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Process optimization by decoupled control of key microbial populations: Distribution of activity and abundance of polyphosphate-accumulating organisms and nitrifying populations in a full-scale IFAS-EBPR plant

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ABSTRACT

This study investigated the abundance and distribution of key functional microbial populations and their activities in a full-scale integrated fixed film activated sludge–enhanced biological phosphorus removal (IFAS-EBPR) process. Polyphosphate accumulating organisms (PAOs) including *Accumulibacter* and EBPR activities were predominately associated with the mixed liquor (>90%) whereas nitrifying populations and nitrification activity resided mostly (>70%) on the carrier media. Ammonia oxidizer bacteria (AOB) were members of the *Nitrosomonas europaea/eutropha/halophila* and the *Nitrosomonas oligotropha* lineages, while nitrite oxidizer bacteria (NOB) belonged to the *Nitrospira* genus. Addition of the carrier media in the hybrid activated sludge system increased the nitrification capacity and stability; this effect was much greater in the first IFAS stage than in the second one where the residual ammonia concentration becomes limiting. Our results show that IFAS-EBPR systems enable decoupling of solid residence time (SRT) control for nitrifiers and PAOs that require or prefer conflicting SRT values (e.g. >15 days required for nitrifiers and <5 days preferred for PAOs). Allowing the slow-growing nitrifiers to attach to the carrier media and the faster-growing phosphorus (P)-removing organisms (and other heterotrophs, e.g. denitrifiers) to be in the suspended mixed liquor (ML), the EBPR-IFAS system facilitates separate SRT controls and overall optimization for both N and P removal processes.

1. Introduction

The increasingly stringent limits imposed on nitrogen (N) and phosphorus (P) discharge in wastewater effluents demand for more reliable and better optimization of Biological Nutrient Removal (BNR) processes that target for simultaneous N and P removal. Efficient and reliable N removal normally requires relatively long solid residence time (SRT > 8–15 days) for nitrification process and sufficient carbon source for denitrification process. Fixed film systems such as Integrated Fixed-Film Activated Sludge (IFAS) or moving bed biofilm reactors (MBBR) have been shown to be successful for the enhancement of nitrification and denitrification in BNR system upgrade (Azimi et al., 2007; Christensson and Welander, 2004; Ødegaard,

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2006; Onnis-Hayden et al., 2007; Sen et al., 1994). IFAS allows for decoupling of the growth rate of nitrifying populations and the suspended mixed liquor phase SRT (MLSRT) and, it provides higher treatment capacity with smaller footprint due to increased solids inventory on the carrier media. In addition, less waste sludge production and improvement in robustness and resistance to load variations were observed (Maas, 2007). These advantages make fixed-film systems such as IFAS or MBBR preferable for N removal.

As for phosphorus removal in a BNR process, one main challenge remaining with the enhanced biological phosphors removal (EBPR), is how to improve its reliability and stability since many EBPR plants experience unpredicted upsets and performance fluctuations (Neethling et al., 2005; Gu et al., 2008). Among the identified factors that affect the stability of EBPR process, maintaining conditions favoring the proliferation of polyphosphate accumulating organisms (PAOs) over Glycogen Accumulating Organisms (GAOs) is critical (Gu et al., 2008; Christensson et al., 1998). Shorter SRT (<3 days), higher pH (>7.25) and certain substrates (e.g. propionate) and feeding strategy seem to favor PAOs over GAOs (Filipe et al., 2001; Oehmen et al., 2005; Rodrigo et al., 1996; Whang and Park, 2006). The possibility of incorporating IFAS into an EBPR process has been recently explored by a few researchers at pilot scale (Christensson and Welander, 2004; Sriwiriyarat and Randall, 2005 and Kim et al., 2010). Effective N and P removal at a full-scale IFAS-BNR plant in Broomfield, Colorado has been recently reported (Onnis-Hayden et al., 2007; Rogalla et al., 2006). These limited number of studies demonstrated the potential of IFAS-EBPR for simultaneous N and P removal, although detailed microbial populations analysis was not carried out in any of these studies. One unrecognized and therefore not fully-investigated advantage of an IFAS-EBPR system is that it potentially enables separate SRT control for the slower-growing nitrifiers and the faster-growing heterotrophs including PAOs and denitrifiers, by allowing the former to attach to the carrier media and the latter to be in the suspended mixed liquor (ML). This hypothesis is based on the understanding that nitrifiers usually prefer to reside on fixed-film carrier media, whereas PAOs and denitrifiers (some denitrifiers may be PAOs) would mostly reside in the circulating mixed liquor because proliferation of PAOs requires alternating anaerobic and aerobic/anoxic conditions as provided by the circulating mixed liquor. This decoupling ability is desirable in full-scale practice since the decoupling and separate SRTs controls of key functionally relevant populations allow for simultaneous optimization for both N and P removal processes. To evaluate the validity of this hypothesis, we conducted and reported for the first time a comprehensive and integrated evaluation of the PAO populations and P removal performance, as well as nitrifying populations (ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB)) and nitrification activity at a full-scale IFAS-EBPR system. The PAOs, AOB and NOB population abundance and distribution, the N and P removal activities and their distribution on the biofilm (carrier media) versus that in the suspended biomass in the IFAS-EBPR system were evaluated. The implication of the results on the IFAS-EBPR process modeling, design and operation were discussed.

