RESEARCH ARTICLE

Substrate-dependent denitrification of abundant probe-defined denitrifying bacteria in activated sludge

Fernando Morgan-Sagastume, Jeppe Lund Nielsen & Per Halkjær Nielsen

Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Aalborg, Denmark

Correspondence: Per Halkjær Nielsen, Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 49, DK-9000 Aalborg, Denmark. Tel.: +45 99408503; fax: +45 9814 1808; e-mail: phn@bio.aau.dk

Present address: Fernando Morgan-Sagastume, AnoxKaldnes AB, Klosterängsvägen 11A, 22647 Lund, Sweden.

Received 17 December 2007; revised 26 March 2008; accepted 9 July 2008.
First published online 22 September 2008.

DOI:10.1111/j.1574-6941.2008.00571.x

Editor: Michael Wagner

Keywords
denitrification rates; denitrifying bacteria; activated sludge FISH; microautoradiography.

Abstract

The denitrification capacity of different phylogenetic bacterial groups was investigated on addition of different substrates in activated sludge from two nutrient-removal plants. Nitrate/nitrite consumption rates (CRs) were calculated from nitrate and nitrite biosensor, *in situ* measurements. The nitrate/nitrite CRs depended on the substrate added, and acetate alone or combined with other substrates yielded the highest rates (3–6 mg N $gVSS^{-1}$ h^{-1}). The nitrate CRs were similar to the nitrite CRs for most substrates tested. The structure of the active denitrifying population was investigated using heterotrophic CO_2 microautoradiography ($HetCO_2$-MAR) and FISH. Probe-defined denitrifiers appeared as specialized substrate utilizers despite acetate being preferentially used by most of them. *Azoarcus* and *Accumulibacter* abundance in the two different sludges was related to differences in their substrate-specific nitrate/nitrite CRs. *Aquaspirillum*-related bacteria were the most abundant potential denitrifiers (*c.* 20% of biovolume); however, *Accumulibacter* (3–7%) and *Azoarcus* (2–13%) may have primarily driven denitrification by utilizing pyruvate, ethanol, and acetate. Activated sludge denitrification was potentially conducted by a diverse, versatile population including not only *Betaproteobacteria* (*Aquaspirillum*, *Thauera*, *Accumulibacter*, and *Azoarcus*) but also some *Alphaproteobacteria* and *Gammaproteobacteria*, as indicated by the assimilation of $^{14}CO_2$ by these probe-defined groups with a complex substrate mixture as an electron donor and nitrite as an electron acceptor in $HetCO_2$-MAR–FISH tests.

Introduction

An efficient removal of nitrate by denitrification in nutrient-removal wastewater treatment plants is often hampered by the lack of suitable organic substrates in the incoming wastewater (Henze *et al.*, 1994; Hallin & Pell, 1998). Therefore, single or complex substrates, such as acetate, methanol, glycols, and sludge hydrolysate, may be added as external carbon sources. Activated sludge from different full-scale municipal plants has commonly yielded different denitrification rates upon addition of the same substrate (Gerber *et al.*, 1987; Hagman, 2003). For instance, a wide range of nitrate consumption rates (CRs) (3–7 mg N per gram volatile suspended solids per hour or mg N $gVSS^{-1}$ h^{-1}) upon acetate addition were measured in seven different European municipal activated sludge samples (Naidoo *et al.*, 1998). Similarly, different oxygen uptake rates (OUR) have been reported for the same carbon source in different activated sludge plants (Dircks *et al.*, 1999; Benes *et al.*, 2002; Hagman, 2003).

An obvious explanation for this difference is that the number of oxygen utilizers/denitrifiers varies among different treatment plants, but variations in the relative abundance of different species with specific substrate utilization capacity may also explain these differences in rates. In methanol- and ethanol-adapted activated sludge, differences in the denitrifying populations have been proposed to explain differences in denitrification rates (Hallin & Pell, 1998; Hallin *et al.*, 2006). The community structure of denitrifiers is affected by external carbon sources, as shown by the enrichment of diverse denitrifying bacteria with methanol and acetate (Ginige *et al.*, 2004, 2005; Hallin *et al.*, 2006; Osaka *et al.*, 2006). Nevertheless, in all such studies on the effect of different substrates on denitrification

in full-scale plants, the denitrifiers have not been identified or have been identified only tentatively based on culture-dependent techniques and phenotypic fingerprinting (Lee & Welander, 1996).

Only recently, have the identity and presence of denitrifying bacteria in nutrient-removing activated sludge plants been better described. In several municipal activated sludge plants operated for carbon removal, nitrification, and denitrification, *Betaproteobacteria* belonging to the genus *Aquaspirillum* were suspected to be the dominant denitrifiers (Thomsen *et al.*, 2004), comprising from 10% to 30% of the total bacteria (Thomsen *et al.*, 2007). Other denitrifying *Betaproteobacteria* from the genera *Thauera* and *Azoarcus* constituted from 1% to 12% and 1% to 6% of the total bacteria, respectively (Thomsen *et al.*, 2007). The proposed denitrifying capacity of polyphosphate-accumulating organisms (PAO) (Dold & Barker, 1996; Zeng *et al.*, 2003) has now been evidenced for *Accumulibacter* PAO, which constitute from 5% to 22% of the total bacteria in municipal and industrial activated sludge with biological P removal (Kong *et al.*, 2004). *Zoogloea* along with *Azoarcus* and *Thauera* are abundant and are believed to be the dominant denitrifiers in some industrial treatment plants (Juretschko *et al.*, 2002). *Betaproteobacteria* from the order of *Methylophilales* and from the genera *Dechloromonas* and *Thauera* were the dominant denitrifiers in methanol-acclimated (Ginige *et al.*, 2004) and acetate-acclimated (Ginige *et al.*, 2005) activated sludge, respectively. Also, recently, other *Betaproteobacteria* from activated sludge have been considered to be able to denitrify, such as various bacteria from *Comamonadaceae* and some *Alphaproteobacteria* from *Rhodobacteraceae* (Osaka *et al.*, 2006). The extent to which these and other bacteria contribute to denitrification in full-scale activated sludge systems is so far unknown.

Although activated sludge denitrification appears to be driven by few denitrifying bacterial groups; the substrate utilization specificity of these denitrifiers in full-scale activated sludge plants is only partially understood. Acetate is widely reported to promote the highest denitrification rates (3–12 mg NO_x-N $gVSS^{-1}$ h^{-1}) for different activated sludge types (Gerber *et al.*, 1987; Eilersen *et al.*, 1995; Lee & Welander, 1996; Hallin & Pell, 1998; Marchetto *et al.*, 2003), but mixed, complex substrates are also reported to yield high rates (Monteith *et al.*, 1980; Lee & Welander, 1996). Recent ecophysiological studies on denitrification have shown that some denitrifying bacteria in activated sludge appear very specialized in their substrate uptake while others are more versatile (Thomsen *et al.*, 2007). The abundant *Aquaspirillum*-related bacteria utilize only mixed amino acids, whereas *Thauera*, *Azoarcus*, and *Accumulibacter* PAO consume a range of short-chain fatty acids and amino acids. Glucose and oleic acid can only be consumed by some *Thauera* (Thomsen *et al.*, 2007). A more selective utilization of methanol has been described for denitrifying *Methylophilales* and of acetate for *Dechloromonas* in acclimated activated sludge (Ginige *et al.*, 2004, 2005). Therefore, it is likely that the relative abundance of the main denitrifiers in full-scale activated sludge plants reflects the denitrification rates with certain substrates due to the substrate utilization specificity of the various denitrifiers.

The aim of this study was to evaluate possible correlations between the community structure of known, probe-defined denitrifiers and their denitrifying capacity based on addition of specific organic substrates in full-scale, nutrient-removal activated sludge plants. Probe-defined denitrifiers refer to denitrifying bacteria targeted and identified by specific FISH probes. Nitrate and nitrite CRs were measured upon addition of different substrates *in situ* in sludge via biosensors capable of measuring electron-acceptor concentrations normally encountered in activated sludge (< 0.8 mM NO_x-N or 11 mg NO_x-N L^{-1}). Furthermore, the presence and activity of other so far unidentified denitrifiers was evaluated under nitrite-reducing conditions using heterotrophic CO_2 microautoradiography and FISH ($HetCO_2$-MAR–FISH).

Materials and methods

Activated sludge samples

Mixed-liquor activated sludge was collected from two full-scale wastewater treatment plants (WWTP) treating mainly domestic wastewater in Northern Jutland, Denmark: Aalborg East WWTP and Aabybro WWTP. Both WWTP consist of carbon removal, nitrification, denitrification, and chemical phosphorus removal with the addition of $FeSO_4$; in addition, Aalborg East WWTP has enhanced-biological phosphorus removal (EBPR). Aalborg East WWTP is designed for 100 000 population equivalents (PE) and Aabybro WWTP operates at 9800 PE. The mean sludge retention time in both plants is 25–30 days, and no external carbon sources are used for denitrification. They have had stable nitrogen removal for several years with effluent concentrations of total nitrogen in the range of 5–8 mg N L^{-1}. The activated sludge mixed liquor was used either within 1–6 h after collection or a day later, after overnight storage at 4 °C. No differences in nitrate/nitrite CRs were measured when using fresh or 1-day-old sludge.

