



A hundred years of activated sludge: time for a rethink

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Biological wastewater treatment plants (BWWTPs) based on the activated sludge (AS) process have dramatically improved worldwide water sanitation despite increased urbanization and industrialization. However, current AS-based operations are considered economically and environmentally unsustainable. In this Perspective, we discuss our current understanding of microbial populations and their metabolic transformations in AS-based BWWTPs in view of developing more sustainable processes in the future. In particular, much has been learned over the course of the past 25 years about specialized microorganisms, which could be more comprehensively leveraged to recover energy and/or nutrients from wastewater streams. To achieve this, we propose a bottom-up design approach, focused around the concept of a “wastewater biorefinery column”, which would rely on the engineering of distinct ecological niches into a BWWTP in order to guarantee the targeted enrichment of specific organismal groups which in turn will allow the harvest of high-value resources from wastewater. This concept could be seen as a possible grand challenge to microbial ecologists and engineers alike at the centenary of the discovery of the AS process.

Keywords: activated sludge, wastewater microbial diversity and function, integrated omics, niche engineering, sustainability and renewable resources, energy reclamation – biodiesel and bioethanol, nutrient recovery and fertilizers, wastewater biorefinery column concept

Modern society is placing considerable strain on essential resources including water and energy (e.g., fossil fuels). Human interferences in biogeochemical cycles has already substantially altered the structure and function of atmospheric, aquatic, and terrestrial ecosystems (Vitousek, 1994). This is projected to worsen over the coming decades, as current estimates predict the human population to grow to more than 9 billion by 2050 (United Nations, 2013). In particular, waste residues may become pollutants if care is not given to their discharge into the environment. Thus, apart from public health considerations, systematic waste management is a central premise of sustainable development.

One hundred years ago, Ardern and Lockett (1914) described the process of activated sludge (AS) for biological remediation of sewage, based on heterotrophic microbial biomass either assimilating or oxidizing dissolved organic matter in influent wastewater. Following an aeration phase, the suspended biomass is separated from the treated wastewater by gravity filtration with a subsequent recycling of the majority of the sludge which is termed activated. To date, numerous variations of the original AS-based biological wastewater treatment plants (BWWTPs) are being operated world-wide including for enhanced biological phosphorus removal (Gujer et al., 1995), nitrification-denitrification (Henze et al., 1999), and anaerobic oxidation of ammonium (Van Dongen et al., 2001) together with anaerobic digestion of excess sludge (Grady et al., 2011). However, given the need for aeration as well as moving biomass between treatment tanks, AS-based BWWTPs consume considerable amounts of fossil fuel-derived energy resulting in considerable anthropogenic greenhouse gas emissions. In addition, the processes themselves also generate potent greenhouse gases such as CH₄ (El-Fadel and Massoud,