2. Material and methods

2.1. Full scale IFAS-EBPR process

Broomfield Wastewater Treatment Plant (WWTP) located in Denver, Colorado is one of the few full-scale municipal wastewater treatment plants that were designed as an IFAS-BNR process in the United States (Fig. 1A). The secondary treatment process consists of a pre-anoxic zone, an anaerobic and an anoxic stage followed by two-staged IFAS aeration basins in series that contain AnoxKaldnes K1 carrier media (see Fig. 1A). K1 media is made of high-density polyethylene (density 0.95 g/cm^3) with an effective surface area of 500 m^2 per m^3 and, the water volume displaced by the carrier media is about 6.4% with a volumetric filling of 30%. Current permit requires monthly average effluent NH_4–N <5 mg/l. Although there is no P limit, the plant has an internal target of effluent TP < 1 mg/l. Operational conditions at the plant for the period of the study are reported in Table 1.

2.2. Batch tests for evaluation of EBPR activity and distribution among different forms of biomass

To investigate the level and distribution of PAOs activities between the suspended mixed liquor (ML) and the biofilm media in the IFAS-EBPR processes, batch P uptake and release tests (in triplicates) were carried out with different forms of biomass drawn from aerobic stage 1 and stage 2 IFAS basins (Fig. 1A), including suspended mixed liquor (ML) alone, combination of ML and carrier media (about 30% fill in mixed liquor) and carrier media alone (30% fill in media solution). The tests were conducted on site to eliminate the potential impact of biomass transport (e.g. at least overnight) on the tests results. Details of the P uptake and release tests can be found elsewhere (Gu et al., 2008). Briefly, sodium acetate was added (80 mg/l as acetate) at the beginning of the anaerobic conditions (maintained with nitrogen gas purging for 45 min), then aerobic phase was maintained for 3 h by supplying air flow to maintain a DO level of 4–5 mg/l. Samples were collected at 15 min intervals for about 3 h, immediately filtered through two series filtration (100 μm then 0.45 μm) before they were analyzed for soluble ortho-P, volatile fatty acid (VFA), COD, NO_2–N and NO_3–N. Dissolved oxygen (DO), pH, temperature and Oxidation Reduction Potential (ORP) were continuously recorded. pH was controlled at values measured in the aeration basin (7–7.3) and the temperature was maintained at 20 ± 0.5 °C. MLSS and MLVSS analysis for ML were conducted at the end of each test. Determination of all parameters were performed according to standard methods (SM 4110 for anions, SM 5220 for COD, SM 5560 for VFA, SM 4500-H^+·B for pH, SM 4500-O·G for DO, SM 2580 for ORP and SM 2540 for MLSS and MLVSS, APHA, 2005). Determination of the total solids (TS) attached to the carrier media was performed according to the procedure suggested by the manufacturer (Anox-Kaldnes, Inc. Sweden). Briefly, 10–20 media carriers were dried in a 105 °C oven and the weights were measured before and after a sulfuric acid treatment, which removes the biomass attached to the carrier media. Biomass on media was then derived from the difference in the weights.

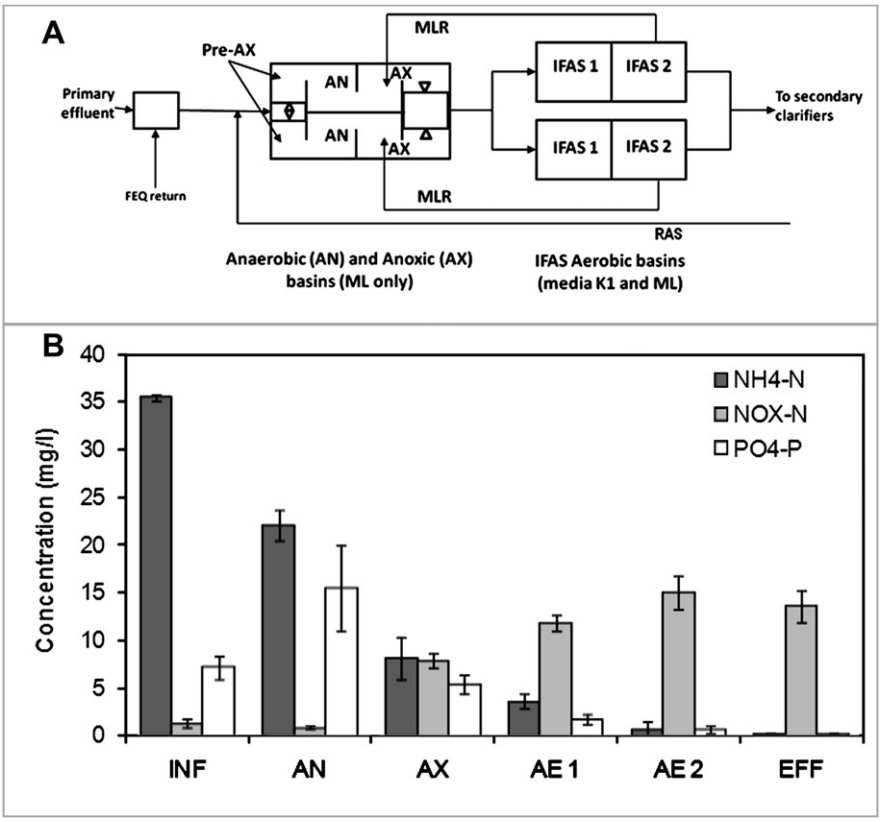

Fig. 1 – (A) Process schematic of the Broomfield WWTP in Denver, Colorado (FEQ: flow equalization return); (B) profiles of nitrogen and phosphorus species along the BNR process at the Broomfield plant, concentration in respect to influent flow (average of three days, the vertical bars represent the standard deviations). INF: influent; AN: anaerobic; AX: anoxic; AE1: IFAS stage 1; AE2: IFAS stage 2; EFF: effluent.