Measurement of nitrate/nitrite CRs

Nitrate/nitrite biosensors

The nitrate and nitrite concentrations in the activated sludge were measured on-line with the simultaneous use of a NO_x^- and a NO_2^- biosensor (Unisense A/S, Aarhus, Denmark) at nitrate and nitrite levels < 11 mg NO_x^--N L^{-1} (800 μM NO_x^--N). These biosensors are based on the diffusion of

nitrate/nitrite through an ion-permeable membrane into a pure culture of denitrifiers, converting the ions into N_2O, which is subsequently electrochemically reduced and detected, as described elsewhere (Larsen *et al.*, 2000; Nielsen *et al.*, 2004). While the NO_2^- biosensor detects nitrite alone, the NO_x^- biosensor detects both nitrate and nitrite together; therefore, the nitrate concentration was determined by subtracting the NO_2^- from the combined NO_x^- concentration. The linear decrease in the measured nitrate/nitrite concentrations ensured that nitrogen availability in the sludge flocs was not diffusion limited at the concentrations used (0.8 mM NO_x^- or 11 mg NO_x^--N L^{-1}). Furthermore, nitrite concentrations < 7 mg NO_2^--N L^{-1} (0.5 mM NO_2^-) were used to avoid nitrite toxicity to the biosensors and activated sludge. Under neutral pH and with 540–3400 mg L^{-1} mixed liquor suspended solids (MLSS), inhibition of nutrient-removal activated sludge by nitrite has been reported at nitrite levels from 5 to 8 mg NO_2^--N L^{-1} (Meinhold *et al.*, 1999) and 20 to 30 mg NO_2^--N L^{-1} (Weon *et al.*, 2002). The concentrations used to measure the nitrate/nitrite consumption rates (NCRs) in this work are within the range of nitrate/nitrite concentrations generally experienced by denitrifiers in full-scale plants (Henze *et al.*, 2002).

Batch measurements

Before measuring the NCRs, the activated sludge temperature was increased from 10–12 or 4 °C (after storage) to 22 °C in a water bath in order to maintain an optimal bacterial activity in the biosensors (operating range 10–38 °C) and to increase the biosensors' NO_x^- detection limits from 0–2.9 mg N-NO_x^- L^{-1} at 10 °C to 0–14 mg N-NO_x^- L^{-1} at 20 °C, as per the manufacturer's specifications. The sludge was aerated for at least 30 min to ensure removal of easily degradable substrate from the mixed liquor. The effect of different carbon sources on the denitrification rates was evaluated in 500-mL batches of undiluted activated sludge under constant magnetic stirring by measuring NCRs after substrate addition (Fig. 1). For each substrate or mixture of substrates, two or three NCRs were sequentially determined in single batch measurements. First, the biosensors' readings were allowed to stabilize, and then the biosensors were calibrated as a five-point calibration in activated sludge under diffused aeration (*c.* 30 min) by spiking with known amounts of nitrate, followed by nitrite (30–60 μL 500 mM NO_3^-/NO_2^-, yielding a final concentration of 30–60 μM NO_3^-/NO_2^-). After the last nitrite spike, nitrite was allowed to be completely nitrified before shifting the air to N_2 (*c.* 40 min) (Fig. 1). All the NCRs were determined under anaerobic conditions ensured by N_2 sparging.

NCRs were measured in a 3-week period and a 2-week period after a lapse of 2 months in activated sludge from Aalborg East WWTP. In the first 3-week period, the series of

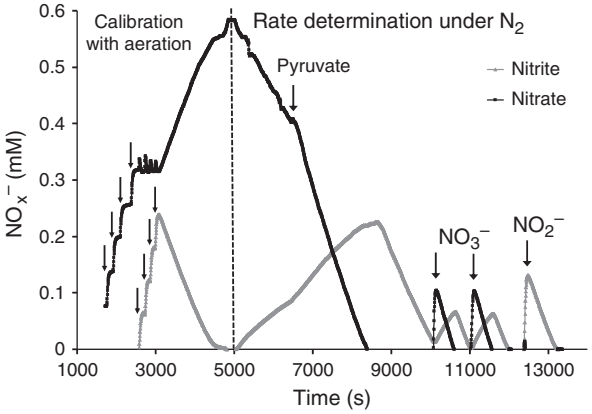

Fig. 1. Typical nitrate and nitrite profiles obtained from a batch experiment with the N-removing sludge (Aabybro) with pyruvate spiking. Nitrate, nitrite, and pyruvate spikes are indicated by arrows.

rates were measured in duplicate batches for each substrate, and the NCRs are therefore reported as means ± SDs of all the rates determined in the two batches. In the later 2-week period, only one series of two or three NCRs were determined (one batch) because high reproducibility in the rates was observed in the first 3-week period. No significant differences in NCRs were observed either within each measurement period or between the first 3-week period and the second 2-week period. For Aabybro WWTP, only series of two or three NCRs were measured during the 2-week period in a single batch measurement. Both the calibrations and the measurements were conducted at the same stirring speed, at a room temperature of 22.5 ± 1 °C, and pH = 7.5 ± 0.5.

Substrate addition

In general, NCRs were determined after substrate spikes, yielding final concentrations in the mixed liquor of 200 mg L^{-1}. However, in order to test against substrate limitation, the sludge was sometimes spiked with substrate two or three times and concentrations of up to 400 or 800–1100 mg L^{-1} (ethanol only) were achieved. The addition of extra substrate had no impact on the NCRs compared with those measured under 200 mg of substrate L^{-1}. The substrates tested (Sigma, except where indicated) were: sodium acetate (ACS Merck, Germany), a complex amino acid mixture: casein hydrolysate (Microbiology, Merck), ethanol (Malteserkors, Denmark), propionic acid, sodium pyruvate (Merck), D-glucose (Merck), sodium oleate, glutamic acid, aspartic acid, glycine (J.T. Baker, Holland), serine, and L-phenylalanine. NCRs were also measured on combined addition of acetate and casein hydrolysate, glutamic and aspartic acids, and serine and glycine. In addition, NCRs were determined without substrate addition in

independent batches and within a batch measurement after shifting to N_2 and before substrate spiking. The addition of substrate always caused a relative increase in NCRs in each batch compared with the endogenous NCRs without substrate addition (Fig. 1).

FISH and HetCO$_2$-MAR–FISH

Paraformaldehyde fixation and FISH probing were conducted based on the protocol by Amann (1995) with homogenized activated sludge. The oligonucleotide probes used were ALF968 for many *Alphaproteobacteria* (Neef, 1997), BET42a for *Betaproteobacteria* (Manz *et al.*, 1992), Aqs997 + competitors for *Aquaspirillum*-related bacteria (Thomsen *et al.*, 2004), Thau646 for *Thauera* (Lajoie *et al.*, 2000), Azo644 for *Azoarcus* (Hess *et al.*, 1997), PAO651 for *Accumulibacter* PAOs (Crocetti *et al.*, 2000), GAM42a for *Gammaproteobacteria* (Manz *et al.*, 1992), Pla46 for *Planctomycetes* (Neef *et al.*, 1998), CF319a+b for *Cytophaga–Flavobacterium*-group of the *Bacteroidetes* (Manz *et al.*, 1996), HGC69a for *Actinobacteria* (Roller *et al.*, 1994), LGC354a+b for many *Firmicutes* (Meier *et al.*, 1999), SRB385+SRB385Db for *Deltaproteobacteria* and *Desulfobacteriaceae* (Amann *et al.*, 1992; Rabus *et al.*, 1996), GNSB941+CFX1223 for Phylum *Chloroflexi* (Gich *et al.*, 2001; Björnsson *et al.*, 2002), Ntspa712 for Phylum *Nitrospira/Leptospirillum* (Daims *et al.*, 2000), and EUBmix (EUB338, EUB338-II, and EUB338-III) for all *Bacteria* (Daims *et al.*, 1999). Further details about the probes and their competitors are provided at probeBase (Loy *et al.*, 2003). EUBmix labelled with FLUOS was used in combination with any of the other probes labelled with Cy3. In the activated sludge sample analysed in this study, those cells targeted by LGC354a+b were also targeted by the EUBmix probe, despite competing for two overlapping bases in the target RNA sequence.

HetCO$_2$-MAR–FISH under nitrite-reducing conditions was conducted following the general approach described previously (Hesselsøe *et al.*, 2005; Nielsen & Nielsen, 2005), but with the following modifications: a sample from Aalborg East activated sludge was incubated for 5 h with 20 μCi of $H^{14}CO_3^-$ mL^{-1} under anaerobic conditions with and without 0.5 mM NO_2^- and with *c.* 800 mg chemical oxygen demand (COD) L^{-1} of a complex mixture of substrates consisting of 14% w/w yeast extract, 14% meat extract, 14% casein hydrolysate, 23% sodium acetate, and 35% glucose (each component yielding 160 mg COD L^{-1}). Before adding the labelled isotope and nitrite, the sludge with the substrate mixture was preincubated for 1 h under anaerobic conditions to remove traces of nitrite and nitrate. In addition, *c.* 73% of the carbonate from the fresh activated sludge used in the preincubation was removed by acidifying to pH 5 with 6 M HCl, allowing the sample stand for 20 min, and then sparging with N_2 for 1 h and restoring the original pH to 7. This ensured an optimal silver-grain MAR signal for assessment and quantification, in which background silver-grain formation was minimized. This pretreatment had no statistically significant impact on the sludge denitrification capacity as assessed by similar NCRs measured in the treated (3.9 ± 2.1 mg N gVSS^{-1} h^{-1}) and untreated (2.9 ± 0.9 mg N gVSS^{-1} h^{-1}) sludge on addition of the complex substrate mixture (paired *t*-test, 95% confidence level, $n = 4$, $P = 0.25$). After isotope labelling incubation, MAR–FISH slide preparations were carried out as described previously (Nielsen & Nielsen, 2005). Homogenization of the biomass was conducted for 5 min with a tissue grinder. The slides were exposed in the dark at 4 °C for 5 days. The effect of longer exposure times on the MAR signal was also tested for 30 and 54 days.

