Assessing bacterial diversity in a seawater-processing wastewater treatment plant by 454-pyrosequencing of the 16S rRNA and amoA genes

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Introduction
Activated sludge constitutes a crucial tool in the biodegradation of organic materials, transformation of toxic compounds into harmless products and nutrient removal in wastewater treatment plants (WWTPs). It contains a highly complex mixture of microbial populations whose composition has been intensively studied in the past decades. By applying culture-dependent methods, many species have been isolated from activated sludge (Dias and Bhat, 1964; Prakasam and Dondero, 1967; Benedict and Carlson, 1971). However, a great majority cannot be obtained by conventional techniques (Wagner et al., 1993) and, consequently, current molecular techniques such as sequence analysis of 16S rRNA gene clone libraries (Snaird et al., 1997), fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE; Boon et al., 2002), thermal gradient gel electrophoresis (TGGE; Eichner et al., 1999) and terminal restriction fragment length polymorphism (Saikaly et al., 2005) along with fluorescence in situ hybridization (FISH) have been employed in wastewater microbiology to analyse and compare the microbial structure of activated sludge. Recently, PCR-based 454 pyrosequencing has been applied to investigate the microbial populations of activated sludge in different WWTPs as well as in full-scale bioreactors (Sanapareddy et al., 2009; Kwon et al., 2010; Kim et al., 2011; Ye et al., 2011; Zhang et al., 2011a; b), greatly expanding our knowledge on activated sludge biodiversity.

An important process in WWTPs is nitrification, in which ammonium is removed by converting it first into nitrite and then to nitrate. Different bacterial species involved in this process have been characterized by means of clone library analysis in addition to FISH (Juretschko et al., 1998; Purkhold et al., 2000; Daims et al., 2001; Zhang et al., 2011b). Several ammonia-oxidizing and nitrite-oxidizing bacterial populations belonging to the phylum Nitrospira and to Beta- and Gammaproteobacteria have been identified as key members in this process, such as the genera Nitrosomonas, Nitrobacter, Nitrospira and Nitrosococcus (Wagner et al., 2002; Zhang et al., 2011b).

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Microbial Biotechnology (2013) 6(4), 435–442
doi:10.1111/1751-7915.12052
Funding Information This work was supported by the Spanish projects Consolider TRAGUA (CSD2006-00044) and CTQ2009-14390-C02-02.

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Nevertheless, most studies of microbial diversity in WWTPs refer to freshwater plants, either domestic or industrial, and yet very little is known about plants that utilize seawater for their operation, mainly because there are still very few of these running in the world. Their utilization responds to the deficiency in hydric resources prevailing in their locations and their use will probably increase in the near future due to water shortage associated to global warming as many areas are experiencing today (Barnett et al., 2005). As a consequence, knowledge of the microbial diversity becomes crucial to identify the key players in these systems.

In a recent survey (Sánchez et al., 2011), the prokaryotic diversity of a seawater-utilizing WWTP from a pharmaceutical industry located in the south of Spain was characterized using a polyphasic approach by means of three molecular tools that targeted the 16S rRNA gene, i.e. DGGE, clone libraries and FISH. The results showed that the composition of the bacterial community differed substantially from other WWTP previously reported, since Betaproteobacteria did not seem to be the predominant group; in contrast, other classes of Proteobacteria, such as Alpha- and Gammaproteobacteria, as well as members of Bacteroidetes and Deinococcus-Thermus contributed in higher proportions. Besides, utilization of specific primers for amplification of the amoA (ammonia monooxygenase subunit A) gene confirmed the presence of nitrifiers corresponding to the Beta- subclass of Proteobacteria, although they were not identified in this study.

In the present article, we further investigated the diversity of this system by applying 454-pyrosequencing, a much more powerful molecular technique, which provides thousands of sequence reads. We analysed the bacterial assemblage by targeting the 16S rRNA gene and increased our knowledge on its diversity by one order of magnitude. Additionally, we characterized the nitrifying members of this sludge by pyrosequencing the amoA gene. As far as we know, this is the first study that analyses the amoA gene diversity in an activated sludge of a WWTPs with the particularity to utilize seawater.