2.3. *Batch tests for evaluation of nitrification activities and distribution among different forms of biomass*

To determine the nitrification rates, batch tests (in duplicate) were conducted on site at the Broomfield WWTP for sludge samples taken from both aerobic IFAS stage 1 and stage 2 (Fig. 1A). Samples were transferred in the 2-L beaker, aerated to obtain a dissolved oxygen (DO) concentration similar to the one in the aeration basin (4 mg/l) and sodium bicarbonate (alkalinity of 200 mg/L as $CaCO_3$) and ammonium chloride (20 mg NH_4–N/L) were added to the reactor. Subsequently samples were collected at 15-min intervals for about 3 h, immediately filtered through two series filtration (100 μm then 0.45 μm) and then analyzed for NH_4–N, NO_2–N and NO_3–N. Dissolved oxygen (DO), Temperature, pH, and Oxidation-Reduction Potential (ORP) were continuously recorded. Analytical methods used for the above mentioned parameters are the same as previously described. At the end of the test, MLTSS, MLVSS and TS attached to the carrier media were also analyzed as previously described.

Table 1 – Operating conditions at the Broomfield WWTP during the period of study. Data are based on those collected for 5 years of operation (frequency of measurements: daily for most parameters, once a week for MLSRT).

Parameter	Value range [average]
Flow rate (m^3/s)	0.11–0.39 [0.2]
Secondary influent bCOD (mg/l)[a]	43–361 [177]
Influent TP (mgP/l)	4–16.5 [8.5]
Influent ammonia (mgN/l)	17–66 [35.2]
Influent NO_x (mgN/l)	0–15.4 [6]
MLSRT (days)	2.6–5.6 [3.78]
Recycle flows in relative to the influent flow	RAS 40% MLR 160%
Temperature [°C]	13–22 [17.4]
C/P (mg bCOD/mgP) [b]	8.4–30[21] [17.5][b]

a bCOD = 1.6 × BOD_5.
b Average C/P available to PAOs, the value is corrected considering the presence of NO_x in influent.

2.4. *Identification and quantification of candidate PAOs and GAOs, AOB and NOB*

Observation and quantification of candidate PAOs and GAOs residing in biomass from suspended mixed liquor and in biomass from scraped biofilm on the carrier media were investigated by Neisser, PHB and DAPI staining (Jenkins et al., 1993; Streichan et al., 1990), as well as by fluorescence in situ hybridization (FISH) targeting known PAOs and GAOs (see FISH probes in Table S1, supplementary information). The FISH protocol and hybridization conditions used were

previously described (He et al., 2008; Zilles et al., 2002). Large sample aggregates were avoided by mild sonication (5W, 1 min) and samples were homogenized passing them through a 27 gage syringe needle for 10–20 times. For the determination of PAO fraction, intracellular polyP was visualized by incubation with 1 ug/mL of 4′,6-Diamidino-2-phenylindole (DAPI) for 60 min (Zilles et al., 2002). Under these conditions, cells containing a large amount of polyP are stained bright yellow while the rest of the cells are blue. The fractions of PAOs (yellow) were determined as the percentage of the total cells (blue + yellow). On separate slides, *Accumulibacter*-related organisms were detected by 16S rRNA-targeted fluorescent FISH. After hybridization, the slide was counterstained with 1 μg/mL of DAPI solution for 3 min to quantify total cells to allow the estimation of the fraction of *Accumulibacter* expressed as the percentage of the total cells. For some selected slides, combined DAPI and FISH were performed to visualize the overlay of Poly-P staining and *Accumulibacter*-type FISH results, with the aim to observe the involvement of *Accumulibacter*-type PAOs in EBPR, and these results, however, were not used for quantification of *Accumulibacter* fraction.

Although nitrifying bacteria are found in at least 6 different phylogenetic groups, only three major groups (AOB within *Betaproteobacteria* and NOB of the genera *Nitrospira* and *Nitrobacter*) are to be expected in wastewater treatment. Therefore, probes specific for these groups were chosen, and additional probes were used to resolve the various lineages within the genus *Nitrosomonas* (Supplementary Table S1). Initial PCR-based analysis had revealed the absence of ammonia-oxidizing Archaea (AOA) in the system (data not shown). Betaproteobacterial AOB, and NOB of the genus *Nitrobacter and Nitrospira* were analyzed in paraformaldehyde (4%)-fixed and homogenized samples by FISH with a suite of published probes according to standard protocols (Pernthaler et al., 2001). Hybridized cells were observed with an epifluorescent microscope (Zeiss Axioplan 2, Zeiss, Oberkochen, Germany). Quantifications of population distributions were carried out using the software DAIME (Daims et al., 2006). Around 20–25 separate randomly chosen images were evaluated with final results reflecting the cumulative biovolumetric fractions of *Accumulibacter*, *Competibacter*, total PAOs, AOB and NOB present in the corresponding samples. Microbial population fractions were expressed as percentage of EUB or DAPI stained cells.