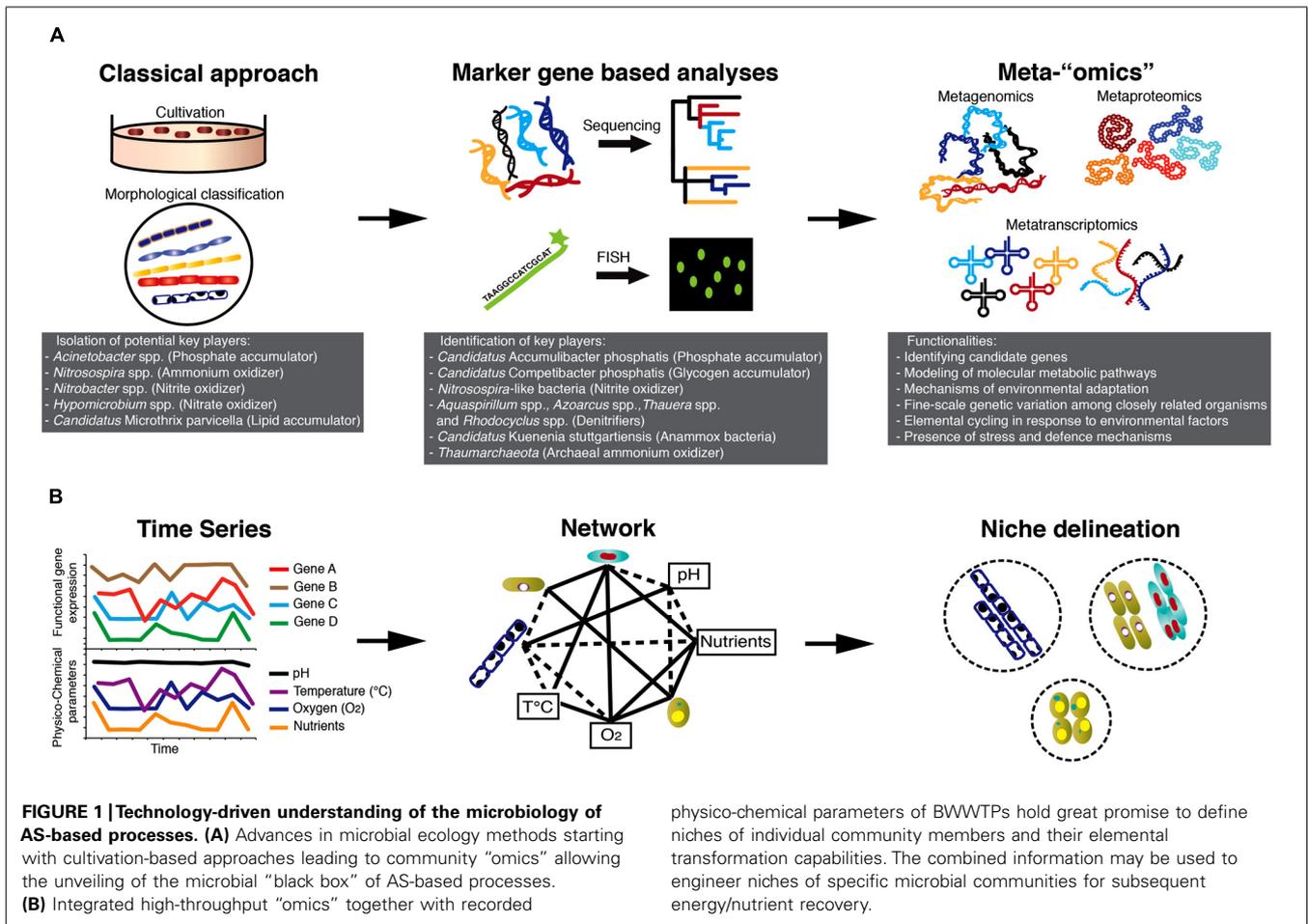
2001) and N₂O (Kampschreur et al., 2008). In this light, the current reliance on AS-based processes is environmentally unsustainable.

Although the organic and inorganic composition of wastewater depends on the influent (either municipal or industrial), it is estimated that the amount of chemical energy contained within wastewater is at least 10-fold higher than the amount of energy currently used to treat it (Shizas and Bagley, 2004; Heidrich et al., 2010). To date, energy recovery yields from anaerobic digestion of excess AS or from microbial fuel cells (MFCs) are significantly lower than the actual chemical energy contained within wastewater (Pham et al., 2006). Therefore, future sustainable strategies to reclaim energy from wastewater should not only reduce our dependence on fossil fuels but could also meet our demands of daily resources such as plastics and fertilizers.

In this Perspective, we introduce the concept of a “wastewater biorefinery column”, an approach for potentially leveraging the existing microbiological and biochemical knowledge of AS-based processes in a bottom-up design approach. This concept is based on the engineering of distinct ecological niches into future wastewater treatment processes, which may in turn allow the targeted enrichment of distinct organismal groups and the comprehensive recovery of energy and biotechnologically relevant molecules.