Results and discussion

We investigated the bacterial community structure and identified the nitrifying members from the activated sludge of a seawater-utilizing WWTP located in Almeria (Southeast Spain). The plant treats wastewater from a pharmaceutical industry. The mean influent flow of the plant is 300 m³ h⁻¹ and has a treatment volume of 32 000 m³. Nitrogen and chemical oxygen demand sludge loads were about 150–170 kg h⁻¹ and 900–1000 kg h⁻¹ respectively. DNA was extracted from samples of aerated mixed activated sludge collected in 2 consecutive years (2007 and 2008).

Diversity of bacterial communities in activated sludge

After a rigorous quality control (see Experimental procedures and Table S1) a total of 16 176 16S rRNA tag sequences of sufficient quality were analysed (8010 sequences corresponding to year 2007 and 8166 sequences to year 2008) and grouped into operational taxonomic units using uclust at 3% cut-off level. The clustering resulted in a total of 320 different OTUs from which 107 were shared between samples (33.3%) as shown in the Venn diagram (Fig. 1A). The number of OTUs in 2007 was 201 and in 2008 was 226. Although the proportion of shared OTUs is rather low, the unique diversity in each sample corresponded mainly to rare OTUs (relative abundance below 1%). In the case of year 2007, the unique clones to that sample represented a 19% of the total reads, from which only three OTUs were above 1%. For the 2008 sample, the unique clones represented a 9% of total sequences and only two OTUs presented an abundance above 1%. These results are in agreement with previous observations in which DGGE analysis from both samples showed virtually the same pattern for universal
primers amplifying Bacteria, suggesting that activated sludge was at a steady state at least for the most abundant phylotypes (Sánchez et al., 2011).

Richness was computed by the Chao1 estimator and analysis by rarefaction showed that the diversity in the two samples was within the same range, although slightly higher in 2008. However, we found that this depth of sequencing was not sufficient to saturate the curve and therefore, the actual diversity is likely much higher (Fig. 2). Nevertheless, if compared with the rarefaction curve from a clone library performed from the 2007 activated sludge sample, we observe that, by applying pyrosequencing we increased our knowledge on the diversity present by one order of magnitude. Rank-abundance curves (Fig. S1A) show that there were only a few abundant phylotypes and a long tail of rare taxa, therefore, most of the unknown diversity probably corresponds to rare diversity (Pedrós-Alió, 2007).

RDP Classifier was used to assign the representative OTU sequences into different phylogenetic bacterial taxa. Figure S2 shows the relative abundances of the different groups at the phylum and class level for both years. *Deinococcus-Thermus*, *Proteobacteria*, *Chloroflexi* and *Bacteroidetes* were abundant in both samples. Comparison with a previous survey (Sánchez et al., 2011) indicates that most of these groups were also retrieved by different molecular methodologies. However, the contribution of each group varied depending on the technique used. The bacterial clone library over-represented the *Deinococcus-Thermus* group, while the rest of procedures showed similar results concerning this phylum. In contrast, the *Alphaproteobacteria* were over-represented by FISH (Fig. S3).

