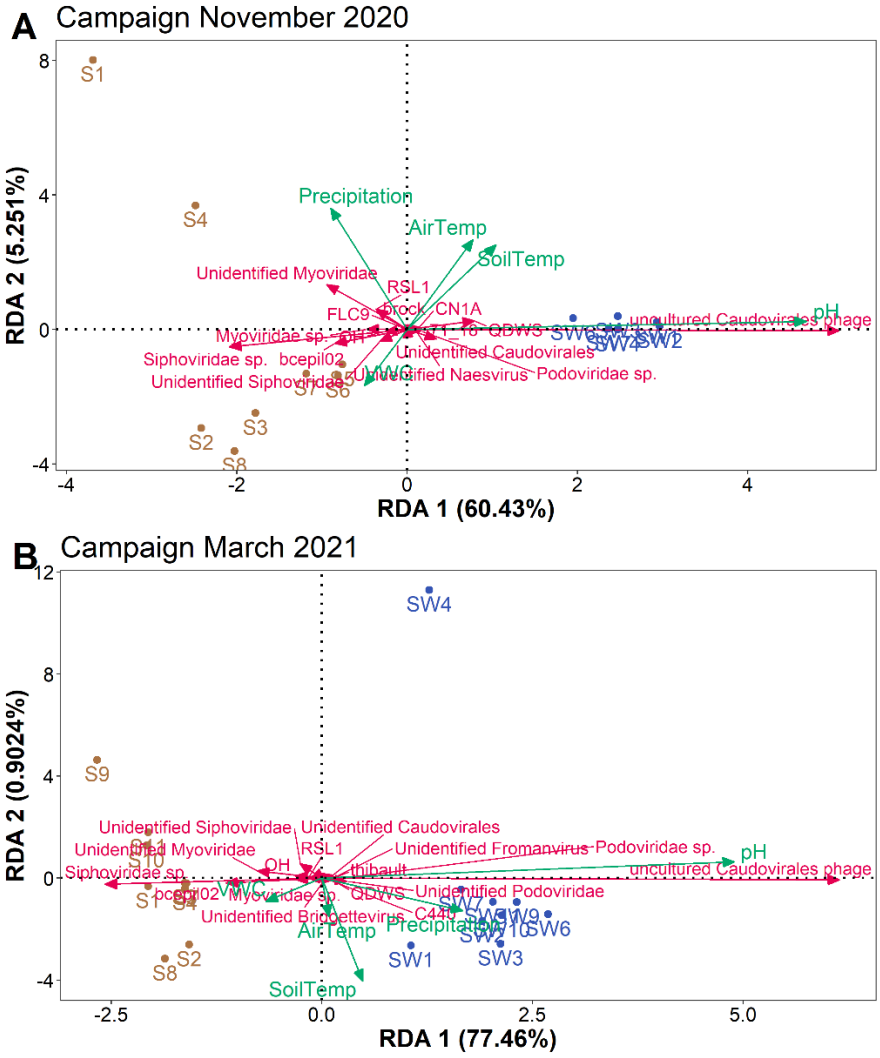
851



852

853

854 *Figure S1: Weierbach soil moisture percentage from October 1, 2019 to April 1, 2021. The*
855 *blue dashed lines represent the first field campaign (from November 13, 2020 to December 14,*
856 *2020) while the red dashed lines represent the second field campaign (from March 2 to March*
857 *25, 2021).*



858

859

860 *Figure S2: Biplot of redundancy analysis (RDA) of the 10 most abundant bacteriophage*
861 *species in relation to the environmental factors during (A) the campaign of November 2020*
862 *and (B) the campaign of March 2021. The pink vectors represent the bacteriophage species,*
863 *and the green vectors display the selected environmental factors. The soil samples are*
864 *represented in brown colour with the letter "S" and the soil water samples in blue with the*
865 *letter "SW". The RDA was built over the two first dimensions, with 77.46% of the fitted*
866 *variations explained by RDA1 and 0.90% by RDA2. (API = Antecedent precipitation index*
867 *followed by the number of days; SoilTemp = soil temperature; AirTemp = air temperature;*
868 *VWC = volumetric water conten*t).

Supplementary tables

Table S1: Physical-chemical properties of soils and soil water on the Weierbach plateau for each collected sample of the two campaigns.

	Collection dates	Soil Water pH	Soil								
		pH	pH	GWC	C/N	C_{org}	N_{tot}	Mg	Na	P_2O_5	K_2O
		-	-	%	-	%	%	mg/100 g ds	mg/100 g ds	mg/100 g ds	mg/100 g ds
November Campaign	13.11.2020	5.8	-	-	-	-	-	-	-	-	-
	16.11.2020	5.1	4.1	40.1	18.4	10.1	0.55	4	2	4	8
	20.11.2020	4.5	4.0	45.7	18.4	10.3	0.56	4	2	4	9
	26.11.2020	5.3	4.1	39.1	19.0	9.7	0.51	4	2	3	7
	01.12.2020	5.4	4.3	36.5	17.9	7.5	0.42	3	1	4	7
	04.12.2020	6.1	4.2	43.4	19.1	10.1	0.53	3	1	3	7
	07.12.2020	6.2	4.3	36.9	17.9	8.6	0.48	3	2	3	7
	10.12.2020	4.9	4.3	43.6	19.0	9.7	0.51	5	2	4	9
	14.12.2020	-	4.3	40.9	20.4	10.4	0.51	4	2	3	8
March Campaign	02.03.2021	5.1	-	-	-	-	-	-	-	-	-
	03.03.2021	5.4	4.1	35.0	20.0	9.6	0.48	5	1	4	9
	05.03.2021	5.5	4.0	36.0	19.2	10.0	0.52	4	1	4	9
	08.03.2021	6.3	4.1	41.3	20.8	7.5	0.36	4	1	4	8
	10.03.2021	6.7	4.1	34.7	20.0	7.4	0.37	3	1	4	7
	11.03.2021	7	4.1	35.3	19.6	9.2	0.47	6	1	4	10
	12.03.2021	5.7	-	-	-	-	-	-	-	-	-
	15.03.2021	5.7	4.1	43.4	20.8	8.3	0.40	4	1	4	9
	18.03.2021	6.4	4.1	42.4	21.2	8.7	0.41	3	1	4	8
	22.03.2021	5.36	4.1	44.8	21.5	8.4	0.39	3	1	4	8
	24.03.2021	6.9	4.0	82.8	19.5	8.6	0.44	3	1	4	8
	25.03.2021	ND	4.1	37.9	20.5	9.0	0.44	4	1	4	8

GWC = gravimetric water content, Corg: organic carbon, Ntot: total nitrogen; ds = dry soil; S = Soil; SW = Soil wate

873

This part of the results focused on the study of the potential mobilisation of previously identified natural populations of bacteriophages. Indeed, a first comparison of the bacteriophage communities identified in the soil and soil water samples from the Weierbach was made and they were different from each other. Moreover, this difference in communities was significatively related to the difference in pH between the two types of samples. The hypothetical mobilisation of bacteriophages between soil and soil water was to be reflected in the visualisation of variation in bacteriophage abundances in response to targeted precipitation events. Significant variations and correlations were indeed observed for some species, and more particularly for the bacteriophages RSL1 and Thibault for the field campaigns in November and March 2021, respectively, significantly varying according to rainfall.

Thus, the results of the first WP allowed the characterisation of soil bacteriophage populations and the study of their distribution as a function of different environmental parameters, and those obtained during this second WP revealed variations in the abundance of certain bacteriophage species. By the complementarity of these two WPs, the objective of the thesis, which is based on the proposal of new environmental tracers among the natural population of bacteriophages, can be answered and discussed. The next part of this manuscript will thus be dedicated to this discussion of the objective as well as the PhD work hypotheses proposed in Chapter 2.

Chapter 4:

DISCUSSION AND PERSPECTIVES

Chapter 4:

DISCUSSION AND PERSPECTIVES

The objective of the present study was to assess the potential of natural soil DNA-bacteriophages to serve as environmental tracers of hydrological processes, with the ambition to search for new potential hydrological tracer candidates. Two complementary work packages (WPs) were built to achieve this objective. The first WP aimed at studying DNA-bacteriophage communities in several types of soils in Luxembourg (WP1). The second WP aimed at determining the ability of the natural soil DNA-bacteriophages previously identified to be mobilised from soil to soil water (WP2).

The characterisation of the natural populations of DNA-bacteriophages in diverse types of soil in Luxembourg has demonstrated the importance of these entities for terrestrial life. Bacteriophages are mainly known to be dependent on microbial communities, they however actively participate in soil life by playing significant roles in bacterial activities (e.g. soil decomposition) (Briones, 2018; Islam et al., 2022; Raynaud and Nunan, 2014). As an integral part of the soil system, the fate and survival of bacteriophages also depend on environmental factors over temporal and spatial scales. Given their importance in the terrestrial compartment and the promising first results on their mobilisation, it is now fully consistent that natural DNA-bacteriophages can be considered hydrological tracers. To deepen the new knowledge acquired during this work, the results obtained on these different aspects lead to three fundamental points of discussion. Despite progress in the development of molecular methods, the characterisation of microbial populations remains a scientific challenge. There are still no standard methods applicable to various types of samples and studies, so method optimisation work had to be carried out in the framework of this thesis project. The methods developed have successfully allowed the characterisation of total microbial populations from complex samples such as soil. However, as in many cases where very specific methods are required, limitations exist, and these will be developed and discussed in this section. The discussion will then turn to the natural populations of bacteriophages identified in soils and their relationship to certain environmental factors and climatic parameters. Using these results, the discussion will continue by reviewing the criteria needed to define an ideal tracer and comparing them with the characteristics of the

natural soil DNA-bacteriophages identified with the final aim of selecting potential candidates. Depending on the knowledge available on the bacteriophages selected as tracers, the last part of the discussion will be devoted to proposals for the development of methodologies to firstly acquire more information on the bacteriophages through laboratory experiments, and secondly to allow the detection of bacteriophages when used as hydrological tracers in experiments.

4.1 Limitations of the current methodology

In the course of this work, many methodological challenges were encountered due to the complexity of the samples, the viruses and the application of the methods, which we tried to overcome as best as possible.

The first obstacle encountered in the methodology for the determination of the bacteriophage population concerns the soil samples themselves. Preparation of soil samples is very often necessary before the bacteriophage populations can be analysed, involving elution, collection and enrichment of the latter (Xavier Fernandez-Cassi et al., 2018; Hjelmsø et al., 2017). The lack of a standard method that can be generally applied has led to many variations of these protocols in the literature (Forés et al., 2022; Monpoeho et al., 2001). Therefore, based on the various possible protocols, optimisation of this virus elution and enrichment methodology was performed (Florent et al., 2022). Although this protocol allowed us to obtain sufficient viral eluates for VLPs enumeration, while DNA quantification revealed too low DNA yield to perform good-quality sequencing techniques. This could mainly be explained by complexity of the soil matrix, which brings together different types of life that interact with each other and with the environment around them (Davis et al., 2006; Joergensen et al., 1995; Kalev and Toor, 2018). In addition, soil characterisation and functioning can vary greatly from one site to another, making multivariate exploration of the datasets difficult (Williamson et al., 2017, 2005). As already described in this manuscript, bacteriophages interact extensively with soil particles. These interactions depend on various variables such as clay content, amount of organic matter or ionic strength (Cheng et al., 2007; Park et al., 2015). This not only gives the bacteriophages an adsorption capacity on the soil particles but also variability in the degree of their adsorption (Rossi and Aragno, 1999). This makes the analysis of soil bacteriophages a real challenge. Indeed, the rate of bacteriophages attached to soil particles is not known and may very likely differ from one soil to another

(Batinovic et al., 2019). It is also impossible to know if all the bacteriophages initially attached to the matrix have actually been detached at the time of elution, potentially resulting in a lower recovery than expected.

The application of this elution protocol is also useful for isolating and extracting viruses from other organisms (i.e. bacteria, eukaryota) in the soil, allowing sequencing on samples containing only viruses (Gaafar and Ziebell, 2020; Monpoeho et al., 2001). This would have resulted in a better representation of viruses in samples and allowed a better depth of sequencing. However, in the case of this PhD work, the low yield in DNA from the viral eluates obtained resulted in either unsuccessful library preparation or poor-quality sequences generated. To overcome this technical issue, DNA extractions were carried out directly on the raw soil samples. This is where we encountered the second obstacle in the methodology. Viruses have high genomic diversity, and few or even no conserved sequences across the species, leading to limitations in the use of targeted metagenomics (Hatfull and Hendrix, 2011). To characterise and identify the entire viral population, shotgun metagenomics must preferably be used (Hiraoka et al., 2016; Zhang et al., 2018). However, this method sequences the totality of the extracted DNA genomes, without distinguishing between the different types of organisms. All taxonomic kingdoms, eukaryotes and archaea, are thus characterised, which presents some issues in the viral analyses. Although viruses are much more abundant than other soil microorganisms, their genomes are much less abundant in order of magnitude, than those of bacteria in particular (Rose et al., 2016). This was reflected in the sequencing results where soil samples were represented by only 0.2 to 1% of generated viral sequences compared to over 85% of bacterial sequences. This low representativeness of viral genomes compared to the totality of genomes sequenced in a sample can be a limitation in the identification of viruses especially when it comes to the implementation of an adequate bioinformatics pipeline. Bioinformatics tools to specifically detect viruses have been developed in recent years, including VirSorter, VirFinder among others (Guo et al., 2021; Kieft et al., 2020; Ren et al., 2017; Roux et al., 2015a). However, their application in the case of DNA extracted directly from soil and soil water samples has not been convincing and has shown to work better on virus-enriched samples. After a series of optimisations, the pipelines using non-specific tools showed more satisfactory results when it came to the taxonomic assignment of viruses. The pipeline used in this thesis project was successful in identifying bacteriophages present in soil and soil water but may require further optimisations to improve and confirm the already promising results.

The recent development of bioinformatics tools for virus detection and identification is important in the scientific advancement of virus ecology (Sanjuán and Domingo-Calap, 2021). However, we are still a long way from what we know about bacterial communities. Viruses are among the least studied ecological communities, for which many investigations are still needed (Rohwer et al., 2009; Sullivan et al., 2017). This need for viral characterisation is particularly reflected in the viral databases, whichare still incomplete and may therefore be responsible for an under-representation of the identified viral population (Rose et al., 2016). However, to select certain bacteriophages as tracers among those characterised, bioinformatics pipelines based on the available databases are preferred since as much information as possible about the bacteriophages will be necessary to use them appropriately in tracing experiments (Dutilh et al., 2012; Mirdita et al., 2021). For this reason, it was more interesting to focus on the known fraction of the viral population that has already been characterised, which is present in the databases (Phadke et al., 2021). These databases have the advantage of being updated quite frequently, allowing access to the most recent viral taxonomy possible (Lefkowitz et al., 2008). Indeed, the latter has evolved very rapidly, particularly over the last two years with a total reorganisation of the viral taxonomy. As of March 2022, the order *Caudovirales*, the most important order in viral taxonomy, as well as the families *Siphoviridae*, *Myoviridae* and *Podoviridae*, were removed and their species were either reclassified into another family or considered temporarily unclassified *Caudoviricetes* (Turner et al., 2021). There is a lag time between the latest changes to the taxonomy and the updating of the databases. In this PhD work, the version of the nr database used in the bioinformatics pipeline was dated April 2022 and did not yet consider these latest taxonomic changes. Thus, the three most representative families of bacteriophage taxonomy, namely *Siphoviridae*, *Myoviridae* and *Podoviridae*, are still relevant to the characterisation of bacteriophage populations in this project. This is where the last methodological limitation is encountered. Many species are classified into these three families, leading to an over-representation of these families in the viral taxonomy (Lopes et al., 2014b; Nelson, 2004). This over-representation has then an impact on the interpretation of the composition of viral populations where these three families will also be the most abundant bacteriophages. However, it can be overcome by exploring the populations at the taxonomic level of the species. The detection of species and their abundance are not dependent on the representation of these three families, thus reducing to some extent the dependence on the composition of the viral classification, which was possible through the implementation of our bioinformatics pipeline.

Despite these limitations, interesting results were obtained, particularly the identification of microbial populations, especially those of bacteriophages, in the soil and soil water. These methods have also revealed that certain environmental and climatic factors play an important role in the distribution and dynamics of natural DNA-bacteriophage populations. Finally, some of the bacteriophages among the characterised populations stood out for their application as hydrological tracers, which was possible thanks to the methodology implemented in this thesis project.

4.2 Dynamics of naturally occurring DNA-bacteriophages in the terrestrial compartment

4.2.1 Characterisation of natural populations of DNA-bacteriophages in various types of soil

To use soil natural populations of DNA-bacteriophages as hydrological tracers, certain analyses are required before the selection of potential candidates. Not only must the site of interest be characterised in terms of bacteriophage diversity, but the influence of some environmental factors must also be considered to best predict the fate of the tracer. The bacteriophage populations characterised in the eight Luxembourg soils studied in this study seem to open the way to a large panel of possible candidates to meet the demand for new hydrological tracers. The choice can then be made among the 10^9 to 10^{10} VLPs per gram of dry soil, in which *Caudovirales* was the most frequently identified order in all sampling sites of the study, and *Siphoviridae*, *Podoviridae* and *Myoviridae* were the most abundant families observed.

The DNA-bacteriophage distribution was analysed at a spatial scale, with the selection of 8 sites covering a total surface of approximately 100 km² in the Northwest of Luxembourg, nearby the Attert river. The site characteristics important for the interpretations of the results obtained in the WP1 are reported in Table 8. No pattern in the DNA-bacteriophage distribution was highlighted according to the vegetation between the grassland and forest sites, while specific soil parameters did influence their distribution. Bacteriophages are known to be strongly dependent on their bacterial host to survive and replicate (Clokie et al., 2011; Duckworth, 1976). Therefore, the idea of selecting a model bacteriophage per type of

land cover is not feasible in this study, and the choice of the candidate(s) to be a hydrological tracer will rather depend on the characteristics of the soil studied.

Table 8: Summary of the most important characteristics of all sites as part of the WP1, where the 8 sites are displayed with their vegetation, geological region location, soil pH, calcium content, carbon/nitrogen (C/N) ratio and the bacteriophage and bacterial diversities

Sites	**Vegetation**	**Geological regions**	**Soil pH**	**Calcium content (Meq kg^{-1})**	**C/N ratio**	**Bacteriophage diversity (Shannon index)**	**Bacterial diversity (Shannon index)**
Weierbach	Forest	Oesling	4.1	21	18	2.83	2.89
Daerent	Forest	Gutland	6.3	126	10	2.39	3.12
Retgenbusch	Forest	Gutland	4.5	16	8.6	2.31	2.78
Pall 1	Grassland	Gutland	6.8	166	9.8	2.65	3.18
Pall 2	Grassland	Gutland	6.2	151	8.8	2.62	3.13
Hueschterbach	Grassland	Oesling	6.4	87	8.8	2.54	3.11
Mollbach	Grassland	Gutland	6.0	131	8.3	2.79	3.11
Koulbich	Grassland	Gutland	5.7	58	8.3	2.58	3.13

Soil pH and calcium concentration were the environmental factors showing a significant influence on the ecological distribution of the identified DNA-bacteriophage among the different studied soils, and they will thus be taken into consideration in the selection process. Calcium content and soil pH are strongly correlated to each other; soils with low pH generally display low calcium availability. It was shown that the lower the pH and the calcium concentration, the lower the microbial species richness, especially valid for the soils of Weierbach and Retgenbusch. These two sites shared a similar pH and calcium concentration but showed differences in their bacteriophage compositions that could be explained by the difference in carbon/nitrogen (C/N) ratio, much higher for the Weierbach soil than for the Retgenbusch soil. Although the C/N ratio did not show a significant influence on the composition of the DNA-bacteriophage populations, this property could still be studied in the selection process to better refine the final choice. In addition to the effect of certain abiotic factors, our results confirmed the correlation between DNA-bacteriophage distributions and bacterial communities. Not only did they share similar distributions, but they were also impacted by the same soil properties (Whittinghill and Hobbie, 2012, 2011). This could confirm a possible indirect effect of soil properties on the variations of the DNA-bacteriophage population composition by impacting first the bacterial population. However, a co-analysis with host bacteria rather than total bacterial populations would be more appropriate to answer this question and would also verify the actual presence of host bacteria corresponding to the identified DNA-bacteriophage populations.