THE CURRENT MICROBIOLOGICAL AND BIOCHEMICAL KNOWLEDGE OF AS-BASED PROCESSES

Microbial communities of BWWTPs have for a long time been viewed as “black boxes”, as their structure and function have remained largely unknown. The majority of early studies on the microbiology of BWWTPs involved classical isolation techniques



(Prakasam and Dondero, 1967) and standard light microscopy for morphological identification of specific bacterial groups, e.g., filamentous bacteria (Eikelboom, 1975; **Figure 1A**). Using classical culture-dependent microbiological techniques, *Acinetobacter* spp. was implicated in phosphorus removal (Fuhs and Chen, 1975), *Nitrosospira* spp. was considered to be the key ammonium oxidizer (Prosser, 1989), *Nitrobacter* spp. (Henze et al., 1997) to be the dominating nitrite oxidizer and *Hypomicrobium* spp. to be a key denitrifier (Timmermans and Van Haute, 1983).

Intense research based on the retrieval of 16S rRNA gene sequences from BWWTPs in the last decades have ruled out the involvement of *Acinetobacter* spp. as a major player in phosphorus removal (Wagner et al., 1994; Kämpfer et al., 1996; Bond et al., 1995; Santos et al., 1999). So far, an uncultured and unclassified genus belonging to the *Beta-proteobacteria*, named *Candidatus Accumulibacter phosphatis* (CAP), has been shown to be a dominating phosphorus accumulating organism (PAO) in laboratory-scale reactors (Hesselmann et al., 1999; Crocetti et al., 2000) as well as in full-scale BWWTPs (Zilles et al., 2002; Wong et al., 2005). Moreover, subsequent molecular investigations have identified glycogen accumulating organisms (GAOs; Mino et al., 1995) as major competitors of CAP in anaerobic/aerobic sludge cycling. This includes a novel group belonging to *Gamma-proteobacteria* named as *Candidatus Competibacter phosphatis* (Crocetti et al.,

2002) and other groups belonging to *Alpha-proteobacteria* (Wong et al., 2004).

Regarding the cycling of nitrogen, new key players identified using molecular approaches included diverse populations of ammonium oxidizers (Juretschko et al., 1998; Purkhold et al., 2000; Daims et al., 2001), *Nitrosospira*-like microorganisms as dominant nitrite oxidizers (Juretschko et al., 1998; Daims et al., 2001; Dionisi et al., 2002; Maixner et al., 2006; Spieck et al., 2006) and denitrifiers belonging to the genera *Aquaspirillum*, *Azoarcus*, *Thauera*, and PAOs such as CAP (Kong et al., 2004; Hesselsoe et al., 2005; Thomsen et al., 2007). More recently, archaeal members of the *Thaumarchaeota* were also found to be capable of catalyzing the aerobic oxidation of ammonium (Park et al., 2006). The complexity of nitrogen cycling in BWWTPs was further brought to the forefront when distinct microorganisms having novel physiological properties, i.e., being able to carry out anaerobic ammonium oxidation (anammox) were identified (Mulder et al., 1995). These organisms belong to the phylum *Planctomycetes* (Strous et al., 1999) and have been putatively named “*Candidatus Kuenenia stuttgartiensis*” (Schmid et al., 2000).