On the other hand, pyrosequencing allowed the detection of other groups that could not be recognized by other molecular techniques, such as the *Chloroflexi*, *Chlorobi*, *Deferribacteres*, *Verrucomicrobia*, *Planctomycetes* and *Spirochaetes*, deepening our knowledge on the diversity of this activated sludge. Also, a certain percentage of sequences remained as unidentified bacteria (6.5% and 10.5% for years 2007 and 2008; Fig. S2). Except the *Chlorobi* and *Deferribacteres*, different pyrosequencing studies have reported the presence of these groups in conventional activated sludge samples (Sanapareddy et al., 2009; Kwon et al., 2010). However, it is remarkable that, in general, the proportions of the different groups in freshwater activated sludge were different from saline samples, and when going deeper into genus composition, the assemblage of our samples differs strongly from that previously reported. In general, prior pyrosequencing studies with different samples of activated sludge are in agreement with the predominance of the classes *Bacteroidetes* and *Proteobacteria* and the phylum *Bacteroidetes* (Sanapareddy et al., 2009; Kwon et al., 2010), while in our saline activated sludge the groups that predominate are, within the phylum *Proteobacteria*, the *Alphaproteobacteria* (8.0% and 7.3% in samples 2007 and 2008 respectively), *Gamma-proteobacteria* (15.2% and 7.2%), as well as the *Deinococcus-Thermus* group (21.8% and 10.9%) and members of the phyla *Chloroflexi* (9.5% and 35.1%) and *Bacteroidetes* (18.3% and 2.8%). In contrast, Ye and colleagues (2011), who analysed by pyrosequencing the bacterial composition of a slightly saline activated sludge from a laboratory-scale nitrification reactor and a WWTP from Hong Kong, found that, in addition to *Proteobacteria* and *Bacteroidetes*, the phylum *Firmicutes* was also abundant in their samples; they also obtained similar groups as in the present study, such as the *Actinobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Deinococcus-Thermus*, *Chloroflexi* and *Spirochaetes*, although at different relative ratios, as well as different phyla not retrieved in the present work, for example the *Nitrospira*, *Chlamydiae* and TM7. Probably, differences are due to the feeding wastewater, since in our case the main influent corresponds to intermediate products of amoxicillin synthesis whereas in the other study the WWTP treated a slightly saline urban sewage from Hong Kong.

As in previous pyrosequencing studies (Keijser et al., 2008; Liu et al., 2008; Claesson et al., 2009), a part of sequences could only be assigned to the phylum/class level and the majority of taxa could not be classified at the genus level (74% for 2007 and 83.5% for 2008), demonstrating the extraordinary microbial diversity of activated sludge that cannot be classified using public 16S rRNA databases. Table S2 shows the taxa found in each sample.
in this study at the genus level, which are different from other reported genus of freshwater or either slightly saline activated sludge studies (Sanapareddy et al., 2009; Kwon et al., 2010; Ye et al., 2011). One of the most abundant genus in both samples is *Truepera*, a member of the phylum *Deinococcus-Thermus*, which includes radiation-resistant and thermophilic species. Although this phylum was also detected by pyrosequencing in a recent study with a slightly saline activated sludge (Ye et al., 2011), it accounted for no more than 0.6% of total community, while we found a significant percentage of sequences from this genus (21.8% and 10.9% for years 2007 and 2008 respectively).

**Diversity of nitrifying community in activated sludge**

A total of 43,297 amoA gene sequences of good quality (11,236 reads for year 2007 and 32,061 reads for year 2008) were grouped into operational taxonomic units using uclust at 6% cut-off level. We selected a 6% cut-off to group closely related phylotypes of the amoA gene without losing potentially valuable information by the inclusion of phylogenetically distinct sequences. Interestingly, the diversity of the nitrifying bacterial community revealed by pyrosequencing of the amoA gene was very low and rarefaction analyses showed the depth of sequencing was sufficient to saturate the curve and recover the great majority. The clustering of 43,297 reads resulted in a total of only eight OTUs from which six were shared between samples as shown in the Venn diagram (Fig. 1B). The shared OTUs corresponded to 97% of total reads, which indicates that the nitrifying community was very similar both years.

All amoA sequences were highly related to previously described sequences in the GenBank database, both environmental and from isolates (Fig. 3). Phylogenetic analysis revealed that eight phylotypes formed two separate clusters. The first cluster, which contains three OTUs, was mostly retrieved in the 2008 library and represented 45.4% of sequences of that sample. The closest relatives in GenBank database (99% similarity) included sequences from organisms that have not been obtained from a WWTP, and *Nitrosomonas* sp. LT-2 and LT-5, isolated from a CANON reactor (98% identity). The second cluster, which contains five of the OTUs and represented the most abundant phylotypes in both samples, was most closely related (94% identity) to cultured representatives of strains of *Nitrosomonas marina* isolated from a biofilter of a recirculating shrimp aquaculture system (GenBank Accession No. HM345621, HM345612 and HM345618) and *Nitrosomonas* sp. NS20 isolated from coastal marine sediments. This cluster virtually represented all sequences (99.99%) in the sample of 2007 whereas in 2008 it comprised 54.6%.

