Impact on nitrifiers of full-scale bioaugmentation
F. Stenström and J. la Cour Jansen

ABSTRACT
Nitrifiers are the slowest growing bacteria used in conventional biological wastewater treatment. Furthermore, their growth rate is seriously hampered by low temperature. As a result, the volume needed for nitrification dominates the volume of the biological reactors at a wastewater treatment plant. As a way of enhancing nitrification and reducing this volume, bioaugmentation can be used. Nitrifiers from a side-stream plant can be inoculated to the mainstream process, which is thereby boosted. The effect of bioaugmentation can be measured in different ways. This full-scale study focuses on the effect of bioaugmentation from a microbial point of view by using 16S rRNA amplicon sequencing. The study reveals how bioaugmentation increases the diversity of nitrifiers in the mainstream process and in the side-stream plant; that the abundance of nitrifiers is increased in the mainstream process; the interaction between nitrifiers from the side-stream plant and mainstream process; and the effect of bioaugmentation on nitrifying genera and species over time. To our knowledge, this detailed microbial information on nitrifying species during a full-scale bioaugmentation study has not been presented before.

Key words | amplicon sequencing, bioaugmentation, biological wastewater treatment, inoculation, nitrifiers

INTRODUCTION
Nitrifiers are the slowest growing bacteria used at conventional wastewater treatment plants (WWTPs). Due to the low growth rate of nitrifiers, biological reactors must be designed with a large volume to accommodate a sufficient amount of biomass in the system. Furthermore, the growth rate of nitrifiers is greatly influenced by temperature, which implies that the reactor volumes intended for nitrification need to be remarkably large in cold climate regions. Nitrifiers typically represent <4% of the biomass at a conventional WWTP with biological nitrogen removal (Ekama & Wentzel 2008). A feasible way of speeding up the nitrification rate at a WWTP is to multiply the number of nitrifiers through bioaugmentation – that is, to inoculate nitrifiers to the mainstream process. The bioaugmentation of nitrifiers has been shown to be an effective means of shortening the necessary sludge retention time (SRT) at WWTPs in cold regions (Salem et al. 2003; Head & Oleszkiewicz 2004; Stenström & la Cour Jansen 2016). Nevertheless, due to differences in temperature, pH, alkalinity, ammonium concentration, and so forth, several studies have shown that there are different types of nitrifiers in different environments (e.g. Podmirseg et al. 2010; Gatti et al. 2015). This means that inoculated nitrifiers from the side-stream process may not be optimal for conditions in the mainstream process. In order to enhance the type of nitrifiers that already exist in the mainstream process, return activated sludge (RAS) from the mainstream process can be pumped into the side-stream process to be multiplied, and thereafter be returned to the mainstream process.
Bacteria are commonly divided into different kinds of strategists depending on their growth rate and substrate affinity. K-strategists have a low growth rate and high substrate affinity, and consequently benefit from low substrate concentrations. In contrast, r-strategists have a high growth rate but a low substrate affinity, and therefore thrive in high substrate concentrations. In terms of ammonia-oxidizing bacteria (AOB), r-strategists include Nitrosomonas europaea, Nitrosonomas eutropha, Nitrosomonas halophile, and Nitrosomonas mobilis (compiled in Gatti et al. 2015), while K-strategists include Nitrosospira (Schramm et al. 1999) and Nitrosomonas oligotropha (Gatti et al. 2015). In terms of nitrite-oxidizing bacteria (NOB), Nitrobacter are r-strategists, while Nitrospira and Nitrotoxa are considered to be K-strategists (Nowka et al. 2015). During a pilot-scale bioaugmentation study performed by Pei et al. (2015), a microbial change was observed in both the side-stream reactor and the mainstream process, from Nitrospira to Nitrosomonas europaea and from Nitrospira to Nitrobacter. In both cases, the changes were from K-strategist to r-strategist.

In the last few decades, advances in molecular methods have provided a new understanding of microorganisms in wastewater treatment. One of these methods, 16S rRNA amplicon sequencing, can be used to analyze and survey the complete microbial composition by reading the 16S rRNA genes of the bacteria and using them as ‘fingerprints’. In the outcome from this method, different bacteria species are presented as operational taxonomic units (OTUs) and their abundances. However, the results and the interpretation of the results from this method are associated with some difficulties, as reviewed by Kim et al. (2015). One such difficulty is that all bacteria cells are counted, whether they are dead or alive; another is that the number of the targeted gene per cell differs between species, implying that different species are counted a different number of times, which indicates that the abundance may be incorrect even if techniques are used to mitigate this effect. Furthermore, new achievements in the last few years have shown that the earlier distinction that was made between AOB and NOB has become complicated. Daims et al. (2015) and van Kessel et al. (2015) found that species from the genus Nitrospira, a classical NOB, are also capable of oxidizing ammonium to nitrite like an AOB. This finding means that errors may result when classifying different types of nitrifiers into the groups of AOB and NOB. In general, the results obtained from a sequencing should be interpreted with caution.