4.2.2 Mobilisation of natural populations of DNA-bacteriophages in the soil of Weierbach

In the selection process of bacteriophages to use as hydrological tracers, their presence and abundance in the soil is an important criterion but not sufficient on its own. Indeed, these bacteriophages must also be able to be mobilised to reach other compartments. To verify this scientific aspect, the populations of DNA-bacteriophages in soil and soil water were investigated to find similar species between the two reservoirs and to test their variations according to rainfall. The distribution of the DNA-bacteriophage populations was then and studied at a temporal scale solely on the site of Weierbach. Of all the soils studied during this PhD work, the soil of the Weierbach catchment area has always proved to be quite different. Not only did it show the most extreme physicochemical properties, but also

displayed the lowest bacterial and DNA-bacteriophage richness. In addition, the Weierbach catchment is the main model catchment investigated within the DTU, in which this PhD project takes part. For these reasons, the focus has been made on this site, instead of all the others from the WP1. The variations of bacteriophage species were studied over 1 month, at strategic periods where the first campaign occurred in November, at the beginning of the cold period when the soil moisture barely reaches its high level, and the second campaign occurred in March, at the end of the same period, where the high level of soil moisture is well settled, and the temperatures increase. The specific case of the Weierbach showed an important abundance of *Ralstonia phage RSL1* (unclassified *Caudoviricetes*, former *Myoviridae*) and *Lessievirus bcepIL02* (unclassified *Caudoviricetes*, former *Podoviridae*). Bacteriophage populations between soil and soil water samples were found to be significantly different from each other, and this difference is mainly driven by pH. This result confirms the role of environmental pH in the establishment of the DNA-bacteriophage populations in the ecosystems studied here, soil and soil water, among all the factors studied. However, when looking more specifically at the variation in abundance of bacteriophages over time, no environmental factors appear to show any influence on the population at the taxonomic level of the family. On the other hand, when looking at the most abundant bacteriophage species, some of them showed significant correlations with certain factors, such as soil moisture or rainfall. The latter is of particular interest in the case of this research work since precipitations can be at the origin of water transport in the terrestrial compartment, notably via the infiltration process. Thus, the investigations mainly focused on the species with a significant correlation to the targeted rainfalls: *Ralstonia phage RSL1* and *Omegavirus Thibault* (unclassified *Caudoviricetes*, former *Siphoviridae*). These two species were not only present in both compartments, but they were also among the most abundant species identified and their variation in abundance was significantly related to rainfall. These different aspects are rather promising for their selection as hydrological tracers. Finally, it is important to note that bacteriophage *RSL1* was detected in both November and March, but its variation in abundance showed a correlation with the precipitations only in November, whereas bacteriophage *Thibault* was detected only in March, suggesting a potential relationship with the seasonality for this bacteriophage.

4.3 Within the process of selecting bacteriophage candidates as hydrological tracers

4.3.1 Understand the catchment of interest to improve the use of natural bacteriophages as hydrological tracers

As explained in the review paper (Chapter 1), taking into consideration every characteristic of the studied catchment is highly recommended for using both artificial (i.e. injected) and environmental (i.e. natural populations) bacteriophages as hydrological tracers. A thorough study of the catchment area concerned before the start of any tracing experiments, particularly about physicochemical properties, will make it possible to reduce the loss of the bacteriophage signal, and thus optimise their use as hydrological tracers. By the same reasoning, the Weierbach catchment area will be described to better understand the fate and behaviour of the bacteriophages, thus allowing us not only to select the best candidates but also to use them to the best of their abilities.

The plateau of this catchment, where the samples were collected, is characterised by a beech forest. Beech trees thrive best on moist, well-drained and acidic to neutral soils, and can grow on a wide range of soil textures. The Weierbach is located in the geological region of the Oesling, which is mainly composed of shale rock, a clayey sedimentary rock with a laminated structure (Hissler et al., 2021). The texture of the Weierbach soil is characterised as clay-loam (34% clay, 42% silt and 24% sand), which defines a loamy soil with a high proportion of clay. The presence of a high level of clay is one of the most important characteristics of this soil since this high level of clay makes the loamy soil particularly dense. This is because clay particles (< 0.002 mm in diameter) tend to swell when wet, reducing the pore size of the soil; but when dry, the particles shrink, creating cracks on the soil surface (Barton and Karathanasis, 2002; Kumari and Mohan, 2021). This gives clay-loam soil a higher capacity to retain water, particularly during rainfall, which results in moderately slow permeability (0.5 – 2.0 cm/hr) (O'Green, 2013). The beech forest of the Weierbach is characterised by a vast and constant presence of leaf litter. When this litter decomposes, it adds organic matter to the soil, thus reducing water retention in the clay-loam soil. The presence of organic matter allows the soil to dry out more slowly and prevents it from forming clods while allowing water and air to pass through more easily when the soil is wet (Bot and Benites, 2005). Among all soils studied in this project, the soil of the

Weierbach displayed a particularly high content of organic carbon (C/N = 18, organic carbon = 9.7), and when analysing its annual soil moisture cycle, it was noticed that soil moisture was at its highest from November to April with approximately 20% to 26% of soil water content. This means that the soil has rather a drainable porosity with a tendency to reach a permanent writing point at the lowest soil water content (Figure 7).

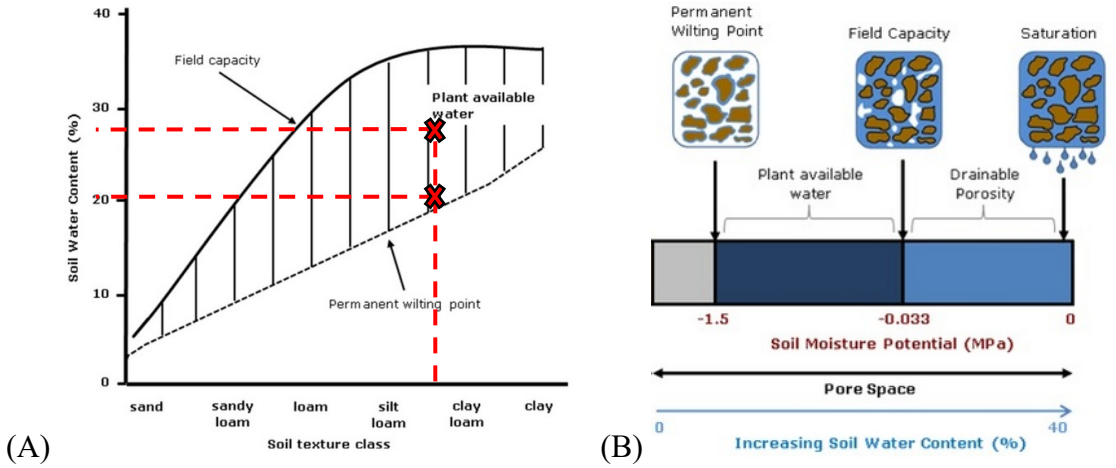


Figure 7: (A) Generalised scheme of the relationship between soil texture and soil water content (%) with the dashed lines representing the range of soil water content measured on the soil of Weierbach (≅ 20-26 %) (O'Green, 2013). (B) Different steps of the drainage according to the increasing soil water content (%), pore space and soil moisture potential (Mpa) (O'Green, 2013).

More precisely on the plateau of the Weierbach, not only runoff processes occurred when precipitations hit the ground, but also a fast subsurface flow through the macropores to then reach the stream (Martínez-Carreras et al., 2016). Therefore, water from precipitation can infiltrate into the soil, but subsequently, the water would be held back from percolating into the aquifer, thus preferentially following a subsurface flow. Bacteriophages are colloidal particles, which are defined as suspended particles in water with a diameter smaller than 10 µm (Fitts, 2013). Due to their small size, their gravity forces are negligible and thus can migrate with the water flow (Bagalkot and Kumar, 2018). Additionally, bacteriophages are colloids with a diameter even smaller than 200 nm, which makes them small enough to migrate through small pathways (e.g. fractures, pores) within soils or aquifers (Fitts, 2013).

Soil organic matter is involved with hydrophobic interactions. It was proven to be positively correlated with the degree of hydrophobicity; the higher the organic matter content, the higher the hydrophobicity (Mataix-Solera and Doerr, 2004). As one of the possible interactions implied in the adsorption of bacteriophages on soil particles, hydrophobic interactions can strongly impact the transport of bacteriophages in the soil. As previously mentioned, the Weierbach soil has a high organic matter content (C/N = 18), which means that the chances for hydrophobic interactions to occur are high. Bacteriophages, especially most families belonging to the order *Caudovirales*, are rather recognised as hydrophilic molecules (Harada et al., 2018). Therefore, they will adsorb less on more hydrophobic molecules, such as the previously mentioned organic matter. However, bacteriophages do not all have the same degree of hydrophobicity, which can influence the degree of adsorption. For example, as the bacteriophage Qβ is more hydrophobic than the bacteriophage MS2 (Sautrey et al., 2018), Qβ will adsorb more on the hydrophobic organic matter than MS2 suggesting that MS2 will be more transported. The hydrophobicity of both the environment and the bacteriophages is crucial in the selection and use of bacteriophages as hydrological tracers (as artificial or environmental tracers), especially for the Weierbach.

The high organic carbon content improves the water drainage, participates in the adsorption process, and is also most likely responsible for the acidity of soil (around 4.1 in the case of the Weierbach). Indeed, the organic carbon content in soils was shown to be negatively correlated with the soil pH (Zhou et al., 2019). Therefore, when the organic carbon in soil increases, the soil becomes more acidic. Considering the natural population of bacteriophages, pH of their initial soil habitat alone might not have a direct effect on their fate, but the change in pH can. When bacteriophages are transported from the soil to another compartment, such as soil water or surface water, they are subject to changes in environmental pH. When this study revealed that the soil pH of the Weierbach was around 4.1 and the pH of the soil water was found to be around 5-6, the literature reported the surface water as being generally around 7 (Clokie et al., 2011; Salmond and Fineran, 2015). Although we can easily imagine that certain bacteriophages naturally present in acidic soils can be sensitive to slightly acidic to neutral pH, experiments performed on laboratory strains showed that bacteriophages were rather sensitive, in terms of inactivation, to extreme pH such as below 2 or above 9 (Jończyk et al., 2011; Kimura et al., 2008). Moreover, the soil pH of the Weierbach did not vary temporally between the three campaigns in September 2019, November 2020 and March 2021, with a pH always around 4. Therefore, the natural

bacteriophage that will be selected as hydrological tracers can be used at any time of year in the soil of the Weierbach. Among the results obtained during this PhD work, the soil pH showed to be the environmental factor influencing the most the DNA-bacteriophage distribution at a spatial scale, first between the different types of soils, as described in section 3.1.2, and also, within the Weierbach, between the soil and the soil water samples, as demonstrated in section 3.2.2.

Besides the hydrophobic interactions, the mechanism of adsorption is also driven by van der Waals electrostatic forces, which occur between atoms or molecules and depend on the distance (Zemb et al., 2013). The soil texture of the Weierbach plateau has a high proportion of clay (≅ 34%). It is a natural soil material structured in layers made of silica and alumina bound by oxygen and hydroxide ions and presents a permanent negative charge on its surface (Barton and Karathanasis, 2002; Brigatti et al., 2006). Viruses have electrical charges on their surface, inducing interactions with other molecules, such as clay minerals in soils. These electrical charges depend strongly on the pH of the surrounding solutions, which is linked to the pI (isoelectric point) of bacteriophages. Thus, in the case of the soil of Weierbach, every bacteriophage with a pI below pH 4.1 will be found to be negatively charged and will rather interact with positively charged surfaces. With a pI above pH 4.1, bacteriophages will be positively charged and will be able to interact with negative charges, such as the one on clay minerals. It is important to note that clay minerals are subjected to isomorphic substitution. This process is the exchange of a cation for another one with a similar size, giving both positive and negative charges to the minerals. The replacement of a cation with another one with a lower valence (Si^{4+} by Al^{3+}) will add negative charges to the mineral. On the contrary, if the substitution involves the replacement of a cation with another one with a higher valence (Mg^{2+} by Fe^{3+}), then a positive charge will be gained (Schoonheydt and Johnston, 2006; Wang et al., 2017). Not only does this mean that clay minerals have a strong cation exchange capacity (CEC), but also that interactions with clay are reversible. However, in soil, isomorphic substitutions resulting in the gain of negative charges are mostly found (Barton and Karathanasis, 2002). The electrostatic interactions can strongly impact the transport of bacteriophages. In general, the change in environmental pH can cause a change in the surface charges of bacteriophages, potentially resulting in a new interaction with the surrounding environment. Moreover, in the case where bacteriophages are already attached to the soil surface, an increase in soil pH could potentially cause their detachment, and thus their release into the environment.

To sum up, the important characteristics to keep in mind in the process of selecting the best bacteriophage candidates as a hydrological tracer in the case of the Weierbach are:

- Water from precipitation will infiltrate, but bacteriophages will then follow subsurface flow rather than percolation,
- The soil in the Weierbach had a high organic matter content, improving the drainage capacity of the soil but also involving hydrophobic interactions in the adsorption process of bacteriophages,
- The soil pH showed low pH changes within the soil over time, but soil pH is significantly different from the nearby compartments,
- The soil is characterised as loam with a high level of clay, which plays an important role in the electrostatic interactions in the adsorption process of bacteriophages

4.3.2 Best-suited bacteriophage candidates according to the criteria of an ideal tracer

As mentioned in Chapter 1, certain criteria have been predefined to best meet the requirements of an ideal tracer. These criteria are reported below in Table 9, for the conventional tracers, but also the bacteriophages as artificial and environmental tracers. These criteria will be used as a guideline to select the most suitable natural DNA-bacteriophages according to the properties of the Weierbach catchment area and those of the DNA-bacteriophages themselves. In this way, the selected DNA-bacteriophages will meet the requirements of the ideal tracer as far as possible and will be consistent with the catchment properties for optimal use of the bacteriophage as tracers.

According to the criteria for ideal tracers, the bacteriophages used as environmental tracers are among those that best meet the requirements. It is important to note that none of the tracers used so far meet all the necessary criteria. Until now, natural isotopes (radioactive or stable) have been the most popular hydrological tracers among hydrologists and thus, the most widely used since they meet five of the eight criteria.

Bacteriophages, as artificial tracers, have already proven to be effective during tracing experiments due to their low limit of detection, their ability to perform tracing experiments involving several different bacteriophages and their environmental friendliness. As explained in Chapter 1, the last statement is however problematic when considering the large

volume of exogenous bacteriophage solutions with very high concentrations injected into the environment. Therefore, to solve to this potential environmental concern, the concept of natural bacteriophages as hydrological tracers has been developed in this study.

The remaining flaws of the natural bacteriophages are their high adsorption, their inactivation in the environment, their sensitivity to environmental changes and their solubility. As colloidal particles, bacteriophages are *per se* not soluble entities, a property that cannot be changed. However, other criteria, such as adsorption, inactivation and sensitivity to environmental changes, are highly dependent on the family, genera or even species of the bacteriophage and can therefore be optimised by selecting the bacteriophages according to their intrinsic properties. It is important to note, however, that two criteria, the limit of detection and the low cost, need to be slightly qualified in the case of bacteriophages. Considering the plaque assay, it is quite valid to say that the detection limit is relatively low, as is the cost of application. The detection limit, whether for the plaque assay or the molecular techniques, will strongly depend on the initial concentration of bacteriophages in the medium studied. If the concentration is too low, a preliminary step of virus concentration can be performed to overcome this issue. Finally, the costs involved in using bacteriophages as tracers will mainly concern molecular techniques. Indeed, the cost varies a lot from one method to another (i.e. qPCR is about 5-10€/sample while the price of a metagenomic shotgun can start at 150 €/sample) (Baffert et al., 2013; Goldrick et al., 2013; Hren, 2018). In addition to this, there are equipment and post-sequencing computer analysis costs to consider, which also vary from one device or software to another depending on the intended use (Muir et al., 2016; Schwarze et al., 2019).

Table 9: The eight criteria predefined to best meet the requirements of an ideal tracer, for the conventional tracers (i.e. dyes, salts and isotopes) and bacteriophages as both artificial (i.e. injected) and environmental (i.e. natural populations) tracers.

Criteria	Dyes	Salts	Radioactive isotopes		Stable isotopes		Bacteriophages	
			Injected	Natural	Injected	Natural	Injected	Natural
Low sorption	No	No	No	No	No	No	No	No
Good chemical and biological stability	No	No	No	No	No	No	No	No
Good solubility	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Low background concentration	No	No	Yes	NC	Yes	NC	Yes	NC
Insensitive to changes	No	No	No	No	No	No	No	No
Efficient detection limit	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes*
Low toxicity and environmental impact	No	No	No	Yes	Yes	Yes	No	Yes
Low cost	Yes	Yes	No	Yes	No	Yes	Yes	Yes**
Total of criteria met	3	2	3	5	4	5	3	4

NC = Not Concerned; *considering sufficient bacteriophage concentrations for detection; **depending on the detection method

As far as microbial diversity is concerned, the soil of the Weierbach plateau has never been characterised in terms of bacteria, eukaryotes or even viruses. Our results revealed a large presence of the genera *Lessievirus* (*Podoviridae*) and *Mieseafarmvirus* (*Myoviridae*), specifically present in the Weierbach soil compared to the other studied sites of the project. These genera were found to be rather correlated to a high level of organic matter content in soils, which is consistent with the catchment of interest. The identification of the species allowed showing that the abundance of *Lessievirus* was due to the high abundance of *Lessievirus BcepIL02*, while only one species belongs to the genera *Mieseafarmvirus*, *Ralstonia phage RSL1*. However, when looking at the DNA-bacteriophage population in the soil water, *Lessievirus BcepIL02* was not detected at all and *Ralstonia phage RSL1* was in low abundance. *Lessievirus BcepIL02* was always found in the highest abundance in soils during every campaign. The ecology of this species is not known at all, however, its bacterial host *Burkholderia cepacia*, without which it cannot replicate, is known to be related to soil pH and to specifically live in acidic soils but to also be found in marine compartment (Hall et al., 2015; Paul et al., 2021). Therefore, it is most likely that the bacteriophage BcepIL02 might not be part of the populations found in the soil water since it was not detected before the rainfall events starts. Moreover, the precipitations did not trigger its mobilisation from the soil to the soil water, potentially suggesting adsorption to the soil particles and incapacity of desorption. This indicates that most likely *Lessievirus BcepIL02* would not be a good candidate for investigating hydrological processes.

Ralstonia phage RSL1 was found in low abundance in soil water but did show a dynamic in both soil and soil water samples. Moreover, a significant effect of the precipitation was detected, particularly in the soil. Indeed, its abundance in soil increased with the peaks of the precipitation in November, while in March, its abundance was found to be more variable in both soil and soil water. The campaign of November occurred when the soil moisture started to reach its high level. Therefore, water from precipitation, once infiltrated into the soil, would spread quite rapidly into the deeper layers of the soil, carrying the bacteriophages with it. Thus, the bacteriophage RSL1 would be detected in higher abundance immediately after the peak of precipitation, and then its signal would be lost due to its infiltration into the lower soil layers. The campaign of March happened at the end of the same period when the high level of soil moisture was well settled. As a result, soil moisture is at its highest in the case of the Weierbach soil at this time, water from precipitation will thus seep more slowly

into the lower soil layers, allowing longer detection of the bacteriophage RSL1 in the soil and soil water. Concerning soil water, it would be interesting to mention that a dilution effect could be the cause of a lower abundance. *Ralstonia phage RSL1* infects the bacterium *Ralstonia solanacearum* and has a head of 150 nm in diameter with a helical non-contractile tail of 138 nm in length (Figure 8) (Effantin et al., 2013; Yamada et al., 2007).