Numerous investigations into the ecophysiology of microorganisms in BWWTPs were carried out using fluorescence *in situ* hybridization coupled with microautoradiography (MAR-FISH), which allowed direct visualization of specific microorganisms and

qualitatively link specific carbon (C), nitrogen (N), and phosphorus (P) substrate transformations (Nielsen and Nielsen, 2002; Wagner et al., 2006). MAR-FISH allowed for example the study of competition between PAOs and GAOs for carbon substrates and allowed the formulation of models (Oehmen et al., 2010). During anaerobic conditions, PAOs assimilate organic carbon substrates, e.g., volatile fatty acids and store them as polyhydroxyalkanoates (PHAs; Kong et al., 2004). When exposed to aerobic conditions, PAOs oxidize PHAs, which provides energy for polyphosphate accumulation, leading to P removal from the wastewater by biomass retrieval. GAOs compete with PAOs for volatile fatty acids under anaerobic conditions for PHA synthesis (Bengtsson et al., 2008) and do not accumulate polyphosphate under aerobic conditions but rather use energy for glycogen accumulation (Crocetti et al., 2002; Kong et al., 2006). Further information on the intra-cellular accumulation of PHA or polyphosphate indicating storage and/or cycling of these polymers was confirmed by combining MAR-FISH with staining methods (Crocetti et al., 2000; Wong et al., 2004). Other MAR-FISH studies demonstrated the physiological potential of lipid accumulating organisms (LAOs), such as *Candidatus* *Microthrix parvicella* to take up and store lipid substrates under anaerobic conditions and highlighted a competitive advantage of these filamentous bacteria compared to other organisms unable to assimilate these energy-rich substrates anaerobically (Andreasen and Nielsen, 1998; Nielsen et al., 2002).

Remarkably, advances in high-resolution molecular approaches, so called “meta-omics”, are now allowing concrete linkages to be drawn between microbial community compositions and functions. For example, metagenomic studies have unraveled some of the enigmatic microbial phenotypic traits by identifying candidate genes for ladderane biosynthesis and biological hydrazine metabolism in *Candidatus* *Kuenenia stuttgartiensis* (Strous et al., 2006). Full-scale BWWTP metagenomic studies have underlined the need for more reference genomes of key species (Albertsen et al., 2011), which can be used as a template to interpret *in situ* data as illustrated by recent laboratory studies (Kristiansen et al., 2013; McIlroy et al., 2013). In this context, metagenomic or single cell sequencing data may be fruitfully employed to infer media formulation and growth conditions for organisms of interest.