Quantification of FISH-based bacterial abundance and HetCO$_2$-MAR–FISH signal

The relative abundance of each probe-defined bacterial group was estimated as the ratio of area fluorescing with each specific probe to the area fluorescing with the EUBmix probe on images of thin sludge samples. For each sample, 20 images of fields of view were taken at × 630 magnification under oil immersion from two different hybridization wells containing the same sample on the slide using an Axioskope II epifluorescent microscope (METAVUE software 6.4; Universal Imaging Corp., Downington, PA). The numbers of cells (cells mL^{-1}) in the activated sludge samples and for the targeted organisms were determined based on 4,6-diamino-2-phenylindoldihydrochloride-dilactate (DAPI) staining, as explained elsewhere (Morgan-Sagastume *et al.*, 2008), where the accuracy of the method and comparisons with the literature are also presented.

The percentage of FISH-probe-defined cells yielding a positive, silver-grain HetCO$_2$-MAR signal was used for quantifying specific microbial activity under nitrite-reducing conditions (incubation with nitrite) and under anaerobic, fermenting conditions (incubation without nitrite). Thus, the ratio of probe-specific fluorescing area with silver-grain coverage to the total area fluorescing with the specific oligonucelotide probe was calculated on MAR–-FISH images. Bright-field images of silver-grain coverage and fluorescing images were taken from 20 fields of view at × 1000 magnification. All image analyses were conducted using the image-processing software IMAGEJ 1.34s (http://rsb.info.nih.gov/ij/; National Institute of Health, US). MAR–FISH quantification was conducted after 5, 30, and 54 days of exposure, and although the longer exposure increased the silver grain density for some probe-defined bacteria, the same trends in results were observed. Nevertheless, reporting the silver-grain density for a range of probe-defined organisms after a 5-day exposure ensured

that the effects of background and neighbouring silver-grain formation were taken into account.

Results

Activated sludge nitrate/nitrite CRs

The nitrate CR without substrate addition was 1.0 ± 0.2 mg NO_3^--N g VSS^{-1} h^{-1} for the N- and P-removing sludge (Aalborg East) in the first 3-week period, as calculated from two batch measurements ($n = 5$). This value was subtracted from the measured NCRs in order to account for background denitrification on sludge substrates (Fig. 2). During the later 2-week period, NCRs without substrate addition were measured during each batch measurement before spiking with a specific substrate in both the N- and P-removing (Aalborg East) and the N-removing sludge (Aabybro). Thus, these values were used to correct the NCRs measured in each specific batch (Fig. 2).

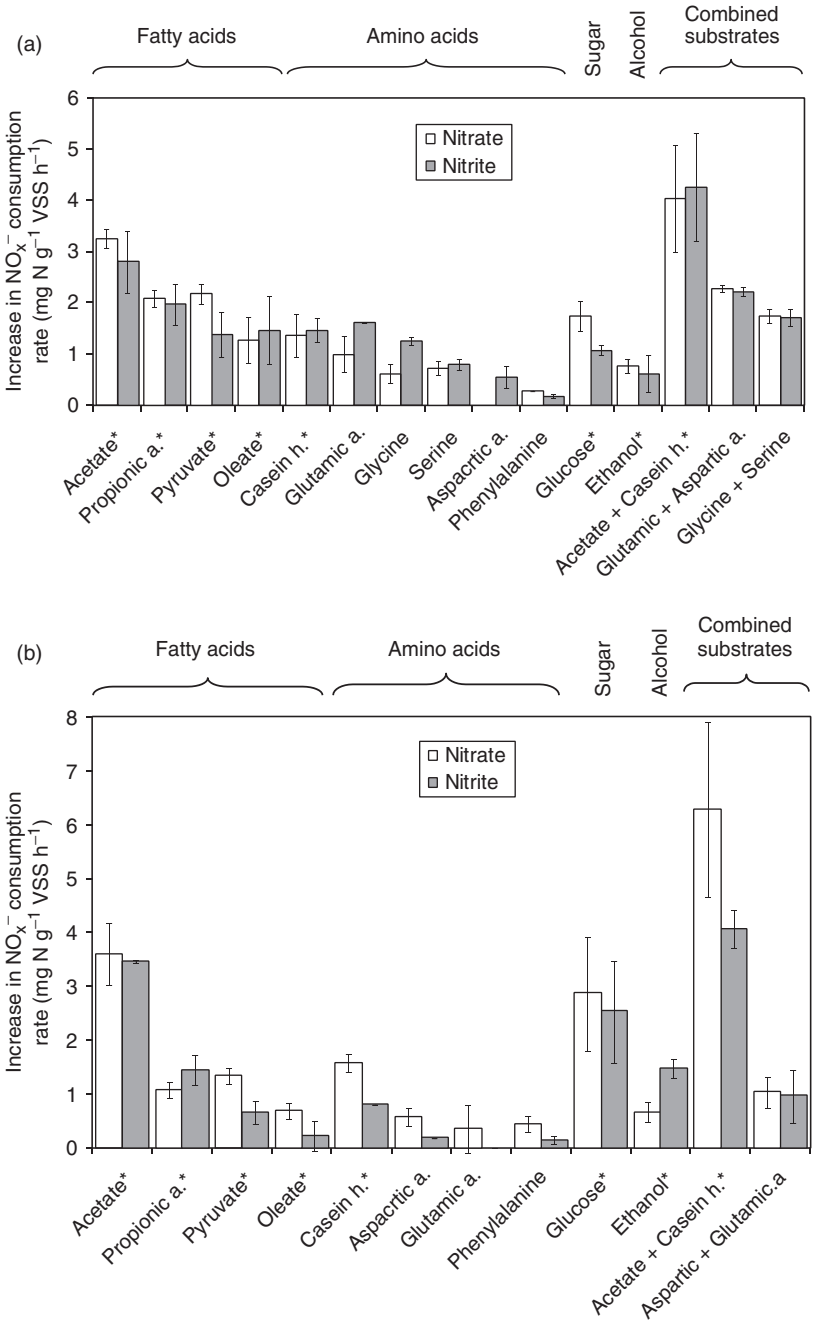

Fig. 2. Increase in denitrification rates as NCRs measured upon substrate addition in (a) the N- and P-removing sludge (Aalborg East) and (b) the N-removing sludge (Aabybro). The increase with respect to the rates without substrate addition is shown for each substrate and the rates were calculated by subtracting the NCRs without substrate addition from the NCRs measured in each batch. For (a), the averages for the NCRs without substrate addition equalled 1.7 ± 0.5 NO_2^--N g VSS^{-1} h^{-1} ($n = 6$) and 1.3 ± 0.4 NO_3^--N g VSS^{-1} h^{-1} ($n = 5$); *set of data from an initial 3-week period, in which the increase in the rates was determined by subtracting a constant average NCR without substrate addition = 1.0 mg NO_3^--N g VSS^{-1} h^{-1} from the measured rates for all substrates. For (b), the averages for the NCRs without substrate addition equalled 1.5 ± 0.1 NO_2^--N g VSS^{-1} h^{-1} ($n = 3$) and 1.7 ± 0.4 NO_3^--N g VSS^{-1} h^{-1} ($n = 12$).

Table 1. Summary of the substrate-uptake specificity for probe-defined (parentheses) denitrifiers with nitrite and nitrate as electron acceptors in Aalborg East activated sludge, based on MAR–FISH studies (Kong *et al*., 2004; Thomsen *et al*., 2004, 2007)

Substrate	*Aquaspirillum*-related bacteria (Aqs997)	*Thauera* (Thau646)	*Azoarcus* (Azo644)	*Accumulibacter* (PAO651)
16-Amino acid mixture	+(20–30%)	+(15–25%)	+(50–60%)	ND
Acetate	–	+(50%)	+(20–30%)	+(22–35%)
Pyruvate	–	+(10–20%)	+(5–10%)	+*
Ethanol	–*	+(10–20%)	+(20–30%)	–*
Propionate	–	+(30–40%)	–	+/–
Oleic acid	–	+(10%)	–	–*
Glucose	–	+(10–20%)	ND	–*

+, Uptake; –, no uptake; +/–, both uptake and no uptake observed; ND, not determined, but likely.
The values in parentheses correspond to percentages from each probe-defined population taking up labelled substrate.
*Expected facultative behaviour for nitrate/nitrite, based on substrate uptake tests conducted with oxygen as electron acceptor. No MAR–FISH tests under anoxic conditions were conducted.

All the substrates tested have been reported to be utilized by one or several of the probe-defined denitrifiers in Aalborg East activated sludge (Table 1). Immediately after substrate addition, a linear reduction in nitrate and an increase in nitrite levels were observed for all substrates tested. Nitrite levels did not start decreasing until all nitrate was removed (Fig. 1). From the substrates tested, the highest NCRs (3–6 mg N $gVSS^{-1}$ h^{-1}) in both activated sludge types were measured with acetate and the combination of acetate and casein hydrolysate (Fig. 2). The lowest NCRs (< 1 mg N g VSS^{-1} h^{-1}) in both sludge types were measured after addition of specific amino acids, such as aspartic acid and phenylalanine. Glutamic acid and oleate also yielded low NCRs in the N-removing sludge (Aabybro) and serine and ethanol in the N- and P-removing sludge (Aalborg). Intermediate NCR values ranging from 1 to 3 mg N $gVSS^{-1}$ h^{-1} were measured with other substrates (Fig. 2).