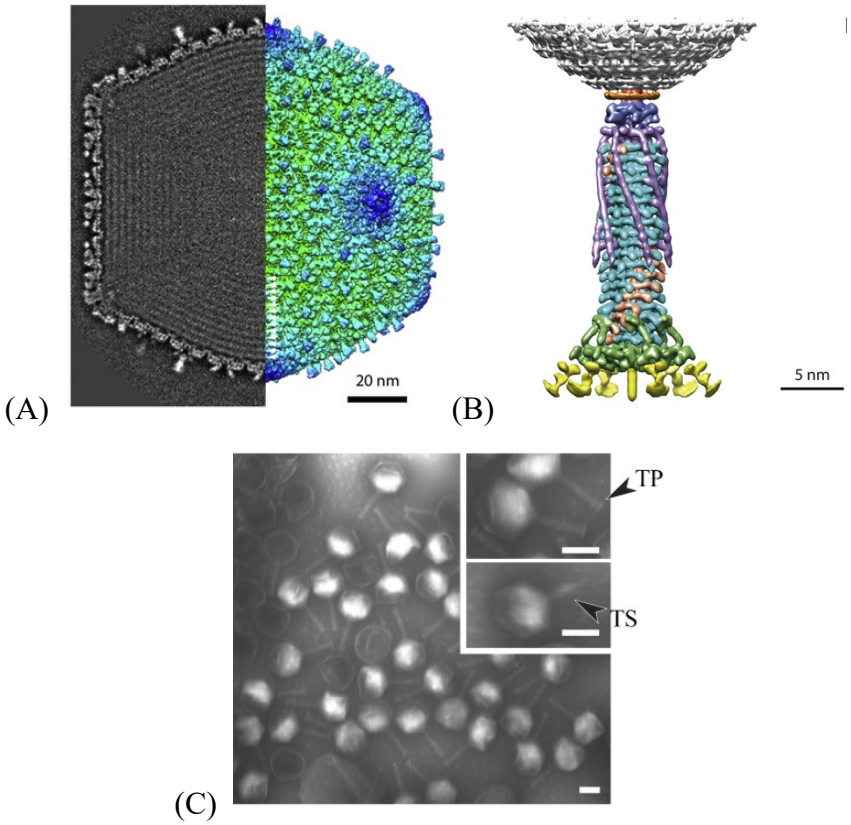


Figure 8: Structure of the bacteriophage RSL1 with (A) the capsid organisation in 3D reconstruction and its concentric DNA genome inside, (B) a 3D reconstruction of the helical tail (Effantin et al., 2013). (C) Morphology of the bacteriophage RSL1 with an electron micrograph (Yamada et al., 2007) (size of bar = 100 nm).

With its 231 kbp genome, bacteriophage RSL1 belongs to the group of the jumbo phage, which gathers tailed bacteriophages with large genomes (> 200 kbp) (Yamada et al., 2010). The host bacterial of RSL1, *Ralstonia solanacearum*, is a soil-born bacterium, which plays a role in nitrogen fixation, carbon decomposition and crop pathogenicity (Fujiwarta et al., 2011; Gill et al., 2011). Used as a biocontrol against *Ralstonia solanacearum* infection on tomato plants, Fujiwara *et al.* studied the persistence of the bacteriophage RSL1 in plants

and soil. Using plaque assay, a large number of the bacteriophages RSL1 were found in the two tested media 4 months after the plaque assay, which shows good persistence of the bacteriophage RSL1 (Fujiwara et al., 2011). Unfortunately, apart from a study showing the persistence of the bacteriophage, no studies have been carried out on its fate or behaviour in the soil. Bacteriophage RSL1 was discovered in 2007 and since, research into its genetic material and its use as a biocontrol agent for plant diseases have been growing, already providing access to some of the bacteriophage's data. The results obtained during the PhD work concerning the dynamics of the bacteriophage RSL1 in soil and soil water and its significant relationship with precipitation events are promising for the use of this DNA-bacteriophage as a hydrological tracer. Thus, some research could be undertaken to learn more about the intrinsic properties of the bacteriophage in the Weierbach soil (e.g. isoelectric point and degree of hydrophobicity), as well as laboratory tracing experiments to assess its transport capacity. This will be explained in more detail in section 4.4 below.

Among the most abundant bacteriophages, one last species has proved to be interesting to be considered as hydrological tracers. Indeed, *Omegavirus Thibault* showed significant variation according to the precipitation event followed in March 2021. *Omegavirus Thibault* is a bacteriophage of the Siphovirus morphotype, i.e. it is a non-enveloped bacteriophage with a head-tail structure with a 100 nm icosahedral capsid and a 230 nm non-contractile tail (Figure 9). Its genome is a linear dsDNA, varying in size from 106 to 112 kb and containing about 237 genes (Swiss Institute of Bioinformatics, 2015). The genus *Omegavirus*, an unclassified *Caudoviricetes*, contains six species including *Omegavirus Thibault*, the bacteriophage of interest which is known to infect *Mycolicibacterium smegmatis MC2 155* (ICTV, 2021).

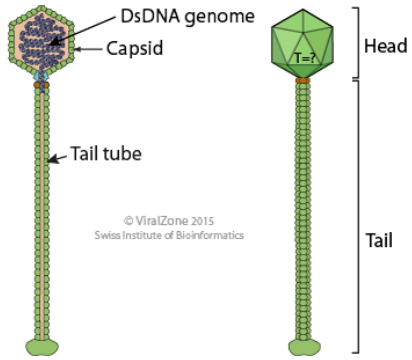


Figure 9: Morphology of the genus *Omegavirus*, to which the species *Omegavirus Thibault* belongs (Swiss Institute of Bioinformatics, 2015).

Unfortunately, besides this structural information, no studies were found in the literature mentioning the presence of this bacteriophage in the descriptions of ecological diversity. Thus, information on its ecological distribution within the different compartment types is not available. On the other hand, its bacterial host, *Mycolicibacterium smegmatis MC2 155*, is better known for its non-pathogenic aspect and is therefore used as a laboratory model strain for studies on pathogenic species of the genus Mycobacterium, a dominant genus in the soil (Ofer et al., 2012). It is also the most commonly used bacterial host strain for growing mycobacteriophages. The species *Mycolicibacterium smegmatis MC2 155* is believed to be present in a variety of environments, such as soil, fresh and marine waters, and has shown to be particularly persistent in environments with high salt concentrations (Berney et al., 2014; Yamada et al., 2018). Knowing the bacterial host of the bacteriophage *Omegavirus Thibault* gives the possibility to carry out experiments and extend the knowledge on this bacteriophage, however too little information is available to confront the results and to apply this bacteriophage in tracing experiments. Moreover, unlike the bacteriophage RSL1, *Omegavirus Thibault* was only detected during the March campaign studied in this thesis. Thus, if there is a strong seasonality of the bacteriophage abundance with periods of complete absence, then this aspect may limit its application as a hydrological tracer at certain times of the year. Bacteriophage Thibault however shares a common feature with bacteriophage RLS1, its morphological structure. Both are bacteriophages with a non-contractile tail, a feature that makes them particularly fragile (Ackermann et al., 2004). This structural element is important to take into account when exploring their transport, as damage to the tail will contribute to the inactivation of the bacteriophages.

To conclude on the potential candidates among the bacteriophages identified in soils, bacteriophage *RSL1* seems to be the most promising natural DNA-bacteriophage as a hydrological tracer in the Weierbach case. This first conclusion based on the results obtained and discussed in this PhD project still needs further analysis and experiments to confirm the interest of bacteriophage RSL1as hydrological tracers in the Weierbach catchment.

4.4 Methodological proposals for further analyses

4.4.1 Development of detection methods specific to the selected bacteriophage

To confirm the potential for the bacteriophage *Ralstonia phage RSL1* to become a hydrological tracer in the Weierbach catchment, further analyses need to be carried out to deepen the knowledge of this promising candidate. During tracing experiments, a tracer cannot be used without the prior development of detection methods (Leibundgut and Seibert, 2010). In the case of the use of bacteriophages, several methods can be used in a complementary way, culture-based techniques and molecular methods. In past tracing experiments using bacteriophages as artificial tracers, the detection method used was always the plaque assay (Kinnunen, 1978; Martin, 1988; Rossi et al., 1998). While this method is a basic reference for detecting a specific bacteriophage, it is not sufficient on its own to give a complete detection. Indeed, bacteriophages are known to be sensitive to environmental factors and therefore can potentially be inactivated during experiments (Clokie et al., 2011). However, their inactivation does not mean that they are not present in the medium. Once inactivated, the whole infectious particles can no longer be detected via the plaque assay, but their genomes can still be detected by molecular methods. For the use of the bacteriophage RSL1, we thus propose to develop a plaque assay as well as a qPCR to be able to detect infectious bacteriophages and total genomes respectively in each environmental sample.

Bacteriophage RSL1 was used as a biocontrol agent to control a plant pathogenic bacterium *Ralstonia solanacearum* known to infect tomato plants (Fujiwara et al., 2011). Thus, laboratory methods to detect this bacteriophage have already been developed, which notably is the case for the plaque assay. A stock solution of the bacteriophage RSL1 is first prepared using the *R. solanacearum M4S* bacterial strain (ATCC® 11696™, BSL 1). Using the same

bacterial host, the plaque assay is performed on Casamino acid-Peptone-Glucose (CPG) medium containing 0.45% agar and incubated for 6 to 24 hours at 28°C (Fujiwara, 2010; Yamada et al., 2007).

In addition to the culture-based technique previously described, a molecular detection method can also be developed for the bacteriophage RSL1. The dsDNA genome of bacteriophage RSL1 (231,255 bp encoding 343 Open reading frame, or ORF) has been well investigated and can allow the development of PCR techniques for the detection and quantification of this specific bacteriophage (Yamada et al., 2010). Bacteriophage RSL1 has already been subjected to PCR in previous studies using the forward primer 5′-AGG ATC CAT GCG GCT CCA TCG GTCCAA GGA and the reverse primer 5′-CGA TAG GAG CGG CCG CTC TAT TCCTGC TT, corresponding to the ORF18 (Fujiwara, 2010). These primers could therefore be used for the development of a molecular detection technique, but the search for another conserved area of the genome may also be possible. Bacteriophage detection and quantification tend to be done using qPCR as a reference (Anderson et al., 2011; Antonelli et al., 2016; Ogorzaly and Gantzer, 2006), however, a new technology, the digital droplet PCR or ddPCR, started to show better results in studying bacteriophages (Kim et al., 2014; Morella et al., 2018). Indeed, the operating system remains the same as for qPCR but ddPCR differs in that it implies partitioning the PCR into a numerous number of nanodroplets where one reaction occurs per droplet (Kim, Jeong and Cho, 2014). In addition, compared to the qPCR, the ddPCR has the advantages of being more accurate, more sensitive to inhibitors (potentially very present in environmental samples) and does not require the establishment of a standard curve for the quantification since it is based on quantum calculations (Ács et al., 2020; Kojabad et al., 2021; Morella et al., 2018).

4.4.2 Towards a better understanding of the fate of RSL1 for future applications in tracing experiments

Once the detection method for bacteriophage RSL1 has been developed, further experiments can be carried out both in the laboratory and in the field to explore the transport of this bacteriophage. As a first step, some important properties, i.e. the hydrophobicity and the surface electric charge, must be characterised. These characteristics can then be used to assess the adsorption rate of the bacteriophage on soil particles via laboratory analysis, and to help to learn more about preferential pathways in the soil structure.

Surface charges and hydrophobicity

The surface electric charge of a bacteriophage is determined by calculating the isoelectric point (pI) of a bacteriophage and it is a crucial criterion to explore the degree of adsorption through electrostatic interactions between environmental particles and the bacteriophage. This characteristic will be a key point in the analysis of the retention rate of the bacteriophage in natural systems to predict the fate and transport of bacteriophages. Classically, several methods have been used to determine the surface electric charges of viruses (Dika et al., 2015; Langlet et al., 2008), where the most known are the zeta (ζ) potential measurement and isoelectric focusing (Heffron and Mayer, 2021). The zeta potential measurement is based on the application of an electric field to a purified viral solution which can be performed using a benchtop zeta potential analyser (i.e. Malvern Zetasizer Nano ZS) (Passaretti et al., 2020). A bacteriophage with a zeta potential between -10 and +10 mV (millivolt) will have a neutral surface charge, while a zeta potential below -30 mV will characterise the bacteriophage as anionic and above +30 mV, as cationic (Clogston and Patri, 2011). Isoelectric focusing is an electrophoresis technique using a pH gradient gel where proteins will migrate to one of two electrodes, a cathode (positively charged) or an anode (negatively charged). Considering that opposite charges attract each other, the migration of proteins on the gel will define their isoelectric point, the pH value at which no electric charge is present (Bjellqvist et al., 1982; Braun et al., 2017). Lately, the development of a novel method to characterise the surface charge of bacteriophages has been proposed, chemical force microscopy (CFM), to overcome the limits encountered with the previously mentioned methods (e.g. high purity and a large number of samples). CFM can measure the surface charge of a single virus particle by measuring the interaction between the particles and the tip of the device used, the atomic force microscope (AFM) (Mi et al., 2020). The pI is an interesting indicator for the study of electrostatic interactions between bacteriophages and their surrounding environment, however, it should be noted that it reveals potential interactions taking place under the conditions tested and interpolation to a wider range of pH conditions should be avoided (Dika et al., 2015).

Among the interactions responsible for the adsorption of bacteriophage in environmental media, there are also hydrophobic/hydrophilic interactions (Sautrey et al., 2018). These interactions take place mainly in the terrestrial compartment with the organic matter (Mataix-Solera and Doerr, 2004). The hydrophobicity measurement is even more important in the case of Weierbach since the soil was characterised by a high organic matter content.

Hydrophobicity is commonly determined through the measurement of the contact angle (θ) of a molecule onto a hydrophobic or hydrophilic surface (Carlson et al., 2021; Lamour et al., 2010). In practice, a solution of bacteriophages is placed onto a treated surface to induce the adsorption process. Then, the samples are analysed under an AFM, based on mathematical calculations of the contact angle (Richter et al., 2021; Sakoda et al., 1997; Wu et al., 2022).

Fate and transport of bacteriophage RSL1 in laboratory experiments

With the development of detection methods and the characterisation of adsorption properties, we have all the cards in hand to set up tracing experiments at the laboratory scale. To be consistent with the study carried out in this PhD work, the fate and transport of the bacteriophage RSL1 can be studied first in soil using columns in the laboratory. This protocol has already been reported in the literature for the same purpose, using different bacteriophage species (Bales et al., 1995; Corapcioglu et al., 2006; Joyce et al., 2007; Rousseau et al., 2004). A solution of the bacteriophage RSL1 can be prepared upstream containing a concentration of about 10^9 particles/mL, the concentration of VLPs calculated for the Weierbach. Soil columns, e.g. between 50 cm and 1 m, directly collected from the Weierbach can be brought to the laboratory, into which the bacteriophage RSL1 solutions are poured. Different collection points along the column could be created to quantify the concentrations at different depths and deduce recovery rates, using the detection methods previously developed (i.e. plaque assay and qPCR). To assess transport under different conditions, artificial rainfall of varying intensity could be subjected to bacteriophage RSL1. This condition would make it possible to analyse the paths preferentially taken by the bacteriophage or the variation in its transport speed (Germann, Alaoui and Riesen, 2002; Rousseau et al., 2004). The sensitivity of the tracer to environmental changes could be studied by varying the soil pH through the addition of a basic solution to reach pH values close to those of soil water (around 6) or surface water (around 7) before the introduction of the RSL1 solution (Kinoshita et al., 1993). By adjusting the pH, the adsorption capacity of the bacteriophage on the soil via electrostatic forces is also affected. The pH gradient used will be optimised according to the pI of bacteriophage RSL1 determined before this experiment. To further the adsorption process, the hydrophobic adsorption capacity of the bacteriophage on the organic matter can be evaluated by adding compost or green manure (Senesi, 1989; Shepherd et al., 2006).

The same types of experiments could also be carried out to assess the flow of surface water by setting up a laboratory-based catchment into which the bacteriophage solutions could be injected (Howe et al., 2019; Qu et al., 2007). In the same way, different conditions could be submitted such as rainfall intensities, and the addition of vegetation cover to evaluate the speed of dispersion. Finally, the two types of experiments could be carried out in a complementary and simultaneous manner to evaluate the relationship between the infiltration capacity in the soil and that of surface runoff after precipitation. This experiment could be carried out using a tank containing soil on which a slope and artificial precipitation would be imposed (Qu et al., 2007; Song and Bai, 2015).

Detection of the bacteriophage RSL1 in further compartments of the Weierbach

To have a complete picture of the presence of bacteriophage RSL1 within the Weierbach catchment, detection methods could be deployed in different types of samples, such as in groundwater, surface water or covering all aspects of the basin, plateau, slope or riparian areas.

These field analyses would in particular make it possible to go to the end of the continuum and to move towards a better understanding of the potential transport of bacteriophage RSL1 from the soil to groundwater or even to rivers. In the same way that previous works have been carried out on diatoms, an exploration of the interactions between hydrology and ecosystems will be necessary for bacteriophages (Foets et al., 2020; Pfister et al., 2017). Indeed, diatoms have been at the centre of temporal and spatial investigations in the soil carried out at larger scales than those present in this PhD work (Foets, 2021). These large-scale temporal and spatial variations could therefore be made for the bacteriophage RSL1 to have a better view of its distribution and its relationships with different environmental factors. However, these large-scale variations could also be conducted on total bacteriophage populations as done in this PhD work at smaller temporal and spatial scales. Even if the bacteriophage RSL1 has shown promising results to be used as a hydrological tracer in the Weierbach catchment, this does not remove the possibility of discovering other candidates potentially as interesting to add to the toolbox of hydrologists.

CONCLUSION

Throughout this PhD project, the research carried out has made it possible to answer the various scientific questions stated in Chapter 2, and above all the objective of extending the toolbox of hydrologists by providing new tracers. The results notably highlighted the potential of natural DNA-bacteriophages as candidates to be hydrological tracers. Through the two WPs built into this PhD project, it was possible to reveal the interest of certain species of DNA-bacteriophages in particular. As part of the first WP, the characterisation of DNA-bacteriophage communities was described as a function of diverse soils presenting different physicochemical properties (i.e. texture, granulometry, organic matter content among others) and different land cover (i.e. forest and grasslands). The composition of natural DNA-bacteriophage communities was therefore explored according to these abiotic factors to find a pattern in their distribution in soil. In addition, the DNA-bacteriophage communities were analysed according to the bacterial communities, which highlighted significant correlations between the two. Among all eight sites selected, the forest of the Weierbach revealed differences in several points: high organic matter, acidic soil, low bacteriophages richness and specifically showed a high abundance of two bacteriophages: *Ralstonia phage RSL1* and *Burkholderia phage bicepIL02*. To get further into exploring the potential of natural bacteriophages, their dynamics need to be investigated since their presence in the soil is not sufficient to make it good tracers. They can be suitable tracers if, in addition to being highly abundant, water can mobilise and carry them downstream. The second WP has for objective to verify whether the identified DNA-bacteriophage species in the soil of the Weierbach, model catchment of the DTU, can be mobilised for the soil to the closest soil component, the soil water triggered by precipitation. The previously mentioned species *Ralstonia phage RSL1* showed promising results in addition to the species *Omegavirus Thibault*, which both varied significantly according to the targeted precipitation events. However, the significant temporal variation of *Omegavirus Thibault*, only detected in March, suggests a potential relationship with seasonality, which reduces its usefulness to limited periods of the year. In contrast, *Ralstonia phage RSL1* was detected in every field campaign, September 2019, November 2020 and March 2021, a ubiquity appreciated in the process of selecting a hydrological tracer. Therefore, we can conclude that the findings of the PhD work allowed revealing the potential for one species, *Ralstonia phage RSL1*, to be

a promising environmental tracer, with the complementary use of *Omegavirus Thibault* when seasonality is involved.

These first findings are an interesting start in the use of natural bacteriophages as hydrological tracers. However, it is important to keep in mind that there is still a long way before we may be able to use them in field-scale experiments. Natural populations of bacteriophages may solve the issue of negative environmental impact that would cause the injection of exogenous bacteriophages, but most bacteriophages identified from natural ecosystems remain too poorly known and are usually not used in laboratory experiments. This results in very little available information and thus requires additional experiments to acquire as much data as needed. Furthermore, the composition of the bacteriophage population at the taxonomical level of the species highly differs from one soil type to another, leading to a high specificity of the tracer. In the case of the Weierbach, *Ralstonia phage RSL1* showed promising results but was very specific to this site since it was not detected in any other sites studied in this PhD work. *Ralstonia phage RSL1* has been used as a control bioagent to fight against bacterial infection in plants. Therefore, some information is already available for developing detection methods, such as plaque assay and genomic information to design a qPCR. In contrast, certain important characteristics are missing, notably surface charges and hydrophobicity, which provide inputs for the investigation of interaction with the surrounding environment during the transport of the bacteriophage.