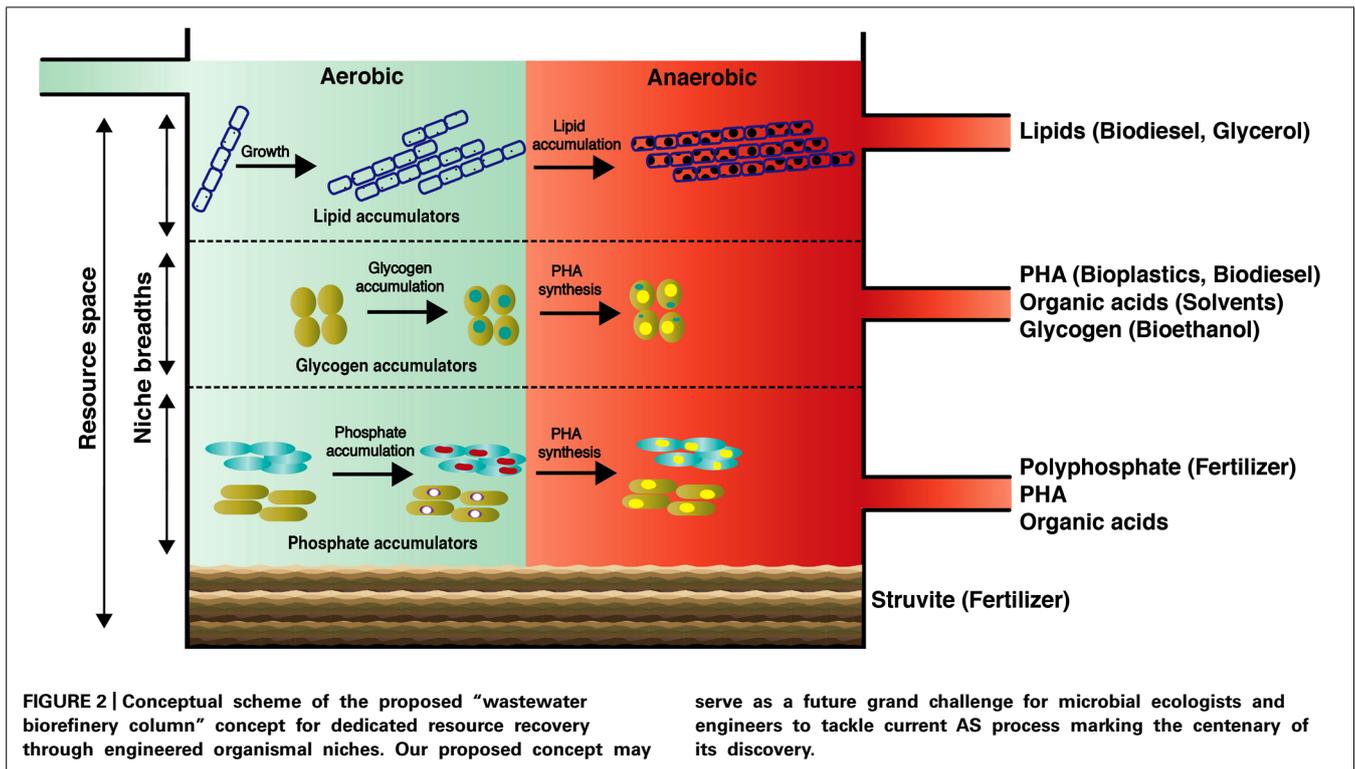
While metagenomic studies have greatly contributed to the clarification of the functional capabilities of specific populations of interest, metaproteomics (Wilmes et al., 2008), metatranscriptomics (Yu and Zhang, 2012), and the combination of these two approaches (Haroony et al., 2013) have furthered our knowledge about the actual expression of relevant genes involved in key processes under different environmental conditions such as alternating anaerobic/aerobic phases. Thus, integrated “omics” over space and time (Muller et al., 2013) in combination with recorded physico-chemical parameters will allow reconstruction of the ecological networks and detailed definition of organismal niches (Figure 1B). Such knowledge may then be used to identify key determinants of overall microbial community structure and function, which in turn may be harnessed for comprehensive reclamation of energy and biotechnologically relevant products from wastewater.

INTO THE FUTURE: USING THE INTELLIGENCE OF WASTEWATER MICROBES TO RECOVER THE HIDDEN TREASURE

Given the numerous organismal groups which have now been identified, as well as the short-term prospect of additional unprecedented data from multi-omic analyses, the time is ripe at the centenary of the AS-process to think about formulation of new biological wastewater treatment processes. In particular, we can start to consider bottom-up design approaches rather than the top-down approaches promulgated thus far.

Here, we introduce the concept of a “wastewater biorefinery column” (Figure 2), based on a hypothetical bottom-up design approach, which takes into account the detailed knowledge of how specific microorganisms behave *in situ* and accumulate different storage compounds of interest over changing BWWTPs environmental conditions. Consequently, the engineering of specific niches would allow the harvest of high-value resources from wastewater. Such niche engineering may be achieved for example by (1) establishing distinct substrate gradients within the entire resource space of wastewater thereby exploiting the individual organismal niche breadths; (2) manipulation of the vertical distribution of key microorganisms by exploiting their respective settling velocities which in turn may be influenced by microbial/floc size and intracellular storage compounds; (3) harvesting dominant members for energy and/or nutrient reclamation. In laboratory-scale reactors, pronounced organismal enrichments have already been obtained for organisms of interest, in particular PAOs and GAOs (Winkler et al., 2011a), highlighting the feasibility of enriching these organisms by providing them with appropriate environmental conditions. With the advent of advanced meta-omic approaches, we will be able to define the niches of the respective organismal groups much more precisely, thereby allowing these to be engineering into future systems. Given the heterogeneous and dynamic composition of wastewater, niches may have to be continually adjusted. Such niche fine-tuning could be based on feedback from microbial fuel cells acting as biosensors (Di Lorenzo et al., 2009), which would allow the continuous monitoring of organic and inorganic composition of influents.

