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Diversity of nitrifying bacteria in a full-scale petroleum refinery wastewater treatment plant experiencing unstable nitrification

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ABSTRACT

We have investigated bacterial populations relevant to nitrification in a full-scale activated sludge plant receiving wastewater from a petroleum refinery showing unstable nitrification. Inhibition of ammonia oxidation was related to phenol concentration according to a model of non-competitive inhibition. While the number of ammonia-oxidizing bacteria (AOB) did not correlate with nitrification performance, the total number of nitrite-oxidizing bacteria (NOB) dropped considerably during periods of nitrite accumulation or no nitrification. Diversity of nitrifiers in the sludge of the full-scale facility was examined at a time of full nitrification with the construction of clone libraries of ammonia monooxygenase (*amoA*) gene and of the 16S rRNA gene of NOB. Nucleotide sequences of *amoA* gene belonged to one dominant population, associated with *Nitrosomonas europaea*, and to a minor population related to the *Nitrosomonas nitrosa* lineage. The majority of sequences retrieved in the NOB-like clone library also clustered within a single operational taxonomic unit. The high dominance of *Nitrobacter* over *Nitrospira* and the low diversity of nitrifying bacteria observed in this wastewater treatment plant might account for the increased risk of failure in the presence of disturbances.

1. Introduction

Ammonium causes eutrophication and oxygen depletion in receiving waters, and exerts direct toxicity effects to aquatic life. Biological wastewater treatment plants achieve the important service of ammonia removal from wastewater via autotrophic ammonium oxidation, a two-step process, carried out by two categories of chemolithotrophic microorganisms: ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). The ways in which the environment affects biological nitrification will ultimately depend on the diversity and susceptibility to the environmental conditions of nitrifiers existing in each particular ecosystem. The growth and the activity of nitrifying bacteria in wastewater treatment plants are influenced by the process temperature, ammonia/nitrite concentration, oxygen concentration, BOD_5/TKN ratio, pH and the presence of toxic compounds [1]. Among the latter, chemical inhibitors of nitrification present in the wastewater produce unreliable process performance, which eventually leads to complete failure [2,3]

Wastewater containing phenol is discharged from several industrial processes, such as petroleum refinement, coking operations, coal processing, and the manufacture of petrochemical, pharmaceutical, plastics, wood products, paint, and pulp and paper industries. A large body of research has focused on the inhibitory effect of phenol on nitrification on isolated bacteria [4,5] and mixed communities [2,6]. However, results from lab-experiments are difficult to extrapolate to real wastewater treatment conditions, and little is known about the microbial ecology of nitrifiers in full-scale processes treating wastewater containing both phenol and high ammonia concentrations. We have used molecular tools to investigate bacterial populations relevant to nitrification in a full-scale activated sludge plant receiving wastewater from a petroleum refinery, which shows unstable nitrification. Diversity surveys on AOB and NOB, and the relationship of their abundance and activity with overall process condition may be useful to understand the basis for process instability. This knowledge would allow one to design better monitoring programs in order to avoid operational failure, and to devise bioaugmentation strategies to enhance nitrification performance in the presence of toxic chemicals.

2. Experimental

2.1. Description of the wastewater treatment plant

Activated sludge samples were collected from the aerated basins of a modified Ludzack-Ettinger (MLE) process receiving pretreated wastewater from an oil refinery industry. The wastewater treatment plant (WWTP) treated daily approximately 3.4 mil-

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Table 1
Oligonucleotide probes used in this study.

Short name	Target	Primer sequence	Specificity	Reference
amoA-1f	*amoA*	GGGGHTTYTACTGGTGGT	AOB	[43]
amoA-2r	*amoA*	CCCCTCKGSAAAGCCTTCTTC	AOB	[43]
CTO-189f	*rRNA*	GGAGRAAAGYAGGGGATCG	*Nitrosomonas/Nitrosospira*	[44]
CTO-654r	*rRNA*	CTAGCYTTGTAGTTTCAAACGC	*Nitrosomonas/Nitrosospira*	[44]
amoG1 f84	*amoA*	CACGATGTATCTGACACGCA	OTU1	This study
amoG1 r284	*amoA*	CGCAGTGAACCTTGCTCAA	OTU1	This study
amoG2a-f109	*amoA*	TTGGTAACGGCGCTGATC	OTU2a	This study
amoG2a-r284	*amoA*	AGTGAACCCTGCTCAATC	OTU2a	This study
AmoG2b-f174	*amoA*	TTTTGGACCGACGCACTT	OTU2b	This study
AmoG2b-r395	*amoA*	AGAAGGCTGTGCAGTAGA	OTU2b	This study
FGPS-872	*rRNA*	TTTTTTGAGATTTGCTAG	*Nitrobacter*	[45]
FGPS-1269	*rRNA*	CTAAAACTCAAAGGAATTGA	*Nitrobacter*	[45]
NSR-1113F	*rRNA*	CCTGCTTTCAGTTGCTACCG	*Nitrospira.*	[10]
NSR-1264R	*rRNA*	GTTTGCAGCGCTTTGTACCG	*Nitrospira*	[10]
F1114	*rRNA*	GCAACGAGCGCAACCC	Bacteria	[46]
R1392	*rRNA*	ACGGGCGGTGTGTRC	Bacteria	[46]