These data are consistent with previous results found by Ye and colleagues (2011) in slightly saline activated sludge, which showed that *Nitrosomonas*, together with *Nitrospira*, was the dominant nitrifying genera, and also with the study by Park and colleagues (2009), who identified that a specific ammonia-oxidizing bacteria belonging to the *Nitrosomonas europaea* lineage was dominant in a full-scale bioreactor treating saline wastewater due to its adaptation to high-salt conditions. In general, nitrifiers are also responsible for ammonia oxidation in conventional WWTPs (Purkhold et al., 2000; Zhang et al., 2011b). However, we did not retrieve *Nitrospira* in the pyrosequencing 16S rRNA libraries or previously in DGGE gels, clone libraries and FISH (Sánchez et al., 2011) probably due to their low abundance. Thus, pyrosequencing of functional genes such as amoA revealed the presence of particular groups which could not be retrieved when analyzing the 16S rRNA, demonstrating its value to deepen into the functionality of microbial populations when targeting specific genes. The only nitrifier that could be retrieved in our samples by 16S rRNA pyrosequencing was *Nitrococcus*, a *Gammaproteobacteria* which just represented 0.3% and 0.5% of the total reads for years 2007 and 2008 respectively, and has also been reported to be an important nitrifier in some activated sludges (Juretschko et al., 1998; Raszka et al., 2011). Thus, since it was actually detectable in the general bacterial 16S rRNA gene population, it could also participate in ammonia oxidation together with the *Betaproteobacteria*, despite previous efforts for amplifying the gammaproteobacterial amoA gene yielded negative results (Sánchez et al., 2011).

On the other hand, we know that nitrification and denitrification are central processes in our system, since a nitrification fraction of 98% and a total nitrogen removal over 80% have been reported (M.I. Maldonado, pers. comm.). In fact, Yu and Zhang (2012), when applying both metagenomic and metatranscriptomic approaches to characterize microbial structure and gene expression of an activated sludge community from a municipal WWTP in Hong Kong found that nitrifiers such as *Nitrosomonas* and *Nitrospira* had a high transcription activity despite presenting a very low abundance (they accounted only 0.11% and 0.02% respectively in their DNA data set), and the results from Zhang and colleagues (2011b) indicated that the abundance of ammonia-oxidizing bacteria in the activated sludge from different WWTPs was very low. Similarly, our results suggest that *Nitrosomonas* could be responsible of nitrification although showing a low abundance. Also, it may be possible that other genera different from the well-known *Betaproteobacteria* could contribute to nitrification activity.

In fact, different studies have reported the autotrophic oxidation of ammonia by members of the domain *Archaea*. The crenarchaeon *Nitrosopumilus maritimus* is...
Fig. 3. Maximum-likelihood tree of amoA gene. The tree was determined using approximately 491 unambiguously aligned positions of nucleic acid amoA sequences. Each sequence from this study (in bold) is representative of clustered amoA sequences in the WWTP activated sludge with an identity of 94%. Reference sequences from GenBank database are indicated by their accession number if they correspond to uncultured organisms or by the strain name if they belong to amoA sequences from bacterial strains. The tree was constructed with RAxML (http://bioinformatics.oxfordjournals.org/content/22/21/2688.full) using the GTRGAMMA model and an alignment made with MUSCLE (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC390337). The sequence of methane monoxygenase subunit A from Methylococcus capsulatus strain Bath served as outgroup (GenBank Accession No. YP_115248). The scale bar indicates substitutions per site.;
able to oxidize ammonia to nitrite under mesophilic conditions (Könneke et al., 2005), while ammonia-oxidizing Archaea occurred in activated sludge bioreactors used to remove ammonia from wastewater (Park et al., 2006). However, amplification of the amoA gene to detect the presence of archaeal nitrifiers yielded negative results in our samples. In fact, Archaea accounted only for 6% of DAPI counts, and all sequences retrieved previously in an archaeal clone library were related to methanogenic archaea (Sánchez et al., 2011). Other studies have also demonstrated the presence of methanogens in aerated activated sludge but, although active, they played a minor role in carbon and nitrogen turnover (Gray et al., 2002; Fredriksson et al., 2012).

