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Genomics update

Genomics of biological wastewater treatment

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Cleaning up wastewater from domestic sewage and industrial processes is essential for safeguarding public health and the environment (for an overview of wastewater treatment processes see http://en.wikipedia.org/wiki/Sewage_treatment). Biological wastewater treatment plants (WWTPs) employing sludge represent one of the most widely used biotechnological processes, with more than 120 billion litres treated daily in the USA alone (Fig. 1). Removal of organic carbon and other nutrients, mainly nitrogen (N) and phosphorus (P), by sludge microbes is essential in order to avoid eutrophication and deterioration of recipient surface waters (Seviour *et al.*, 2003). The complexity of wastewater microbial communities, based on the analysis of 16S rRNA sequences, is known to be enormous (Chouari *et al.*, 2005a,b) (Fig. 2). As we have very limited knowledge of composition, dynamics and stability of microbial communities, several processes in wastewater treatment are generally regarded as being a 'black box'. Their improvement is often based on a trial-and-error approach, which leads to inconsistent results and unexpected behaviours (Daims *et al.*, 2006a). In recent years, with the development of several new high-throughput sequencing platforms (Hall, 2007; Marsh, 2007), metagenome sequencing strategies (Schloss and Handelsman, 2005; Tringe and Rubin, 2005; Schmeisser *et al.*, 2007) and bioinformatics toolboxes (Chen and Pachter, 2005; Tringe *et al.*, 2005; Foerstner *et al.*, 2006; Raes *et al.*, 2007), the genome analysis of complex communities has become much more accessible, albeit by no means easier. Here we give a brief update of the current status of (meta)genome sequencing and mining of wastewater-related microorganisms and communities.

Trends in wastewater genomics

For decades, the putative key players in wastewater treatment processes were those that were culturable and well studied, while the really important species remained non-culturable or unknown (Crocetti *et al.*, 2000; Chouari *et al.*, 2005a). A variety of genomes of cultured microbes have been sequenced in the past 5 years, but now the focus is shifting towards culture-independent metagenome sequencing. Table 1, extracted from the GOLD

Fig. 1. Wastewater treatment plant near Arnhem, the Netherlands (photo M. Galardini).

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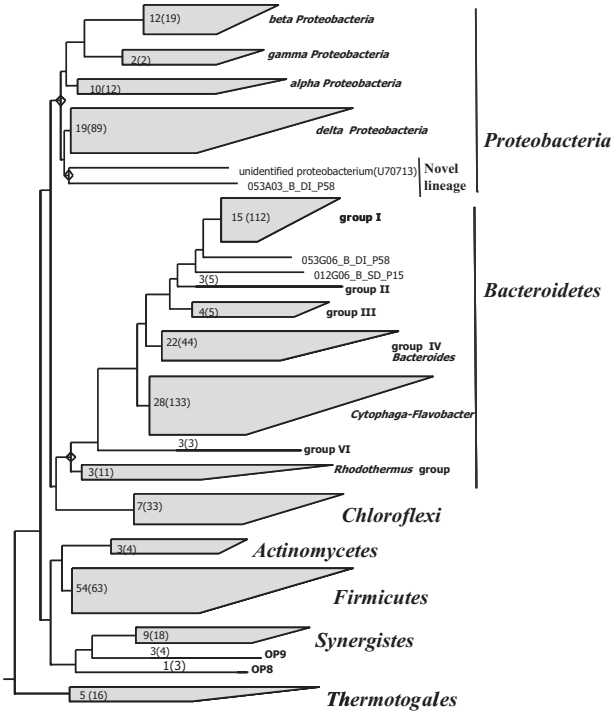

Fig. 2. Bacterial diversity analysis in an anaerobic sludge digester. Neighbour-joining tree of bacterial 16S rRNA sequences showing different operational taxonomic units (OTUs). Number of OTUs is indicated on the branches, and total number of clones is indicated in brackets. Out-group is not shown. Adapted with permission from Chouari and colleagues (2005a). Copyright 2005, Society for Applied Microbiology and Wiley-Blackwell Publishers.