Bacteriophages have proven their ability to investigate the water cycle as both artificial and environmental tracers. They both display advantages and inconveniences and should be used according to the desired applications. Bacteriophages as environmental tracers is a novel concept that showed promise in this PhD work and despite the limitations that may encounter their use, ecosystems, particularly terrestrial compartment, have a large diversity of bacteriophages to offer and thus, an indefinite number of potential candidates. For the optimal long-term use of bacteriophages, some scientific gaps still need to be addressed. Some intrinsic characteristics of the selected bacteriophages need to be determined, such as hydrophobicity and surface charges, and could be the next step to be carried out after this thesis work. In addition, their dynamics as a function of changing parameters (e.g. pH) as well as their transport through particular media (e.g. soil) should be explored in laboratory experiments. Thus, the use of bacteriophages in the field during tracing experiments could be a way forward in the exploration of hydrological systems.

REFERENCES[1]

Abedon, S.T., Yin, J., 2009. Bacteriophage plaques: theory and analysis. Methods in molecular biology 501, 161–174. https://doi.org/10.1007/978-1-60327-164-6_17/COVER/

Ackermann, H.W., 2012. Bacteriophage electron microscopy. Adv Virus Res 82, 1–32. https://doi.org/10.1016/B978-0-12-394621-8.00017-0

Ackermann, H.W., 2011. Bacteriophage taxonomy. Under the microscope 90–94.

Ackermann, H.-W., Tremblay, D., Moineau, S., 2004. Long-term bacteriophage preservation. WFCC Newslett 38, 35–40.

Ács, N., Gambino, M., Brøndsted, L., 2020. Bacteriophage Enumeration and Detection Methods. Front Microbiol 11, 1–7. https://doi.org/10.3389/FMICB.2020.594868/FULL

Adriaenssens, E.M., van Zyl, L., de Maayer, P., Rubagotti, E., Rybicki, E., Tuffin, M., Cowan, D.A., 2015. Metagenomic analysis of the viral community in Namib Desert hypoliths. Environ Microbiol 17, 480–495. https://doi.org/10.1111/1462-2920.12528

Adriaenssens, E.M., Wittmann, J., Kuhn, J.H., Turner, D., Sullivan, M.B., Dutilh, B.E., Jang, H. bin, van Zyl, L.J., Klumpp, J., Lobocka, M., Moreno Switt, A.I., Rumnieks, J., Edwards, R.A., Uchiyama, J., Alfenas-Zerbini, P., Petty, N.K., Kropinski, A.M., Barylski, J., Gillis, A., Clokie, M.R.C., Prangishvili, D., Lavigne, R., Aziz, R.K., Duffy, S., Krupovic, M., Poranen, M.M., Knezevic, P., Enault, F., Tong, Y., Oksanen, H.M., Rodney Brister, J., 2018. Taxonomy of prokaryotic viruses: 2017 update from the ICTV Bacterial and Archaeal Viruses Subcommittee. Arch Virol 163, 1125–1129. https://doi.org/10.1007/s00705-018-3723-z

[1] This list takes into consideration all the references present in this manuscript, but does not include those present in the different articles. These references are listed at the end of each article.

Ageno, M., Donelli, G., Guglielmi, F., 1973. Structure and physico-chemical properties of bacteriophage G. II, The shape and symmetry of the capsid. Micron (1969) 4, 376–403. https://doi.org/10.1016/0047-7206(73)90002-2

Aishwarya, P. v., Mohan, A.S., Philip, R., Antony, S.P., 2021. Metagenomic phage biodiversity: New insight into the tropical estuarine ecosystem process and microbial risk assessment. Reg Stud Mar Sci 46, 101894. https://doi.org/10.1016/J.RSMA.2021.101894

Anderson, B., Rashid, M.H., Carter, C., Pasternack, G., Rajanna, C., Revazishvili, T., Dean, T., Senecal, A., Sulakvelidze, A., 2011. Enumeration of bacteriophage particles: Comparative analysis of the traditional plaque assay and real-time QPCR- and nanosight-based assays. Bacteriophage 1, 86–93. https://doi.org/10.4161/BACT.1.2.15456

Andersson, T., Lood, R., 2019. Plaque Assay : A Method to Determine Viral Titer as Plaque Forming Units (PFU). Visualized experiments 1–5.

Angly, F.E., Felts, B., Breitbart, M., Salamon, P., Edwards, R.A., Carlson, C., Chan, A.M., Haynes, M., Kelley, S., Liu, H., Mahaffy, J.M., Mueller, J.E., Nulton, J., Olson, R., Parsons, R., Rayhawk, S., Suttle, C.A., Rohwer, F., 2006. The Marine Viromes of Four Oceanic Regions. PLoS Biol 4, e368. https://doi.org/10.1371/JOURNAL.PBIO.0040368

Antonelli, M., Balasubramanian, M.N., Ogorzaly, L., Pfister, L., 2016. qPCR (quantitative polymerase chain reaction) for the quantification of bacteriophages in stream water samples to investigate hydrological processes: a proof-of-concept study in the Huewelerbach experimental catchment (Luxembourg), in: EGU General Assembly. p. 1.

Armon, R., 2011. Soil bacteria and bacteriophages, in: Witzany, G. (Ed.), Biocommunication in Soil Microorganisms. Springer-Verlag, pp. 67–112. https://doi.org/10.1007/978-3-642-14512-4

Ashelford, K.E., Day, M.J., Fry, J.C., 2003. Elevated Abundance of Bacteriophage Infecting Bacteria in Soil. Appl Environ Microbiol 69, 285. https://doi.org/10.1128/AEM.69.1.285-289.2003

Babich, H., Stotzky, G., 1980. Reductions in inactivation rates of bacteriophages by clay minerals in lake water. Water Res 14, 185–187. https://doi.org/10.1016/0043-1354(80)90236-5

Bae, H.G., Nitsche, A., Teichmann, A., Biel, S.S., Niedrig, M., 2003. Detection of yellow fever virus: A comparison of quantitative real-time PCR and plaque assay. J Virol Methods 110, 185–191. https://doi.org/10.1016/S0166-0934(03)00129-0

Baffert, S., Italiano, A., Pierron, G., Traoré, M.A., Rapp, J., Escande, F., Ghnassia, J.P., Terrier, P., Voegeli, A.C., Ranchere-Vince, D., Coindre, J.M., Pedeutour, F., 2013. [Comparative cost analysis of molecular biology methods in the diagnosis of sarcomas]. Bull Cancer 100, 963–971. https://doi.org/10.1684/BDC.2013.1822

Bagalkot, N., Kumar, G.S., 2018. Colloid Transport in a Single Fracture–Matrix System: Gravity Effects, Influence of Colloid Size and Density. Water 2018, Vol. 10, Page 1531 10, 1531. https://doi.org/10.3390/W10111531

Bagheri, H., Dyer, R., Severin, A., Rajan, H., Severin, A.J., 2020. Comprehensive Analysis of Non Redundant Protein Database. https://doi.org/10.21203/rs.3.rs-54568/v1

Bales, R.C., Li, S., Maguire, K.M., Yahya, M.T., Gerba, C.P., Harvey, R.W., 1995. Virus and Bacteria Transport in a Sandy Aquifer, Cape Cod, MA. Groundwater. https://doi.org/10.1111/j.1745-6584.1995.tb00321.x

Ballaud, F., Dufresne, A., Francez, A.J., Colombet, J., Sime-Ngando, T., Quaiser, A., 2016. Dynamics of viral abundance and diversity in a sphagnum-dominated peatland: Temporal fluctuations prevail over habitat. Front Microbiol 6, 1494. https://doi.org/10.3389/FMICB.2015.01494/BIBTEX

Barnes, M.A., Turner, C.R., 2015. The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics 2015 17:1 17, 1–17. https://doi.org/10.1007/S10592-015-0775-4

Barton, C.D., Karathanasis, A.D., 2002. CLAY MINERALS.

Bastviken, D., Thomsen, F., Svensson, T., Karlsson, S., Sandén, P., Shaw, G., Matucha, M., Öberg, G., 2007. Chloride retention in forest soil by microbial uptake and by natural chlorination of organic matter. Geochim Cosmochim Acta 71, 3182–3192. https://doi.org/10.1016/j.gca.2007.04.028

Batinovic, S., Wassef, F., Knowler, S.A., Rice, D.T.F., Stanton, C.R., Rose, J., Tucci, J., Nittami, T., Vinh, A., Drummond, G.R., Sobey, C.G., Chan, H.T., Seviour, R.J., Petrovski, S., Franks, A.E., 2019. Bacteriophages in Natural and Artificial Environments. Pathogens 8. https://doi.org/10.3390/PATHOGENS8030100

Behrens, H., Beims, U., Dieter, H., Dietze, G., Eikmann, T., Grummt, T., Hanisch, H., Henseling, H., Käss, W., Kerndorff, H., Leibundgut, C., Müller-Wegener, U., Rönnefahrt, I., Scharenberg, B., Schleyer, R., Schloz, W., Tilkes, F., 2001. Toxicological and ecotoxicological assessment of water tracers. Hydrogeol J 9, 321–325. https://doi.org/10.1007/s100400100126

Bergh, O., Børsheim, K.Y., Bratbak, G., Heldal, M., 1989. High abundance of viruses found in aquatic environments. Nature 340, 467–468. https://doi.org/10.1038/340301a0

Berney, M., Greening, C., Conrad, R., Jacobs, W.R., Cook, G.M., 2014. An obligately aerobic soil bacterium activates fermentative hydrogen production to survive reductive stress during hypoxia. Proc Natl Acad Sci U S A 111, 11479–11484. https://doi.org/10.1073/PNAS.1407034111/-/DCSUPPLEMENTAL/PNAS.201407034SI.PDF

Berryman, C.J., 2005. Tracer tests for investigating flow and transport in the hyporheic zone.

Bertrand, I., Schijven, J.F., Sánchez, G., Wyn-Jones, P., Ottoson, J., Morin, T., Muscillo, M., Verani, M., Nasser, A., de Roda Husman, A.M., Myrmel, M., Sellwood, J., Cook, N., Gantzer, C., 2012. The impact of temperature on the inactivation of enteric viruses in food and water: a review. J Appl Microbiol 112, 1059–1074. https://doi.org/10.1111/J.1365-2672.2012.05267.X

Bi, L., Yu, D.T., Han, L.L., Du, S., Yuan, C.Y., He, J.Z., Hu, H.W., 2022. Unravelling the ecological complexity of soil viromes: Challenges and

opportunities. Science of The Total Environment 812, 152217. https://doi.org/10.1016/J.SCITOTENV.2021.152217

Bitton, G., 1975. Adsorption of Viruses Onto Surfaces Soil and Water. Water Res 9, 473–484.

Bitton, G., Mitchell, R., 1974. Effect of colloids on the survival of bacteriophages in seawater. Water Res 8, 227–229. https://doi.org/10.1016/0043-1354(74)90159-6

Bjellqvist, B., Ek, K., Giorgio Righetti, P., Gianazza, E., Görg, A., Westermeier, R., Postel, W., 1982. Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. J Biochem Biophys Methods 6, 317–339. https://doi.org/10.1016/0165-022X(82)90013-6

Bolduc, B., Youens-Clark, K., Roux, S., Hurwitz, B.L., Sullivan, M.B., 2017. PERSPECTIVE iVirus: facilitating new insights in viral ecology with software and community data sets imbedded in a cyberinfrastructure Viral metagenomics: an ecological tool with increasing impact. ISME J 11, 7–14. https://doi.org/10.1038/ismej.2016.89

Bot, A., Benites, J., 2005. Chapter 2. Organic matter decomposition and the soil food web. The importance of soil organic matter 3–9.

Braga, L.P.P., Spor, A., Kot, W., Breuil, M.C., Hansen, L.H., Setubal, J.C., Philippot, L., 2020. Impact of phages on soil bacterial communities and nitrogen availability under different assembly scenarios. Microbiome 8, 1–14. https://doi.org/10.1186/s40168-020-00822-z

Bratbak, G., Heldal, M., Norland, S., Thingstad, T.F., 1990. Viruses as Partners in Spring Bloom Microbial Trophodynamics. Appl Environ Microbiol 56, 1400. https://doi.org/10.1128/AEM.56.5.1400-1405.1990

Braun, M., Gebauer, W., Krczal, G., Ziegler, C., Müller-Renno, C., Boonrod, K., 2017. A simple method to estimate the isoelectric point of modified Tomato bushy stunt virus (TBSV) particles. Electrophoresis 38, 2771–2776. https://doi.org/10.1002/ELPS.201700209

Breitbart, M., Bonnain, C., Malki, K., Sawaya, N.A., 2018. Phage puppet masters of the marine microbial realm. Nat Microbiol 3, 754–766. https://doi.org/10.1038/s41564-018-0166-y

Brigatti, M.F., Galan, E., Theng, B.K.G., 2006. Chapter 2 Structures and Mineralogy of Clay Minerals. Dev Clay Sci 1, 19–86. https://doi.org/10.1016/S1572-4352(05)01002-0

Briones, M.J.I., 2018. Book Review: Fundamentals of Soil Ecology (Third Edition). Front Environ Sci 0, 91. https://doi.org/10.3389/FENVS.2018.00091

Brown, M.R., Camézuli, S., Davenport, R.J., Petelenz-Kurdziel, E., Øvreås, L., Curtis, T.P., 2015. Flow cytometric quantification of viruses in activated sludge. Water Res 68, 414–422. https://doi.org/10.1016/J.WATRES.2014.10.018

Bruder, K., Malki, K., Cooper, A., Sible, E., Shapiro, J.W., Watkins, S.C., Putonti, C., 2016. Freshwater metaviromics and bacteriophages: A current assessment of the state of the art in relation to Bioinformatic challenges. Evolutionary Bioinformatics 12, 25–33. https://doi.org/10.4137/EBO.S38549

Buerkert, A., Joergensen, R.G., Ludwig, B., Schlecht, E., 2012. Chapter 18 - Nutrient and Carbon Fluxes in Terrestrial Agro-Ecosystems, in: Marschner, P.B.T.-M.M.N. of H.P. (Third E. (Ed.), . Academic Press, San Diego, pp. 473–482. https://doi.org/https://doi.org/10.1016/B978-0-12-384905-2.00018-2

Cai, L., Feng, C., Xie, L., Xu, B., Wei, W., Jiao, N., Zhang, R., 2022. Ecological dynamics and impacts of viruses in Chinese and global estuaries. Water Res 226, 119237. https://doi.org/10.1016/J.WATRES.2022.119237

Callanan, J., Stockdale, S.R., Adriaenssens, E.M., Kuhn, J.H., Pallen, M., Rumnieks, J., Shkoporov, A.N., Draper, L.A., Ross, R.P., Hill, C., 2021. Rename one class (Leviviricetes - formerly Allassoviricetes), rename one order (Norzivirales - formerly Levivirales), create one new order (Timlovirales), and expand the class to a total of six families, 420 genera and 883 species. https://doi.org/10.13140/RG.2.2.25363.40481

Callanan, J., Stockdale, S.R., Shkoporov, A., Draper, L.A., Ross, R.P., Hill, C., 2018. RNA Phage Biology in a Metagenomic Era. Viruses 10, 1–17. https://doi.org/10.3390/V10070386

Carlson, S., Becker, M., Brünig, F.N., Ataka, K., Cruz, R., Yu, L., Tang, P., Kanduč, M., Haag, R., Heberle, J., Makki, H., Netz, R.R., 2021. Hydrophobicity of Self-Assembled Monolayers of Alkanes: Fluorination, Density, Roughness, and Lennard-Jones Cutoffs. Langmuir 37, 13846–13858. https://doi.org/10.1021/ACS.LANGMUIR.1C02187/SUPPL_FILE/LA1C02187_SI_001.PDF

Ceballos, R.M., Marceau, C.D., Marceau, J.O., Morris, S., Clore, A.J., Stedman, K.M., 2012. Differential virus host-ranges of the Fuselloviridae of hyperthermophilic Archaea: Implications for evolution in extreme environments. Front Microbiol 3, 295. https://doi.org/10.3389/FMICB.2012.00295/BIBTEX

Chattopadhyay, S., Puls, R.W., 2000. Forces dictating colloidal interactions between viruses and soil. Chemosphere 41, 1279–1286. https://doi.org/10.1016/S0045-6535(99)00519-6

Chen, L., Xun, W., Sun, L., Zhang, N., Shen, Q., Zhang, R., 2014. Effect of different long-term fertilization regimes on the viral community in an agricultural soil of Southern China. Eur J Soil Biol 62, 121–126. https://doi.org/10.1016/j.ejsobi.2014.03.006

Cheng, L., Chetochine, A.S., Pepper, I.L., Brusseau, M.L., 2007. Influence of DOC on MS-2 bacteriophage transport in a sandy soil. Water Air Soil Pollut 178, 315–322. https://doi.org/10.1007/s11270-006-9200-4

Clogston, J.D., Patri, A.K., 2011. Zeta potential measurement. Methods Mol Biol 697, 63–70. https://doi.org/10.1007/978-1-60327-198-1_6/COVER

Clokie, M.R.J., Millard, A.D., Letarov, A. v., Heaphy, S., 2011. Phages in nature. Bacteriophage 1, 31–45. https://doi.org/10.4161/bact.1.1.14942

Colombet, J., Billard, H., Viguès, B., Balor, S., Boulé, C., Geay, L., Benzerara, K., Menguy, N., Ilango, G., Fuster, M., Enault, F., Bardot, C., Gautier, V., Pradeep Ram, A.S., Sime-Ngando, T., 2019. Discovery of High Abundances of Aster-Like Nanoparticles in Pelagic Environments: Characterization and Dynamics. Front Microbiol 10, 2376. https://doi.org/10.3389/fmicb.2019.02376

Cook, P., 2020. Introduction to Isotopes and Environmental Tracers as Indicators of Groundwater Flow. The Groundwater Project.

Corapcioglu, M.Y., Vogel, J.R., Munster, C.L., Pillai, S.D., Dowd, S., Wang, S., 2006. Virus transport experiments in a sandy aquifer. Water Air Soil Pollut 169, 47–65. https://doi.org/10.1007/s11270-006-0942-9

Cota-Robles, E.H., Coffman, M.D., 1963. Fine structure of the bacteriophage attachment process. J Bacteriol 86, 266–273. https://doi.org/10.1128/JB.86.2.266-273.1963

Dalcin Martins, P., Danczak, R.E., Roux, S., Frank, J., Borton, M.A., Wolfe, R.A., Burris, M.N., Wilkins, M.J., 2018. Viral and metabolic controls on high rates of microbial sulfur and carbon cycling in wetland ecosystems. Microbiome 6, 1–17. https://doi.org/10.1186/S40168-018-0522-4/FIGURES/6

Daniels, R.S., Harvey, R., Ermetal, B., Xiang, Z., Galiano, M., Adams, L., McCauley, J.W., 2021. A Sanger sequencing protocol for SARS-CoV-2 S-gene. Influenza Other Respir Viruses 15, 707. https://doi.org/10.1111/IRV.12892

Davies, E. v., Winstanley, C., Fothergill, J.L., James, C.E., 2016. The role of temperate bacteriophages in bacterial infection. FEMS Microbiol Lett 363, 15. https://doi.org/10.1093/FEMSLE/FNW015

Davis, J.A., Farrah, S.R., Wilkie, A.C., 2006. Adsorption of viruses to soil: impact of anaerobic treatment. Water Science and Technology 54, 161–167. https://doi.org/10.2166/WST.2006.464

Dawson, D.J., Paish, A., Staffell, L.M., Seymour, I.J., Appleton, H., 2005. Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus.

J Appl Microbiol 98, 203–209. https://doi.org/10.1111/J.1365-2672.2004.02439.X

Delmas, B., Attoui, H., Ghosh, S., Malik, Y.S., Mundt, E., Vakharia, V.N., 2019. Ictv virus taxonomy profile: Picobirnaviridae. Journal of General Virology 100, 133–134. https://doi.org/10.1099/JGV.0.001186

Dika, C., Duval, F.L., Francius, G., Perrin, A., Gantzer, C., 2015. Isoelectric point is an inadequate descriptor of MS2, Phi X 174 and PRD1 phages adhesion on abiotic surfaces. J Colloid Interface Sci 446, 327–334. https://doi.org/10.1016/j.jcis.2014.08.055

Dion, M.B., Oechslin, F., Moineau, S., 2020. Phage diversity, genomics and phylogeny. Nat Rev Microbiol 18, 125–138.