lion liters of wastewater containing an average influent BOD_5 of $146 \pm 78\,mg\,l^{-1}$, hydrocarbons average of $6.1 \pm 3.9\,mg\,l^{-1}$ and a high load of ammonia, $81 \pm 33\,mg\,l^{-1}$. No sanitary sewage was discharged into this WWTP. Phosphoric acid was supplemented as the single source of phosphorus. The basin pH was maintained at 7.1 ± 0.3 by the addition of spent caustic soda. Process temperature was $34 \pm 3\,^{\circ}C$. The system was operated with a hydraulic retention time of 36 h and a mean cell residence time of 49 days. Although originally designed with an anoxic zone for total nitrogen removal, the WWTP has not produced significant denitrification due to the low COD/N ratio

Relationships between operational parameters and performance indicators were analysed using two-tailed Pearson correlation coefficients (at significance <0.05). Calculations were carried out using GraphPad Prism, version 2.01 (GraphPad Inc., San Diego, CA).

2.2. Inhibition of nitrification by phenol

Phenol inhibition of nitrification was described according to a model for non-competitive inhibition on the assumption that the nitrification is zero-order with respect to oxygen and ammonia [3]:

$$k_{PhOH} = k_{\max} \frac{[PhOH]_c}{[PhOH]_c + [PhOH]} \quad (1)$$

where k_{PhOH} is the observed ammonia transformation rate when phenols are present, $[PhOH]_c$ is the inhibition constant for which k_{PhOH} is half of the maximum ammonia transformation rate $k_{\max}$.

The inhibition of nitrification was defined as

$$\%\,\text{inhibition} = 100 \times \frac{k_{\max} - k_{PhOH}}{k_{\max}} \quad (2)$$

It follows that the inhibition is related to the concentration of phenol as:

$$\frac{100}{\%\,\text{inhibition}} = 1 + \frac{[PhOH]_c}{[PhOH]} \quad (3)$$

This expression is similar to the expression derived from the extended non-competitive inhibition model used to fit the inhibition of nitrification for combined municipal and industrial wastewater [7].

2.3. DNA extraction

Activated sludge samples were transported to the laboratory at RT and stored at −20 °C until analysis. Extraction of genomic DNA from sludge was performed as in [8]

2.4. Real-time PCR with SYBR green I and melting curve analysis

Gene copy numbers were quantified by real-time PCR, using a Sybr Green assay on an Opticon2 (MJ Research). The primer sets used for betaproteobacterial AOB (CTO and *amoA*), *Nitrobacter*-like NOB (FGPS) and *Nitrospira*-like NOB (NRS) are indicated in Table 1. Reactions were carried out using 25 μl reaction mixtures in 8-well reaction strips with optical caps. PCR mixtures contained 1 U *Taq Platinum* DNA polymerase (Invitrogen), 1× *Taq Platinum* buffer, a 0.25 mM concentration of each deoxynucleoside triphosphate, SYBR green I (1:50,000; Molecular Probes), DMSO 5%, primers 0.4 μM and 3 mM $MgCl_2$. Samples were analysed in triplicate at three different concentrations, using 3 ng, 0.6 ng and 0.3 ng of DNA template, except for the bacterial 16S rRNA gene, where DNA was added to the PCR reaction at two concentrations in a 10-fold dilution (6 ng and 0.6 ng of DNA template). The level of contamination, the absence of inhibitory effect and the efficiency of the PCR were checked for each assay. Calculation of cell numbers were perfomed assuming one 16S rRNA gene copy/AOB cell [9], three *amoA* gene copies/AOB cell [9], one 16S rRNA gene copy/*Nitrobacter* cell [10], one 16S rRNA gene copy/*Nitrospira* cell [10] and 3.3 16S rRNA gene copies/cell [11].

Primer sequences developed in this study for quantification of ammonia-oxidizing bacteria OTU1, OTU2a and OTU2b are listed in Table 1. The PCR conditions for the newly designed primers were optimized with clones containing the target sequence. All primer sets were tested for their specificities using clones belonging to one representative of each other non-target groups as negative controls (10^6 copies of competing gene fragments per assay).

2.5. Estimation of in situ substrate turnover rates

The amount of ammonia oxidized to nitrite per unit of time was estimated according to the formula modified from [12]

$$NH_{4_t}^{+} = \{NH_{4_i}^{+} - N_R \Delta COD - NH_{4e}^{+}\} \times r \quad (4)$$

where $NH_{4_t}^{+}$ is the transformed ammonia–nitrogen (in milligrams per hour), NH_{4i}^{+} is the ammonia–nitrogen concentration in the influent, NH_{4e}^{+} is the ammonia–nitrogen concentration in the effluent, r is the reactor influent rate [12], and N_R is the ammonia requirement for biomass growth [13], calculated as:

$$N_R = \frac{0.087(1 + f_D b_H \theta_x) Y_H}{1 + b_H \theta_x} \quad (5)$$

where Y_H is the true growth yield for heterotrophs, f_D is the fraction of active biomass contributing to biomass debris, b_H is the decay coefficient for heterotrophs and θ_x is the solid retention time

(49 days). Values for $Y_H = 0.45$ mg MLSS (mg BOD)$^{-1}$, $f_D = 0.2$ and $b_H = 0.1$ d^{-1} were obtained from the literature [13].