database (Liolios *et al*., 2008), gives an overview of completed and ongoing sequencing projects, divided into relevant steps in wastewater treatment. Clearly the focus is still on bacteria, with virtually no information on archaea and eukarya, even though they are known to play a role in wastewater processes (Liu *et al*., 2008; Rehman *et al*., 2008). In many cases, wastewater microbes have first been enriched in laboratory bioreactors prior to metagenome sequencing (Strous *et al*., 2006; Maixner *et al*., 2008; Pelletier *et al*., 2008). In addition, metagenome sequences have provided a first insight into metabolic requirements of specific microbes, allowing further optimization of enrichment conditions (Garcia Martin *et al*., 2006). 16S rRNA sequences from metagenome libraries have been used to identify new bacterial divisions and species (Guermazi *et al*., 2008; Pelletier *et al*., 2008) and to visualize them using molecular techniques such as FISH (Roh *et al*., 2008; Yoon *et al*., 2008). Some recent breakthroughs are highlighted in more detail below.

Anaerobic digestion: *Candidatus Cloacamonas acidaminovorans*

Metagenomics projects involve not only species that are predominant in the wastewater treatment, but also minor members of the population. For instance, a new bacterial division (WWE1) belonging to the *Spirochaetes*, with no cultured representative, has been characterized (Chouari *et al*., 2005b). Members of this division are present in anaerobic digesters, generally as a subdominant group (< 10% of the population), where they perform the decomposition of organic matter into methane and CO_2. Starting from a metagenomic analysis of sludge microbes from such a digester, Pelletier and colleagues (2008) succeeded in reconstructing the complete genome of a WWE1 bacterium, represented by only 2% of the fosmid library reads, using a novel sequencing and assembly strategy specifically designed during this project. The reconstruction started with seed sequences (fully sequenced fosmids on which WWE1 16S rRNA sequences were found), to which > 99% identical fosmid paired-end sequences from the metagenome library were added in an iterative fashion (Fig. 3). A circular genome of 2.2 Mb was then obtained by standard finishing techniques; its reconstruction was probably made possible by the extremely low sequence polymorphism of the dominant phylotype (Pelletier *et al*., 2008). This is the first time that such an iterative procedure had been used, and the first time ever a 'complete' genome from a previously unknown, uncultured and subdominant bacterium was reconstructed out of such

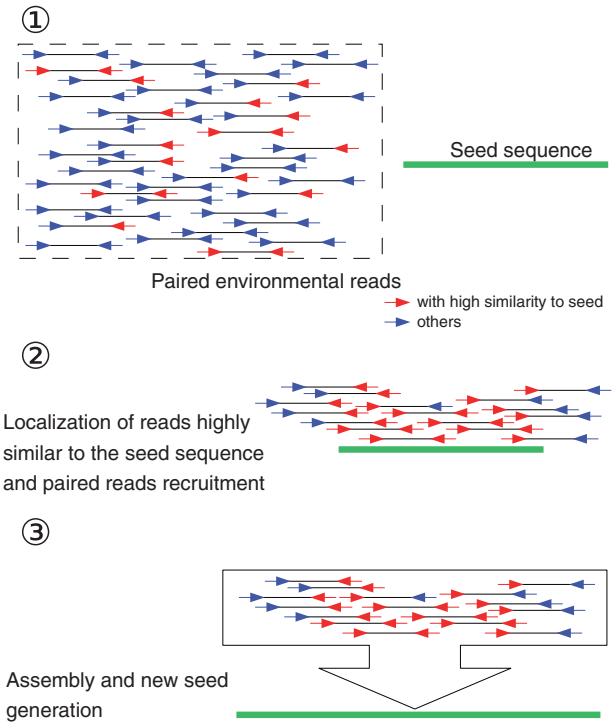

Fig. 3. Iterative assembly process of fosmid paired-end sequences used for reconstruction of the complete genome of *Candidatus* Cloacamonas acidaminovorans. Reprinted with permission from Pelletier and colleagues (2008); for details see their paper. Copyright 2008, American Society for Microbiology.