In both sludge types, the mixture of acetate and casein hydrolysate yielded NCRs that corresponded approximately to the addition of the single substrate rates with acetate and casein hydrolysate alone (Fig. 2). Glucose and ethanol produced higher NCRs in the N-removing sludge (Aabybro) than in the N- and P-removing sludge (Aalborg East). The NCRs in the N-removing sludge with the other short-chain fatty acids and amino acids were lower than in the N- and P-removing sludge.

For most of the substrates tested, no significant differences in nitrate and nitrite CRs were observed (Fig. 2). However, nitrate CRs were higher than the nitrite CRs for pyruvate and glucose, and for sludge from Aabybro WWTP also oleate, aspartate, phenylalanine, and casein hydrolysate, which, alone or in combination with acetate, also gave higher rates. Only for a few substrates (ethanol in Aabybro and glutamate and glycine in Aalborg East) were the nitrite-removal rates higher than the nitrate-removal rate.

Abundance of potential denitrifying bacteria in activated sludge

Group-specific oligonucleotide probes were used to quantify the relative abundance of the dominant and physiologically important bacterial groups in the sludge (Fig. 3). The number of group-specific cells in the sludge (cells mL^{-1}) was estimated based on average DAPI cell numbers (Aalborg East: 2.5×10^9 cells mL^{-1}; Aabybro: 1.9×10^9 cells mL^{-1}), an average EUBmix/DAPI ratio (0.76) (Morgan-Sagastume *et al*., 2008) assumed to be similar for both sludge types, and the group-specific-probe/EUBmix ratios were determined. The five betaproteobacterial genera previously shown to inhabit denitrifying activated sludge, i.e., *Aquaspirillum*, *Thauera*, *Accumulibacter*, *Azoarcus*, and *Zoogloea* were found in both sludge types, with the *Aquaspirillum*-related bacteria, *Thauera*, *Accumulibacter*, and *Azoarcus* as the most abundant (Fig. 3a). *Zoogloea* was estimated to be present in low abundance ($< 1 \times 10^7$ cells mL^{-1}, < 0.5–1%).

The abundance of *Aquaspirillum*-related *Azoarcus* and *Accumulibacter* bacteria was different in the two sludge types (Fig. 3a). The *Azoarcus* were more abundant in the N-removing sludge (Aabybro: $19 \pm 2 \times 10^7$ cells mL^{-1}, $13 \pm 5\%$) than in the N- and P-removing sludge (Aalborg East: $3.7 \pm 0.9 \times 10^7$ cells mL^{-1}, $2 \pm 1\%$), and the *Aquaspirillum*-related bacteria and *Accumulibacter* were more abundant in the N- and P-removing sludge (Aalborg East: $39 \pm 4 \times 10^7$ cells mL^{-1}, $20 \pm 2\%$ and $12 \pm 1 \times 10^7$ cells mL^{-1}, $7 \pm 2\%$) than in the N-removing sludge (Aabybro: $2.5 \pm 0.2 \times 10^7$ cells mL^{-1}, $18 \pm 2\%$ and $3.9 \pm 0.7 \times 10^7$ cells mL^{-1}, $3 \pm 1\%$). The relative abundance of *Thauera* was similar in both sludge types ($10 \pm 2 \times 10^7$ cells mL^{-1}, $5 \pm 1\%$ in Aalborg East and $8.5 \pm 1 \times 10^7$ cells mL^{-1}, 6 ± 1, Aabybro).

In terms of broader probe-defined groups, *Betaproteobacteria* ($36 \pm 3\%$), *Chloroflexi* ($33 \pm 4\%$), and *Alphaproteobacteria*

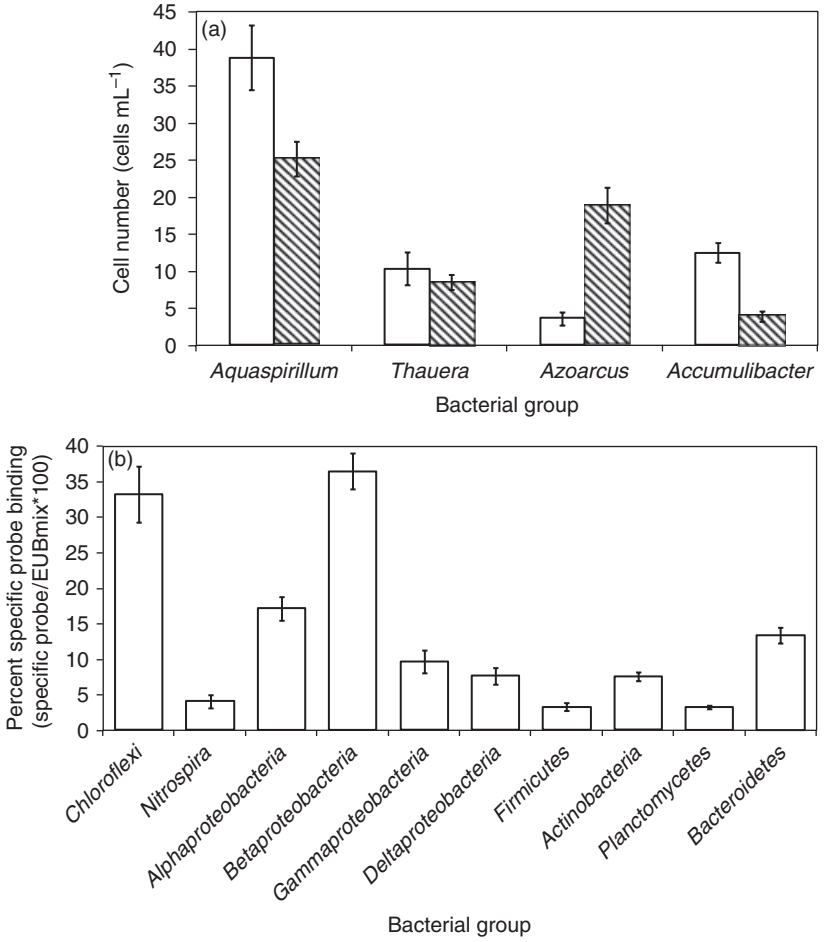

Fig. 3. Abundance of (a) known denitrifiers in the N- and P-removing sludge (Aalborg East) and the N-removing sludge (Aabybro; shaded bars) and of (b) broad bacterial groups in the N- and P-removing sludge (Aalborg East), as determined using FISH biovolume measurements. The error bars correspond to averages ± 95% confidence limits.

(17 ± 2%) were the most dominant bacterial groups, and *Bacteroidetes* (13 ± 1%) and *Gammaproteobacteria* (10 ± 2%) were present in medium abundance in Aalborg East WWTP (Fig. 3b). The addition of the percent-specific probe binding for all the groups, except *Chloroflexi*, was close to 90%, indicating that the nine probe-targeted bacterial groups accounted for the majority of the EUBmix-positive bacteria in the sludge. An overestimation of the abundance for some groups, as the specific case of *Chloroflexi*, is possible because many filamentous bacteria belonging to *Chloroflexi* were not targeted by the EUBmix, but gave a clear signal with the group-specific probes GNSB941 and CFX1223. This yielded an artificially higher percentage of the relative abundance of *Chloroflexi*, which, however, appear inactive under nitrite-reducing conditions, as discussed below.

$^{14}CO_2$ assimilation under nitrite-reducing conditions

In order to gain insights into the diversity of potentially active denitrifiers (nitrite reducers) in the activated sludge, assimilation of $^{14}CO_2$ with nitrite as an electron acceptor and with a complex substrate mixture as an electron donor was investigated in Aalborg East activated sludge. A broad mixture of substrates, including acetate and amino acids, was used to target most of the bacteria with potential denitrifying capacity and in the same sludge on which previous single-substrate, MAR–FISH studies had been conducted (Table 1). Active cells assimilating CO_2 were visualized using MAR and their identity was determined using FISH. Parallel MAR incubations were conducted to test the assimilation of $^{14}CO_2$ under anaerobic conditions without nitrite but with the complex substrate mixture in order to exclude the possible activity of nondenitrifiers under nitrite-reducing conditions, such as fermenting bacteria. The semi-quantitative HetCO_2-MAR–FISH results are summarized in Table 2.

Assimilation of $^{14}CO_2$ with the complex substrate mixture under nitrite-reducing conditions was detected in some of the probe-defined bacterial groups (Table 2). Assimilation of $^{14}CO_2$, as determined by a medium (30–50% of targeted bacteria) to high (> 50%) silver-grain coverage of probe-hybridized cells, was detected for *Alphaproteobacteria*,

Table 2. Potential denitrifiers as determined by $HetCO_2$-MAR–FISH

Oligonucleotide probe-targeted bacteria	Density of silver grain formation	
	Incubation with nitrite (nitrite-reducing conditions)	Incubation without nitrite (anaerobic conditions)
Chloroflexi	−	−
Nitrospira	−	−
Alphaproteobacteria	+	+
Betaproteobacteria	+	+
Aquaspirillum-related	+	−
Azoarcus	+	−/+
Thauera	+	−
Accumulibacter	++	+
Gammaproteobacteria	+	+
Deltaproteobacteria	−	−
Firmicutes	++	++
Actinobacteria	−/+	−/+
Planctomycetes	−	−
Bacteroidetes	−	−

−, no or insignificant silver grain coverage: < 5–10% of probe-defined organisms; −/+, slight silver-grain coverage: < 10–20%; +, medium silver grain coverage: 30–50%; ++, high silver-grain coverage: > 50%. Summary of the assimilation of $^{14}CO_2$ with a complex substrate mixture as electron donor and carbon source, and under nitrite-reducing and anaerobic conditions by different probe-defined bacterial groups in Aalborg East activated sludge. Quantification was conducted based on the ratio of probe-specific fluorescing area with silver grain coverage to the total area fluorescing with the specific oligonucleotide probe.