2.6. Libraries construction and phylogenetic analysis

Two clone libraries were constructed using DNA extracted at the time of full nitrification (week 54). One for ammonia-oxidizing bacteria, using primers amoA1f and amoA-2R targeted highly conserved regions, which amplify a 490 bp fragment of the gene coding for the subunit A of the enzyme ammonia monooxygenase (Table 1). Nitrite-oxidizing bacteria (NOB) were characterized by constructing a clone library of a 400 bp fragment of the 16S rRNA of *Nitrobacter*-like NOB, amplified using primers FGPS 872 and FGPS 1269 (Table 1).

The purified amplicons were ligated into the pGem T-Easy vector (Promega Corp., Madison, WI), and transformed into *E. coli* DH10B cells by electroporation. Plasmid templates DNA were prepared by standard alkaline lysis method. DNA sequencing was performed at the Macrogen sequencing facility (Seoul, Korea).

ClustalX was used to align gene sequences to one another and to selected related sequences obtained from the GenBank. Distance matrix was built using DNADIST, and used to construct a phylogenetic tree with the NEIGHBOR program, which implement the neighbor joining method. Bootstrap confidence analysis was performed by generating 100 replicated data sets with the SEQBOOT program and generating the consensus tree with the CONSENSE program. Phylogenetic trees were also constructed with the DNAML (maximum likelihood) and DNAPARS (parsimony) programs (all in the PHYLIP software package version 3.5). Trees were visualized using the ARB program package [14]. Sequence assignment to OTUs was performed with the furthest neighbor algorithm of DOTUR [15]. OTUs were defined using a genetic distance between sequences of 0.06 for amoA and 0.04 for NOB [15].

2.7. Nucleotide sequence accession numbers

The sequences obtained in this study have been deposited in the GenBank database under accession number FJ812415-FJ812514 and GU562970-GU563050.

3. Results

3.1. Nitrification performance

Ammonia removal fluctuated widely in the full-scale activated sludge (Fig. 1a). Nitrification inhibition resulted in accumulation of ammonia, and occasionally of nitrite. Periods of low nitrification were coincident with absence of filamentous bacteria and heavily reduced number of higher life forms, an implicit hint of an inhibitory or toxic environment. The profiles of phenol during the studied period suggested a link between phenol concentration and the inhibition of nitrification (Fig. 1b). The Pearson test revealed a significant correlation between phenol and ammonia concentration ($p = 0.030$) and also between phenol and nitrate ($p = 0.014$). The inhibition of phenol could be fitted to a pattern of non-competitive inhibition (Fig. 2). According to the model, the concentration of phenol for which the rate of ammonium oxidation was one half of the maximum rate was 0.5 mg l^{-1}.

Nitrification failure could not in principle be attributed to lack of oxygen, because dissolved oxygen levels were maintained at all times above 2 mg l^{-1}. Interestingly, there was a significant correlation ($p = 0.009$) albeit positive, between ammonia concentration and dissolved oxygen concentration, a likely indication of the inhibition of oxygen consumption by heterotrophic and/or nitrifying bacteria. In agreement with this suggestion, phenol concentration also displayed positive correlation, although not significant at the 95% level, with dissolved oxygen concentration ($p = 0.112$) and effluent hydrocarbon concentration ($p = 0.105$). Loss of nitrification did not correlate significantly with changes in process pH ($p = 0.869$).