Interestingly, different heterotrophic bacteria, such as Bacillus sp. (Kim et al., 2005), Alcaligenes faecalis (Liu et al., 2012), Marinobacter sp. (Hai-Yan et al., 2012), Achromobacter xylosoxidans (Kundu et al., 2012) and Pseudomonas sp. (Su et al., 2006) have been described as potential nitrifiers, and remarkably, some of these genera have been isolated from our activated sludge by culture-dependent techniques (data not shown); for instance, some strains were identified as Bacillus sp., Alcaligenes sp., Marinobacter hydrocarbonoclasticus and Pseudomonas sp.

In contrast, sequences of Nitratireductor sp., a denitrifying microorganism, have been retrieved with different molecular methods (pyrosequencing in this study and DGGE and clone library in Sánchez et al., 2011), while other sequences from potential denitrifiers have been recovered only by 454-pyrosequencing, such as Leucobacter sp., Caldithrix sp., Castellaniella sp. and Halomonas sp. Besides, other candidates for denitrifying bacteria have been isolated by culture-dependent techniques, such as Alcaligenes sp., Bacillus sp., Paracoccus sp., Pseudomonas sp. and Marinobacter sp. (data not shown). Other genera retrieved by pyrosequencing were related to the nitrogen fixation process, that is, Microbacterium sp., Aminobacter sp. and Spirochaeta sp., while Sphingomonas was detected by clone library and culture-dependent methods.

Summarizing, we can conclude that the bacterial diversity in the activated sludge of the seawater-processing plant was high as previously observed in conventional WWTPs. However, the composition of the bacterial community differed strongly from other plants, and was dominated by Deinococcus-Thermus, Proteobacteria, Chloroflexi and Bacteroidetes. Previous analyses by clone library, DGGE and FISH were not enough to reflect the profile of the bacterial community in wastewater sludge and although pyrosequencing was a powerful tool to define the microbial composition deeper sequencing is required. Despite nitrification rates were high in the system, known ammonia-oxidizing bacteria were not identified by means of 16S rRNA studies and analysis of the specific functional gene amoA was required to reveal the presence and identity of the bacteria responsible for this process. These results suggest that only a few populations of low abundant but specialized bacteria likely with high transcription activity are responsible for removal of ammonia in these systems. However, further studies to isolate the key microorganisms involved in ammonia-oxidation will be essential in order to understand this process in saline WWTPs.

Acknowledgements
We thank the Bioinformatics Platform UAB (BioinfoUAB), Ramiro Logares and Guillem Salazar for help and support with sequence analyses.

Conflict of interest
None declared.