Table 1. Selected wastewater-relevant microbial genome sequencing projects. Adapted from the GOLD Database v2.0 (http://www.genomesonline.org).

Phylum	Organism(s)	Type	Sequencing	Habitat/isolation	Oxygen	Source
Anaerobic digestion						
Unclassified	*Candidatus Cloacamonas acidaminovorans*	Metagenome	Complete	Sludge, wastewater	Anaerobe	Pelletier *et al.* (2008)
Proteobacteria	*Rubrivivax gelatinosus* IL144	Genome	Incomplete	Aquatic, sludge	Facultative	NITE
Firmicutes	*Aminomonas paucivorans* GLU-3	Genome	Incomplete	Aquatic, wastewater	Anaerobe	DSMZ, JGI
Firmicutes	*Aminobacterium colombiense* ALA-1	Genome	Incomplete	Aquatic, wastewater	Anaerobe	DSMZ, JGI
Anaerobic digestion and hydrogen production						
Firmicutes	*Ethanoligenens harbinense* YUAN-3T	Genome	Incomplete	Sludge	Anaerobe	DSMZ, JGI
Phosphorus removal						
Proteobacteria	*Candidatus Accumulibacter phosphatis*	Metagenome	Complete	Sludge	Facultative	Garcia Martin *et al.* (2006)
Gemmatimonadetes	*Gemmatimonas aurantiaca* T-27T	Genome	Complete	Wastewater	Aerobe	NITE
Unclassified	Wastewater EBPR community	Metagenome	Incomplete	Sludge	Facultative	JGI
Actinobacteria	*Microlunatus phosphovorus* NM-1	Genome	Incomplete	Sludge	Aerobe	NITE
Anaerobic ammonia oxidation (anammox)						
Planctomycetes	*Candidatus Kuenenia stuttgartiensis*	Metagenome	Incomplete	Wastewater	Anaerobe	Strous *et al.* (2006)
Planctomycetes	*Brocadia anammoxidans*	Genome	Incomplete	Aquatic	Anaerobe	University of Nijmegen, NL
Planctomycetes	*Gemmata obscuriglobus* UQM 2246	Genome	Draft	Aquatic	Aerobe	JCVI
Unclassified	Anaerobic ammonium-oxidizing community	Metagenome	Incomplete	Aquatic	Anaerobe	JGI
Ammonia oxidation						
Proteobacteria	*Nitrosomonas europaea* IFO	Genome	Complete	Soil, aquatic	Aerobe	Chain *et al.* (2003)
Proteobacteria	*Nitrosomonas eutropha* C91 (C71)	Genome	Complete	Aquatic	Aerobe	Stein *et al.* (2007)
Proteobacteria	*Nitrosomonas oligotropha* Nm45	Genome	Incomplete	Soil	Aerobe	JGI
Proteobacteria	*Nitrosomonas* sp. 17	Genome	Incomplete	Marine	Aerobe	JCVI
Nitrite oxidation						
Proteobacteria	*Nitrobacter hamburgensis* X14	Genome	Complete	Soil	Aerobe	Starkenburg *et al.* (2008)
Proteobacteria	*Nitrobacter winogradskyi* Nb-255	Genome	Complete	Soil	Facultative	Starkenburg *et al.* (2006)
Nitrospirae	*Nitrospira marina* Nb-295	Genome	Incomplete	Aquatic	Facultative	JCVI
Nitrospirae	*Candidatus Nitrospira defluvii*	Genome	Incomplete	Aquatic	Facultative	Genoscope, France
Proteobacteria	*Nitrospina gracilis* Nb-211	Genome	Incomplete	Aquatic	Facultative	JCVI
Denitrification						
Proteobacteria	*Paracoccus denitrificans* PD1222	Genome	Complete	Soil	Aerobe	JGI
Proteobacteria	*Delftia acidovorans* SPH-1	Genome	Complete	Aquatic, sludge, soil	Aerobe	JGI
Proteobacteria	*Thiobacillus denitrificans* ATCC 25259	Genome	Complete	Soil	Facultative	Beller *et al.* (2006)
Biofilm formation and flocculation						
Proteobacteria	*Acidovorax temperans*	Genome	Incomplete	Sludge	Aerobe	University of Auckland, NZ
Bulking						
Chloroflexi	*Herpetosiphon aurantiacus* ATCC 23779	Genome	Complete	Aquatic	Aerobe	JGI
Chloroflexi	*Anaerolinea thermophila* UNI-1	Genome	Incomplete	Sludge	Anaerobe	NITE