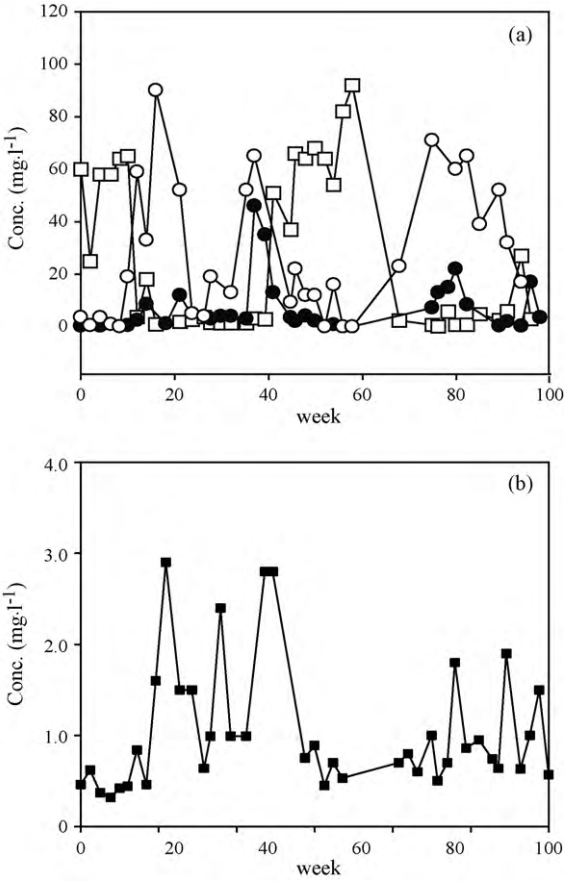

Fig. 1. (a) Operational data of ammonia–N (○), nitrite–N (●) and nitrate–N (□) concentrations in the effluent samples taken from the full-scale activated sludge. (b) Variation in the effluent phenol concentration (■).

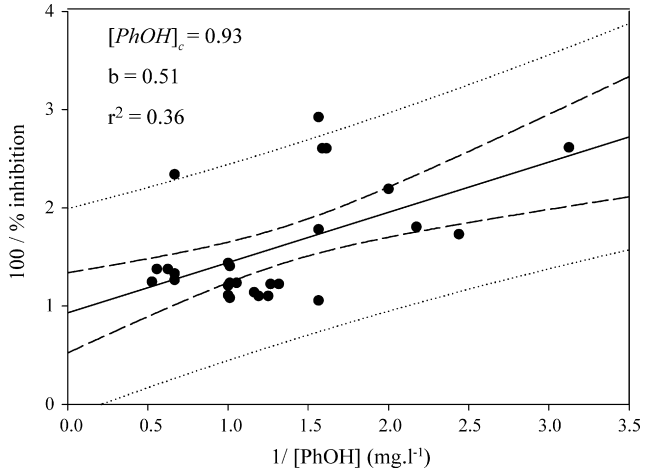

Fig. 2. Inhibition of nitrification by phenol. Data were fitted to Eq. (3), which describes the inhibition of nitrification according to a model for non-competitive inhibition on the assumption that nitrification is zero-order with respect to oxygen and ammonia. The linear regression of the double reciprocal plot (solid line) is shown with 95% confidence limits (dashed lines) and 95% prediction limits (dotted lines). $[PhOH]_c$ is the inhibition constant for which k_{PhOH} is half of the maximum ammonia transformation rate k_{max}; b is the y-intercept.

Table 2
Quantification of gene copy number and conversion to cell number.

Target gene	Copies l^{-1}	Cells l^{-1}
AOB 16S rRNA	$(9.3 \pm 2.9) \times 10^{10}$	$(9.3 \pm 2.9) \times 10^{10}$
amoA	$(2.8 \pm 1.0) \times 10^{11}$	$(9.2 \pm 3.2) \times 10^{10}$
Nitrobacter 16S rRNA	$(1.8 \pm 0.7) \times 10^{11}$	$(1.8 \pm 0.7) \times 10^{11}$
Nitrospira 16S rRNA	$(1.4 \pm 0.5) \times 10^{9}$	$(1.4 \pm 0.5) \times 10^{9}$
Total bacteria	$(3.1 \pm 2.0) \times 10^{13}$	$(9.5 \pm 6.0) \times 10^{12}$

3.2. Quantification of AOB and NOB

The total number of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria *Nitrobacter* sp. and *Nitrospira* sp. were determined for available sludge samples at three different periods, exhibiting marked differences in nitrification. The total number of ammonium oxidizing bacteria was determined using quantitative PCR of a fragment of the ammonia monooxygenase gene (*amoA*) and of a fragment targeting the V2–V4 region of the 16S rRNA gene of β-subclass AOB (CTO, Table 2). *Nitrobacter*-like and *Nitrospira*-like cells were quantified using primers targeting conserved sequences in the 16S rRNA gene FGPS and NRS, respectively (Table 2). The relative proportions of AOB to the total bacterial density did not correlate with nitrifying activity of the sludge (Fig. 3). Larger variations were observed for the NOB comparing periods of high and low nitrification. *Nitrobacter*-like NOB represented approximately 2% of

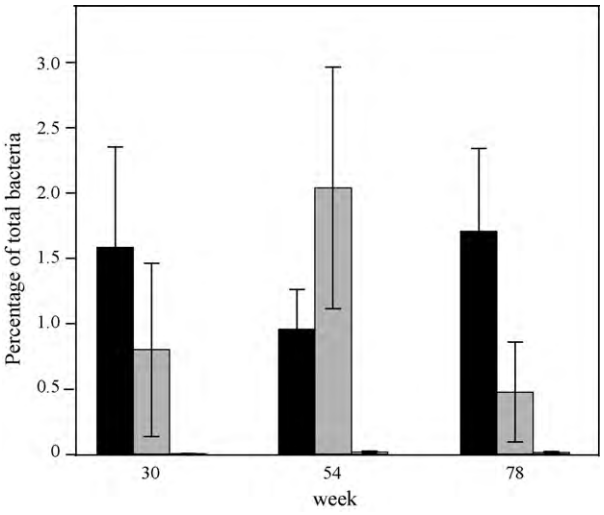

Fig. 3. Abundances of AOB (black), *Nitrobacter*-like NOB (gray) and *Nitrospira*-like NOB (white) in the full-scale activated sludge at three different periods, exhibiting marked differences in nitrification (see Fig. 1). Error bars indicate standard deviation, $n = 3$.