A study examining how AOB were affected by bioaugmentation stated that the presence of AOB through 16S rDNA sequencing should not be set equal with active AOB biomass, and that samples with low gene copy numbers of the 16S rDNA gene can still represent highly active AOB communities (Podmirseg et al. 2010). Furthermore, it was suggested that bioaugmentation seems to have a larger effect on the community composition than on the total abundance of AOB. This finding implies that abundance fluctuations are not necessarily directly connected to the nitrification rate. The growth rates of AOB and NOB are strongly dependent on temperature. Hellina et al. (1998) observed that AOB dominate NOB at temperatures above 20–25 °C, whereas NOB dominate AOB at temperatures below this range. The diversity of bacteria in a nitrifying/denitrifying system has been found to decrease with lower temperature (Karkman et al. 2011). In addition, Urakawa et al. (2008) suggested that temperature is a key factor for the species richness of AOB, and that the AOB diversity decreases with a drop in temperature.

Various laboratory-scale and pilot-scale studies have been performed in order to examine the inoculation effect on nitrifiers, although the number of full-scale studies in the literature is limited. Moreover, only a few full-scale studies have been combined with an evaluation of the microbial communities using molecular tools. Podmirseg et al. (2010) showed in a full-scale study how AOB in the mainstream process were influenced by bioaugmentation. However, this evaluation did not examine how the NOB were affected. This paper presents the benefits of bioaugmentation and reveals how the nitrifiers in the mainstream process of a full-scale municipal plant can be enhanced by conveying nitrifiers from a side-stream reactor. Furthermore, 16S rRNA amplicon sequencing is used to show that the number of AOB and NOB species are increased in the mainstream process, and to demonstrate how different nitrifying genera and species are affected by bioaugmentation over time. To our knowledge, such detailed information on nitrifying species during a full-scale bioaugmentation study has not been presented before.

**MATERIALS AND METHODS**

**Slotshagen WWTP and normal SBR operation**

This work was performed at Slotshagen WWTP, a plant that treats municipal wastewater from the city of Norrköping, Sweden. The plant is designed for 200,000 population equivalents (PE), although the actual load corresponds to 135,000 PE. The WWTP is an activated sludge plant comprising pre-precipitation, biological reactors with pre-denitrification and contact stabilization, and post-precipitation. The biological
treatment in the mainstream process consists of two separated trains, referred to in this study as the ‘augmented train’ and the ‘reference train’. Two-thirds of the influent flow rate is directed to the augmented train and one-third is directed to the reference train, corresponding to the share of each out of the total volume. From former studies (Stenström & la Cour Jansen 2016), an inherent difference in the behavior of the two trains has been observed. The load (according to the volume), SRT and operation of the trains are similar but the reference train has, for example, proved to reach higher nitrification rates. The reason for this is unknown. The RAS flow rate averages approximately 95% of the influent flow rate. During this study, the ammonium concentration in the influent and effluent to/from the biological step in the mainstream process (for both the augmented train and the reference train) averaged 23 mg L⁻¹ and 2.5 mg L⁻¹, respectively, implying an ammonium reduction of about 90%. The estimated SRT in the mainstream process during this period was 16–20 d. The sludge is stabilized in mesophilic digesters. It is thereafter dewatered in centrifuges, and the digester supernatant is directed to a buffer tank, from which it is pumped to the SBR.

The SBR is typically in operation from November to May in order to guarantee a low effluent concentration of total nitrogen (TN) from the WWTP during the winter. During the start-up, the SBR is partly filled with excess sludge from the mainstream process (from both trains). Parts of the digester supernatant are cautiously added, and the SBR cycle is initiated. After 1–2 weeks, the SBR receives all produced digester supernatant. The covered SBR has a volume of 1,000 m³ and constitutes 3% of the total volume of the biological treatment in the mainstream process. It is operated with post-denitrification and has a cycle length of 8 h. Ethanol is added during denitrification. Typical characteristics of the digester supernatant in the influent to the SBR are as follows: TN, 1,500 mg L⁻¹; NH₄⁺–N, 1,200 mg L⁻¹; NO₃⁻–N, 3 mg L⁻¹; COD, 2,000 mg L⁻¹; PO₄³⁻–P, 40 mg L⁻¹; HCO₃⁻, 90 mM; pH, 8.0; and temperature, 50 °C. In an ordinary cycle for the SBR, the influent flow is 70 m³. The flow rate of digester supernatant typically constitutes 0.5% of the flow rate of influent to the WWTP, whereas the TN load to the SBR averages about 15% of the TN load to the plant.