NITE, National Institute of Technology and Evaluation, Japan; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; JGI, Joint Genome Institute, USA; NL, the Netherlands; JCVI, J. Craig Venter Institute, USA; NZ, New Zealand.

a complex environment. Their procedure clearly shows promise for application to other wastewater-sequencing projects as well as to other environmental samples.

Analysis of the *in silico* proteome revealed that the primary energy source for this bacterium is derived from the fermentation of amino acids and thus the provisional name '*Candidatus* Cloacamonas acidaminovorans' was coined. Survival under minimal oxygen concentrations appears to be due to the presence of various enzymes that give protection against oxidative stress. Moreover, predictions about the consortium composition was made knowing that *C. acidaminovorans* can generate H_2 and CO_2 from the oxidation of propionate, because this pathway is possible only when H_2-scavenging bacteria are present. An attempt to isolate this bacterium was made starting from the predicted nutrient needs and adding the putative syntrophic partners: unfortunately the attempt failed, probably because the real syntrophic bacteria are also non-culturable. Although it appears that this bacterial division is widespread in many anaerobic digesters, the importance of this species for the function of the digester is not clear yet.

Enhanced biological phosphorus removal (EBPR)

Phosphorus removal is one of the key steps in wastewater treatment. Low inorganic phosphate (P_i) levels in treated water do not allow algal growth and thereby prevent eutrophication of water systems. The possibility to use so-called PAOs (polyphosphate-accumulating organisms) instead of more expensive chemical treatments is a strong incentive to improve our understanding of their physiology. WWTPs for enhanced biological phosphorus removal (EBPR) have an anaerobic treatment phase that precedes an aerobic phase, and selects for bacteria which accumulate large amounts of polyphosphate (Blackall *et al*., 2002). The consensus model of EBPR assumes that in the aerobic stage P_i is removed from wastewater by uptake and storage in PAOs as polyphosphate. In the anaerobic stage, the stored polyphosphate is consumed to provide energy for uptake and transformation of volatile fatty acids (VHAs, mostly acetate and propionate) into polyhydroxyalkanoates (PHAs). From a macroscopic point of view, the objective is to let the PAOs accumulate the phosphate inside their cells and then remove the bacteria by settling in a clarifier.

For a long time, culture-dependent analyses have pointed at members of the genus *Acinetobacter* as the key organism for the EBPR process (Deinema *et al*., 1985; Streichan *et al*., 1990). In recent years, new molecular techniques had revealed that in fact the most important member of the PAO community, called '*Candidatus* Accumulibacter phosphatis', in EBPR reactors belongs to the order *Rhodocyclales* which has no cultured representatives (Hesselmann *et al*., 1999; Crocetti *et al*., 2000). Now, this organism has been highly enriched (60–80% of biomass) in two independent lab-scale reactors seeded with sludge from EBPR plants in the USA and Australia (Garcia Martin *et al*., 2006). Metagenome analysis of these two samples allowed assembly of a nearly complete composite genome of *A. phosphatis*, and indicated that the dominant strains in both reactors are closely related (> 95% identity at nucleotide level) (Garcia Martin *et al*., 2006). A comprehensive metabolic reconstruction was performed, and the EBPR-relevant anaerobic metabolism inferred from the genome is shown in Fig. 4. Moreover, gene sets for N fixation, CO_2 fixation and high-affinity P_i transporters were identified, suggesting that *A. phosphatis* is also well adapted to nutrient-limited habitats such as freshwater. Cobalt dependence was inferred from the presence of cobalamin biosynthesis genes, allowing an enrichment of this species by growing in an N-free, cobalt-rich medium. Finally, a gene-centric analysis of all metagenome sequences obtained from the wastewater plant sample revealed an over-representation of genes related to the EBPR process (e.g. phosphate transporters, cobalt transporters and VFA-handling genes).