References

Barnett, T.P., Adam, J.C., and Lettenmaier, D.P. (2005) Potential impacts of a warming climate on water availability in snow-dominated regions. Nature 438: 303–309.
Benedict, R.G., and Carlson, D.A. (1971) Aerobic heterotrophic bacteria in activated sludge. Water Res 5: 1023–1030.
Boon, N., De Windt, W., Verstraete, W., and Top, E.M. (2002) Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiol Ecol 39: 101–112.
Claesson, M.J., O’Sullivan, O., Wang, Q., Nikkilä, J., Marchesi, J.R., Smidt, H., et al. (2009) Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS ONE 4: e6669.
Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer, K.H., and Wagner, M. (2001) In situ characterization of Nitrospira-like nitrite-oxidation bacteria active in wastewater treatment plants. Appl Environ Microbiol 67: 5273–5284.
Dias, F.G., and Bhat, J.V. (1964) Microbial ecology of activated sludge. Appl Environ Microbiol 12: 412–417.
Eichner, C.A., Erb, R.W., Timmis, K.N., and Wagner-Döbler, I. (1999) Thermal gradient gel electrophoresis analysis of bioprotection from pollutant shocks in the activated sludge microbial community. Appl Environ Microbiol 65: 102–109.
Fredriksson, N.J., Hermansson, M., and Wilén, B.-M. (2012) Diversity and dynamics of Archaea in an activated sludge wastewater treatment plant. BMC Microbiol 12: 140.
Gray, N.D., Miskin, E.P., Kornilova, O., Curtis, T.P., and Head, I.M. (2002) Occurrence and activity of Archaea in aerated activated sludge wastewater treatment plants. Environ Microbiol 4: 158–168.
Hai-Yan, Z., Ying, L., Xi-Yan, G., Guo-Min, A., Li-Li, M., and Zhi-Wei, L. (2012) Characterization of a marine origin aerobic nitrifying–denitrifying bacterium. J Biosci Bioeng 114: 33–37.

Juretschko, S., Timmerman, G., Schmid, M., Schleifer, K.-H., Pommerening-Roser, A., Koops, H., and P., and Wagner, M. (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrososoccus mobilis and Nitrospira-like bacteria as dominant populations. Appl Environ Microbiol 64: 3042–3051.

Keijser, B.J., Zaura, E., Huse, S.M., van der Vossen, J.M., Schuren, F.H., Montijn, R.C., et al. (2008) Pyrosequencing analysis of the oral microflora of healthy adults. J Dent Res 87: 1016–1020.

Kim, J.K., Park, K.P., Cho, K.S., Nam, S.W., Park, T.J., and Bajpai, R. (2005) Aerobic nitrification-denitrification by heterotrophic Bacillus strains. Bioreasour Technol 96: 1897–1906.

Kim, T.-S., Kim, H.-S., Kwon, S., and Park, H.-D. (2011) Nitrifying bacterial community structure of a full-scale integrated fixed-film activated sludge process as investigated by pyrosequencing. J Microbiol Biotechnol 21: 293–298.

Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., and Stahl, D.A. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437: 543–546.

Kundu, P., Pramanik, A., Mitra, S., Choudhury, J.D., Mukherjee, J., and Mukherjee, S. (2012) Heterotrophic nitrification by Achromobacter xylosoxidans S18 isolated from a small-scale slaughterhouse wastewater. Bioprocess Biosyst Eng 35: 721–728.

Kwon, S., Kim, T.-S., Yu, G.H., Jung, J.-H., and Park, H.-D. (2010) Bacterial community composition and diversity of a full-scale integrated fixed-film activated sludge system as investigated by pyrosequencing. J Microbiol Biotechnol 20: 1717–1723.

Liu, Y., Li, Y., and Lv, Y. (2012) Isolation and characterization of a heterotrophic nitrifier from coke plant wastewater. Water Sci Technol 65: 2084–2090.

Liu, Z., deSantis, T.Z., Andersen, G.L., and Knight, R. (2008) Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. Nucleic Acids Res 36: e120.

Park, H.-D., Wells, G.F., Bae, H., Criddle, C.S., and Francis, C.A. (2006) Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors. Appl Environ Microbiol 72: 5643–5647.

Park, H.-D., Lee, S.-Y., and Hwang, S. (2009) Redundancy analysis demonstration of the relevance of temperature to ammonia-oxidizing bacterial community compositions in a full-scale nitrifying bioreactor treating saline wastewater. J Microbiol Biotechnol 19: 346–350.

Pedrós-Alló, C. (2007) Ecology. Dipping into the rare biosphere. Science 315: 192–193.

Prakash, T.B.S., and Dondono, N.C. (1967) Aerobic heterotrophic populations of sewage and activated sludge. I. Enumeration. Appl Environ Microbiol 15: 461–467.

Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M.C., Koops, H.P., and Wagner, M. (2000) Phylogenetic analysis of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. Appl Environ Microbiol 66: 5368–5382.