Divine, C.E., McDonnell, J.J., 2005. The future of applied tracers in hydrogeology. Hydrogeol J 13, 255–258. https://doi.org/10.1007/s10040-004-0416-3

Drury, D.F., Wheeler, D.C., 1982. Applications of a Serratia marcescens bacteriophage as a new microbial tracer of aqueous environments. Journal of Applied Bacteriology 53, 137–142. https://doi.org/10.1111/j.1365-2672.1982.tb04668.x

Duckworth, D.H., 1976. "Who Discovered Bacteriophage?" American society for microbiology 40, 793–802.

Duhamel, S., Jacquet, S., 2006. Flow cytometric analysis of bacteria- and virus-like particles in lake sediments. J Microbiol Methods 64, 316–332. https://doi.org/10.1016/j.mimet.2005.05.008

Dutilh, B.E., Schmieder, R., Nulton, J., Felts, B., Salamon, P., Edwards, R.A., Mokili, J.L., 2012. Reference-independent comparative metagenomics using cross-assembly: crAss. Bioinformatics 28, 3225–3231. https://doi.org/10.1093/BIOINFORMATICS/BTS613

Duyvejonck, H., Merabishvili, M., Pirnay, J.P., De Vos, D., Verbeken, G., Van Belleghem, J., Gryp, T., De Leenheer, J., Van der Borght, K., Van Simaey, L., Vermeulen, S., Van Mechelen, E., Vaneechoutte, M., 2019. Development of a qPCR platform for quantification of the five bacteriophages within

bacteriophage cocktail 2 (BFC2). Sci Rep 9, 1–10. https://doi.org/10.1038/s41598-019-50461-0

Effantin, G., Hamasaki, R., Kawasaki, T., Bacia, M., Moriscot, C., Weissenhorn, W., Yamada, T., Schoehn, G., 2013. Cryo-electron microscopy three-dimensional structure of the jumbo phage ΦrSL1 infecting the phytopathogen Ralstonia solanacearum. Structure 21, 298–305. https://doi.org/10.1016/J.STR.2012.12.017

Elbreki, M., Ross, R.P., Hill, C., O'Mahony, J., McAuliffe, O., Coffey, A., 2014. Bacteriophages and Their Derivatives as Biotherapeutic Agents in Disease Prevention and Treatment. J Viruses 2014, 1–20. https://doi.org/10.1155/2014/382539

Environment Agency, 2021. Tracer tests and remediation schemes: environmental permit exemption [WWW Document]. GOV.UK. URL https://www.gov.uk/guidance/tracer-tests-and-remediation-schemes-environmental-permit-exemption#full-publication-update-history (accessed 10.17.22).

Farnell-Jackson, E.A., Ward, A.K., 2003. Seasonal patterns of viruses, bacteria and dissolved organic carbon in a riverine wetland. Freshw Biol 48, 841–851. https://doi.org/10.1046/J.1365-2427.2003.01052.X

Fauvel, B., Cauchie, H.M., Gantzer, C., Ogorzaly, L., 2019. Influence of physico-chemical characteristics of sediment on the in situ spatial distribution of F-specific RNA phages in the riverbed. FEMS Microbiol Ecol 95. https://doi.org/10.1093/femsec/fiy240

Fazzino, L., Anisman, J., Chacón, J.M., Heineman, R.H., Harcombe, W.R., 2020. Lytic bacteriophage have diverse indirect effects in a synthetic cross-feeding community. ISME J 14, 123–134. https://doi.org/10.1038/s41396-019-0511-z

Feng, Y.Y., Ong, S.L., Hu, J.Y., Tan, X.L., Ng, W.J., 2003. Effects of pH and temperature on the survival of coliphages MS2 and Qβ. J Ind Microbiol Biotechnol 30, 549–552. https://doi.org/10.1007/s10295-003-0080-y

Fernandez-Cassi, Xavier, Rusinol, M., Martinez-Puchol, S., 2018. Viral concentration and amplification from human serum samples prior to application of next-generation sequencing analysis, in: The Human Virome: Methods and Protocols. pp. 173–188. https://doi.org/10.31826/9781463230777-013

Fernandez-Cassi, X., Timoneda, N., Martínez-Puchol, S., Rusiñol, M., Rodriguez-Manzano, J., Figuerola, N., Bofill-Mas, S., Abril, J.F., Girones, R., 2018. Metagenomics for the study of viruses in urban sewage as a tool for public health surveillance. Science of the Total Environment 618, 870–880. https://doi.org/10.1016/j.scitotenv.2017.08.249

Field, M.S., Wilhelm, R.G., Quinlan, J.F., Aley, T.J., 1995. An assessment of the potential adverse aroperties of fluorescent tracer dyes used for groundwater tracing. Environ Monit Assess 38, 75–96.

Filée, J., Tétart, F., Suttle, C.A., Krisch, H.M., 2005. Marine T4-type bacteriophages, a ubiquitous component of the dark matter of the biosphere. Proc Natl Acad Sci U S A 102, 12471–12476. https://doi.org/10.1073/PNAS.0503404102/SUPPL_FILE/03404FIG4.JPG

Filippini, M., Buesing, N., Bettarel, Y., Sime-Ngando, T., Gessner, M.O., 2006. Infection paradox: High abundance but low impact of freshwater benthic viruses. Appl Environ Microbiol 72, 4893–4898. https://doi.org/10.1128/AEM.00319-06/ASSET/B6F068F5-38B0-4305-9AD6-6D23B79457B2/ASSETS/GRAPHIC/ZAM0070669120002.JPEG

Fitts, C.R., 2013. Groundwater Contamination. Groundwater Science 499–585. https://doi.org/10.1016/B978-0-12-384705-8.00011-X

Flannery, J., Keaveney, S., Rajko-Nenow, P., O'flaherty, V., Doré, W., 2013. Norovirus and FRNA bacteriophage determined by RT-qPCR and infectious FRNA bacteriophage in wastewater and oysters. Water Res 47, 5222–5231. https://doi.org/10.1016/j.watres.2013.06.008

Florent, P., Cauchie, H.-M., Herold, M., Jacquet, S., Ogorzaly, L., 2022. Soil pH, Calcium Content and Bacteria as Major Factors Responsible for the Distribution of the Known Fraction of the DNA Bacteriophage Populations

in Soils of Luxembourg. Microorganisms 10, 1458. https://doi.org/10.3390/MICROORGANISMS10071458

Flury, M., Wai, N.N., 2003a. Dyes as tracers for vadose zone hydrology. Reviews of Geophysics 41. https://doi.org/10.1029/2001RG000109

Flury, M., Wai, N.N., 2003b. Dyes as tracers for vadose zone hydrology. Reviews of Geophysics 41. https://doi.org/10.1029/2001RG000109

Focardi, A., Ostrowski, M., Goossen, K., Brown, M. v., Paulsen, I., 2020. Investigating the Diversity of Marine Bacteriophage in Contrasting Water Masses Associated with the East Australian Current (EAC) System. Viruses 12. https://doi.org/10.3390/V12030317

Foets, J., 2021. Ecohydrological characterization of terrestrial diatoms. https://doi.org/10.18174/538623

Foets, J., Wetzel, C.E., Teuling, A.J., Pfister, L., 2020. Temporal and spatial variability of terrestrial diatoms at the catchment scale: Controls on communities. PeerJ 2020, e8296. https://doi.org/10.7717/PEERJ.8296/SUPP-2

Foppen, J.W., Orup, C., Adell, R., Poulalion, V., Uhlenbrook, S., 2011. Using multiple artificial DNA tracers in hydrology. Hydrol Process 25, 3101–3106. https://doi.org/10.1002/hyp.8159

Fordyce, S.L., Kampmann, M.L., van Doorn, N.L., Gilbert, M.T.P., 2013. Long-term RNA persistence in postmortem contexts. Investig Genet 4. https://doi.org/10.1186/2041-2223-4-7

Forés, E., Rusiñol, M., Itarte, M., Martínez-Puchol, S., Calvo, M., Bofill-Mas, S., 2022. Evaluation of a virus concentration method based on ultrafiltration and wet foam elution for studying viruses from large-volume water samples. Science of The Total Environment 829, 154431. https://doi.org/10.1016/J.SCITOTENV.2022.154431

Fortier, L.C., Sekulovic, O., 2013. Importance of prophages to evolution and virulence of bacterial pathogens. Virulence 4, 354–365. https://doi.org/10.4161/VIRU.24498

Francis, F., Miller, R., Nagra, M., 2017. Phage overlay assay for quantification of infectious bacteriophage particles. JEMI methods 1, 18–21. https://doi.org/10.1007/978-1-60327-164-6_7

Frese, N., Schmerer, P., Wortmann, M., Schürmann, M., König, M., Westphal, M., Weber, F., Sudhoff, H., Gölzhäuser, A., 2021. Imaging of SARS-CoV-2 infected Vero E6 cells by helium ion microscopy. Beilstein Journal of Nanotechnology 12:13 12, 172–179. https://doi.org/10.3762/BJNANO.12.13

Friedman, S.D., Genthner, F.J., Gentry, J., Sobsey, M.D., Vinjé, J., 2009. Gene Mapping and Phylogenetic Analysis of the Complete Genome from 30 Single-Stranded RNA Male-Specific Coliphages (Family Leviviridae). J Virol 83, 11233–11243. https://doi.org/10.1128/JVI.01308-09

Fuhrman, J.A., Noble, R.T., 1995. Viruses and protists cause similar bacterial mortality in coastal seawater. Limnol Oceanogr 40, 1236–1242. https://doi.org/10.4319/LO.1995.40.7.1236

Fujiwara, A., 2010. Characterization and utilization of bacteriophages infecting Ralstonia solanacearum.

Fujiwara, A., Fujisawa, M., Hamasaki, R., Kawasaki, T., Fujie, M., Yamada, T., 2011. Biocontrol of Ralstonia solanacearum by treatment with lytic bacteriophages. Appl Environ Microbiol 77, 4155–4162. https://doi.org/10.1128/AEM.02847-10

Furiga, A., Pierre, G., Glories, M., Aimar, P., Roques, C., Causserand, C., Berge, M., 2011. Effects of ionic strength on bacteriophage MS2 behavior and their implications for the assessment of virus retention by ultrafiltration membranes. Appl Environ Microbiol 77, 229–236. https://doi.org/10.1128/AEM.01075-10

Gaafar, Y.Z.A., Ziebell, H., 2020. Comparative study on three viral enrichment approaches based on RNA extraction for plant virus/viroid detection using high-throughput sequencing. PLoS One 15, 1–17. https://doi.org/10.1371/JOURNAL.PONE.0237951

Gallet, R., Kannoly, S., Wang, I.N., 2011. Effects of bacteriophage traits on plaque formation. BMC Microbiol 11, 1–16. https://doi.org/10.1186/1471-2180-11-181

Garin-Fernandez, A., Pereira-Flores, E., Glöckner, F.O., Wichels, A., 2018. The North Sea goes viral: Occurrence and distribution of North Sea bacteriophages. Mar Genomics 41, 31–41. https://doi.org/10.1016/J.MARGEN.2018.05.004

Germann, P.F., Alaoui, A., Riesen, D., 2002. Drag force approach to the transport of colloids in unsaturated soils. Water Resour Res 38, 18-1-18–15. https://doi.org/10.1029/2001wr000744

Ghanem, N., Kiesel, B., Kallies, R., Harms, H., Chatzinotas, A., Wick, L.Y., 2016. Marine phages as tracers: Effects of size, morphology, and physico−chemical surface properties on transport in a porous medium. Environ Sci Technol 50, 12816–12824. https://doi.org/10.1021/acs.est.6b04236

Gibson, J.J., Edwards, T.W.D., Birks, S.J., St Amour, N.A., Buhay, W.M., McEachern, P., Wolfe, B.B., Peters, D.L., 2005. Progress in isotope tracer hydrology in Canada. Hydrol Process 19, 303–327. https://doi.org/10.1002/hyp.5766

Gill, J.J., Summer, E.J., Russell, W.K., Cologna, S.M., Carlile, T.M., Fuller, A.C., Kitsopoulos, K., Mebane, L.M., Parkinson, B.N., Sullivan, D., Carmody, L.A., Gonzalez, C.F., LiPuma, J.J., Young, R., 2011. Genomes and characterization of phages Bcep22 and BcepIL02, founders of a novel phage type in Burkholderia cenocepacia. J Bacteriol 193, 5300–5313. https://doi.org/10.1128/JB.05287-11

Gobler, C.J., Hutchins, D.A., Fisher, N.S., 1998. Release and bioavailability of C, N, P, Se, and Fe following viral lysis of a marine chrysophyte. Oceanographic literature review 45, 1523–1524.

Goldrick, M., Kamp, B.P., Lepovitz, L., 2013. Reduce microRNA RT-qPCR Assay Costs by More Than 10-fold Without Compromising Results. Uniwersytet śląski 7, 343–354. https://doi.org/10.2/JQUERY.MIN.JS

Goldscheider, N., Haller, L., Poté, J., Wildi, W., Zopfi, J., 2007. Characterizing water circulation and contaminant transport in Lake Geneva using bacteriophage tracer experiments and limnological methods. Environ Sci Technol 41, 5252–5258. https://doi.org/10.1021/es070369p

Gong, Z., Liang, Y., Wang, M., Jiang, Y., Yang, Q., Xia, J., Zhou, X., You, S., Gao, C., Wang, J., He, J., Shao, H., McMinn, A., 2018. Viral Diversity and Its Relationship With Environmental Factors at the Surface and Deep Sea of Prydz Bay, Antarctica. Front Microbiol 9, 1–17. https://doi.org/10.3389/FMICB.2018.02981/FULL

Gonzalez-Martin, C., Teigell-Perez, N., Lyles, M., Valladares, B., Griffin, D.W., 2013. Epifluorescent direct counts of bacteria and viruses from topsoil of various desert dust storm regions. Res Microbiol 164, 17–21. https://doi.org/10.1016/J.RESMIC.2012.08.009

Grose, J.H., Casjens, S.R., 2014. Understanding the enormous diversity of bacteriophages: the tailed phages that infect the bacterial family Enterobacteriaceae. Virology 0, 421. https://doi.org/10.1016/J.VIROL.2014.08.024

Grujcic, V., Nuy, J.K., Salcher, M.M., Shabarova, T., Kasalicky, V., Boenigk, J., Jensen, M., Simek, K., 2018. Cryptophyta as major bacterivores in freshwater summer plankton. The ISME Journal 2018 12:7 12, 1668–1681. https://doi.org/10.1038/s41396-018-0057-5

Guixa-Boixereu, N., Vaqué, D., Gasol, J.M., Sánchez-Cámara, J., Pedrós-Alió, C., 2002. Viral distribution and activity in Antarctic waters. Deep Sea Research Part II: Topical Studies in Oceanography 49, 827–845. https://doi.org/10.1016/S0967-0645(01)00126-6

Guo, J., Bolduc, B., Zayed, A.A., Varsani, A., Dominguez-Huerta, G., Delmont, T.O., Pratama, A.A., Gazitúa, M.C., Vik, D., Sullivan, M.B., Roux, S., 2021. VirSorter2: a multi-classifier, expert-guided approach to detect diverse DNA and RNA viruses. Microbiome 9, 1–13. https://doi.org/10.1186/S40168-020-00990-Y/FIGURES/5

Hall, C.M., Busch, J.D., Shippy, K., Allender, C.J., Kaestli, M., Mayo, M., Sahl, J.W., Schupp, J.M., Colman, R.E., Keim, P., Currie, B.J., Wagner, D.M., 2015. Diverse Burkholderia Species Isolated from Soils in the Southern United States with No Evidence of B. pseudomallei. PLoS One 10. https://doi.org/10.1371/JOURNAL.PONE.0143254

Han, L.L., Yu, D.T., Bi, L., Du, S., Silveira, C., Cobián Güemes, A.G., Zhang, L.M., He, J.Z., Rohwer, F., 2022. Distribution of soil viruses across China and their potential role in phosphorous metabolism. Environmental Microbiomes 17, 1–11. https://doi.org/10.1186/S40793-022-00401-9

Han, M.S., Byun, J.H., Cho, Y., Rim, J.H., 2021. RT-PCR for SARS-CoV-2: quantitative versus qualitative. Lancet Infect Dis 21, 165. https://doi.org/10.1016/S1473-3099(20)30424-2

Haq, I.U., Chaudhry, W.N., Akhtar, M.N., Andleeb, S., Qadri, I., 2012. Bacteriophages and their implications on future biotechnology: a review. Virol J 9, 9. https://doi.org/10.1186/1743-422x-9-9

Harada, L.K., Silva, E.C., Campos, W.F., del Fiol, F.S., Vila, M., Dąbrowska, K., Krylov, V.N., Balcão, V.M., 2018. Biotechnological applications of bacteriophages: State of the art. Microbiol Res 212–213, 38–58. https://doi.org/10.1016/J.MICRES.2018.04.007

Harris, H.M.B., Hill, C., 2021. A Place for Viruses on the Tree of Life. Front Microbiol 11, 1–16. https://doi.org/10.3389/FMICB.2020.604048/BIBTEX

Harrison, E., Brockhurst, M.A., 2017. Ecological and Evolutionary Benefits of Temperate Phage: What Does or Doesn't Kill You Makes You Stronger. BioEssays 39, 1700112. https://doi.org/10.1002/BIES.201700112

Harvey, R.W., Ryan, J.N., 2004. Use of PRD1 bacteriophage in groundwater viral transport, inactivation, and attachment studies. FEMS Microbiol Ecol 49, 3–16. https://doi.org/10.1016/j.femsec.2003.09.015

Hatfull, G.F., Hendrix, R.W., 2011. Bacteriophages and their Genomes. Curr Opin Virol 1, 298–303. https://doi.org/10.1016/J.COVIRO.2011.06.009

Hayes, S., Mahony, J., Nauta, A., Van Sinderen, D., 2017. Metagenomic approaches to assess bacteriophages in various environmental niches. Viruses 9, 1–22. https://doi.org/10.3390/v9060127

Heffron, J., Mayer, B.K., 2021. Virus Isoelectric Point Estimation: Theories and Methods. Appl Environ Microbiol 87, 1–17. https://doi.org/10.1128/AEM.02319-20/SUPPL_FILE/AEM.02319-20-S0001.PDF

Heinrichs, M.E., De Corte, D., Engelen, B., Pan, D., 2021. An Advanced Protocol for the Quantification of Marine Sediment Viruses via Flow Cytometry. Viruses 13, 1–14. https://doi.org/10.3390/V13010102

Helsley, K.R., Brown, T.M., Furlong, K., Williamson, K.E., 2013. Applications and limitations of tea extract as a virucidal agent to assess the role of phage predation in soils. Biol Fertil Soils 50, 263–274. https://doi.org/10.1007/s00374-013-0855-x

Hennes, K.P., Simon, M., 1995. Significance of bacteriophages for controlling bacterioplankton growth in a mesotrophic lake. Appl Environ Microbiol 61, 333–340. https://doi.org/10.1128/AEM.61.1.333-340.1995

Hillary, L.S., Adriaenssens, E.M., Jones, D.L., McDonald, J.E., 2022. RNA-viromics reveals diverse communities of soil RNA viruses with the potential to affect grassland ecosystems across multiple trophic levels. ISME Communications 2022 2:1 2, 1–10. https://doi.org/10.1038/s43705-022-00110-x

Hiraoka, S., Yang, C.C., Iwasaki, W., 2016. Metagenomics and Bioinformatics in Microbial Ecology: Current Status and Beyond. Microbes Environ 31, 204. https://doi.org/10.1264/JSME2.ME16024

Hissler, C., Martínez-Carreras, N., Barnich, F., Gourdol, L., Iffly, J.F., Juilleret, J., Klaus, J., Pfister, L., 2021. The Weierbach experimental catchment in Luxembourg: A decade of critical zone monitoring in a temperate forest - from hydrological investigations to ecohydrological perspectives. Hydrol Process 35, 1–7. https://doi.org/10.1002/HYP.14140

Hjelmsø, M.H., Hellmér, M., Fernandez-Cassi, X., Timoneda, N., Lukjancenko, O., Seidel, M., Elsässer, D., Aarestrup, F.M., Löfström, C., Bofill-Mas, S., Abril, J.F., Girones, R., Schultz, A.C., 2017. Evaluation of methods for the concentration and extraction of viruses from sewage in the context of metagenomic sequencing. PLoS One 12, 1–17. https://doi.org/10.1371/journal.pone.0170199

Ho, J., Seidel, M., Niessner, R., Eggers, J., Tiehm, A., 2016. Long amplicon (LA)-qPCR for the discrimination of infectious and noninfectious phix174 bacteriophages after UV inactivation. Water Res 103, 141–148. https://doi.org/10.1016/J.WATRES.2016.07.032

Howard-Varona, C., Hargreaves, K.R., Abedon, S.T., Sullivan, M.B., 2017. Lysogeny in nature: mechanisms, impact and ecology of temperate phages. ISME J 11, 1511–1520. https://doi.org/10.1038/ISMEJ.2017.16

Howe, K., Kaluna, L., Lozano, A., Fischer, B.T., Tagami, Y., McHugh, R., Jarvi, S., 2019. Water transmission potential of Angiostrongylus cantonensis: Larval viability and effectiveness of rainwater catchment sediment filters. PLoS One 14. https://doi.org/10.1371/JOURNAL.PONE.0209813

Hren, M., 2018. Buying a qPCR Machine? Here's What You Should Know [WWW Document]. BioSistemika. URL https://biosistemika.com/blog/buying-new-qpcr-machine-2/ (accessed 10.30.22).