Metaproteomics studies of sludge microbes from a similar EBPR reactor in the UK confirmed the activities of these EBPR processes, but also highlighted the importance of fatty acid cycling, denitrification and the glyoxylate bypass in EBPR (Wilmes *et al*., 2008a,b). Many proteins were identified with the help of the template composite *A. phosphatis* genome (Garcia Martin *et al*., 2006), but numerous protein variants were also found, which presumably came from related species and strains, highlighting the likely importance of genetic variation for overall community homeostasis. These insights should provide leads to optimize consortia and maintain stable performance of EBPR systems.

Nitrogen removal

Nitrogen elimination from wastewater involves three main processes: (i) nitrification, the oxidation of ammonia (NH_3) via nitrite (NO_2^-) to nitrate (NO_3^-), (ii) denitrification, the reduction of nitrate to nitrogen gas (N_2), and (iii) anaerobic ammonium oxidation (anammox), the direct combination of ammonia and nitrite into N_2. Nitrogen gas is released to the air and thus removed from the wastewater.

Metagenome sequencing was used to decipher the 4.2 Mb genome of the uncultured anammox bacterium *Kuenenia stuttgartiensis*; this bacterium dominated the complex community in a laboratory bioreactor 1 year after inoculation with wastewater sludge (Strous *et al*., 2006). More than 200 genes relevant to anammox catabolism and respiration were detected in the genome, and con-