3. Results and discussion

3.1. *Effective P and N removals in the IFAS-EBPR system*

The IFAS system at Broomfield has performed very well consistently over the past 5 years and monthly average data for influent and effluent nitrogen and phosphorus species are presented in Figure S1. The effluent ammonia concentration averaged 0.37 ± 0.5 mg/l, indicating efficient and complete nitrification, even during the winter months at low temperature of 11–15 °C at a relatively short MLSRT of 3.5–4 d. This demonstrates the advantage of IFAS system that can retain the majority of nitrifiers on the biofilm media, provides high treatment capacity and improves stability at low temperatures. The variation in the effluent TN reflects the seasonal varying requirements for denitrification at the plant.

The average effluent ortho-P for the five years of operation was found to be 0.87 ± 0.62 mgP/l and the average TP was 1.2 ± 0.78 mg/l, indicating good P removal at the plant with influent TP varying from 6 to 21.7 mg/l (Figure S1). One point worthy of mentioning is that this plant has relatively high nitrate and nitrite level in the influent (average influent NO_3–N and NO_2–N at the plant were 3.1 mg/l and 2.92 mg/l, respectively). A pre-anoxic zone was included in the plant design to reduce the introduction of nitrate to the following anaerobic stage; however, an average of 0.9 mgNO_3–N/l and 7.83 NO_3–Nmg/l was measured in the anaerobic zone and in the anoxic zone during the time of this study (see Fig. 1B). Analysis of the samples taken in the different zones at the plant indicates P release occurring in the "anaerobic" zone despite the presence of nitrate (see Fig. 1B). Several studies have shown that nitrate has a negative effect on the EBPR performance during the anaerobic phase, due to the competition for carbon between the denitrifying population and the PAOs (Lopez-Vazquez et al., 2008; Yagci et al., 2003), and possible inhibitory effect of nitric oxide produced during denitrification (Van Niel et al., 1998). Therefore, in case where there is nitrate being introduced into the anaerobic zone, sufficient carbon source to satisfy the denitrifiers and ensure a true anaerobic carbon-rich zone is considered to be required for effective EBPR. It is interesting that effective P removal was achieved at Broomfield plant despite the relatively lower COD/P (mg/mg) ratio (17.5, see Table 1) than recommended (C/P > 20–25), (Gu et al., 2008; Randall et al., 1992) and the lack of a true anaerobic zone. The much shorter MLSRT (3.5–4 days) at this plant compared to that for a typical suspended BNR system (10–15 days) might favor the PAOs over GAOs as we hypothesized and it warrants further investigation.

3.2. *Distribution of PAOs between the suspended biomass and biofilm on media*

Table 2 summarizes the abundance and distribution of PAOs associated with different forms/portions of the biomass in the IFAS-EBPR system. Most PAOs were found to be associated with suspended mixed liquor biomass. In contrast to the intuitive expectation that PAOs may only reside in the suspended mixed liquor that is exposed to alternating anaerobic and aerobic condition in an IFAS system, some PAOs were also found in biofilm biomass scraped from carrier media obtained from the aerobic zones in the IFAS-EBPR system. The abundance of cells containing poly-P granules in the media biofilm biomass, however, was much less than that observed in the suspended mixed liquor biomass. The relative abundance of PAOs in the mixed liquor and in biofilm biomass was estimated to be 20–30% and 3–8% of total bacterial cells, respectively.

FISH was used to visualize and enumerate *Accumulibacter*-related organisms in mixed liquor sludge and in biofilm scraped from the IFAS carrier media (Fig. 2A and B). *Accumulibacter*-like PAOs accounted for 15.8 ± 1.4% of the total bacterial population in the mixed liquor sample, whereas they represented less than 4 ± 1% of the total bacterial population in the biofilm sample. The abundance of *Accumulibacter* in ML is comparable to that observed in conventional EBPR plants in the range of 9–24% of total bacterial population (He et al.,

Table 2 – PAOs and EBPR activity distribution among different biomass fractions at the Broomfield WWTP.

Biomass fraction	Population distribution		EBPR activity	
	PAOs fractions [%][a]	Accumulibacter fraction [%][a]	P-release/P-uptake rate (stage 1)[b]	Contribution to overall EBPR activity [%][c]
Suspended biomass (ML)	25 ± 5	15.8 ± 1.4	12.2/3.8 [mgP/gMLVSS/h] 10.1/3.3 [mgP/gMLSS/h]	96.5%
Attached biomass (Media)	5 ± 3	4 ± 1	0.09 [gP/m^2/d] 0.49 [mgP/gTS/h]	3.5%

a The numbers represent the percentages of the total bacterial population in the respective sample, determined from the cumulative area identified by quantitative image analysis (Daime) ± the standard deviation.
b The numbers represent the average rates for stage 1.
c The % of EBPR activity was calculated considering the fraction of EBPR associated with a specific biomass, respect to the overall EBPR activity of the system.

2008; Kong et al., 2004; Gu et al., 2008). Overlay of DAPI and FISH showed that the *Accumulibacter*-related organisms and other type of PAOs attached to the IFAS carrier media contained poly-P granules (data not shown), therefore may have been active in EBPR. However, it is difficult to determine whether these PAOs were growing on the IFAS carrier media or simply adhered to the biofilm via contact and exchange with those in the mixed liquor.