Hua, J., Huet, A., Lopez, C.A., Toropova, K., Pope, W.H., Duda, R.L., Hendrix, R.W., Conway, J.F., 2017. Capsids and genomes of jumbo-sized bacteriophages reveal the evolutionary reach of the HK97 fold. mBio 8, 1–15. https://doi.org/10.1128/MBIO.01579-17/SUPPL_FILE/MBO005173536ST2.PDF

Hurst, C.J., Gerba, C.P., Cech, I., 1980. Effects of environmental variables and soil characteristics on virus survival in soil. Appl Environ Microbiol 40, 1067–1079.

Hyman, P., Trubl, G., Abedon, S.T., 2021. Virus-Like Particle: Evolving Meanings in Different Disciplines. https://home.liebertpub.com/phage 2, 11–15. https://doi.org/10.1089/PHAGE.2020.0026

ICTV, 2021. Taxon Details | Omegavirus [WWW Document]. ICTV. URL https://ictv.global/taxonomy/taxondetails?taxnode_id=202101094 (accessed 10.20.22).

ICTV, 2020. Viral taxonomy [WWW Document].

Islam, W., Saqib, H.S.A., Adnan, M., Wang, Z., Tayyab, M., Huang, Z., Chen, H.Y.H., 2022. Differential response of soil microbial and animal communities along the chronosequence of Cunninghamia lanceolata at different soil depth levels in subtropical forest ecosystem. J Adv Res 38, 41–54. https://doi.org/10.1016/J.JARE.2021.08.005

Jacobs-Sera, D., Abad, L.A., Alvey, R.M., Anders, K.R., Aull, H.G., Bhalla, S.S., Blumer, L.S., Bollivar, D.W., Alfred Bonilla, J., Butela, K.A., Coomans, R.J., Cresawn, S.G., D'Elia, T., Diaz, A., Divens, A.M., Edgington, N.P., Frederick, G.D., Gainey, M.D., Garlena, R.A., Grant, K.W., Gurney, S.M.R., Hendrickson, H.L., Hughes, L.E., Kenna, M.A., Klyczek, K.K., Kotturi, H., Mavrich, T.N., McKinney, A.L., Merkhofer, E.C., Parker, J.M., Molloy, S.D., Monti, D.L., Pape-Zambito, D.A., Pollenz, R.S., Pope, W.H., Reyna, N.S., Rinehart, C.A., Russell, D.A., Shaffer, C.D., Sivanathan, V., Stoner, T.H., Stukey, J., Nicole Sunnen, C., Tolsma, S.S., Tsourkas, P.K., Wallen, J.R., Ware, V.C., Warner, M.H., Washington, J.M., Westover, K.M., Whitefleet-Smith, J.A.L., Wiersma-Koch, H.I., Williams, D.C., Zack, K.M., Hatfull, G.F., 2020. Genomic diversity of bacteriophages infecting Microbacterium spp. PLoS One 15, 1–41. https://doi.org/10.1371/JOURNAL.PONE.0234636

Jacquet, S., Miki, T., Noble, R., Peduzzi, P., Wilhelm, S., 2010. Viruses in aquatic ecosystems: Important advancements of the last 20 years and prospects for the future in the field of microbial oceanography and limnology. Adv Oceanogr Limnol 1, 97–141. https://doi.org/10.1080/19475721003743843

Jaskulska, A., Šulčius, S., Kokociński, M., Koreivienė, J., Nájera, A.F., Mankiewicz-Boczek, J., 2022. Cyanophage Distribution Across European Lakes of the Temperate-Humid Continental Climate Zone Assessed Using PCR-Based Genetic Markers. Microb Ecol 83, 284–295. https://doi.org/10.1007/S00248-021-01783-Y/FIGURES/4

Joergensen, R.G., Anderson, T.H., Wolters, V., 1995. Carbon and nitrogen relationships in the microbial biomass of soils in beech (Fagus sylvatica L.) forests. Biol Fertil Soils 19, 141–147. https://doi.org/10.1007/BF00336150

Jończyk, E., Kłak, M., Miedzybrodzki, R., Górski, A., 2011. The influence of external factors on bacteriophages-review. Folia Microbiol (Praha) 56, 191–200. https://doi.org/10.1007/s12223-011-0039-8

Joyce, E., Rueedi, J., Cronin, A., Pedley, S., Tellam, J.H., Greswell, R.B., 2007. Fate and transport of phage and viruses in UK Permo-Triassic Sandstone aquifers, Environment Agency of England and Wales Science Report.

Jurczak-Kurek, A., Gasior, T., Nejman-Faleńczyk, B., Bloch, S., Dydecka, A., Topka, G., Necel, A., Jakubowska-Deredas, M., Narajczyk, M., Richert, M., Mieszkowska, A., Wróbel, B., Węgrzyn, G., Węgrzyn, A., 2016. Biodiversity of bacteriophages: Morphological and biological properties of a large group of phages isolated from urban sewage. Sci Rep 6, 1–17. https://doi.org/10.1038/srep34338

Kagzi, K., Hechler, R.M., Fussmann, G.F., Cristescu, M.E., 2022. Environmental RNA degrades more rapidly than environmental DNA across a broad range of pH conditions. Mol Ecol Resour 22, 2640–2650. https://doi.org/10.1111/1755-0998.13655

Kaletta, J., Pickl, C., Griebler, C., Klingl, A., Kurmayer, R., Deng, L., 2020. A rigorous assessment and comparison of enumeration methods for environmental viruses. Scientific Reports 2020 10:1 10, 1–12. https://doi.org/10.1038/s41598-020-75490-y

Kalev, S.D., Toor, G.S., 2018. The Composition of Soils and Sediments, in: Green Chemistry: An Inclusive Approach. Elsevier, pp. 339–357. https://doi.org/10.1016/B978-0-12-809270-5.00014-5

Kallies, R., Kiesel, B., Schmidt, M., Kacza, J., Ghanem, N., Narr, A., Zopfi, J., Wick, L.Y., Hackermüller, J., Harms, H., Chatzinotas, A., 2017. Complete genome sequence of Pseudoalteromonas phage vB_PspS-H40/1 (formerly H40/1) that infects Pseudoalteromonas sp. strain H40 and is used as biological tracer in hydrological transport studies. Stand Genomic Sci 12, 1–7. https://doi.org/10.1186/s40793-017-0235-5

Kasman, L.M., Porter, L.D., 2021. Bacteriophages, Brenner's Encyclopedia of Genetics: Second Edition. StatPearls Publishing. https://doi.org/10.1016/B978-0-12-374984-0.00131-5

Kauffman, K.M., Hussain, F.A., Yang, J., Arevalo, P., Brown, J.M., Chang, W.K., Vaninsberghe, D., Elsherbini, J., Sharma, R.S., Cutler, M.B., Kelly, L., Polz, M.F., 2018. A major lineage of non-tailed dsDNA viruses as unrecognized killers of marine bacteria. Nature 2018 554:7690 554, 118–122. https://doi.org/10.1038/nature25474

Kavagutti, V.S., Andrei, A.Ş., Mehrshad, M., Salcher, M.M., Ghai, R., 2019. Phage-centric ecological interactions in aquatic ecosystems revealed through ultra-deep metagenomics. Microbiome 7, 1–15. https://doi.org/10.1186/S40168-019-0752-0/FIGURES/5

Keen, E.C., 2015. A century of phage research: bacteriophages and the shaping of modern biology. Bioessays 37, 6–9. https://doi.org/10.1002/bies.201400152.A

Kieft, K., Zhou, Z., Anantharaman, K., 2020. VIBRANT: Automated recovery, annotation and curation of microbial viruses, and evaluation of viral community function from genomic sequences. Microbiome 8, 1–23. https://doi.org/10.1186/S40168-020-00867-0/TABLES/2

Kim, K.H., Chang, H.W., Nam, Y. do, Roh, S.W., Kim, M.S., Sung, Y., Jeon, C.O., Oh, H.M., Bae, J.W., 2008. Amplification of uncultured single-stranded DNA viruses from rice paddy soil. Appl Environ Microbiol 74, 5975–5985. https://doi.org/10.1128/AEM.01275-08

Kim, T.G., Jeong, S.Y., Cho, K.S., 2014. Comparison of droplet digital PCR and quantitative real-time PCR for examining population dynamics of bacteria in

soil. Appl Microbiol Biotechnol 98, 6105–6113. https://doi.org/10.1007/S00253-014-5794-4

Kimura, M., Jia, Z.J., Nakayama, N., Asakawa, S., 2008. Ecology of viruses in soils: Past, present and future perspectives. Soil Sci Plant Nutr 54, 1–32. https://doi.org/10.1111/j.1747-0765.2007.00197.x

Kinnunen, K., 1978. Tracing water movement by means of Eschirichia coli bacteriophages. The water research institute, Helsinki.

Kinoshita, T., Bales, R.C., Maguire, K.M., Gerba, C.P., 1993. Effect of pH on bacteriophage transport through sandy soils. J Contam Hydrol 14, 55–70. https://doi.org/10.1016/0169-7722(93)90041-P

Kirchner, J.W., Tetzlaff, D., Soulsby, C., 2010. Comparing chloride and water isotopes as hydrological tracers in two Scottish catchments. Hydrol Process 24, 1631–1645. https://doi.org/10.1002/hyp.7676

Kłopot, A., Zakrzewska, A., Lecion, D., Majewska, J.M., Harhala, M.A., Lahutta, K., Kazmierczak, Z., Łaczmanski, Ł., Kłak, M., Dabrowska, K., 2017. Real-Time qPCR as a method for detection of antibody-neutralized phage particles. Front Microbiol 8, 1–10. https://doi.org/10.3389/FMICB.2017.02170/BIBTEX

Knezevic, P., Adriaenssens, E.M., Siddell, S.G., Lefkowitz, E.J., Sabanadzovic, S., Simmonds, P., Zerbini, F.M., Smith, D.B., Orton, R.J., Adriaenssens, E.M., 2021. ICTV virus taxonomy profile: Plectroviridae. Journal of General Virology 102, 001597. https://doi.org/10.1099/JGV.0.001597/CITE/REFWORKS

Kojabad, A.A., Farzanehpour, M., Galeh, H.E.G., Dorostkar, R., Jafarpour, A., Bolandian, M., Nodooshan, M.M., 2021. Droplet digital PCR of viral DNA/RNA, current progress, challenges, and future perspectives. J Med Virol 93, 4182. https://doi.org/10.1002/JMV.26846

Kolundžija, S., Cheng, D.Q., Lauro, F.M., 2022. RNA Viruses in Aquatic Ecosystems through the Lens of Ecological Genomics and Transcriptomics. Viruses 14. https://doi.org/10.3390/V14040702

Koonin, E. v., Yutin, N., 2020. The crAss-like Phage Group: How Metagenomics Reshaped the Human Virome. Trends Microbiol 28, 349–359. https://doi.org/10.1016/J.TIM.2020.01.010

Krishnamurthy, S.R., Janowski, A.B., Zhao, G., Barouch, D., Wang, D., 2016. Hyperexpansion of RNA Bacteriophage Diversity. PLoS Biol 14, e1002409. https://doi.org/10.1371/JOURNAL.PBIO.1002409

Krupovic, M., Consortium, I.R., 2018. ICTV Virus Taxonomy Profile: Plasmaviridae. J Gen Virol 99, 617–618. https://doi.org/10.1099/JGV.0.001060

Kumari, N., Mohan, C., 2021. Basics of Clay Minerals and Their Characteristic Properties, in: Clay and Clay Minerals. IntechOpen, pp. 1–30. https://doi.org/10.5772/INTECHOPEN.97672

Laanto, E., Mäntynen, S., de Colibus, L., Marjakangas, J., Gillum, A., Stuart, D.I., Ravantti, J.J., Huiskonen, J.T., Sundberg, L.R., 2017. Virus found in a boreal lake links ssDNA and dsDNA viruses. Proc Natl Acad Sci U S A 114, 8378–8383. https://doi.org/10.1073/PNAS.1703834114/SUPPL_FILE/PNAS.201703834SI.PDF

Labat, D., Mangin, A., 2015. Transfer function approach for artificial tracer test interpretation in karstic systems. J Hydrol (Amst) 529, 866–871. https://doi.org/10.1016/j.jhydrol.2015.09.011

Lamour, G., Hamraoui, A., Buvailo, A., Xing, Y., Keuleyan, S., Prakash, V., Eftekhari-Bafrooei, A., Borguet, E., 2010. Contact Angle Measurements Using a Simplified Experimental Setup. J Chem Educ 87, 1403–1407. https://doi.org/10.1021/ED100468U

Langlet, J., Gaboriaud, F., Duval, J.F.L., Gantzer, C., 2008. Aggregation and surface properties of F-specific RNA phages: Implication for membrane filtration processes. Water Res 42, 2769–2777. https://doi.org/10.1016/J.WATRES.2008.02.007

Langlet, J., Gaboriaud, F., Gantzer, C., 2007. Effects of pH on plaque forming unit counts and aggregation of MS2 bacteriophage. J Appl Microbiol 103, 1632–1638. https://doi.org/10.1111/J.1365-2672.2007.03396.X

Lappänen, M., Sundberg, L.-R., Laanto, E., Almeida, G.M. de F., Papponen, P., Maasilta, I.J., 2017. Imaging bacterial colonies and phage-bacterium interaction at sub-nanometer resolution using helium-ion microscopy. Adv Biosyst 1, 1–8.

le Vay, K., Salibi, E., Song, E.Y., Mutschler, H., 2020. Nucleic Acid Catalysis under Potential Prebiotic Conditions. Chem Asian J 15, 214–230. https://doi.org/10.1002/ASIA.201901205

Lee, C.Y., Chiu, Y.C., Wang, L.B., Kuo, Y.L., Chuang, E.Y., Lai, L.C., Tsai, M.H., 2013. Common applications of next-generation sequencing technologies in genomic research. Transl Cancer Res 2, 33–45. https://doi.org/10.3978/J.ISSN.2218-676X.2013.02.09

Lefkowitz, E.J., Dempsey, D.M., Hendrickson, R.C., Orton, R.J., Siddell, S.G., Smith, D.B., 2018. Virus taxonomy: the database of the International Committee on Taxonomy of Viruses (ICTV). Nucleic Acids Res 46, D708–D717. https://doi.org/10.1093/NAR/GKX932

Lefkowitz, E.J., Odom, M.R., Upton, C., 2008. Virus Databases. Encyclopedia of Virology 348. https://doi.org/10.1016/B978-012374410-4.00719-6

Leibundgut, C., Seibert, J., 2010. Tracer Hydrology. Treatise on Water Science 2, 215–236. https://doi.org/10.1016/B978-0-444-53199-5.00036-1

Leroy, M., Prigent, M., Dutertre, M., Confalonieri, F., Dubow, M., 2008. Bacteriophage morphotype and genome diversity in Seine River sediment. Freshw Biol 53, 1176–1185. https://doi.org/10.1111/j.1365-2427.2008.01985.x

Levy, B.S., Chambers, R.M., 1987. Bromide as a conservative tracer for soil-water studies. Hydrol Process 1, 385–389. https://doi.org/10.1002/HYP.3360010406

Li, C.X., Shi, M., Tian, J.H., Lin, X.D., Kang, Y.J., Chen, L.J., Qin, X.C., Xu, J., Holmes, E.C., Zhang, Y.Z., 2015. Unprecedented genomic diversity of RNA viruses in arthropods reveals the ancestry of negative-sense RNA viruses. Elife 4, 1–26. https://doi.org/10.7554/ELIFE.05378

Liang, X., Wagner, R.E., Zhuang, J., DeBruyn, J.M., Wilhelm, S.W., Liu, F., Yang, L., Staton, M.E., Sherfy, A.C., Radosevich, M., 2019. Viral abundance and diversity vary with depth in a southeastern United States agricultural ultisol. Soil Biol Biochem 137, 107546. https://doi.org/10.1016/J.SOILBIO.2019.107546

Liao, R., Yang, P., Wu, W., Luo, D., Yang, D., 2018. A DNA Tracer System for Hydrological Environment Investigations. Environ Sci Technol 52, 1695–1703. https://doi.org/10.1021/acs.est.7b02928

Lind, K., Ståhlberg, A., Zoric, N., Kubista, M., 2006. Combining sequence-specific probes and DNA binding dyes in real-time PCR for specific nucleic acid quantification and melting curve analysis. Biotechniques 40, 315–319. https://doi.org/10.2144/000112101/ASSET/IMAGES/LARGE/TABLE1.JPEG

Lischer, H.E.L., Shimizu, K.K., 2017. Reference-guided de novo assembly approach improves genome reconstruction for related species. BMC Bioinformatics 18, 1–12. https://doi.org/10.1186/S12859-017-1911-6/FIGURES/6

Lopes, A., Tavares, P., Petit, M.A., Guérois, R., Zinn-Justin, S., 2014a. Automated classification of tailed bacteriophages according to their neck organization. BMC Genomics 15, 1–17. https://doi.org/10.1186/1471-2164-15-1027/FIGURES/5

Lopes, A., Tavares, P., Petit, M.A., Guérois, R., Zinn-Justin, S., 2014b. Automated classification of tailed bacteriophages according to their neck organization. BMC Genomics 15. https://doi.org/10.1186/1471-2164-15-1027

López-Pérez, M., Haro-Moreno, J.M., Gonzalez-Serrano, R., Parras-Moltó, M., Rodriguez-Valera, F., 2017. Genome diversity of marine phages recovered

from Mediterranean metagenomes: Size matters. PLoS Genet 13, e1007018. https://doi.org/10.1371/JOURNAL.PGEN.1007018

Louten, J., 2016. Virus Structure and Classification. Essential Human Virology 19–29. https://doi.org/10.1016/B978-0-12-800947-5.00002-8

Luo, E., Aylward, F.O., Mende, D.R., Delong, E.F., 2017. Bacteriophage Distributions and Temporal Variability in the Ocean's Interior. mBio 8, 1–13. https://doi.org/10.1128/MBIO.01903-17

Luque, A., Reguera, D., 2010. The Structure of Elongated Viral Capsids. Biophys J 98, 2993–2003. https://doi.org/10.1016/J.BPJ.2010.02.051

Lynch, K.H., Stothard, P., Dennis, J.J., 2012. Characterization of DC1, a Broad-Host-Range Bcep22-Like Podovirus. Appl Environ Microbiol 78, 889. https://doi.org/10.1128/AEM.07097-11

Maghsoodi, A., Chatterjee, A., Andricioaei, I., Perkins, N.C., 2019. How the phage T4 injection machinery works including energetics, forces, and dynamic pathway. Proc Natl Acad Sci U S A 116, 25097–25105. https://doi.org/10.1073/PNAS.1909298116/-/DCSUPPLEMENTAL

Malki, K., Kula, A., Bruder, K., Sible, E., Hatzopoulos, T., Steidel, S., Watkins, S.C., Putonti, C., 2015. Bacteriophages isolated from Lake Michigan demonstrate broad host-range across several bacterial phyla. Virol J 12. https://doi.org/10.1186/S12985-015-0395-0

Maltseva, M., Langlois, M.A., 2022. Flow Virometry for Characterizing the Size, Concentration, and Surface Antigens of Viruses. Curr Protoc 2, 1–26. https://doi.org/10.1002/CPZ1.368

Mangin, A., 1984. Pour une meilleure connaissance des systèmes hydrologiques à partir des analyses corrélatoires et spectrale. J Hydrol (Amst) 67, 25–43.