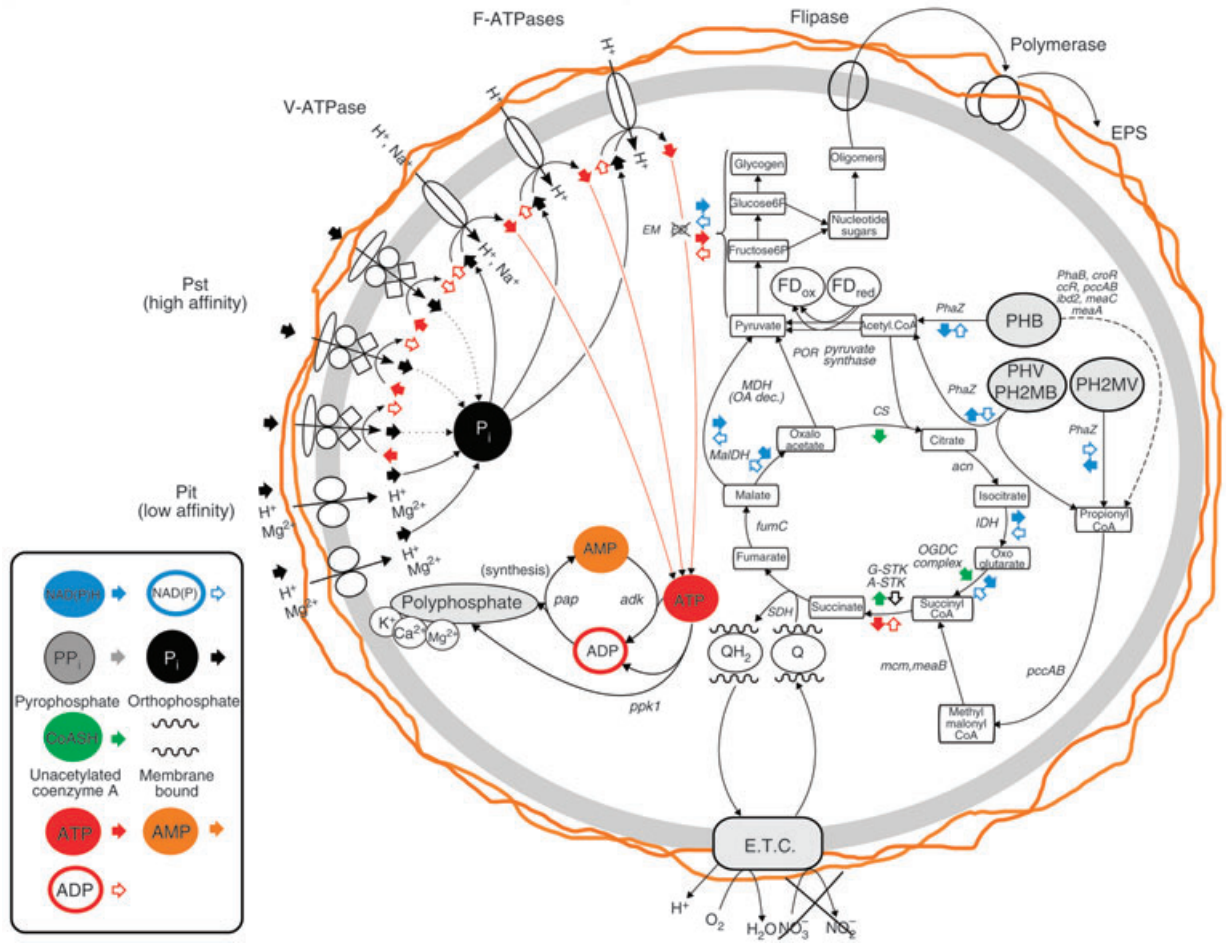

Fig. 4. EBPR-relevant aerobic metabolism inferred from the *Accumulibacter phosphatis* genome. In the aerobic phase, the lack of acetate prevents growth of other species, while the PHA reserves ensure its dominance in the reactor microbial ecosystem. The restoration of polyphosphate reserves via ATP depletes the water of P_i, thus giving rise to EBPR. Reprinted with permission from Garcia Martin and colleagues (2006). Copyright 2006, Macmillan Publishers.

firmed earlier hypotheses that hydrazine (N_2H_4) and hydroxylamine (NH_2OH) are intermediates in anammox catabolism. Now the question is how to culture this bacterium more rapidly, as controllable anammox is a highly desirable process in N removal. Analysis of the genome for nutrient requirements may provide the answers for overcoming their slow grow rate.

Nitrification is a two-step process, catalysed sequentially by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Nitrifying bacterial communities in flocs and biofilms in WWTPs are extremely complex and elucidation of their composition and dynamics offers unique opportunities for studying interactions, competition and niche differentiation (Juretschko *et al.*, 1998; Daims *et al.*, 2006a,b; Maixner *et al.*, 2006; Moussa *et al.*, 2006) (Fig. 5). A few genome sequences of some culturable members are available now (Table 1). The ammonia-oxidizing *Nitrosomonas eutropha* was found to have many genes for heavy metal and drug resistance, which could explain its presence in a polluted environment such as a WWTP (Stein *et al.*, 2007). Members of the genus *Nitrosomonas* and *Nitrobacter* were long believed to be the main nitrifiers in wastewater treatment, but culture-independent techniques have now revealed a large diversity of yet uncultured and poorly characterized AOB and NOB, and suggest that the more important members may in fact be *Nitrosococcus* and *Nitrospira* (Juretschko *et al.*, 1998; Moussa *et al.*, 2006). An environmental metagenomics approach was used to sequence, identify and assemble a 137 kb genome fragment of '*Candidatus* Nitrospira defluvii', which had been enriched from activated sludge from a domestic WWTP (Maixner *et al.*, 2008). This contig, harbouring a 16S rRNA gene of *N. defluvii*, was surprisingly found to encode a chlorite dismutase, which converts chlorite (ClO_2^-) to chloride (Cl^-) and O_2. Enzymatic tests then