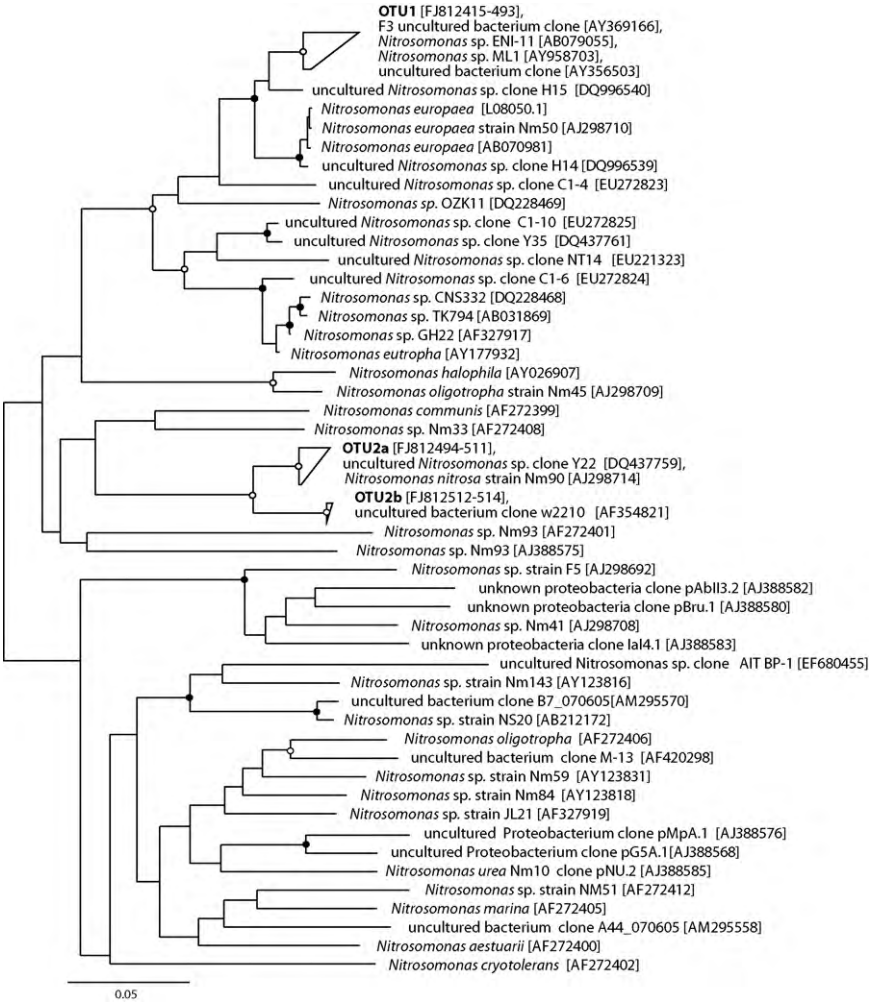

Fig. 4. Dendrogram showing the phylogenetic position of the clones obtained after amplification of a fragment of the *amoA* gene from genomic DNA of the activated sludge. Filled circles correspond to branches conserved for all methods tested (maximum parsimony and neighbor joining with more than 70% bootstrap, and maximum likelihood). Open circles correspond to branches conserved for at least two of the methods tested. The scale bar represents a 0.05 substitution per nucleotide position.