Betaproteobacteria, including all the four potential denitrifiers (*Aquaspirillum*, *Azoarcus, Thauera*, and *Accumulibacter*), *Gammaproteobacteria*, and *Firmicutes* under nitrite-reducing conditions. A small fraction (< 10–20%) of bacteria belonging to *Actinobacteria* presented a positive $HetCO_2$-MAR signal. *Chloroflexi* filaments, *Deltaproteobacteria/Desulfobacteriaceae*, *Nitrospira*, *Planctomycetes*, and *Bacteroidetes* were unable to assimilate $^{14}CO_2$ (no silver-grain coverage observed) under such conditions (Table 2). The expected lack of $^{14}CO_2$ assimilation by autotrophic nitrite oxidizers *Nitrospira* served as a negative control for the $HetCO_2$-MAR experiments under nitrite-reducing conditions.

Some probe-targeted bacteria assimilating $^{14}CO_2$ under nitrite-reducing conditions were also able to assimilate $^{14}CO_2$ under anaerobic conditions without nitrite (Table 2). Some *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Firmicutes*, and *Actinobacteria* showed similar silver-grain coverage under anaerobic conditions as under nitrite-reducing conditions. However, the previously reported betaproteobacterial denitrifiers *Aquaspirillum*, *Azoarcus*, and *Thauera* showed an insignificant silver-grain coverage (< 5–10%) under anaerobic conditions without nitrite. The MAR-positive *Accumulibacter* were also more active under nitrite-reducing conditions than under anaerobic conditions (Table 2). From the probe-defined bacterial groups assessed, only *Firmicutes* showed high activity (> 50% grain coverage) in both incubations. None of the bacterial groups examined and found capable of assimilating $^{14}CO_2$ were completely MAR-positive with a 100% silver-grain coverage, which indicates the presence of inactive, non-$^{14}CO_2$-assimilating, and/or non-substrate-utilizing members within these probe-defined groups.

Discussion

Measurement of denitrification as NCRs

The NCRs of this study reflect the process of denitrification *in situ* and at electron-acceptor concentrations to which activated sludge is generally exposed in a full-scale plant. Although the NO_X^- (combined nitrate and nitrite) and nitrite biosensors had been used previously in activated sludge (Larsen *et al.*, 2000; Nielsen *et al.*, 2004), they had not been systematically used to determine both nitrate and nitrite CRs in bulk activated sludge. The NO_X^- biosensor has only been applied in denitrification studies with diatoms in freshwater sediment (Lorenzen *et al.*, 1998) and with activated sludge (NO_X^- CRs only) (McMurray *et al.*, 2004; Sin & Vanrolleghem, 2004). The online measurement of nitrate and nitrite allowed for NCR measurements in activated sludge at concentrations three to six times lower than those used in conventional approaches (*c.* 20–60 mg NO_3^--N L^{-1}) with periodic sampling and analysis (Kristensen *et al.*, 1992; Oh & Silverstein, 1999; Hagman, 2003). Therefore, these *in situ* measurements reflect more realistically the ecophysiology of the denitrifiers in activated sludge. Although the denitrification rates determined in this study at $22.5 \pm 1\ ^\circ C$ will be higher than those expected in the full-scale plant (10–12 °C), no significant impact on the relative activity of the different bacterial groups assessed is expected. No interference in the biosensor measurements by N_2O was expected because N_2O concentrations have been observed to be negligible in activated sludge under anoxic conditions (Larsen *et al.*, 2000; McMurray *et al.*, 2004).

Detectable nitrite consumption only occurred once the nitrate levels were limiting (Fig. 1). This denitrification behaviour involving nitrite accumulation has also been reported for activated sludge from other nutrient removal plants (Wilderer *et al.*, 1987; Ekama & Wentzel, 1999). Nitrate reduction without nitrite accumulation has only been reported for acclimated sludge, as for the case of an acetate-fed sludge (Oh & Silverstein, 1999), or for pure or mixed cultures of known denitrifying bacterial strains under specific substrate and environmental conditions (Blaszczyk, 1993; van Rijn *et al.*, 1996).

The NCRs measured in both sludge types (Fig. 2) are in agreement with denitrification rates reported in the

literature for some of the substrates used, i.e., acetate, propionate, and ethanol (Table 3). For the case of glucose, however, the NCRs from this study are comparatively higher. Compared with denitrification rates from sludge acclimated to specific substrates (e.g. ethanol or methanol), the NCRs from this study are relatively lower because no microbial population actively utilizing the specific substrates was enriched. Denitrification rates with the addition of different substrates and from different activated sludge plants are limited in the literature (Table 3). Nevertheless, based on the similarity between the NCRs published and measured in this study for acetate, propionate, and ethanol, the NCRs for other substrates are believed to reflect real denitrification rates in activated sludge.

The NCRs measured in the activated sludge are also comparable with those measured in pure cultures of

Table 3. Comparison of available activated sludge denitrification rates for some of the substrates used in this study

	Denitrification rates (mg N gVSS^{-1} h^{-1})						
Activated Sludge	Acetate	Propionate	Ethanol	Glucose	No substrate	Observations	References
Goudkoppies sewage plant, SA	*c*. 2.5	*c*. 1.7	*c*. 1.8	*c*. 0.9		Washed, filtered, resuspended sludge, (mg N g MLSS^{-1} h^{-1})	Gerber *et al*. (1987)
Northern works sewage plant, SA	*c*. 2.2	*c*. 2.1	*c*. 0.6	*c*. 0.7			
Henriksdal sewage plant, Stockholm	–				*c*. 0.8–1.3	Full-scale plants	Hansson & Gunnarson (1990)
Odense North-West plant	–				1.4		
Slotshagen sewage plant, Norrköping	4.7				–		
Chemically pretreated wastewater	3.4				–	Pilot plant fed with differently pretreated domestic wastewater	Kristensen *et al*. (1992)
Chemically pretreated+hydrolysate	4.8				1.7		
Chemically pretreated+hydrolysed sludge	3.4				1.5	Rates determined at $T = 12$–$13\ ^\circ$C and	
Chemically pretreated (twice)+hydrolysate	3.5				–	corrected for $T = 20\ ^\circ$C	
Primary settled wastewater	4.3				1.3		
Frederikssund (nitrification-denitrification)	7.4				2.2		
Sjælso (nitrifying sludge)	1.1				0.4		
Pilot plant	4.5					$T = 20 \pm 2\ ^\circ$C	Isaacs *et al*. (1994)
Kungsängen Municipal plant, Uppsala	3–4		–		0.5–1	As N_2O-N released at $T = 18\ ^\circ$C	Hallin & Pell (1994)
	5.1 ± 0.2		*c*. 3		–	As N_2O-N released at $T = 15\ ^\circ$C	Rates read from figures
	5.0 ± 0.2		–		–	As nitrate uptake rates at $T = 15\ ^\circ$C	
Nonacclimated sewage plant	3.6 ± 0.9					University of Florida treatment plant	Lee *et al*. (1995)
Acclimated to acetate sewage plant	12.1 ± 0.4					$T = 22 \pm 1.5\ ^\circ$C	
Acclimated to methanol			4.3			Municipal sludge	Nyberg *et al*. (1996)
Acclimated to ethanol			10			$T = 10\ ^\circ$C	
Sludge acclimated to acetate	76 ± 7					$T = 15\ ^\circ$C	Lee & Welander (1996)
Kungsängen Municipal plant, Uppsala	6.2–7.5	2.8–3.8	2.3–3.5	1.5–2.3	0.3–0.9	Municipal sludge, predenitrification	Hallin & Pell (1998)
Full-scale adapting to methanol	4.2	2.2	5.5	1.3	0.7	As N_2O-N released at $T = 15\ ^\circ$C	Hallin *et al*. (2006)
Pilot plant	5	2	2.5	0.5	0		(Graphical source)
Pilot plant adapting to ethanol	12.8	3.5	12	1.5	0.2		
Acclimated to ethanol Pilot plant, Uppsala			4–8			As N_2O-N released at $T = 15\ ^\circ$C	Hasselblad & Hallin (1998)

Table 3. Continued.