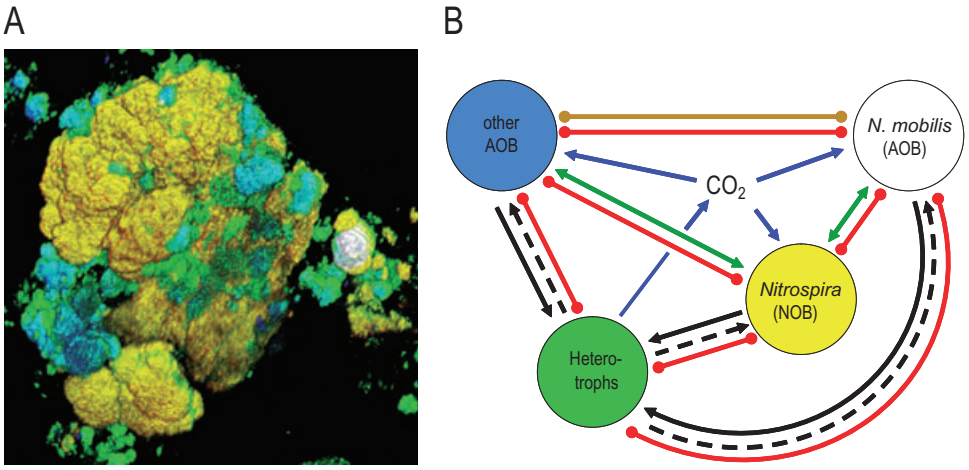

Fig. 5. Diversity and interactions in the nitrifying biofilm of a pilot-scale, sequencing batch biofilm reactor.
A. Confocal micrograph showing co-aggregated nitrifying and other bacteria, detected by FISH with 16S rRNA-targeted oligonucleotide probes; different groups of bacteria are labelled with different colours.
B. Simplification of interactions among different nitrifiers and heterotrophic bacteria in the biofilm. *N. mobilis*, *Nitrosococcus mobilis*.
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confirmed that this highly active chlorite dismutase is expressed *in situ* in *Nitrospira*, indicating that this nitrite oxidizer may be involved in bioremediation of perchlorate (ClO_4^-) and chlorite. Survival of this species in a nitrifying WWTP may depend on this somewhat unique activity, as (per)chlorate-containing fertilizers, disinfectants and bleaching agents are all discharged into the environment. Furthermore, hypochlorite (ClO^-) is often added to activated sludge to reduce the outgrowth of filamentous bacteria (Seka *et al.*, 2003).

Understanding biofilm formation and flocculation

Good settling properties of activated sludge are important for the separation of sludge and treated wastewater. Setting problems, such as bulking and foaming, are often caused by excessive growth of filamentous bacteria (Martins *et al.*, 2004). When the formation of so-called 'flocs', generally regarded as the centre of the desired metabolic reactions, is not controlled, this can result in excessive foaming and problems in oxygenation and settling. To better understand cell–cell interactions in wastewater treatment, the 4.2 Mb genome has been sequenced of the low-abundant bacterium *Acidovorax temperans* strain CB2, a model organism for biofilm and floc formation (Turner *et al.*, 2008). *Acidovorax temperans* is present in the wastewater environment in microcolonies or as single cells. It exhibits two distinct morphological colony types with different properties in terms of biofilm and floc formation; for instance, it would appear that biofilm formation leads to poor settling ability. Data mining of the genome suggests that cell–cell interactions in biofilm formation are mediated by numerous factors, including nutritional signals, extracellular DNA and cell structures such as pili and exopolysaccharides.

Future focus

Clean water is one of the most important aspects of life. Its availability is sometimes taken for granted, but when rivers and lakes turn eutrophic action is required. In our 'developing world' we need to focus on avoiding these problems. Opening the black box of biological wastewater treatment is not the daunting experience that is once was. The viable but non-culturable is no longer the non-sequencable, non-characterizable enigma that existed at one time. Metagenomics is leading the way for more specific studies in related fields. New microbial species can be named and classified, while associations, dependencies and hierarchies can be described. New intriguing enzymes and metabolic pathways will be discovered, and interactions between consortium members will be unravelled. Many bacterial and archaeal divisions will be studied in greater detail, and it is expected that many of them will become culturable, allowing a better use in the construction of bioreactors specific for different types of wastewater treatment. This new knowledge may someday lead to starter cultures for wastewater treatment in the same manner as yogurt and cheese starter cultures for the dairy industry. And finally, genomic studies of wastewater treatment microbes, apart from their biotechnological applications, are also an excellent test field for a variety of other burning ecological and environmental questions.

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