Rasza, A., Surnacz-Górksa, J., Zabczyński, S., and Miksch, K. (2011) The population dynamics of nitrifiers in ammonium-rich systems. Water Environ Res 83: 2159–2169.

Saikaly, P.E., Stroot, P.G., and Oerther, D.B. (2005) Use of 16S rRNA gene terminal restriction fragment analysis to assess the impact of solids retention time on the bacterial diversity of activated sludge. Appl Environ Microbiol 71: 5814–5822.

Sanapareddy, N., Hamp, T.J., González, L.C., Hilger, H.A., Fodor, A.A., and Clinton, S.M. (2009) Molecular diversity of a North Carolina wastewater treatment plant as revealed by pyrosequencing. Appl Environ Microbiol 75: 1688–1696.

Sánchez, O., Garrido, L., Forn, I., Massana, R., Maldonado, M.I., and Mas, J. (2011) Molecular characterization of activated sludge from a seawater-processing wastewater treatment plant. Microb Biotechnol 4: 628–642.

Snайдер, J., Ammann, R., Huber, I., Ludwig, W., and Schleifer, K.-H. (1997) Phylogenetic analysis and in situ identification of bacteria in activated sludge. Appl Environ Microbiol 63: 2884–2896.

Su, J.J., Yeh, K.S., and Tseng, P.W. (2006) A strain of Pseudomonas sp. Isolated from piggery wastewater treatment systems with heterotrophic nitrification capability in Taiwan. Curr Microbiol 53: 77–81.

Wagner, M., Amman, R., Lemmer, H., and Schleifer, K.-H. (1993) Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl Environ Microbiol 59: 1520–1525.

Wagner, M., Loy, A., Nogueira, R., Purkhold, U., Lee, N., and Daims, H. (2002) Microbial community composition and function in wastewater treatment plants. Antonie Van Leeuwenhoek 81: 665–680.

Ye, L., Shao, M.-S., Zhang, T., Tong, A.H.Y., and Lok, S. (2011) Analysis of the bacterial community in a laboratory-scale nitrification reactor and a wastewater treatment plant by 454-pyrosequencing. Water Res 45: 4390–4398.

Yu, K., and Zhang, T. (2012) Metagenomic and metatranscriptomic analysis of microbial community structure and gene expression of activated sludge. PLoS ONE 7: e38183. doi:10.1371/journal.pone.0038183.

Zhang, T., Shao, M.F., and Ye, L. (2011a) 454 pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. ISME J 6: 1137–1147.

Zhang, T., Ye, L., Tong, A.H.Y., Shao, M.-F., and Lok, S. (2011b) Ammonia-oxidizing archaea and ammonia-oxidizing bacteria in six full-scale wastewater treatment bioreactors. Appl Microbiol Biotechnol 91: 1215–1225.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Experimental procedures.
Table S1. Summary of clean sequences, singletons and operational taxonomic units (OTUs) in the two data sets analysed.
Table S2. Genera identified from pyrosequencing and cloning in the saline activated sludge.
Fig. S1. Rank abundance curves of OTUs defined by a 3% sequence variation for 16S rRNA (A) and 6% for amoA (B) genes in the activated sludge.
Fig. S2. Taxonomic composition by phylum and class for the sequences retrieved in 2 consecutive years.
Fig. S3. Percentage of relative intensity of DGGE bands, clones (library), probe positive cells scaled to Eub probes (FISH) and reads (454) affiliated to different phylogenetic groups from samples D07 and D08. Data from DGGE, cloning and FISH have been extracted from Sánchez and colleagues (2011). [Alphaproteobacteria (Alpha), Betaproteobacteria (Beta), Gammaproteobacteria (Gam), Deltaproteobacteria (Delta), Bacteroidetes (Bact), Firmicutes (Firm), Deinococcus-Thermus (DT), Actinobacteria (Actino), Chloroflexi (Chloroflexi), Def (Deferribacteres), Chlorobi (Chlorobi), Not determined (Not det).]