	Denitrification rates (mg N gVSS^{-1} h^{-1})						
Activated Sludge	Acetate	Propionate	Ethanol	Glucose	No substrate	Observations	References
Rostock, Germany	7.3 ± 0.4				1.1 ± 0.1	Municipal sludge, full-scale plants	Naidoo *et al*. (1998)
Orense, Spain	6.8 ± 1.6				–	*T* = 20 °C	
Crespières, France	5.3 ± 0.1				–		
Brno, Czech Rep.	5.2 ± 0.2				–		
Berwick, UK	4.5 ± 0.1				–		
Boran, France	4.4 ± 0.1				1.0 ± 0.1		
Morainvilliers, France	3.2 ± 0.1				–		
Fed with acetate	20 ± 2 23 ± 4					*T* = 21 °C	Oh & Silverstein (1999)
Overall activated sludge	4–12					*T* = from 15 to 30 °C	Henze *et al*. (2002)
Fed with effluent from an anaerobic fluidized bed reactor treating domestic sewage	16			10		As N_2O-N released at *T* = 30 °C	Marchetto *et al*. (2003)
Källby, Lund (predenitrification, postchemical treatment)	6		2.5			Municipal sludge, full-scale plants	Hagman (2003)
						T = 20 °C	Hagman *et al*. (in press)
Klagshamn, Malmö (prechemical treatment, postdenitrification)	8.2		9				
Sjölunda pilot plant, Malmö (N and P removal)	4.8		3				
N- and P-removing sludge						*T* = 22.5 ± 1 °C	This study
(Aalborg East)	4.0–6.4	2.9–3.3	1.6–1.9	2.4–3.1	0.4–1.7		
	3.0–5.1	*2.5–3.4*	*1.4–2.1*	*1.9–2.2*	0.8–2.5		
N-removing sludge (Aabybro)	4.8–6.1	2.6–2.9	1.8–2.1	2.7–5.8	1.2–2.2		
	4.9	*2.8–3.4*	*2.5–2.9*	*2.7–4.6*	*1.4–1.6*		

Rates correspond to nitrate consumption rates except where indicated (nitrite consumption rates are in italics).

denitrifiers with acetate and propionate addition. Assuming that 10–20% of the sludge organic biomass (VSS) are cells (Nielsen, 2002) and that 20% of the bacterial cells may be able to denitrify based on an estimation of the active *Betaproteobacteria* able to reduce nitrite in MAR–FISH tests (refer to next section) or that 50% of the bacterial cells may denitrify based on acetate utilization with nitrate as an electron acceptor (Nielsen & Nielsen, 2002), denitrification rates for acetate of 5 mg N g VSS^{-1} h^{-1} would correspond to 125 or 50 mg N (g cell dry wt)$^{-1}$ h^{-1}, respectively. These values are comparable with the nitrite reduction rates reported for pure cultures of *Pseudomonas* spp. with acetate: 137 ± 7 mg NO_2^--N (g cell dry wt)$^{-1}$ h^{-1} (Almeida *et al*., 1995) and 75 ± 13 mg NO_2^--N (g cell dry wt)$^{-1}$ h^{-1} (van Rijn *et al*., 1996) for *P. fluorescens* and *P. stutzeri*, respectively. Higher nitrate CRs are reported (318 ± 21 and 173 ± 17 mg NO_3^--N (g cell dry wt)$^{-1}$ h^{-1}, respectively) in the same pure cultures, which points to the importance of assessing denitrification rates *in situ* involving all the sludge-denitrifying populations because no significant difference between nitrate and nitrite CRs upon acetate addition was observed with the sludge of this study.

Denitrification rates and substrate utilization

In accordance with several studies (Table 3), acetate yielded the highest nitrate/nitrite CRs as a sole substrate in both sludge types (Fig. 2). This could be explained by acetate-based selection in treatment plants and a common capacity for acetate assimilation among denitrifiers. Acetate in wastewater may enrich and select for acetate degraders in full-scale plants because acetate typically comprises 5–10% of the total COD in municipal wastewater, whereas the other soluble organic compounds occur in lower concentrations (Henze *et al*., 1994). In addition, acetate uptake under anoxic conditions, i.e. with the supply of nitrate, is a trait of facultative denitrifying bacteria in activated sludge (Nielsen & Nielsen, 2002). Acetate is utilized under anoxic conditions by three (*Thauera*, *Azoarcus*, and *Accumulibacter*) out of the four abundant potential denitrifying groups present in the activated sludge investigated (Table 1), and, therefore, the overall denitrification rate would correspond to the addition of the rate contribution from each group.

The substrate-based NCRs (Fig 1, Table 1) suggest that denitrifiers in activated sludge are specialized utilizers of

substrates as electron donors and carbon sources, and that the denitrifiers all combined yield an overall activated sludge-denitrifying capacity based on utilizing specific substrates from a pool of organic compounds. Two combined substrates always yielded higher NCRs than the substrates alone, as in the cases of acetate and casein hydrolysate, and aspartic and glutamic acids (Fig. 2). This is in agreement with higher NCRs reported for a mixture of acetate and methanol (Hagman *et al.*, 2008) and several combined substrates (Dionisi *et al.*, 2004) compared with NCRs for the single substrates. A broad substrate, for example sludge hydrolysate, has also yielded higher NCRs than acetate alone in different sludge samples (Kristensen *et al.*, 1992). The increased NCR with combined substrates could be due to a larger number of denitrifying species utilizing different substrates and/or increased specific denitrification rates per cell basis due to substrate coutilization. MAR–FISH studies on several activated sludge microorganisms, such as *Accumulibacter* and glycogen-accumulating organisms (*Competibacter* and *Defluviicoccus*), have shown that these bacteria are able to take up two substrates simultaneously (Kong *et al.*, 2006; Burow *et al.*, 2007). Furthermore, the MAR–FISH studies with denitrifiers in activated sludge (Table 1) suggest that no universal single substrate exists that can be utilized by all the known probe-defined denitrifiers in activated sludge.

The relatively high NCRs based on glucose (Fig. 2) could result from not only denitrifiers using glucose directly but also denitrifiers using glucose fermentation products, such as pyruvate and acetate, as assessed previously for *Accumulibacter* (Kong *et al.*, 2004).

Community structure of denitrifiers and denitrification capacity in activated sludge

Aquaspirillum-related bacteria appear as the most abundant potential denitrifying *Betaproteobacteria* in municipal activated sludge (Fig. 3a), confirming previous results (Thomsen *et al.*, 2007). The relative abundance of the probe-defined denitrifying *Betaproteobacteria* in the N- and P-removing sludge (Aalborg East) agrees with recent quantifications for these potential denitrifiers in sludge from the same plant (*Aquaspirillum*-related bacteria $29 \pm 5\%$, *Thauera* $7 \pm 4\%$, *Accumulibacter* $7 \pm 2\%$, and *Azoarcus* $4 \pm 2\%$) (Thomsen *et al.*, 2007). In contrast, the same groups, especially *Azoarcus*, appear to be more abundant in the N-removing sludge (Aabybro) than reported previously (*Aquaspirillum*-related bacteria $14 \pm 6\%$, *Thauera* $2 \pm 1\%$, *Azoarcus* $2 \pm 2\%$, and *Accumulibacter* 0%) (Thomsen *et al.*, 2007) perhaps due to changes undergone in the 2 years between the assessments. The lower abundance of *Accumulibacter* in the N-removing sludge (Aabybro) agrees with the lack of significant biological phosphorus removal in this plant, but underlines the role of some *Accumulibacter* as denitrifiers.

Differences in the abundance of *Azoarcus* and *Accumulibacter* between the two sludge types coincided with differences in NCRs for specific substrates known to be utilized by these bacterial groups (Table 1), which suggests that the structure of the denitrifying community and bacterial group substrate specificity determine the denitrification rates. The 33% lower abundance of *Accumulibacter* in the N-removing sludge (Aabybro) than in the N- and P-removing sludge (Aalborg East) coincided with lower pyruvate-based (52% and 58% lower for both nitrite and nitrate, respectively) and propionate-based (48% lower for nitrate) NCRs (Fig. 2). Pyruvate and propionate can also be taken up by some *Thauera* and *Azoarcus* under nitrate-reducing conditions (Table 1), but no lower abundances were observed in the N-removing sludge for these organisms. Consequently, *Accumulibacter* may play an important role as nitrate reducers utilizing pyruvate. The fourfold higher abundance of *Azoarcus* in the N-removing sludge than in the N- and P-removing sludge can be related to 1.5-fold and 23% higher nitrite CRs based on ethanol- and acetate, respectively (Fig. 2). Although the lower abundance of *Aquaspirillum* and *Accumulibacter* coincided with lower propionate- and casein hydrolysate-based nitrite CRs, these rate differences were not statistically significant. Therefore, both *Azoarcus* and *Accumulibacter* may drive nitrite reduction because *Azoarcus*' ability to utilize ethanol and acetate (Table 1) agrees well with their higher abundance in the sludge and the higher ethanol and acetate-based nitrite CRs. The capacity to utilize pyruvate by *Accumulibacter* may have reflected lower pyruvate-based nitrite CRs due to lower abundance.

The results suggest that *Aquaspirillum*-related bacteria, although abundant and involved in denitrification, are not the only main driving organisms of the process in the two plants assessed because their inability to utilize acetate (Table 1) contrasts with the highest acetate-based NCRs measured in this study. In addition, casein hydrolysate and specific amino acids (glycine, serine, glutamic, and aspartic acids), shown to be selectively utilized by *Aquaspirillum*-related bacteria under denitrifying conditions in MAR-FISH studies (Table 1), produced the lowest NCRs. However, an important difference between these studies and the actual conditions in full-scale N-removal plants is the concentration of organic substrate. Under *in situ* conditions, amino acids are provided by hydrolysis of proteins and thus are only present in very low concentrations implying that microorganisms able to take up substrate at low concentrations and at low rates may be important for the denitrification process. Indeed, typical denitrification rates in full-scale plants with wastewater or due to endogenous metabolism at 12–15 °C are low (0.7–1.25 mg NO_3^--N $g\,VSS^{-1}\,h^{-1}$) (Metcalf & Eddy, 2003). In any case, the large biomass of

Aquaspirillum-related bacteria indicates that they may consume a significant part of the incoming organic matter coming from proteins. Furthermore, relative differences in denitrification rates with different substrates may depend not only on the abundance of the different types of denitrifiers but also on cell-specific denitrification rates and the fraction of active cells for each probe-bacterial group.