Among the renewable sources of energy currently being explored, biodiesel holds significant promise as a potential alternative to partially replace petroleum-based fuels. At present, up to 85% of the overall biodiesel production cost is associated with the feedstocks (Knothe et al., 2005; Mondala et al., 2009). Consequently, biomolecules from wastewater of immediate bioenergy interest are saponifiable lipids since they can be easily transformed into biodiesel (Fukuda et al., 2001; Chisti, 2007; Mondala et al., 2009). In municipal wastewater, lipids can represent up to 41% of the total organic pools (Raunkjær et al., 1994), with a vast majority being triacylglycerides (TAGs) and a minor part free long chain fatty acids (Quemeneur and Marty, 1994). Importantly, the long chain fatty acids comprising TAGs within wastewater sludge are predominantly in the range of C₁₄–C₁₈ which are ideal for the production of methyl esters (Dufreche et al., 2007; Mondala et al., 2009). Due to their hydrophobicity, wastewater lipids are usually sorbed onto particles and not readily extractable (Dueholm et al., 2000). However, LAOs excrete extracellular lipases, which allow hydrolysis of lipids and efficient bacterial assimilation. Following



our “wastewater biorefinery column” concept, the buildup of filamentous LAO biomass could be favored at the top of the column (Figure 2). Given the high levels of lipid accumulation by filamentous organisms and straightforward production of biodiesel from this lipid-rich biomass, a large potential exists in wastewater biodiesel production as its synthesis is economically viable (Knothe et al., 2005). Interestingly, a byproduct of TAG-derived biodiesel production is glycerol, which can be further used to produce PHA (Mothes et al., 2007), thereby leading to a complete and high-value valorization of the wastewater TAG fraction.

PHA synthesis naturally occurs within the microbial biomass of AS. Wastewater-derived PHAs are currently being used to synthesize biodegradable bioplastics on an industrial scale (Chen, 2009; Morgan-Sagastume et al., 2010) and they exhibit similar thermomechanical properties compared to chemically synthesized polypropylenes (Curran, 1996). Additionally, PHAs can be chemically transformed into the biofuel hydroxybutyrate methyl ester by acid catalyzed hydrolysis (Zhang et al., 2009). Therefore, wastewater-derived PHAs represent a suitable renewable resource for plastic production, as resource expenses in the entire PHA production chain can account up to 50% of the total production costs (Choi and Lee, 1999). The microbial accumulation of PHAs from wastewater can be very rapid (ca. 5 h) and pronounced (PHAs can constitute up to 77% of cell dry weight (Jiang et al., 2012)). Both PAOs and GAOs are known to accumulate PHAs under alternating anaerobic/aerobic conditions. Given tailored environmental conditions, targeted enrichment of these organisms would therefore allow reclamation of PHA and fermentation products along with polyphosphate or glycogen (see below). PAOs in full-scale BWWTPs can contribute up to 35% of the total bacterial biomass

(Oehmen et al., 2010) and laboratory reactor studies suggest that PAOs exhibit fast settling rates when compared to GAOs because of their differing cell densities (Vlaeminck et al., 2010; Winkler et al., 2011b; Volcke et al., 2012). These properties could be harnessed for separating both organismal groups and carrying out targeted resource reclamation (Figure 2). Through the establishment of density gradients, e.g., through manipulation of settling times, it could therefore be envisaged that GAOs could occupy the middle layers followed by PAOs in the lower section of the “wastewater biorefinery column” (Figure 2).