**Experimental setup**

This study was performed from December 2013 to May 2014. Two process conditions were altered in the SBR during the full-scale study: the SRT, and the flow rate of RAS to the SBR compared with the flow rate of the digester supernatant to the SBR (the RAS/digester supernatant flow rate ratio). The SRT in the SBR was decreased in steps of approximately 10 d, 8 d, 6 d, 4 d, and finally 2.5 d. Over the same time period, the flow rate ratio of RAS/digester supernatant to the SBR was increased in a stepwise manner from 0% to 10%, to 35%, and finally to 100%. This process is illustrated in Figure 1, which also provides a process scheme for the full-scale experimental setup. In parallel to this study, nitrification rate tests were performed. For further information regarding the normal operation, the experimental setup, and the results from the nitrification rate tests, see Stenström & la Cour Jansen (2016). Treated water from the SBR (i.e. decanted water and excess sludge) was directed to the augmented train of the mainstream process. The augmented train could then be compared with the non-bioaugmented reference train. Because the mainstream process was operated with nearly complete nitrification, the effect from bioaugmentation could not be discerned by the ammonium concentration in the effluent from the plant. The temperature in the SBR during the study was 28–35 °C except for a week-long period in week 11 when the temperature dropped to 24 °C at its lowest.

**Molecular methods**

Grab samples were taken approximately every second week from the SBR and from the two separate trains in the mainstream process. The samples were homogenized and immediately frozen. They were later analyzed with 16S rRNA amplicon sequencing targeting the bacterial variable region 1–3 (V1–3). This method comprises DNA extraction from all bacteria through a molecular process, followed by DNA sequencing. The bacterial DNA have specific ‘fingerprints’ that are used to identify species by matching with a database. DNA was extracted using the FastDNA SPIN Kit for soil (MP Biomedicals, USA); 4× the normal bead beating was used in order to enable the recovery of bacteria that are difficult to lyse (Albertsen et al. 2015). In the same way as described in Matturro et al. (2016), 16S rRNA amplicon library preparation (V1–3), DNA sequencing, and 16S rRNA amplicon bioinformatics processing were performed. All Nitrospira species were classified as NOB in the study.

**RESULTS AND DISCUSSION**

A total of 33 samples from all three reactors were analyzed by means of 16S rRNA amplicon sequencing in order to identify and count the bacteria species and their
abundances. The number of reads per sample varied between 24,755 and 57,870. The relative abundances of all species were determined, that is, the number of reads of every species were compared with the total number of reads per sample. A total of 3,549 species were identified. Most species were observed at a very low abundance and only in occasional samples. In general, the 50 most common species comprised about 50% of the relative abundance in all samples. The number of species in the three reactors ranged from 899 (in the SBR) to 1,996 (in the augmented train). The relative abundances of AOB and NOB were examined thoroughly. In the augmented train, reference train, and SBR, the relative abundance of nitrifiers varied from 1.5–3.5%, 1.2–3.2%, and 1.4–7.8%, respectively. Eleven different AOB species were read; 10 of these were of the genus *Nitrosomonas*, one was of the genus *Nitrospira* (in a few samples only, and at very low abundance). Five different NOB species were detected; three were of the genus *Nitrosospira* and two were of the genus *Candidatus Nitrotoga*. No change from r-strategists to K-strategists was observed for AOB or NOB in any of the three reactors, in contrast to the results of a pilot-plant study that were reported by Pei et al. (2015). The nitrification rate tests revealed that the nitrification rate was 41% higher in the augmented train than in the reference train, on average, for a period of 3 weeks with the lowest water temperature (Stenström & la Cour Jansen 2016). The nitrification rate was found to be 25% higher, on average, in the augmented train than in the reference train, over the whole bioaugmentation period. In this microbiological study, no obvious effect could be discerned from the deliberate variations of the SRT in the SBR, or from the variations in the RAS flow rate to the SBR.