The $HetCO_2$-MAR–FISH results confirm the denitrifying capacity of a fraction of *Betaproteobacteria* related to *Aquaspirillum*, *Azoarcus, Thauera*, and *Accumulibacter* in activated sludge, as indicated by the medium to high silver-grain coverage under nitrite-reducing conditions (Table 2). These probe-defined bacteria appeared to be inactive under anaerobic conditions without nitrite, with the exception of *Accumulibacter*, which showed some activity, as expected, as polyphosphate-accumulating organisms (Kong *et al.*, 2004). Some *Alphaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria* showed a similar activity under both nitrite-reducing and anaerobic conditions, indicating that some members from these classes are possible glycogen-accumulating organisms, fermenters, Fe(III) reducers, or denitrifiers. Indeed, denitrifiers from the *Alphaproteobacteria* and *Gammaproteobacteria* have been isolated from activated sludge (Heylen *et al.*, 2006) and found in clone libraries based on stable isotope probing under nitrate-reducing conditions (Osaka *et al.*, 2006), but their abundance remains to be established. Among the gammaproteobacterial glycogen-accumulating bacteria, some of the *Competibacter* may be denitrifiers (Kong *et al.*, 2006). *Firmicutes* displaying the highest activity under nitrite-reducing and anaerobic conditions are most likely displaying the high activity of fermenting bacteria known to be abundant in this phylum (Madigan *et al.*, 2006), but some denitrifiers may also be present in this group (Heylen *et al.*, 2006). *Chloroflexi*, *Planctomycetes*, and *Bacteroidetes* did not show denitrification capacity. To the authors' knowledge, no denitrifiers have been reported within these groups in activated sludge, except for one *Bacteroidetes* strain isolated recently (Heylen *et al.*, 2006). Anammox activity within *Planctomycetes* is ruled out because no Anammox activity or probe-defined bacteria have ever been detected in the sludge from the Aalborg East plant (J.L. Nielsen & P.H. Nielsen, unpublished results). The relatively high NCRs measured with oleate in both sludge types (Fig. 2) suggest the presence of additional denitrifiers because oleate is only utilized by *Thauera* of the known denitrifiers (Table 1). For instance, the filamentous bacterium *Candidatus* 'Microthrix parvicella' could contribute to nitrate CRs (Nielsen *et al.*, 2002), but not to nitrite CRs (Hesselsøe *et al.*, 2005).

An estimate of the active denitrifiers within each probe-defined group based on the fraction of MAR-positive cells under nitrite-reducing conditions (Table 2: *Alphaproteobacteria c.* 30–50%, *Betaproteobacteria c.* 30–50%, *Gammaproteobacteria c.* 30–50%, and *Actinobacteria c.* 5–10%) and their relative abundance in the sludge (Fig. 3b) suggests that the active denitrifying bacteria from *Alphaproteobacteria* (*c.* 5–9%), *Gammaproteobacteria* (*c.* 3–5%), and *Actinobacteria* (*c.* 1%) combined (9–15%) would be as important as *Betaproteobacteria* (*c.* 11–18%).

The combined denitrifying capacity of activated sludge revealed a microbial versatility with multiple phylogenetic groups involved. These denitrifying consortia constitute a dynamic ecosystem built upon the presence of a broad range of available substrates, with each group having specialized nutritional niches. Similar to what may occur in other environments, such diversity may stabilize the system upon external or internal changes. It is, however, relatively easy to enrich denitrifiers in low abundance with specific substrates, for example methanol in lab-scale reactors (Ginige *et al.*, 2004; Osaka *et al.*, 2006) and full-scale treatment plants (Hagman *et al.*, 2008). Interestingly, in the lab-scale reactors, bacteria related to *Methylophilaceae* and *Hyphomicrobiaceae* were dominant, whereas *Azoarcus* was strongly enriched in the full-scale plant and constituted 30% of the bacteria. This confirms the common observation that results from lab-scale reactors are difficult to extrapolate to full-scale plants. The polyphyletic distribution of the denitrifying microorganisms makes them well suited for further investigations on their survival strategies in a mixed community. The ecophysiological characterization of microorganisms responsible for biological nutrient removal is anticipated to be a helpful tool for process optimization, for example when determining the treatment feasibility of waste streams with a specific organic content, when assessing external substrates for enhancing denitrification, or when determining/applying denitrification rates for optimizing or modelling nutrient-removal processes.

Acknowledgements

This work was supported by a Postdoctoral Fellowship from the Natural Sciences and Engineering Research Council (NSERC) of Canada to F. M.-S. and by Aalborg University.

References

Almeida JS, Reis MAM & Carrondo MJT (1995) Competition between nitrate and nitrite reduction in denitrification by *Pseudomonas fluorescens*. *Biotechnol Bioeng* **46**: 476–484.

Amann RI (1995) *In situ* identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. *Molecular Microbial Ecology Manual* (Akkermans ADL, Elsas JDv & Bruijn FJd, eds), pp. 3.3.6/1–3.3.6/15. Kluwer Academic Publications, London.

Amann RI, Stromley J, Devereux R, Key R & Stahl DA (1992) Molecular and microscopic identification of sulfate-reducing

bacteria in multispecies biofilms. *Appl Environ Microbiol* **58**: 614–623.

Benes O, Spanjers H & Holba M (2002) Respirometry techniques and activated sludge models. *Water Sci Technol* **46**: 1–6.

Björnsson L, Hugenholtz P, Tyson GW & Backall LL (2002) Filamentous *Chloroflexi* (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal. *Microbiology* **148**: 2309–2318.

Blaszczyk M (1993) Effect of medium composition on the denitrification of nitrate by *Paracoccus denitrificans*. *Appl Environ Microbiol* **59**: 3951–3953.

Burow LC, Kong Y, Nielsen JL, Blackall LL & Nielsen PH (2007) Abundance and ecophysiology of *Defluviicoccus* spp., glycogen-accumulating organisms in full-scale wastewater treatment processes. *Microbiology* **153**: 178–185.

Crocetti GR, Hugenholtz P, Bond PL, Schuler A, Keller J, Jenkins D & Blackall LL (2000) Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Appl Environ Microbiol* **66**: 1175–1182.

Daims H, Brühl A, Amann R, Schleifer K-H & Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *System Appl Microbiol* **22**: 434–444.

Daims H, Nielsen PH, Nielsen JL, Juretschko S & Wagner M (2000) Novel *Nitrospira*-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: diversity and *in situ* physiology. *Wat Sci Technol* **41**: 85–90.

Dionisi D, Renzi V, Majone M, Beccari M & Ramadori R (2004) Storage of substrate mixtures by activated sludges under dynamic conditions in anoxic or aerobic environments. *Water Res* **38**: 2196–2206.

Dircks K, Pind PF, Mosbæk H & Henze M (1999) Yield determination by respirometry – the possible influence of storage under aerobic conditions in activated sludge. *Water SA* **25**: 69–74.

Dold PL & Barker PS (1996) Denitrification behaviour in biological excess phosphorus removal activated sludge systems. *Water Res* **30**: 769–780.

Eilersen AM, Henze M & Kløft L (1995) Effect of volatile fatty acids and trimethylamine on denitrification in activated sludge. *Water Res* **29**: 1259–1266.

Ekama GA & Wentzel MC (1999) Denitrification kinetics in biological N and P removal activated sludge systems treating municipal wastewaters. *Water Sci Technol* **39**: 69–77.

Gerber A, Mostert ES, Winter CT & de Villiers RH (1987) Interactions between phosphate, nitrate and organic substrate in biological nutrient removal processes. *Water Sci Technol* **19**: 183–194.

Gich F, Garcia-Gil J & Overmann J (2001) Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. *Arch Microbiol* **177**: 1–10.

Ginige MP, Hugenholtz P, Daims H, Wagner M, Keller J & Blackall LL (2004) Use of stable-isotope probing, full-cycle rRNA analysis, and fluorescence *in situ* hybridization-microautoradiography to study a methanol-fed denitrifying microbial community. *Appl Environ Microbiol* **70**: 588–596.

Ginige MP, Keller J & Blackall LL (2005) Investigation of an acetate-fed denitrifying microbial community by stable isotope probing, full-cycle rRNA analysis, and fluorescent *in situ* hybridization-microautoradiography. *Appl Environ Microbiol* **71**: 8683–8691.

Hagman M (2003) COD degradation and the impact on population dynamics in activated sludge – Applications of OUR measurements. Licentiate Thesis, Lund Institute of Technology/Lund University, Lund, Sweden.

Hagman M, Nielsen JL, Nielsen PH & Jansen JIC (2008) Mixed carbon sources for nitrate reduction in activated sludge-identification of bacteria and process activity studies. *Water Res* **42**: 1539–1546.

Hallin S & Pell M (1994) Acetylene inhibition for measuring denitrification rates in activated sludge. *Water Sci Technol* **30**: 161–167.

Hallin S & Pell M (1998) Metabolic properties of denitrifying bacteria adapting to methanol and ethanol in activated sludge. *Water Res* **32**: 13–18.

Hallin S, Throbäck IN, Dicksved J & Pell M (2006) Metabolic profiles and genetic diversity of denitrifying communities in activated sludge after addition of methanol and ethanol. *Appl Environ Microbiol* **72**: 5445–5452.