3.3. *Distribution of PAOs activities between the suspended biomass and the biofilm on media*

To further evaluate the EBPR activities and contributions of PAOs residing on the biofilm or in the mixed liquor, a series of P release and uptake batch tests were conducted with different forms of biomass in the IFAS-EBPR systems, including suspended mixed liquor (ML), biofilm on the carrier media (media) and combination of suspended mixed liquor and media (ML + media). Fig. 3A shows an example of the P profiles obtained during three batch tests with different forms of biomass from the aerobic stage 1 at the Broomfield WWTP. The P release amount in the test with media + ML was slightly higher than that with ML alone, indicating some level of EBPR activity associated with the carrier media. However, for the test with biofilm media alone, there was no trend of P uptake and release as for EBPR process, instead, there was a slight and continues P release during the test at a rate of 0.61 mgP/L/h. The reason for the absence of P uptake during the test is unclear, however, one possible explanation is that the COD dosed was left unconsumed at the end of the anaerobic phase due to the very low EBPR and denitrifying activities associated with the biofilm, which inhibited the P uptake due to competition for oxygen and/or carbon between the PAOs and other heterotrophs in the biofilm.

Previous studies have demonstrated that EBPR activity can occur within fixed-film provided with alternating anaerobic/aerobic conditions (Goncalves and Rogalla, 2000; Helness and Odegaard, 1999). In a full-scale system, often there is diurnal fluctuation (such as diurnal changes in the influent COD and NH_4–N loadings) that may cause micro-scale local and periodical alternating aerobic or anaerobic condition due to DO level and diffusion depth variations, which may allow for growth of PAOs within the biofilm. However, we believe that the presence of a rather low relative abundance of PAOs in the biofilm is most likely due to attachment and detachment exchange of biomass between the mixed liquor and the biofilm because similar observations were found in our lab-scale IFAS-EBPR process that had rather consistent loading conditions (data not shown). Further investigation is therefore needed to better understand the exchange and interaction of

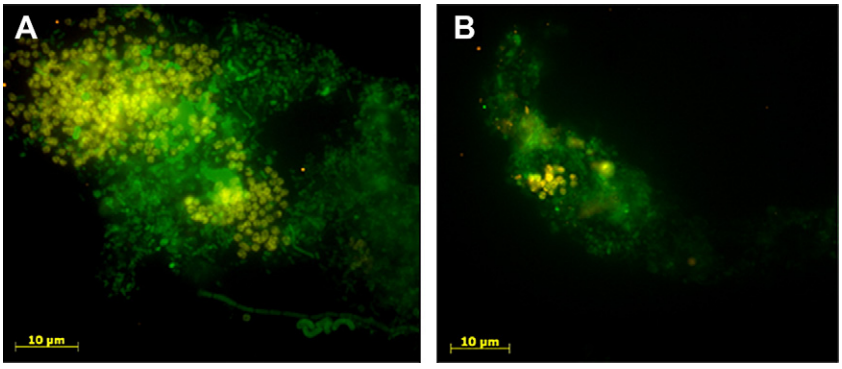

Fig. 2 – FISH Micrographs for samples from IFAS stage #1 of the Broomfield WWTP. (A) Suspended mixed liquor and (B) biofilm from carrier media; the samples were hybridized with Cy3-labeled PAO-mix probe and FAM-labeled EUB probe. *Accumulibacter* are shown in yellow and all other bacteria are shown in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