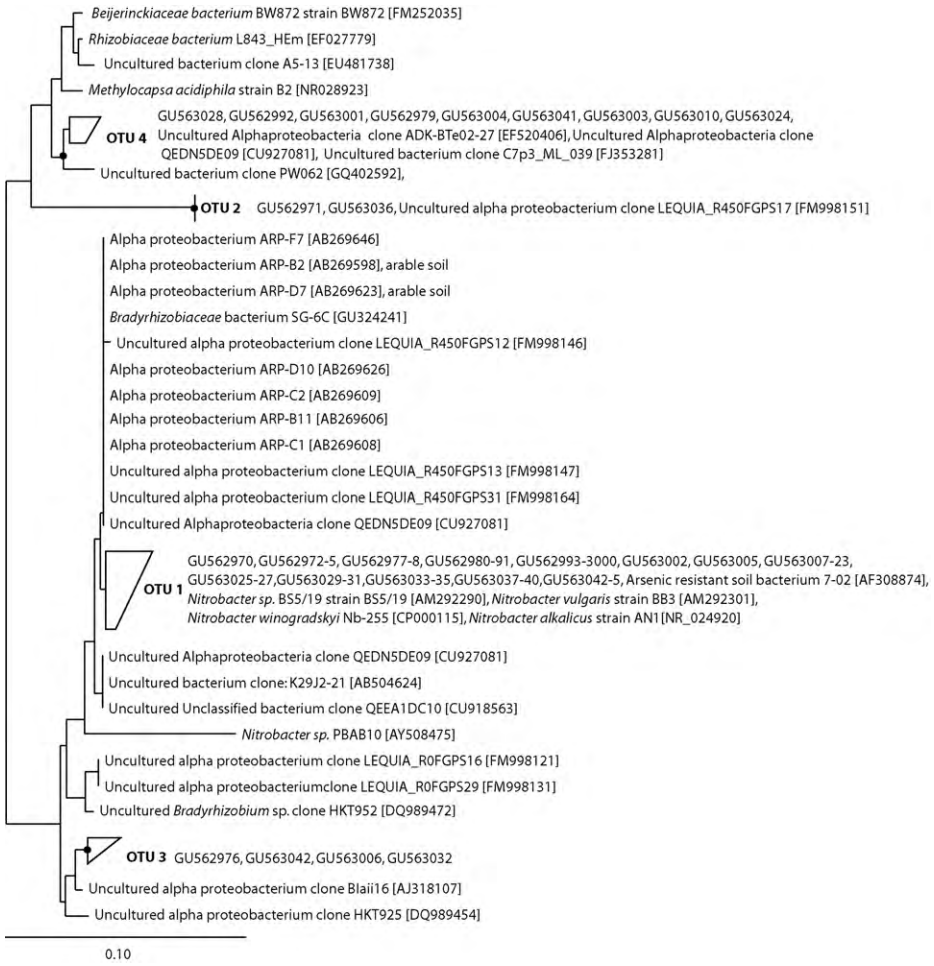

Fig. 5. Dendrogram showing the phylogenetic position of the clones obtained after amplification of a fragment of the 16S rRNA gene using primers specific for *Nitrobacter*-like NOB. Filled circles correspond to branches conserved for all methods tested (maximum parsimony and neighbor joining with more than 70% bootstrap, and maximum likelihood). Open circles correspond to branches conserved for at least two of the methods tested. The scale bar represents a 0.05 substitution per nucleotide position.

bacterial populations during time of full nitrification. *Nitrospira*-like NOB were detected only during time of full nitrification, albeit in very low amount (Fig. 3). The total number of NOB dropped considerably during periods of nitrite accumulation or no nitrification. Accordingly, AOB/NOB ratio was 3.6 under conditions of nitrification failure and shifted down to 0.5 at the time of good nitrification.

3.3. Cloning, sequencing and phylogeny for AOB and Nitrobacter-like NOB bacteria

A diversity analysis of nitrifiers in the sludge of the full-scale facility was performed at a time of full nitrification. Nucleotide sequences of *amoA* gene fragments and the derived amino acid sequences were determined for a total of 110 clones. All clones were classified as *Nitrosomonas* species, according to the phylogenetic tree given in Fig. 4. *AmoA* genes belonged to two populations separated by a genetic distance of 0.06. The dominant population (77% of the clones) was related to an ammonia oxygenase gene belonging to *N. europaea*, whereas the second population showed 94% similarity with the *amoA* of *N. nitrosa* (AF272404).

In addition to the use of primers targeting conserved fragments of the *amoA* gene, novel PCR primer sets were designed to target signature DNA sequences in the *amoA* gene of the two OTUs detected in the clone library, and used to quantify both taxa using real-time PCR. The number of bacteria determined as belonging to the *N. europaea*-related taxa (OTU 1) was $(8.5 \pm 0.6) \times 10^{10}$ cells ml^{-1}, in close agreement with total AOB. Populations belonging to *N. nitrosa*-related taxa were separately quantified in two groups and accounted for only $(5.4 \pm 2.7) \times 10^{9}$ cells ml^{-1} for OTU2a and $(1.4 \pm 0.6) \times 10^{8}$ for OTU2b, meaning that *N. nitrosa*-like bacteria represented less than 5% of total AOB. Thus, the quantitative data confirm the dominance of *N. europaea*-like clones determined in the clone library.

The populations of nitrite-oxidizing bacteria (NOB) were characterized by constructing a clone library of a PCR-amplified fragment of the 16S rRNA of *Nitrobacter* species. The vast majority (82%) of the 80 clone sequences retrieved from the *Nitrobacter* clone library clustered within a single operational taxonomic unit (OTU1, Fig. 5), suggesting that nitrite oxidation, as well as ammonia oxidation, depended on a highly dominant population. The closest matches retrieved from a BLAST search corresponded to a *Nitrobacter* strain isolated from a soil in Galapagos Islands (Ecuador) [16], a clone derived from a nitritation reactor [17], and an enrichment culture of nitrite-oxidizing bacteria from activated sludge source [18].