Hansson B & Gunnarson L (1990) The use of industrial by-products as a carbon source for denitrification. *Chemical Water and Wastewater Treatment* (Hahn HH & Klute R, eds), pp. 531–541. Springer-Verlag, Berlin.

Hasselblad S & Hallin S (1998) Intermittent addition of external carbon to enhance denitrification in activated sludge. *Water Sci Technol* **37**: 227–233.

Henze M, Kristensen GH & Strube R (1994) Rate-capacity characterization of wastewater for nutrient removal processes. *Water Sci Technol* **29**: 101–107.

Henze M, Harremoës P, Jansen JlC & Arvin E (2002) *Wastewater Treatment: Biological and Chemical Processes*. Springer-Verlag, Berlin.

Hess A, Zarda B, Hahn D, Häner A, Stax D, Höhener P & Zeyer J (1997) *In situ* analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. *Appl Environ Microbiol* **63**: 2136–2141.

Hesselsøe M, Nielsen JL, Roslev P & Nielsen PH (2005) Isotope labeling and microautoradiography of active heterotrophic bacteria on the basis of assimilation of $^{14}CO_2$. *Appl Environ Microbiol* **71**: 646–655.

Heylen K, Vanparys B, Wittebolle L, Verstraete W, Boon N & De Vos P (2006) Cultivation of denitrifying bacteria: optimization of isolation conditions and diversity study. *Appl Environ Microbiol* **72**: 2637–2643.

Isaacs SH, Henze M, Søeberg H & Kümmel M (1994) External carbon source addition as a means to control an activated sludge nutrient removal process. *Water Res* **28**: 511–520.

Juretschko S, Loy A, Lehner A & Wagner M (2002) The microbial community composition of a nitrifying-denitrifying activated sludge from and industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *System Appl Microbiol* **25**: 84–99.

Kong Y, Nielsen JL & Nielsen PH (2004) Microautoradiography study of *Rhodocyclus*-related polyphosphate-accumulating bacteria in full-scale enhances biological phosphorus removal plants. *Appl Environ Microbiol* **70**: 5383–5390.

Kong Y, Xia Y, Nielsen JL & Nielsen PH (2006) Ecophysiology of a group of uncultured Gammaproteobacterial glycogen-accumulating organisms in full-scale enhanced biological phosphorus removal wastewater treatment plants. *Environ Microbiol* **8**: 479–489.

Kristensen GH, Jørgensen PE & Henze M (1992) Characterization of functional microorganism groups and substrate in activated sludge and wastewater by AUR, NUR and OUR. *Water Sci Technol* **25**: 43–57.

Lajoie CA, Layton AC, Gregory IR, Sayler GS, Taylor DE & Meyers AJ (2000) Zoogleal clusters and sludge dewatering potential in an industrial activated sludge wastewater treatment plant. *Water Environ Res* **72**: 56–64.

Larsen LH, Damgaard LR, Kjær T, Stenstrøm T, Lyngaard-Jensen A & Revsbech NP (2000) Fast responding biosensor for on-line determination of nitrate/nitrite in activated sludge. *Water Res* **34**: 2463–2468.

Lee NM & Welander T (1996) The effect of different carbon sources on respiratory denitrification in biological wastewater treatment. *J Ferment Bioeng* **82**: 277–285.

Lee S III, Koopman B, Park S-K & Cadee K (1995) Effect of fermented wastes on denitrification in activated sludge. *Water Environ Res* **67**: 1119–1122.

Lorenzen J, Larsen LH, Kjær T & Revsbech NP (1998) Biosensor determination of the microscale distribution of nitrate, nitrite assimilation, nitrification, and denitrification in a diatom-inhabited freshwater sediment. *Appl Environ Microbiol* **64**: 3264–3269.

Loy A, Horn M & Wagner M (2003) probeBase: an online resource for rRNA-targeted oligonucleotide probes. *Nucleic Acids Res* **31**: 514–516.

Madigan MT, Martinko JM & Parker J (2006) *Brock Biology of Microorganisms*. Pearson Prentice Hall.

Manz W, Amann R, Ludwig W, Wagner M & Schleifer K-H (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. *System Appl Microbiol* **15**: 593–600.

Manz W, Amann R, Ludwig W, Vancanneyt M & Schleifer K-H (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga–Flavobacter–Bacteroides* in the natural environment. *Microbiology* **142**: 1097–1106.

Marchetto M, Gianotti EP, Campos JR, Pires RC & Moraes ED (2003) Estimate of denitrifying microbiota in tertiary sewage treatment and kinetics of the denitrification process using different sources of carbon. *Braz J Microbiol* **34**: 104–110.

McMurray SH, Meyer RL, Zeng RJ, Yuan Z & Keller J (2004) Integration of titrimetric measurement, off-gas analysis and NO_x^- biosensors to investigate the complexity of denitrification processes. *Water Sci Technol* **50**: 135–141.

Meier H, Amann R, Ludwig W & Schleifer K-H (1999) Specific oligonucleotide probes for *in situ* detection of a major group of Gram-positive bacteria with low DNA G+C content. *System Appl Microbiol* **22**: 186–196.

Meinhold J, Arnold E & Isaacs S (1999) Effect of nitrite on anoxic phosphate uptake in biological phosphorus removal activated sludge. *Water Res* **33**: 1871–1883.

Metcalf & Eddy Inc. (2003) *Wastewater Engineering: Treatment and Reuse*, 4th edn. McGraw-Hill, New York.

Monteith HD, Bridle TR & Sutton PM (1980) Industrial waste carbon sources for biological denitrification. *Prog Water Technol* **12**: 127–141.

Morgan-Sagastume F, Larsen P, Nielsen JL & Nielsen PH (2008) Characterization of the loosely attached fraction of activated sludge bacteria. *Water Res* **42**: 843–854.

Naidoo V, Urbain V & Buckley CA (1998) Characterization of wastewater and activated sludge from European municipal wastewater treatment plants using the NUR test. *Water Sci Technol* **38**: 303–310.

Neef A (1997) Awendung der *in situ*-Einzelzell-Identifizierung von Bakterien zur Popultionsanalyse in komplexen mikrobiellen Biozönosen. PhD Thesis, Technische Universität München, Munich.

Neef A, Amann R, Schlesner H & Schleifer K-H (1998) Monitoring a widespread bacterial group: *in situ* detection of *Planctomycetes* with 16S rRNA-targeted probes. *Microbiology* 3257–3266.

Nielsen JL & Nielsen PH (2002) Enumeration of acetate-consuming bacteria by microautoradiography under oxygen and nitrate respiring conditions in activated sludge. *Water Res* **36**: 421–428.

Nielsen JL & Nielsen PH (2005) Advances in microscopy: microautoradiography of single cells. *Methods Enzymol* **397**: 237–256.

Nielsen M, Larsen LH, Jetten MSM & Revsbech NP (2004) Bacterium-based NO_2^- biosensor for environmental applications. *Appl Environ Microbiol* **70**: 6551–6558.

Nielsen PH (2002) Activated sludge – the floc. *Encyclopedia of Environmental Microbiology* (Bitton G, ed), pp. 54–61. John Wiley & Sons Inc., Chichester.

Nielsen PH, Roslev P, Dueholm TE & Nielsen JL (2002) *Microthrix parvicella*, a specialized lipid consumer in anaerobic-aerobic activated sludge plants. *Water Sci Technol* **46**: 73–80.

Nyberg U, Andersson B & Aspergen H (1996) Long-term experiences with external carbon sources for nitrogen removal. *Water Sci Technol* **33**: 109–116.

Oh J & Silverstein J (1999) Acetate limitation and nitrite accumulation during denitrification. *J Environ Eng-ASCE* **125**: 234–242.

Osaka T, Yoshie S, Tsuneda S, Hirata A, Iwami N & Inamori Y (2006) Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing. *Microb Ecol* **52**: 253–266.

Rabus R, Fukui M, Wilkes H & Widdel F (1996) Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. *Appl Environ Microbiol* **62**: 3605–3613.

Roller C, Wagner M, Amann R, Ludwig W & Schleifer K-H (1994) *In situ* probing of gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. *Microbiology* **140**: 2849–2858.

Sin G & Vanrolleghem PA (2004) A nitrate biosensor based methodology for monitoring anoxic activated sludge activity. *Water Sci Technol* **50**: 125–133.

Thomsen TR, Nielsen JL, Ramsing NB & Nielsen PH (2004) Micromanipulation and further identification of FISH-labelled microcolonies of a dominant denitrifying bacterium in activated sludge. *Environ Microbiol* **6**: 470–479.

Thomsen TR, Kong Y & Nielsen PH (2007) Ecophysiology of dominant denitrifying bacteria in activated sludge. *FEMS Microbiol Ecol* **60**: 370–382.

van Rijn J, Tal Y & Barak Y (1996) Influence of volatile fatty acids on nitrite accumulation by a *Pseudomonas stutzeri* strain isolated from a denitrifying fluidized bed reactor. *Appl Environ Microbiol* **62**: 2615–2620.

Weon S-Y, Lee C-W, Lee S-I & Koopman B (2002) Nitrite inhibition of aerobic growth of *Acinetobacter* sp. *Water Res* **36**: 4471–4476.

Wilderer PA, Jones WL & Dau U (1987) Competition in denitrification systems affecting reduction rate and accumulation of nitrite. *Water Res* **21**: 239–245.

Zeng RJ, Saunders AM, Yuan Z, Blackall LL & Keller J (2003) Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms. *Biotechnol Bioeng* **83**: 140–148.