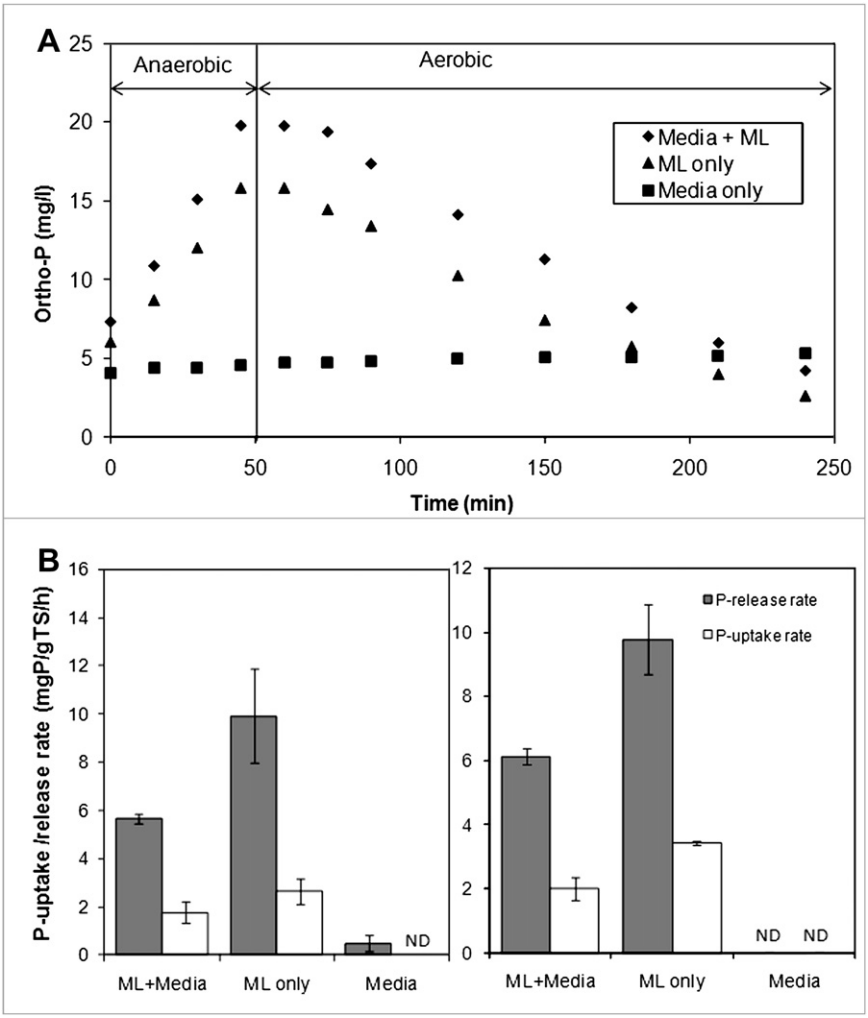

Fig. 3 – (A) Comparison of soluble ortho-P profiles during the anaerobic (AN) P release and aerobic (AE) P uptake batch testing among three different forms of biomass from IFAS aerobic stage 1 at the Broomfield WWTP. (B) P release and uptake rates for various forms of biomass samples (media plus mixed liquor (ML), mixed liquor only (ML) and media only) from IFAS at the Broomfield WWTP for stage 1 (left), and stage 2 (right). Represented are average values of the triplicate tests, while the vertical bars represent the corresponding standard deviations.

microbial cells (e.g. PAOs) in the biofilm with those in the mixed liquor in an IFAS-EBPR process.

Table 2 summarizes the results of the EBPR activity for the different biomass fractions, for stage 1. The results were consistent with the population abundance observed, therefore supporting the hypothesis that most PAOs activity is associated with the suspended biomass.

Over 96% of the EBPR activity was indeed associated with the mixed liquor, where the majority of PAOs were found, but only a very small percentage (less than 4%) of EBPR activity was associated with the biofilm. The aerobic P uptake rates and anaerobic P release rates with the mixed liquor biomass of stage 1 were 3.9 ± 0.43 and 12.1 ± 2.1 mgP/gVSS/h, respectively, which were comparable to the values found in other studies for full-scale EBPR plants (Gu et al., 2008; Neethling et al., 2005; Lopez-Vazquez et al., 2008). Similar values were also obtained, as expected, for tests using ML from stage 2 (Fig. 3B).

3.4. *Distribution of nitrifying microbial populations in the IFAS-EBPR system*

Abundance of AOB and NOB on carrier media and those in mixed liquor were determined for biomass from aerobic stage 1. Table 3 summarizes the abundance of AOB and NOB estimated for various fractions of biomass in the IFAS-BNR system, as well as the nitrification activities obtained for those fractions. The combined results clearly demonstrate that nitrification is mainly associated with the biofilm attached to the carrier media, where nitrifiers can be maintained even at lower MLSRT and temperature. FISH analysis of biomass scraped off the carrier media showed heterogeneous distribution of AOB and NOB: in some patches (most likely from the anoxic deeper layers of the biofilm) nitrifiers were nearly absent while other areas (most likely from the oxic, nitrifying surface) were densely colonized with AOB and NOB

(Fig. 4). AOB were identified as members of the *N. europaea/eutropha/halophila* and the *N. oligotropha* lineages based on an hierarchical set of probes (BET42a, NSO1225, NEU23a and Nmo218). These lineages have been previously found in sequencing batch biofilm reactors (Gieseke et al., 2001) and other systems with high ammonium/high salt environments (Juretschko et al., 1998; Koops et al., 2003) and highly fluctuating conditions (especially oxic/anoxic cycles). None of the other five probes targeting betaproteobacterial AOB (NSV443, NSE1472, NmV, NmIV, NmII) yielded any positive results, therefore indicating that the AOB community of the biofilm consists entirely of the two populations mentioned above.

In the presumably oxic, nitrifying part of the biofilm, both AOB together accounted for less than 10% of the total population. In the mixed liquor only few single cells or small aggregates of AOB were detected, most likely originating from the biofilm by detachment.

NOB were rather abundant in the presumably oxic, nitrifying part of the biofilm (10–20% of the total population), forming typical cell clusters with extremely small cells (<1 μm) of *Nitrospira* sp., as identified by probes Ntspa712 and Ntspa662 (Fig. 4). *Nitrobacter* sp. was not detected. Such high abundance of *Nitrospira* sp. has been previously reported from sequencing batch biofilm reactors (Gieseke et al., 2001; Schramm, 2003). Similar to AOB, only few NOB were detected in the mixed liquor, mostly as very small aggregates, again indicating that they originated from the biofilm by detachment.

3.5. *Distribution of nitrification activity between the suspended biomass and the biofilm on media*

Nitrification activities associated with various forms of biomass in the IFAS-BNR system including the mixed liquor, the carrier media in the aerobic zone and the mixture of ML and media, were evaluated with batch tests. Figure S2 shows exemplary nitrate profiles obtained with the different biomass fractions. A summary of the nitrification rates obtained from the batch tests is presented in Table 3 and Table 4. The nitrification rate per carrier media surface area for the stage 1 was consistent with those obtained by others (Rusten et al., 1995; Rusten et al., 2003).