Maranger, R., Bird, D.F., 1995. Viral abundance in aquatic systems: a comparison between marine and fresh waters. Mar Ecol Prog Ser 121, 217–226. https://doi.org/10.3354/MEPS121217

Martin, C., 1988. The Application of Bacteriophage Tracer Techniques in South West Water. Water and Environment Journal 2, 638–642. https://doi.org/10.1111/j.1747-6593.1988.tb01352.x

Martin, R., Thomas, A., 1974. An example of the use of bacteriophage as a groundwater tracer. J Hydrol (Amst) 23, 73–78. https://doi.org/10.1016/0022-1694(74)90024-9

Martínez-Carreras, N., Hissler, C., Gourdol, L., Klaus, J., Juilleret, J., Iffly, J.F., Pfister, L., 2016. Storage controls on the generation of double peak hydrographs in a forested headwater catchment. J Hydrol (Amst) 543, 255–269. https://doi.org/10.1016/J.JHYDROL.2016.10.004

Martínez-Carreras, N., Wetzel, C.E., Frentress, J., Ector, L., McDonnell, J.J., Hoffmann, L., Pfister, L., 2015. Hydrological connectivity inferred from diatom transport through the riparian-stream system. Hydrol Earth Syst Sci 19, 3133–3151. https://doi.org/10.5194/hess-19-3133-2015

Martinez-Hernandez, F., Garcia-Heredia, I., Gomez, M.L., Maestre-Carballa, L., Martínez, J.M., Martinez-Garcia, M., 2019. Droplet digital PCR for estimating absolute abundances of widespread pelagibacter viruses. Front Microbiol 10. https://doi.org/10.3389/FMICB.2019.01226/FULL

Massali, F.Z., Bouaninba, S., 2016. Etudes sur les bactériophages du sol et leurs méthodes d'isolement.

Mataix-Solera, J., Doerr, S.H., 2004. Hydrophobicity and aggregate stability in calcareous topsoils from fire-affected pine forests in southeastern Spain. Geoderma 118, 77–88. https://doi.org/10.1016/S0016-7061(03)00185-X

Matsushita, I., Yanase, H., 2009. The Genomic Structure of Thermus Bacteriophage ϕIN93. The Journal of Biochemistry 146, 775–785. https://doi.org/10.1093/JB/MVP125

Matsushita, I., Yanase, H., 2008. A novel thermophilic lysozyme from bacteriophage φIN93. Biochem Biophys Res Commun 377, 89–92. https://doi.org/10.1016/J.BBRC.2008.09.101

Mavrich, T.N., Hatfull, G.F., 2017. Bacteriophage evolution differs by host, lifestyle and genome. Nat Microbiol 2, 1–9. https://doi.org/10.1038/nmicrobiol.2017.112

McDonnell, J.J., Rowe, L., Stewart, M.K., 1999. A combined tracer-hydrometric approach to assess the effect of catchment scale on water flow path, source and age, in: Integrated Methods in Catchment Hydrology: Tracer, Remote Sensing and New Hydrometric Techniques. pp. 265–273.

McKay, L.D., Cherry, J.A., Bales, R.C., Yahya, M.T., Gerba, C.P., 1993. A Field Example of Bacteriophage as Tracers of Fracture Flow. Environ Sci Technol 27, 1075–1079. https://doi.org/10.1021/es00043a006

Mercanti, D.J., Guglielmotti, D.M., Patrignani, F., Reinheimer, J.A., Quiberoni, A., 2012. Resistance of two temperate Lactobacillus paracasei bacteriophages to high pressure homogenization, thermal treatments and chemical biocides of industrial application. Food Microbiol 29, 99–104. https://doi.org/10.1016/J.FM.2011.09.003

Mi, X., Bromley, E.K., Joshi, P.U., Long, F., Heldt, C.L., 2020. Virus Isoelectric Point Determination Using Single-Particle Chemical Force Microscopy. Langmuir 36, 370–378. https://doi.org/10.1021/ACS.LANGMUIR.9B03070/SUPPL_FILE/LA9B03070_SI_001.PDF

Michen, B., Graule, T., 2010. Isoelectric points of viruses. J Appl Microbiol 109, 388–397. https://doi.org/10.1111/j.1365-2672.2010.04663.x

Miranda, J.A., Steward, G.F., 2017. Variables influencing the efficiency and interpretation of reverse transcription quantitative PCR (RT-qPCR): An empirical study using Bacteriophage MS2. J Virol Methods 241, 1–10. https://doi.org/10.1016/J.JVIROMET.2016.12.002

Mirdita, M., Steinegger, M., Breitwieser, F., Sö Ding, J., Levy Karin, E., 2021. Fast and sensitive taxonomic assignment to metagenomic contigs. Bioinformatics 37, 3029–3031. https://doi.org/10.1093/BIOINFORMATICS/BTAB184

Mohiuddin, M., Schellhorn, H.E., 2015. Spatial and temporal dynamics of virus occurrence in two freshwater lakes captured through metagenomic analysis. Front Microbiol 6, 1–13. https://doi.org/10.3389/fmicb.2015.00960

Moineau, S., 2013. Bacteriophage, Brenner's Encyclopedia of Genetics: Second Edition. Elsevier Inc. https://doi.org/10.1016/B978-0-12-374984-0.00131-5

Monpoeho, S., Maul, A., Mignotte-Cadiergues, B., Schwartzbrod, L., Billaudel, S., Ferré, V., 2001. Best Viral Elution Method Available for Quantification of Enteroviruses in Sludge by Both Cell Culture and Reverse Transcription-PCR. Appl Environ Microbiol 67, 2484–2488. https://doi.org/10.1128/AEM.67.6.2484-2488.2001

Moon, K., 2017. Ecological and Genomic Study on Freshwater Bacteriophages.

Moon, K., Jeon, J.H., Kang, I., Park, K.S., Lee, K., Cha, C.J., Lee, S.H., Cho, J.C., 2020a. Freshwater viral metagenome reveals novel and functional phage-borne antibiotic resistance genes. Microbiome 8, 1–15. https://doi.org/10.1186/S40168-020-00863-4/FIGURES/6

Moon, K., Kim, S., Kang, I., Cho, J.C., 2020b. Viral metagenomes of Lake Soyang, the largest freshwater lake in South Korea. Scientific Data 2020 7:1 7, 1–6. https://doi.org/10.1038/s41597-020-00695-9

Morella, N.M., Yang, S.C., Hernandez, C.A., Koskella, B., 2018. Rapid quantification of bacteriophages and their bacterial hosts in vitro and in vivo using droplet digital PCR. J Virol Methods 259, 18–24. https://doi.org/10.1016/J.JVIROMET.2018.05.007

Muir, P., Li, S., Lou, S., Wang, D., Spakowicz, D.J., Salichos, L., Zhang, J., Weinstock, G.M., Isaacs, F., Rozowsky, J., Gerstein, M., 2016. The real cost of sequencing: Scaling computation to keep pace with data generation. Genome Biol 17, 1–9. https://doi.org/10.1186/S13059-016-0917-0/FIGURES/4

Muniesa, M., Imamovic, L., Jofre, J., 2011. Bacteriophages and genetic mobilization in sewage and faecally polluted environments. Microb Biotechnol 4, 725–734. https://doi.org/10.1111/j.1751-7915.2011.00264.x

Nasser, A.M., Oman, S.D., 1999. Quantitative assessment of the inactivation of pathogenic and indicator viruses in natural water sources. Water Res 33, 1748–1752. https://doi.org/10.1016/S0043-1354(98)00380-7

Naureen, Z., Dautaj, A., Anpilogov, K., Camilleri, G., Dhuli, K., Tanzi, B., Maltese, P.E., Cristofoli, F., Antoni, L. De, Beccari, T., Dundar, M., Bertelli, M., 2020. Bacteriophages presence in nature and their role in the natural selection of bacterial populations. Acta Bio Medica : Atenei Parmensis 91, 1–13. https://doi.org/10.23750/ABM.V91I13-S.10819

Nelson, D., 2004. Phage Taxonomy: We Agree To Disagree. J Bacteriol 186, 7029. https://doi.org/10.1128/JB.186.21.7029-7031.2004

Nielsen, K.M., Johnsen, P.J., Bensasson, D., Daffonchio, D., 2007. Release and persistence of extracellular DNA in the environment. Environ Biosafety Res 6, 37–53. https://doi.org/10.1051/EBR:2007031

Nishide, M., Tsujimoto, K., Uozaki, M., Ikeda, K., Yamasaki, H., Koyama, A.H., Arakawa, T., 2011. Effects of electrolytes on virus inactivation by acidic solutions. Int J Mol Med 27, 803–809. https://doi.org/10.3892/ijmm.2011.668

Novik, G., Ladutska, A., Rakhuba, D., 2004. Phage Taxonomy and Classification. Encyclopedia of Virology 1221–1228. https://doi.org/10.1006/rwvi.1999.0024

Nunez, R., 2001. DNA Measurement and Cell Cycle Analysis by Flow Cytometry. Current Issues in Molecular Biology 2001, Vol. 3, Pages 67-70 3, 67–70. https://doi.org/10.21775/CIMB.003.067

O'Brien, R.T., Newman, J.S., 1977. Inactivation of polioviruses and coxsackieviruses in surface water. Appl Environ Microbiol 33, 334–340.

Ofer, N., Wishkautzan, M., Meijler, M., Wang, Y., Speer, A., Niederweis, M., Gur, E., 2012. Ectoine biosynthesis in Mycobacterium smegmatis. Appl Environ Microbiol 78, 7483–7486. https://doi.org/10.1128/AEM.01318-12/SUPPL_FILE/ZAM999103756SO1.PDF

Ogorzaly, L., Gantzer, C., 2006. Development of real-time RT-PCR methods for specific detection of F-specific RNA bacteriophage genogroups: Application to urban raw wastewater. J Virol Methods 138, 131–139. https://doi.org/10.1016/j.jviromet.2006.08.004

O'Green, A.T., 2013. Soil Water Dynamics. Nature Education Knowledge 4, 9.

Oksanen, H.M., Consortium, I.R., 2017. ICTV Virus Taxonomy Profile: Corticoviridae. J Gen Virol 98, 888. https://doi.org/10.1099/JGV.0.000795

Ortmann, A.C., Suttle, C.A., 2005. High abundances of viruses in a deep-sea hydrothermal vent system indicates viral mediated microbial mortality. Deep Sea Research Part I: Oceanographic Research Papers 52, 1515–1527. https://doi.org/10.1016/J.DSR.2005.04.002

Park, J.A., Kang, J.K., Kim, J.H., Kim, S.B., Yu, S., Kim, T.H., 2015. Bacteriophage removal in various clay minerals and clay-amended soils. Environmental Engineering Research 20, 133–140. https://doi.org/10.4491/EER.2014.084

Parmar, K., Dafale, N., Pal, R., Tikariha, H., Purohit, H., 2018. An Insight into Phage Diversity at Environmental Habitats using Comparative Metagenomics Approach. Curr Microbiol 75, 132–141. https://doi.org/10.1007/s00284-017-1357-0

Passaretti, P., Sun, Y., Dafforn, T.R., Oppenheimer, P.G., 2020. Determination and characterisation of the surface charge properties of the bacteriophage M13 to assist bio-nanoengineering. RSC Adv 10, 25385–25392. https://doi.org/10.1039/D0RA04086J

Paul, S.I., Rahman, M.M., Salam, M.A., Khan, M.A.R., Islam, M.T., 2021. Identification of marine sponge-associated bacteria of the Saint Martin's island of the Bay of Bengal emphasizing on the prevention of motile Aeromonas septicemia in Labeo rohita. Aquaculture 545, 737156. https://doi.org/10.1016/J.AQUACULTURE.2021.737156

Perez Sepulveda, B., Redgwell, T., Rihtman, B., Pitt, F., Scanlan, D.J., Millard, A., 2016. Marine phage genomics: the tip of the iceberg. FEMS Microbiol Lett 363, 1–8. https://doi.org/10.1093/FEMSLE/FNW158

Pfister, L., McDonnell, J.J., Hlubikova, D., Matgen, P., Fenicia, F., Ector, L., Hoffmann, L., 2009. Watershed Hydrology and Surface Hydraulics Hydrometeorology and the Impacts of Climate Change Geochemistry and Solute Transport Sediments and Geomorphology Ecohydrology and Water Management. Hydrol Process 20, 1–5. https://doi.org/10.1002/hyp

Pfister, L., Wetzel, C.E., Klaus, J., Martínez-Carreras, N., Antonelli, M., Teuling, A.J., McDonnell, J.J., 2017. Terrestrial diatoms as tracers in catchment hydrology: a review. Wiley Interdisciplinary Reviews: Water 4, e1241. https://doi.org/10.1002/wat2.1241

Phadke, S., Macherla, S., Scheuermann, R.H., 2021. Database and Analytical Resources for Viral Research Community. Encyclopedia of Virology 141. https://doi.org/10.1016/B978-0-12-809633-8.20995-3

Pieper, A.P., Ryan, J.N., Harvey, R.W., Amy, G.L., Illangasekare, T.H., Metge, D.W., 1997. Transport and recovery of bacteriophage PRD1 in an unconfined sand aquifer: effect of anthropogenic organic material. Environ Sci Technol. 31, 1163–1170.

Poranen, M.M., Mäntynen, S., 2017. ICTV Virus Taxonomy Profile: Cystoviridae. J Gen Virol 98, 2423. https://doi.org/10.1099/JGV.0.000928

Prangishvili, D., Krupovic, M., 2012. A new proposed taxon for double-stranded DNA viruses, the order "Ligamenvirales." Arch Virol 157, 791–795. https://doi.org/10.1007/S00705-012-1229-7

Proctor, L.M., Fuhrman, J.A., 1990. Viral mortality of marine bacteria and cyanobacteria. Nature 1990 343:6253 343, 60–62. https://doi.org/10.1038/343060a0

Qu, L., Lei, T., Zhao, J., Gao, P., Yan, L., 2007. Laboratory experimental study on runoff and erosion processes from a small catchment under rainfallsimulator.

2007 ASABE Annual International Meeting, Technical Papers 3 BOOK. https://doi.org/10.13031/2013.23214

Raynaud, X., Nunan, N., 2014. Spatial Ecology of Bacteria at the Microscale in Soil. PLoS One 9. https://doi.org/10.1371/JOURNAL.PONE.0087217

Reavy, B., Swanson, M.M., Cock, P.J.A., Dawson, L., Freitag, T.E., Singh, B.K., Torrance, L., Mushegian, A.R., Taliansky, M., 2015. Distinct circular single-stranded DNA viruses exist in different soil types. Appl Environ Microbiol 81, 3934–3945. https://doi.org/10.1128/AEM.03878-14

Refardt, D., 2012. Real-time quantitative PCR to discriminate and quantify lambdoid bacteriophages of Escherichia coli K-12. Bacteriophage 2, 98–104. https://doi.org/10.4161/BACT.20092

Ren, J., Ahlgren, N.A., Lu, Y.Y., Fuhrman, J.A., Sun, F., 2017. VirFinder: a novel k-mer based tool for identifying viral sequences from assembled metagenomic data. Microbiome 5, 69. https://doi.org/10.1186/S40168-017-0283-5/TABLES/2

Renner, T.M., Tang, V.A., Burger, D., Langlois, M.-A., 2020. Intact Viral Particle Counts Measured by Flow Virometry Provide Insight into the Infectivity and Genome Packaging Efficiency of Moloney Murine Leukemia Virus. J Virol 94. https://doi.org/10.1128/JVI.01600-19

Richert-Pöggeler, K.R., Franzke, K., Hipp, K., Kleespies, R.G., 2019. Electron microscopy methods for virus diagnosis and high resolution analysis of viruses. Front Microbiol 10, 3255. https://doi.org/10.3389/FMICB.2018.03255/BIBTEX

Richter, Ł., Księżarczyk, K., Paszkowska, K., Janczuk-Richter, M., Niedziółka-Jönsson, J., Gapiński, J., Łoś, M., Hołyst, R., Paczesny, J., 2021. Adsorption of bacteriophages on polypropylene labware affects the reproducibility of phage research. Scientific Reports 2021 11:1 11, 1–12. https://doi.org/10.1038/s41598-021-86571-x

Rohwer, F., Prangishvili, D., Lindell, D., 2009. Roles of viruses in the environment. Environ Microbiol 11, 2771–2774. https://doi.org/10.1111/J.1462-2920.2009.02101.X

Roingeard, P., Raynal, P.I., Eymieux, S., Blanchard, E., 2019. Virus detection by transmission electron microscopy: Still useful for diagnosis and a plus for biosafety. Rev Med Virol 29. https://doi.org/10.1002/RMV.2019

Rose, R., Constantinides, B., Tapinos, A., L. Robertson, D., Prosperi, M., 2016. Challenges in the analysis of viral metagenomes. Virus Evol 2, 1–11. https://doi.org/10.1093/VE/VEW022

Rossi, P., Aragno, M., 1999. Analysis of bacteriophage inactivation and its attenuation by adsorption onto colloidal particles by batch agitation techniques. Can J Microbiol 45, 9–17. https://doi.org/10.1139/w98-117

Rossi, P., Doerfliger, N., Kennedy, K., Müller, I., Aragno, M., 1998. Bacteriophages as surface and ground water tracers. Hydrol Earth Syst Sci 2, 101–110.

Rossi, P., Käss, W., 1998. Phagen, in: Markierungsmittel. pp. 209–232.

Rousseau, M., di Pietro, L., Angulo-Jaramillo, R., Tessier, D., Cabibel, B., 2004. Preferential Transport of Soil Colloidal Particles: Physicochemical Effects on Particle Mobilization. Vadose Zone Journal 3, 247–261. https://doi.org/10.2113/3.1.247

Roux, S., Enault, F., Hurwitz, B.L., Sullivan, M.B., 2015a. VirSorter: Mining viral signal from microbial genomic data. PeerJ 2015, e985. https://doi.org/10.7717/PEERJ.985/SUPP-2

Roux, S., Hallam, S.J., Woyke, T., Sullivan, M.B., 2015b. Viral dark matter and virus–host interactions resolved from publicly available microbial genomes. Elife 4, 1–20. https://doi.org/10.7554/elife.08490

Roux, S., Krupovic, M., Poulet, A., Debroas, D., Enault, F., 2012. Evolution and Diversity of the Microviridae Viral Family through a Collection of 81 New Complete Genomes Assembled from Virome Reads. PLoS One 7, e40418. https://doi.org/10.1371/JOURNAL.PONE.0040418

Roux, S., Tournayre, J., Mahul, A., Debroas, D., Enault, F., 2014. Metavir 2: New tools for viral metagenome comparison and assembled virome analysis. BMC Bioinformatics 15, 1–12. https://doi.org/10.1186/1471-2105-15-76/FIGURES/6

Sabir, I.H., Haldorsen, S., Torgersen, J., Aleström, P., Gaut, S., Colleuille, H., Pedersen, T.S., Kitterød, N.-O., 2000. Synthetic DNA tracers: Examples of their application in water related studies. IAHS-AISH Publication 262, 159–165.