Figure 2(a) shows the fluctuations in the total number of bacteria species in each reactor. At first, there were barely 1,000 different species in the SBR. After the bioaugmentation started, that is, once RAS from the mainstream process began being pumped to the SBR, the number of species increased to more than 1,700 in a few weeks—an obvious effect of bioaugmentation on the SBR. When comparing the total number of bacteria in the two trains in the mainstream process, the total number of bacteria did not obviously increase in the augmented train compared with the reference train. However, the smallest difference between the two trains was found just before bioaugmentation started, at week 51. The difference

![Figure 1](https://iwaponline.com/wst/article-pdf/76/11/3079/210630/wst076113079.pdf)
between the two mainstream trains becomes more apparent when the numbers of different AOB and NOB species are examined, as shown in Figure 2(b). Before the bioaugmentation started, there were a maximum of eight nitrifying species in each reactor. When the bioaugmentation was running, the average number of species in the non-bioaugmented reference train decreased, as was also found by Urakawa et al. (1999). The opposite pattern was found in the augmented train over the same time period, with increasing numbers of different nitrifying species (which also occurred in the SBR), even during the coldest season. Both the AOB and the NOB species increased. These findings contrast with the results of Urakawa et al. (2008), who stated that the nitrifier diversity decreases when the temperature drops; however, they align with the findings of Gatti et al. (2015), who pointed out that bioaugmentation enhances microbial diversity. The increased number of nitrifying species in the augmented train and in the SBR is the result of an interaction from bioaugmentation, as they boost each other in a ‘win-win’ system.

Figure 3 presents the average relative abundance of AOB and NOB in the three different reactors during the total bioaugmentation period. As expected, the highest abundance of nitrifiers is found in the SBR, due to the high nitrogen concentration in the digester supernatant. When the augmented train is compared with the reference train, it is seen that both AOB and NOB are more abundant in the augmented train. The average relative abundances of AOB and NOB are 32% and 17% higher in the augmented train, respectively. Thus, the average relative abundance of nitrifiers is 25% higher in the augmented train over the whole bioaugmentation period of four months. This result aligns with the differences in average nitrification rates of the two trains (Stenström & la Cour Jansen 2016).

The dominating species of NOB in all three reactors were Nitrotoga and Nitrospira. Nitrotnoga was recently
discovered as an NOB that is adapted to a colder climate, with a temperature optimum at 10 °C. It is also adapted to low nitrite concentrations (Alawi et al. 2007). It has been found that Nitrotoga occurs in activated sludge systems, and that its abundance is supported by a low temperature (Alawi et al. 2009; Karkman et al. 2011; Saunders et al. 2013); it is suggested that Nitrotoga be classified as a K-strategist. Furthermore, Nitrotoga has been found to outgrow Nitrospira during long-term cultivation at a temperature of 10 °C (Alawi et al. 2009). Nitrospira is also classified as a K-strategist, but it grows in a broad temperature range with a higher temperature optimum than Nitrotoga (Alawi et al. 2009). Lücker et al. (2015) found that Nitrotoga and Nitrospira often coexist. The distribution of NOB species during the experiment revealed that the bioaugmentation had an impact on both the augmented train and the SBR. During the first period of bioaugmentation, the abundance of Nitrospira dominated in all three reactors. After a few weeks, the abundance of Nitrotoga began to increase in the reference train. At that point, the abundance of Nitrotoga in the augmented train and in the SBR was still very low. A few weeks later, the abundance of Nitrotoga began to increase simultaneously in the augmented train and in the SBR. This delay of the Nitrotoga entrance in the augmented train and in the SBR could be explained by the fact that Nitrospira is favored by a higher temperature in the SBR, and is thereby retained in the system. When Nitrotoga finally began growing in the augmented system, its abundance in the augmented train and in the SBR increased at the same pace, as shown in Figure 4. The reason for the temporarily increased abundance of Nitrotoga in the SBR at week 17 is unknown. The change in species did not result in any change in different strategists, unlike the results described by Pei et al. (2015). Surprisingly, Nitrobacter was not found in any of the reactors.

CONCLUSIONS

The following results were observed in this full-scale study:

- As a result of bioaugmentation, the diversity of nitrifiers increased in the mainstream process as well as in the side-stream plant, even during the coldest season.
- The abundance of nitrifiers in the mainstream process increased by 25%, on average, over the whole bioaugmentation period of 4 months. This finding aligns with an earlier study on nitrification rates at the same WWTP.
- The recently discovered NOB, Nitrotoga, was highly abundant in the mainstream process during the cold season. In contrast, Nitrobacter was not found at all.

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