It is clear that the nitrification rate associated with the biomass attached to the carrier media for stage 1 is much higher than the one obtained with only ML, indicating higher abundance and/or activities of nitrifiers associated with carrier media than those with ML. The specific nitrification rates in the tests conducted with only mixed liquor from stage 1 and stage 2 were very similar as expected since it is the same ML. Note that the specific nitrification rates obtained in the test with carrier media only from stage 1 was 145% higher than the one from stage 2. The biofilm thickness was also substantially different in the two reactors by visual inspection (thinner and darker biofilm in carrier media from stage 2 than

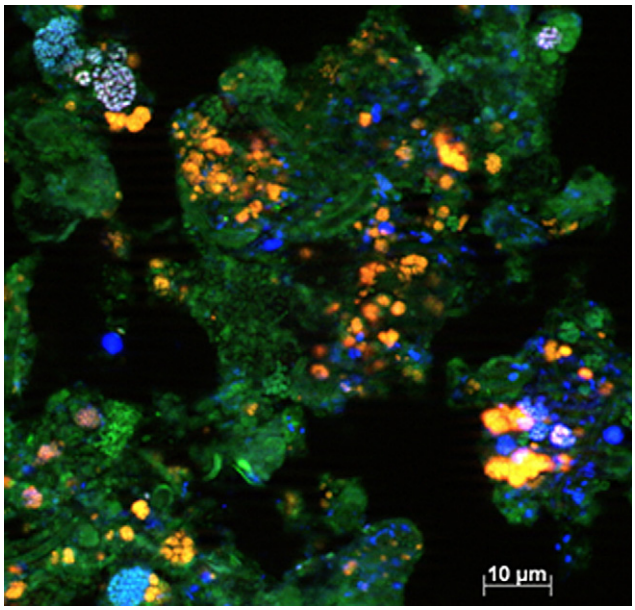

Fig. 4 – FISH images of biomass from the nitrifying part of the biofilm from stage 1. Orange-red, *Nitrospira*-like NOB (hybridized with probe Ntspa662-CY3); whitish (pink-)-blue, *Nitrosomonas oligotropha* -like AOB (hybridized with probes Nmo218-CY3, NSO1225-FITC, and BET42a-CY5); light (green-) blue, other AOB (*Nitrosomonas europaea/eutropha/halophila*-like, hybridized with probes NSO1225-FITC, and BET42a-CY5); dark blue, other Betaproteobacteria (hybridized with probe BET42a-CY5); green, background fluorescence of the biofilm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3 – Nitrifiers and nitrification activities distribution among different biomass fractions at the Broomfield WWTP.

Biomass fraction	Population distribution		Nitrification activity	
	AOB fraction [%][a]	NOB fraction [%][a]	Nitrification rate (stage 1)	Contribution to overall nitrification activity [%][b]
Suspended biomass (ML)	<1%	<1%	2.5 [mgN/gMLVSS/h] 2.0 [mgN/gMLSS/h]	25.4%
Attached biomass (Media)	7 ± 4	15 ± 6	1.1 [gN/m^2/d] 5.87 [mgN/gTS/h]	75.6%

a The numbers represent the percentages of the total bacterial population in the respective sample, determined from the cumulative area identified by quantitative image analysis Daime ± the corresponding standard deviation.
b The % of nitrification activity was calculated considering the fraction of nitrification associated with a specific biomass, respect to the overall nitrification activity of the system.

stage 1) and, this was further confirmed by the higher value of biomass attached on unit media surface area in stage 1 than that of stage 2 (7.93 versus 4.97 g/m^2).

Nitrification rates and nitrifier presence on the fixed film are affected by dissolved oxygen (DO), organic loading and ammonium concentration. Bulk ammonium concentration can be limiting at concentration lower than 1–3 mgNH_4–N/l (Ødegaard, 2006) or when the oxygen to ammonium concentration ratio is higher than 2–5 (Hem et al., 1994). In the case of Broomfield, organic loading and dissolved oxygen concentrations are similar for both stages however the average NH_4–N concentrations were found to be 3.6 mg/L and 0.6 mg/L in stage 1 and 2, respectively and the DO to ammonia ratio was 1.3 for stage 1 and 7.5 for stage 2. Therefore, the differences in the nitrification rates are likely attributed to the difference in ammonium level in the two stages, resulting in ammonia-limiting condition for stage 2. The results also show that the addition of the carrier media in the hybrid system increased the nitrification capacity by about 155% for stage 1 and by 25% for stage 2. This indicates that the benefits of maintaining higher nitrification activity on the fixed-film start to diminish as the residual ammonia concentration becomes really low and is limiting the reaction rates. Another point worth mentioning is the presence of nitrite (up to 2.5 mg/l) in all the tests performed with the ML only, whereas in the tests containing carrier media, the concentration of nitrite remained very low (below 0.2 mg/l); this fact can be seen as a sign of more stable and coupled AOB and NOB population on carrier media than in mixed liquor. This observation is consistent with the population analysis results, which showed that the majority of AOB and NOBs reside on the carrier media and only a very limited number of AOB and NOB are present in the mixed liquor. During the time of this study, the aerobic MLSRT was about 3.5 days and the average plant temperature was 17 °C. The minimal SRT required for nitrification at 17 °C was calculated to be 5.96 days (Tchobanoglous et al., 2003), therefore the nitrification activities observed with ML in the IFAS-EBPR system is likely due to sloughing off of nitrifiers from the biofilm.