Sakoda, A., Sakai, Y., Hayakawa, K., Suzuki, M., 1997. Adsorption of viruses in water environment onto solid surfaces. Water Science and Technology 35, 107–114. https://doi.org/10.1016/S0273-1223(97)00120-0

Salmond, G.P.C., Fineran, P.C., 2015. A century of the phage: Past, present and future. Nat Rev Microbiol 13, 777–786. https://doi.org/10.1038/nrmicro3564

Sanger, F., Air, G.M., Barrell, B.G., Brown, N.L., Coulson, A.R., Fiddes, J.C., Hutchison, C.A., Slocombe, P.M., Smith, M., 1977. Nucleotide sequence of bacteriophage φX174 DNA. Nature 1977 265:5596 265, 687–695. https://doi.org/10.1038/265687a0

Sanjuán, R., Domingo-Calap, P., 2021. Genetic Diversity and Evolution of Viral Populations. Encyclopedia of Virology 53. https://doi.org/10.1016/B978-0-12-809633-8.20958-8

Sautrey, G., Brié, A., Gantzer, C., Walcarius, A., 2018. MS2 and Qβ bacteriophages reveal the contribution of surface hydrophobicity on the mobility of non-enveloped icosahedral viruses in SDS-based capillary zone electrophoresis. Electrophoresis 39, 377–385. https://doi.org/10.1002/ELPS.201700352

Schmidt, M., Byrne, J.M., Maasilta, I.J., 2021. Bio-imaging with the helium-ion microscope: A review. Beilstein Journal of Nanotechnology 12, 1. https://doi.org/10.3762/BJNANO.12.1

Schoonheydt, R.A., Johnston, C.T., 2006. Chapter 3 Surface and Interface Chemistry of Clay Minerals. Dev Clay Sci 1, 87–113. https://doi.org/10.1016/S1572-4352(05)01003-2

Schudel, B., Biaggi, D., Dervey, T., Kozel, R., Müller, I., Ross, J.H., Schindler, U., 2002. Utilisation des traceurs artificiels en hydrogéologie - Guide pratique. Série Géologique 77.

Schulz, C., Fast, C., Wernery, U., Kinne, J., Joseph, S., Schlottau, K., Jenckel, M., Höper, D., Patteril, N.A.G., Syriac, G., Hoffmann, B., Beer, M., 2019. A Novel Genus of Actinobacterial Tectiviridae. Viruses 11. https://doi.org/10.3390/V11121134

Schwarze, K., Buchanan, J., Fermont, J.M., Dreau, H., Tilley, M.W., Taylor, J.M., Antoniou, P., Knight, S.J.L., Camps, C., Pentony, M.M., Kvikstad, E.M., Harris, S., Popitsch, N., Pagnamenta, A.T., Schuh, A., Taylor, J.C., Wordsworth, S., 2019. The complete costs of genome sequencing: a microcosting study in cancer and rare diseases from a single center in the United Kingdom. Genetics in Medicine 2019 22:1 22, 85–94. https://doi.org/10.1038/s41436-019-0618-7

Sedlar, K., Kupkova, K., Provaznik, I., 2017. Bioinformatics strategies for taxonomy independent binning and visualization of sequences in shotgun metagenomics. Comput Struct Biotechnol J 15, 48–55. https://doi.org/10.1016/J.CSBJ.2016.11.005

Selleck, B., Koff, D., 2008. Stable Isotope Signature of Middle Devonian Seawater From Hamilton Group Brachiopods, Central New York State. Northeastern Geology & Environmental … 30, 330–343.

Senesi, N., 1989. Composted materials as organic fertilizers. Science of The Total Environment 81–82, 521–542. https://doi.org/10.1016/0048-9697(89)90161-7

Serôdio, J., Lavaud, J., 2020. Diatoms and Their Ecological Importance, in: Life Below Water. Encyclopedia of the UN Sustainable Development Goals, pp. 1–9. https://doi.org/10.1007/978-3-319-71064-8_12-1ï

Shepherd, M.A., Harrison, R., Webb, J., 2006. Managing soil organic matter – implications for soil structure on organic farms. Soil Use Manag 18, 284–292. https://doi.org/10.1111/J.1475-2743.2002.TB00270.X

Sierra, M.A., Li, Q., Pushalkar, S., Paul, B., Sandoval, T.A., Kamer, A.R., Corby, P., Guo, Y., Ruff, R.R., Alekseyenko, A. v., Li, X., Saxena, D., 2020. The Influences of Bioinformatics Tools and Reference Databases in Analyzing the Human Oral Microbial Community. Genes (Basel) 11, 1–12. https://doi.org/10.3390/GENES11080878

Silverman, A.I., Boehm, A.B., 2020. Systematic Review and Meta-Analysis of the Persistence and Disinfection of Human Coronaviruses and Their Viral Surrogates in Water and Wastewater. Environ Sci Technol Lett 7, 544–553. https://doi.org/10.1021/ACS.ESTLETT.0C00313/SUPPL_FILE/EZ0C00313_SI_001.XLSX

Silverman, A.I., Peterson, B.M., Boehm, A.B., McNeill, K., Nelson, K.L., 2013. Sunlight inactivation of human viruses and bacteriophages in coastal waters containing natural photosensitizers. Environ Sci Technol 47, 1870–1878. https://doi.org/10.1021/es3036913

Sime-Ngando, T., 2014. Environmental bacteriophages: viruses of microbes in aquatic ecosystems. Front Microbiol 5. https://doi.org/10.3389/FMICB.2014.00355

Sime-Ngando, T., Colombet, J., 2009. Virus et prophages dans les écosystèmes aquatiques. Can J Microbiol 55, 95–109. https://doi.org/10.1139/W08-099

Sinton, L.W., Ching, S.B., 1987. An evaluation of two bacteriophages as sewage tracers. Water Air Soil Pollut 35, 347–356.

Skilton, H., Wheeler, D., 1988. Bacteriophage tracer experiments in groundwater. Journal of Applied Bacteriology 65, 387–395. https://doi.org/10.1111/j.1365-2672.1988.tb01907.x

Skvortsov, T., De Leeuwe, C., Quinn, J.P., McGrath, J.W., Allen, C.C.R., McElarney, Y., Watson, C., Arkhipova, K., Lavigne, R., Kulakov, L.A., 2016. Metagenomic Characterisation of the Viral Community of Lough

Neagh, the Largest Freshwater Lake in Ireland. PLoS One 11, e0150361. https://doi.org/10.1371/JOURNAL.PONE.0150361

Slatko, B.E., Gardner, A.F., Ausubel, F.M., 2018. Overview of Next Generation Sequencing Technologies. Curr Protoc Mol Biol 122, e59. https://doi.org/10.1002/CPMB.59

Smettem, K.R.J., Trudgill, S.T., 1983. An evaluation of some fluorescent and non-fluorescent dyes in the identification of water transmission routes in soils. Journal of Soil Science 34, 45–56. https://doi.org/10.1111/j.1365-2389.1983.tb00811.x

Smith, J.L., McNeal, B.L., Cheng, H.H., 1985. Estimation of soil microbial biomass: An analysis of the respiratory response of soils. Soil Biol Biochem 17, 11–16. https://doi.org/https://doi.org/10.1016/0038-0717(85)90083-5

Song, X., Bai, P., 2015. Research experiment on infiltration and runoff in Jujube land of northern Shaanxi Province. IAHS-AISH Proceedings and Reports 368, 174–179. https://doi.org/10.5194/PIAHS-368-174-2015

Sotomayor-Garcia, A., Sala, M.M., Ferrera, I., Estrada, M., Vázquez-Domínguez, E., Emelianov, M., Cortés, P., Marrasé, C., Ortega-Retuerta, E., Nunes, S., Castillo, Y.M., Cuerva, M.S., Sebastián, M., Dall'osto, M., Simó, R., Vaqué, D., 2020. Assessing Viral Abundance and Community Composition in Four Contrasting Regions of the Southern Ocean. Life 10, 1–20. https://doi.org/10.3390/LIFE10070107

Sullivan, M.B., Weitz, J.S., Wilhelm, S., 2017. Viral ecology comes of age. Environ Microbiol Rep 9, 33–35. https://doi.org/https://doi.org/10.1111/1758-2229.12504

Sun, M., Zhan, Y., Marsan, D., Páez-Espino, D., Cai, L., Chen, F., 2021. Uncultivated Viral Populations Dominate Estuarine Viromes on the Spatiotemporal Scale. mSystems 6. https://doi.org/10.1128/MSYSTEMS.01020-20/SUPPL_FILE/MSYSTEMS.01020-20-ST002.DOCX

Sundaramoorthy, N.S., Thothathri, S., Bhaskaran, M., GaneshPrasad, A.K., Nagarajan, S., 2021. Phages from Ganges River curtail in vitro biofilms and planktonic growth of drug resistant Klebsiella pneumoniae in a zebrafish infection model. AMB Express 11, 1–11. https://doi.org/10.1186/S13568-021-01181-0/FIGURES/6

Suttle, C.A., 2005. Viruses in the sea. Nature 437, 356–361. https://doi.org/10.1038/nature04160

Suttle, C.A., 1994. The significance of viruses to mortality in aquatic microbial communities. Microbial Ecology 1994 28:2 28, 237–243. https://doi.org/10.1007/BF00166813

Swanson, M.M., Fraser, G., Daniell, T.J., Torrance, L., Gregory, P.J., Taliansky, M., 2009. Viruses in soils: Morphological diversity and abundance in the rhizosphere. Annals of Applied Biology 155, 51–60. https://doi.org/10.1111/j.1744-7348.2009.00319.x

Swiss Institute of Bioinformatics, 2015. Omegavirus [WWW Document]. ViralZone. URL https://viralzone.expasy.org/6336 (accessed 10.20.22).

Taj, M.K., Ling, J.X., Bing, L.L., Qi, Z., Taj, I., Hassani, T.M., Samreen, Z., Yunlin, W., 2014. Effect of dilution, temperature and pH on the lysis activity of T4 phage against E. coli BL21. The journal of animal and plant sciences 24, 1252–1255.

Tars, K., 2020. ssRNA Phages: Life Cycle, Structure and Applications. Biocommunication of Phages 261. https://doi.org/10.1007/978-3-030-45885-0_13

Tetzlaff, D., Buttle, J., Carey, S.K., Mcguire, K., Laudon, H., Soulsby, C., 2015. Tracer-based assessment of flow paths, storage and runoff generation in northern catchments: a review. Hydrol Process 29, 3475–3490. https://doi.org/10.1002/HYP.10412

Tithi, S.S., Jensen, R. v., Zhang, L., 2017. Fast Virome Explorer: A Pipeline for Virus and Phage Identification and Abundance Profiling in Metagenomics Data. bioRxiv 196998. https://doi.org/10.1101/196998

Tolstoy, I., Kropinski, A.M., Brister, J.R., 2018. Bacteriophage taxonomy: an evolving discipline, in: Azeredo, J., Sillankorva, S. (Eds.), Bacteriophage Therapy: From Lab to Clinical Practice. Springer Science, pp. 57–71. https://doi.org/10.1007/978-1-4939-7395-8

Tran, P.Q., Anantharaman, K., 2021. Biogeochemistry Goes Viral: towards a Multifaceted Approach To Study Viruses and Biogeochemical Cycling. mSystems 6. https://doi.org/10.1128/MSYSTEMS.01138-21/ASSET/E2F6D409-21E0-4823-B080-2C3373BE9933/ASSETS/IMAGES/LARGE/MSYSTEMS.01138-21-F002.JPG

Trubl, G., Roux, S., Solonenko, N., Li, Y.F., Bolduc, B., Rodríguez-Ramos, J., Eloe-Fadrosh, E.A., Rich, V.I., Sullivan, M.B., 2019. Towards optimized viral metagenomes for double-stranded and single-stranded DNA viruses from challenging soils. PeerJ 2019. https://doi.org/10.7717/PEERJ.7265/SUPP-7

Turner, D., Kropinski, A.M., Adriaenssens, E.M., 2021. A roadmap for genome-based phage taxonomy. Viruses 13, 1–10. https://doi.org/10.3390/v13030506

Valiente-Mullor, C., Beamud, B., Ansari, I., Frances-Cuesta, C., Garcia-Gonzalez, N., Mejia, L., Ruiz-Hueso, P., Gonzalez-Candelas, F., 2021. One is not enough: On the effects of reference genome for the mapping and subsequent analyses of short-reads. PLoS Comput Biol 17, e1008678. https://doi.org/10.1371/JOURNAL.PCBI.1008678

Walker, P.J., Siddell, S.G., Lefkowitz, E.J., Mushegian, A.R., Adriaenssens, E.M., Dempsey, D.M., Dutilh, B.E., Harrach, B., Harrison, R.L., Hendrickson, R.C., Junglen, S., Knowles, N.J., Kropinski, A.M., Krupovic, M., Kuhn, J.H., Nibert, M., Orton, R.J., Rubino, L., Sabanadzovic, S., Simmonds, P., Smith, D.B., Varsani, A., Zerbini, F.M., Davison, A.J., 2020. Changes to virus taxonomy and the Statutes ratified by the International Committee on Taxonomy of Viruses (2020). Arch Virol 165, 2737–2748. https://doi.org/10.1007/S00705-020-04752-X/TABLES/1

Walshe, G.E., Pang, L., Flury, M., Close, M.E., Flintoft, M., 2010. Effects of pH, ionic strength, dissolved organic matter, and flow rate on the co-transport of MS2 bacteriophages with kaolinite in gravel aquifer media. Water Res 44, 1255–1269. https://doi.org/10.1016/J.WATRES.2009.11.034

Wang, Q., Zhu, C., Yun, J., Yang, G., 2017. Isomorphic Substitutions in Clay Materials and Adsorption of Metal Ions onto External Surfaces: A DFT Investigation. Journal of Physical Chemistry C 121, 26722–26732. https://doi.org/10.1021/ACS.JPCC.7B03488/SUPPL_FILE/JP7B03488_SI_001.PDF

Weinbauer, M.G., Wilhelm, S.W., Suttle, C.A., Pledger, R.J., Mitchell, D.L., 1999. Sunlight-induced DNA damage and resistance in natural viral communities. Aquatic Microbial Ecology 17, 111–120. https://doi.org/10.3354/ame017111

White, H.E., Orlova, E. V., 2019. Bacteriophages: Their Structural Organisation and Function, in: Bacteriophages - Perspectives and Future. IntechOpen, pp. 1–32. https://doi.org/10.5772/INTECHOPEN.85484

Whitmer, S., Baker, L., Wass, R., 2000. Loss of Bromide in a Wetland Tracer Experiment. J Environ Qual 29, 2043–2045. https://doi.org/10.2134/JEQ2000.00472425002900060043X

Whittinghill, K.A., Hobbie, S.E., 2012. Effects of pH and calcium on soil organic matter dynamics in Alaskan tundra. Biogeochemistry 111, 569–581. https://doi.org/10.1007/S10533-011-9688-6/FIGURES/5

Whittinghill, K.A., Hobbie, S.E., 2011. Effects of pH and calcium on soil organic matter dynamics in Alaskan tundra. Biogeochemistry 111, 569–581. https://doi.org/10.1007/S10533-011-9688-6

Wichels, A., Biel, S.S., Gelderblom, H.R., Brinkhoff, T., Muyzer, G., Schütt, C., 1998. Bacteriophage diversity in the North Sea. Appl Environ Microbiol 64, 4128–4133. https://doi.org/10.1128/AEM.64.11.4128-4133.1998/ASSET/CF3E58A1-C46D-4FBA-B6B6-73801074BDDF/ASSETS/GRAPHIC/AM1180921005.JPEG

Williamson, K.E., Fuhrmann, J.J., Wommack, K.E., Radosevich, M., 2017. Viruses in Soil Ecosystems: An Unknown Quantity Within an Unexplored Territory. Annu Rev Virol 4, 201–219. https://doi.org/10.1146/annurev-virology-101416-041639

Williamson, K.E., Radosevich, M., Smith, D.W., Wommack, K.E., 2007. Incidence of lysogeny within temperate and extreme soil environments. Environ Microbiol 9, 2563–2574. https://doi.org/10.1111/J.1462-2920.2007.01374.X

Williamson, K.E., Radosevich, M., Wommack, K.E., 2005. Abundance and diversity of viruses in six Delaware soils. Appl Environ Microbiol 71, 3119–3125. https://doi.org/10.1128/AEM.71.6.3119

Williamson, K.E., Wommack, K.E., Radosevich, M., 2003. Sampling Natural Viral Communities from Soil for Culture-Independent Analyses. Appl Environ Microbiol 69, 6628–6633. https://doi.org/10.1128/AEM.69.11.6628-6633.2003

Wolf, A., Wiese, J., Jost, G., Witzel, K.P., 2003. Wide Geographic Distribution of Bacteriophages That Lyse the Same Indigenous Freshwater Isolate (Sphingomonas sp. Strain B18). Appl Environ Microbiol 69, 2395. https://doi.org/10.1128/AEM.69.4.2395-2398.2003

Wolf, Y.I., Kazlauskas, D., Iranzo, J., Lucía-Sanz, A., Kuhn, J.H., Krupovic, M., Dolja, V. V., Koonin, E. V., 2018. Origins and Evolution of the Global RNA Virome. mBio 9. https://doi.org/10.1128/MBIO.02329-18

Wolkersdorfer, C., LeBlanc, J., 2012. Regulations, legislation, and guidelines for artificial surface water and groundwater tracer tests in Canada. Water Quality Research Journal of Canada 47, 42–55. https://doi.org/10.2166/wqrjc.2012.042

Wommack, K.E., Hill, R.T., Kessel, M., Russek-Cohen, E., Colwell, R.R., 1992. Distribution of viruses in the Chesapeake Bay. Appl Environ Microbiol 58, 2965–2970.

Wu, R., Davison, M.R., Nelson, W.C., Graham, E.B., Fansler, S.J., Farris, Y., Bell, S.L., Godinez, I., Mcdermott, J.E., Hofmockel, K.S., Jansson, J.K., 2021. DNA Viral Diversity, Abundance, and Functional Potential Vary across Grassland Soils with a Range of Historical Moisture Regimes. mBio 12. https://doi.org/10.1128/MBIO.02595-21

Wu, Y., Liu, S., Liu, Z., Liu, B., Du, B., Tong, Z., Xu, J., 2022. A new method for quantitative analysis of M13 bacteriophage by atomic force microscopy. Synth Syst Biotechnol 7, 1066–1072. https://doi.org/10.1016/J.SYNBIO.2022.07.001

Yamada, H., Yamaguchi, M., Igarashi, Y., Chikamatsu, K., Aono, A., Murase, Y., Morishige, Y., Takaki, A., Chibana, H., Mitarai, S., 2018. Mycolicibacterium smegmatis, Basonym Mycobacterium smegmatis, Expresses Morphological Phenotypes Much More Similar to Escherichia coli Than Mycobacterium tuberculosis in Quantitative Structome Analysis and CryoTEM Examination. Front Microbiol 9, 1992. https://doi.org/10.3389/FMICB.2018.01992/BIBTEX

Yamada, T., Kawasaki, T., Nagata, S., Fujiwara, A., Usami, S., Fujie, M., 2007. New bacteriophages that infect the phytopathogen Ralstonia solanacearum. Microbiology (N Y) 153, 2630–2639. https://doi.org/10.1099/mic.0.2006/001453-0

Yamada, T., Satoh, S., Ishikawa, H., Fujiwara, A., Kawasaki, T., Fujie, M., Ogata, H., 2010. A jumbo phage infecting the phytopathogen Ralstonia solanacearum defines a new lineage of the Myoviridae family. Virology 398, 135–147. https://doi.org/10.1016/j.virol.2009.11.043

Yates, M. V., Gerba, C.P., Kelley, L.M., 1985. Virus persistence in groundwater. Appl Environ Microbiol 49, 778–781.

Yuan, Y., Gao, M., 2017. Jumbo Bacteriophages: An Overview. Front Microbiol 8, 403. https://doi.org/10.3389/FMICB.2017.00403

Zemb, O., Manefield, M., Thomas, F., Jacquet, S., 2013. Phage adsorption to bacteria in the light of the electrostatics: A case study using E. coli, T2 and

flow cytometry. J Virol Methods 189, 283–289. https://doi.org/10.1016/j.jviromet.2013.02.007

Zhang, Y.Z., Shi, M., Holmes, E.C., 2018. Using Metagenomics to Characterize an Expanding Virosphere. Cell 172, 1168–1172. https://doi.org/10.1016/j.cell.2018.02.043

Zhou, W., Han, G., Liu, M., Li, X., 2019. Effects of soil pH and texture on soil carbon and nitrogen in soil profiles under different land uses in Mun River Basin, Northeast Thailand. PeerJ 2019. https://doi.org/10.7717/PEERJ.7880/SUPP-1

Zhulin, I.B., 2015. Databases for Microbiologists. J Bacteriol 197, 2458. https://doi.org/10.1128/JB.00330-15

Zimmermann, A.U., Münnich, K.O., Roether, W., Kreutz, W., Schubach, K., 1966. Tracers Determine Movement of Soil Moisture and Evapotranspiration Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/1718314. Science (1979) 152, 346–347.

Zipper, H., Brunner, H., Bernhagen, J., Vitzthum, F., 2004. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. Nucleic Acids Res 32, 1–10. https://doi.org/10.1093/NAR/GNH101