3.4. In situ activity of AOB

The in situ rate of ammonia oxidation per ammonia oxidizer cell within the activated sludge was calculated during the period of full nitrification (week 54), when nitrification was rate-limited by the first step. The average concentration of NH_4–N in the influent and effluent during the investigated period was 81 ± 33 mg l^{-1} and 9.0 ± 8.9 mg l^{-1} respectively. During that time the nitrogen required for assimilation (Eq. (5)) was estimated as 2.1 mg l^{-1}.

From Eq. (4), AOB oxidized $(1.0 \pm 0.9) \times 10^7$ mg of ammonia h^{-1} or $(7.1 \times 10^{17} \pm 6.4) \times 10^{17}$ fmol of ammonia h^{-1}. The average number of AOB determined by quantitative PCR using three different set of primers was $(9.2 \pm 3.2) \times 10^{10}$ cells l^{-1}. Considering that the total reactor volume was 5430 m^3, the total amount of ammonia oxidizers in the reactor was $(5.0 \pm 1.7) \times 10^{17}$ cells. Thus, the cell-specific ammonia oxidation rate was 0.96 ± 0.71 fmol of ammonia to nitrite per hour.

4. Discussion

4.1. Inhibition by phenol

The inhibitory effect of phenol on nitrification is well known. Our data show that periods of nitrification failure coincided with increased phenol concentrations in the effluent wastewater. Hydrocarbons and phenols were the main carbonaceous components of the influent wastewater. *N. europaea* nitrification displayed non-competitive inhibition by aromatic hydrocarbons and halogenated hydrocarbons, whereas phenol showed 90% inhibition at a concentration of 4.7 mg l^{-1} [19]. Large amount of phenols enter the activated sludge of petroleum refineries mainly via spent caustic soda. The inhibitory effect of low concentration of phenol on nitrification has been extensively documented. Earlier studies reported 75% and full inhibition of ammonium oxidation in nitrifying sludge at phenol concentrations of 5.6 mg l^{-1} [20] and 3.7 mg l^{-1} [3]. In a study of the effect of phenol on the nitrifying activity of aerobically grown microbial granules produced in a sequencing batch reactor, it was reported that ammonium oxidation activity of phenol-exposed granules decreased significantly in comparison with the unexposed control. Nitrification could occur only after phenol in wastewater was degraded to a very low value [21]. Similarly, it was found that phenol was inhibitory to the nitrification process in a concentration-dependent manner in a batch assay, where nitrification started only after phenol was completely degraded [2]. In the same study, the authors did not observed inhibition of ammonium removal by phenol in a lab-scale activated sludge reactor, presumably due to rapid dilution or degradation [2]. It was also shown in an activated sludge process with cross-flow filtration that ammonium oxidation depended on the complete removal of phenol [22]. Despite the activated sludge from the petroleum refinery sludge contained high diversity of bacteria with the capacity of degrading phenol [23], complete degradation of phenol was not achieved, possibly due to inhibitory effects of phenol on the activity of phenol hydroxylase. In that regard, the use of biofilm systems and dilution of the industrial wastewater, as in submerged fixed-film reactor technology, might provide better capabilities for removing high concentration of phenol from wastewater, thus minimizing toxic effects on nitrifiers [24].

We have previously shown that the bacterial community in this full-scale industrial activated sludge had a strong dominance of a few species [8], suggesting that a few factors dominate the ecology of the assemblage. Moreover, the pattern of species abundance distribution of heterotrophs was consistent with a geometric series, a typical descriptor of species-poor communities subjected to harsh environmental conditions [8]. Similarly to the case of leachate from creosote-contaminated sand [3], the ammonia oxidation in the industrial activated sludge process was closely correlated with the concentrations of phenolic compounds, even though the adding contributions of other inhibitory compounds, e.g. hydrocarbons may explain the scatter in the fitting observed in Fig. 2.

4.2. AOB diversity

Analysis of microbial diversity may contribute to gain insight into the relationship between environmental variables and ecosystem functioning. Nitrification in the studied industrial activated sludge depended on the activity of a low diverse group of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria belonging to the *Nitrosomonas* and *Nitrobacter*. The phylogenetic alignment of the *amoA* gene indicated that dominant AOB populations in the wastewater treatment plant were related to the *N. europaea*/*Nitrosococcus mobilis* cluster [25]. The closest relative to the *amoA* sequences in the clone library was an uncultured bacterial clone (AY369166) found in a lab-scale activated sludge deammonification reactor. A minor population of AOB was related to the *amoA* gene of *N. nitrosa*.

It has been previously shown that the majority of *amoA* sequences derived from habitats containing high substrate concentrations, like activated sludge, belonged to the genus *Nitrosomonas* [25]. Members of the *N. europaea*/*N. mobilis* [26,27], *N. oligotropha* [10,26], *N. marina* [25] and *N. cryotolerans* [27] clusters have been the AOB most frequently detected in wastewater treatment plants. Yet AOB diversity and composition vary considerably among different wastewater treatment plants [25,28]. Some plants contain a single class of AOB, whereas other display coexistence of different betaproteobacterial AOB. Solid residence time mainly influenced the total numbers of ammonia-oxidizing bacteria and has been associated with lower diversity [26,27]. The high ammonium concentration in the influent, the high volumetric ammonia removals and the rather long SRT are all factors that may support the dominance of the *N. europaea* cluster in our study [29].