3.6. Implication of the results on design operation and modeling of IFAS-EBPR systems

Our results demonstrated that for an IFAS-EBPR process, PAOs and EBPR activity is predominately associated with the mixed liquor (96% of the total P removal activity) and in contrast, most nitrifiers and nitrification activity (>75%) reside on the carrier media. These findings have several implications for BNR design and operation. First, the MLSRT that controls most of the EBPR populations can be varied and optimized to favor EBPR activities and potentially improve stabilities without being restrained by nitrifying populations. For example, SRT was suggested to be a possible factor that impacts the competition between PAOs and GAOs, with the former preferring short SRT (less than 3.5 days) (Whang and Park, 2006). Although other factors, such as substrate type, pH and temperature, affect the EBPR populations dynamics as well as previously described, these parameters are very difficult, if possible at all, to be adjusted for real practice. The flexibility of adjusting the MLSRT for optimizing the EBPR process, without affecting the nitrification performances, is therefore a valuable advantage of an IFAS-EBPR system for achieving simultaneous and optimal P and N removal. The dynamics of the PAO activity and its distribution between ML and the carrier media are not currently considered and addressed in the IFAS-EBPR process design and modeling. Particularly, the factors that affect the extent of PAO populations and EBPR activities associated with the biofilm, although shown to be a relatively small fraction for this study, are not fully understood and require further investigation. The contribution from the PAOs residing on the biofilm media to the overall P removal and

Table 4 – Summary of results from the nitrification batch testing.

	Stage 1 tests				Stage 2 tests			
	ML only	Media only	ML + media[a]	Σ(ML + media)[b]	ML only	Media only	ML + media[a]	Σ(ML + media)[b]
Dissolved oxygen (mg/L)	3.68	4.20	4.20	–	3.58	3.98	3.27	–
Temperature (°C)	20.1	19.9	20.0	–	20.3	20.0	20.2	–
MLSS (mgTSS/l)	1538	–	698	–	1546	–	1425	–
Attached biomass (g/m^2)	–	7.93	7.74	–	–	4.97	4.46	–
Total biomass (mgTS/l)	1538	1488	2225	2225	1546	1010	2239	2239
Nitrate formation Rate gNO_x-N/m^2/d	–	1.12	–	–	–	0.28	–	–
Ammonia oxidation rate mgNH_4-N/gTS/h	2.64	5.89	4.77	4.87	2.00	2.36	2.30	2.13
Nitrate formation rate mgNO_x-N/gTS/h	2.02	5.87	5.16	4.66	1.91	2.39	2.38	2.08
Ammonia oxidation rate mgNH_4 -N/l/h	4.06	8.76	10.63	12.40	3.10	2.39	5.16	5.55
Nitrate formation rate mgNO_x-N/l/h	3.10	8.73	11.47	12.34	2.96	2.41	5.34	5.36

a Measured value with both media and mixed liquor (ML) in the reactor.
b Calculated value using test results with either media alone or ML alone, separately.

their potential impact on nitrification performance on the IFAS media (e.g. competition for oxygen) need to be quantified and incorporated into IFAS-EBPR process modeling and design.

For N removal in a IFAS-EBPR system, the dominant residence of nitrifying populations on the biofilm media decouples the growth of nitrifiers from the MLSRT, therefore potentially allows for a more robust and reliable nitrification process that is more resistant to hydraulic loading fluctuation and toxic shocks, as well as temperature changes. It also seems to lead to more stable and coupled AOB and NOB population on carrier media than in mixed liquor, as indicated by the higher nitrite accumulation observed during the batch testing with the ML than those with biofilm media, as previously discussed.

Another possible advantage of having shorter MLSRT in IFAS-EBPR system is related to denitrification rate. Specific denitrification rate (SDNR) is affected by readily biodegradable COD, nitrate concentration and biomass SRT (F/M ratio) and at any given COD and NO_3–N levels, the SDNR is reversely correlated to SRT (Tchobanoglous et al., 2003). Comparing to a conventional suspended nitrogen removal activated sludge process, the IFAS-EBPR process that allows for much shorter MLSRT (2–4 days versus 8–15 days) for the suspended mixed liquor where most of the denitrifiers would reside, would lead to higher SDNR than conventional BNR plants.

4. Conclusions

In conclusion:

1. In the full-scale IFAS-EBPR process studied, PAOs and EBPR activity is predominately associated with the mixed liquor rather than the biofilm media. The relative abundance of PAOs and *Accumulibacter*-like PAOs was estimated to be 20–30% and 15.8 ± 1.4% in the mixed liquor and, 3–8% and 4 ± 1% in the biofilm media, respectively.
2. Abundance and distribution of nitrifying populations and their activities showed that most nitrifiers and nitrification activity (>75%) reside on the carrier media rather than in the mixed liquor. In addition, more coupled AOB and NOB populations and more stable nitrate removal (indicated by less nitrite accumulation) was observed with biofilm than with mixed liquor.
3. The addition of the carrier media in the hybrid system increased the nitrification capacity, but it was found that the benefits of maintaining higher nitrification activity on the fixed-film starts to diminish in secondary stages where the residual ammonia concentration becomes limiting.
4. The results demonstrated that in the IFAS-EBPR process, the N–removing and P–removing populations that require or prefer conflicting SRT values (e.g. > 15 days for slow-growing nitrifiers and <5 days for fast-growing PAOs) can be decoupled, therefore allowing for separate SRT control and overall optimization for both N and P removal processes.
5. The results from this study contribute to the fundamental understanding and further development of comprehensive mathematical models for IFAS-EBPR process design and modeling.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.watres.2011.04.039.

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