Although poorly studied in AS-based BWWTPs, fermentative bacteria contribute to the hydrolysis of complex organic macromolecules into low-molecular-weight substrates providing energy and carbon sources to other microbes. Produced molecules include industrially relevant commodities such as propionic acid, lactic acid, acetic acid, and formic acid (Kong et al., 2008). Interestingly, the production of alcohols and/or organic acids using fermentative treatment phases could be combined with biodiesel and bioplastic production since both processes require organic solvents. Most importantly, harvesting glycogen from GAO biomass could also be used for bioethanol production, an important biofuel (Hahn-Hägerdal et al., 2006).

Renewable fertilizer production using nutrient recovery from wastewater (primarily N and P) could cover up to 30% of the current agricultural fertilizer demand (Verstraete et al., 2009). A global estimate suggests that fertilizer production consumes up to 1.2% of the world’s energy (out of which 92.5% for N and 3% for P), contributing about 1.2% of the total anthropogenic greenhouse gas emissions (Kongshaug, 1998). In particular, PAO-rich biomass holds great promise as fertilizer. A major limitation of

its current use as an agricultural fertilizer is that the biomass is typically rich in heavy metals (Veeken and Hamelers, 1999). However, there is increasing evidence that wastewater derived adsorbents (e.g., blast furnace slag) possesses high heavy metal adsorption capacities and offers a low-cost alternative treatment of metal-contaminated wastewater (Kurniawan et al., 2006) prior to further processing of the wastewater.

Apart from PAO-enriched biomass, struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$), a commonly occurring mineral found as precipitates in BWWTPs (Rawn et al., 1939) is a commercially produced fertilizer (Mavinic et al., 2007). Estimates suggest that within BWWTPs, 100 m³ of wastewater can yield up to 1 kg of struvite (Shu et al., 2006). The low solubility and the presence of high abundances of N and P in struvite is advantageous as a fertilizer since it reduces nutrient run-off in turn limiting eutrophication in receiving water bodies (Shu et al., 2006; El Diwani et al., 2007). Presence of low concentrations of suspended solids and high concentrations of ammonium and phosphate enhances struvite production (Doyle and Parsons, 2002). Thus, accelerating struvite production by retaining high levels of these ions in the wastewater while continuously reclaiming biomass fractions, e.g., from fast-growing populations could be another aim to be achieved in the “wastewater biorefinery column.”

In the context of N recovery for fertilizer production, nitrate accumulating organisms which are known to occur in varied habitats (McHatton et al., 1996; Schulz et al., 1999) may also be of pronounced interest. Although nitrate accumulators in AS-processes have so far not yet been described, they could represent a significant N source for fertilizer production. A selective enrichment strategy (Spieck et al., 2006) may provide a plausible approach to identify potential nitrate accumulators within full-scale AS-based BWWTPs.

In the past few decades, increasing environmental concerns have triggered the formulation and development of new strategies for energy and nutrient recovery from wastewater through AS-based processes (Verstraete et al., 2009). However, the yields in terms of recovery of either energy and/or biotechnological resources have so far been limited. Our proposed concept of a “wastewater biorefinery column” would use the existing and future wealth of information concerning the genetic repertoire of microorganisms and their metabolic transformations for sustainable production of bioenergy, bioplastics and fertilizers. However, in order to have this come to fruition, it is essential that we first obtain detailed descriptions of the niches of the individual community members. Once such knowledge has been obtained, wastewater treatment processes should be (re-)engineered taking into account the individual organismal niches using bottom-up design approaches rather the top-down strategies pursued so far. The optimization of processes may involve an iterative bottom-up design approach based on a discovery-driven planning approach (Muller et al., 2013) which would involve systematic omic analyses based on which ecological niches may be fine-tuned and, thus, the process continually tweaked to guarantee optimal resource recovery. We still have a long way to go to bring this vision to fruition but it may represent a grand challenge for microbial ecologists and engineers to tackle at the centenary of the discovery of the AS process.

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