For the purpose of calculations of the in situ activity of ammonia-oxidizing bacteria, we have quantified AOB cells using three different primer sets. Approaches targeting the 16S rDNA gene [10,27], as well as the ammonia oxygenase subunit A (*amoA*) gene [30] have been previously used to measure the presence and composition of AOB in wastewater treatment plants. The primers targeting the *amoA* gene are more inclusive, but not completely specific for betaproteobacterial ammonia oxidizers [31]. In order to improve target specificity, we have designed novel primers, which targeted specifically *amoA* sequences retrieved from the clone library. The fact that the three estimates obtained with different set of primers delivered similar values for AOB abundance suggests strongly real-time PCR analysis have correctly estimated the AOB population size. Therefore, despite high ammonia content in the influent wastewater, only around 1% of cells in the activated sludge consist of ammonia-oxidizing bacteria. This value agrees with the proportion of total AOB within the total bacterial population found in a survey of communities of ammonia-oxidizing bacteria in activated sludge of sewage treatment plants in Tokyo [27], but is low compared with the value of 8% that should be present according to a theoretical model, which estimates the expected net AOB biomass production from the ammonia consumed [32,33]:

$$\frac{(X_a)_{ao}}{(X_a)_{het}} = \frac{Y_{oa}(1 + b_{het}\theta_x)\Delta\text{ammonia}}{Y_{het}(1 + b_{nit}\theta_x)\Delta\text{BOD}} \quad (6)$$

According to this model the theoretical ratio of active autotrophic $(X_a)_{ao}$ to total heterotrophic bacteria and $(X_a)_{het}$ depends on the ratio of the ammonia removal (Δammonia) to BOD removal (ΔBOD). q_x is the solid retention time, Y_{het} and Y_{oa} are yield and b_{het} and b_{nit} are the endogenous respiration rate of heterotrophic bacteria and AOB respectively.

Yet the in situ rate of ammonia oxidation per ammonia oxidizer cell estimated from the process parameters and AOB quantification was approximately 0.9 fmol of ammonia per hour, which is within the typical nitrification rates of *Nitrosomonas* sp. [34].

4.3. NOB diversity

The process of nitrite oxidization, and how nitrite-oxidizing bacteria (NOB) are affected by organic carbon, has been much less

investigated than ammonia oxidation. *Nitrospira* sp. are the most common nitrite oxidizers in nitrifying wastewater treatment plants [28,35]. *Nitrobacter* species, previously thought to be the key NOB in wastewater treatment plants, on the basis of cultivation-depended studies [13], are more rarely found in full-scale nitrification systems [32].

A large body of literature indicates that nitrite concentration appears to be the major factor controlling the selection between *Nitrospira* and *Nitrobacter*, the latter considered r-strategist, which outcompetes the former at high nitrogen loading [36,37]. But although *Nitrobacter* has been shown to coexist with *Nitrospira* in plants with high nitrite concentrations [12], very seldom has been found as the only nitrite oxidizers. To our knowledge, the present work is the first report of a *Nitrobacter* population dominating almost entirely the NOB community in a full-scale wastewater treatment plant. We speculate that the absence of *Nitrospira* may reduce the ability of the system to cope with conditions of low oxygen [37] and low nitrite concentrations [38].

4.4. AOB/NOB ratio

It is not well established whether there is a ratio of AOB to NOB that is consistent with good nitrification. Because the energy generated from ammonia oxidization by AOB is higher than the energy produced from nitrite oxidization by NOB, a ratio of AOB/NOB of 2.0–3.5 was suggested appropriately related to the inherent high growth rates of AOB [37,39]. Yet other studies revealed that the populations of AOB were lower than NOB [10,40]. Under the conditions of the activated sludge from the petroleum refinery, nitrification was achieved when NOB was two times greater than AOB and was deficient for AOB/NOB ratios greater than 1.

The apparent inconsistency represented by the high number of AOB during periods in which nitrification was inhibited is not without precedents. Considerable numbers of AOB cells have been reported previously under conditions of nitrification failure [37,41,42]. This has been attributed to the adaptation ability of AOB to survive periods of energy-source deprivation, when they cannot perform their normal task [41].

5. Conclusions

Surveys of diversity in full-scale wastewater treatment plants are important to further understand nitrifying processes developing under stress. The results of this study provide insight into the types of AOB and NOB responsible for nitrification in an industrial activated sludge system exposed to toxic levels of phenol. It is generally believed that a greater level of redundancy is favorable for the maintenance of a stable process. Under the conditions of operation of this wastewater treatment plant, dominance of NOB over AOB appeared to correlate with good nitrification. Additionally, the low diversity of nitrifying bacteria and particularly the high dominance of *Nitrobacter* over *Nitrospira* is consistent with the increase risk of failure in the presence of disturbances.

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