OXYGEN UPTAKE RATE (OUR) TESTS FOR ASSESSMENT OF NITRIFYING ACTIVITIES IN THE DEAMMONIFICATION SYSTEM

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ABSTRACT
The two-step deammonification system for nitrogen removal was established in a technical-scale pilot plant with moving-bed reactors with Kaldnes rings as biofilm carriers. The study consisted in observing the presence of nitrifiers in both activated sludge and biofilm in the partial nitritation reactor and in the Anammox reactor. Oxygen Uptake Rate (OUR) tests were used as a tool to assess qualitatively and quantitatively ammonia- and nitrite-oxidizing bacteria as well as heterotrophic activity. Thirteen series of OUR tests focussed on monitoring the activity of nitrifying microorganisms in the deammonification system. It was demonstrated that the nitrifying culture mostly forms biofilm in the partial nitritation reactor. The results confirmed that there is only insignificant nitrifying activity present in the Anammox reactor and it is mainly concentrated in the activated sludge. It is most likely that nitrifiers sustain oxygen-limited conditions for the Anammox bacteria. The moving-bed system is therefore appropriate to obtain efficient removal of nitrogen with taking advantage of the cooperation of many bacterial cultures.

KEYWORDS
Anammox; activated sludge; biofilm; deammonification; oxygen uptake rate (OUR) test; partial nitritation

INTRODUCTION

Over the last decade many authors described the implementation of a novel process of anaerobic ammonium oxidation (Anammox) in treating highly ammonium-concentrated streams (Van Hulle, 2005; Schmidt et al., 2003; Jetten et al., 1999). Due to a low growth rate of the Anammox bacteria and its sensitivity to nitrite nitrogen concentration (Jetten et al., 1999), a biofilm concept turned out to be especially relevant. Publications demonstrated that the deammonification system with nitritation and Anammox processes could be established successfully in a moving bed biofilm reactor (Rosenwinkel and Cornelius, 2005; Hippen et al., 2001).

In the deammonification system, there are different bacterial cultures active. Table 1 summarizes the reactions performed by the groups of microorganisms that are involved in the ammonia and nitrite uptake: ammonium oxidizers (reaction 1) and nitrite oxidizers (reaction 2), aerobic denitrification (reaction 3) and anaerobic ammonium oxidation (reaction 4). The anaerobic ammonium oxidation (Anammox) is performed by a new group of nitrite-depended Anammox bacteria (van Dongen et al., 2001; Mulder et al., 1995). The Anammox bacteria enlarge their biofilm population (start-up period) on an existing nitrifying biofilm. Initial aerobic operation with high loadings followed by oxygen-limited conditions results in gaining a mixed biocenosis consisting of aerobic and anaerobic ammonia oxidizers. Many authors demonstrated stable nitrite build-up in a separate reactor (Gut et al., 2005a, b; Jianlong and Ning, 2003; Surmacz-Górska et al.,

1997). Rosenwinkel and Cornelius (2005) assessed performance of a moving-bed partial nitritation reactor. They pinpointed three strategies for selective inhibition of nitrite oxidizers. One can set temperature of the process above 25°C, use the effect of lower sensibility of ammonia oxidizers to free ammonia (NH_3) or set the dissolved oxygen concentration in the bulk liquid low enough to enable ammonia-oxidizing bacteria to grow faster.

Table 1. Overview of nitrogen form conversions.

No.	Process	Reaction	Bacteria	References
1	Nitritation	$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$	Nitrosomonas, e.g. N. eutropha, N. europea; Nitrosospira; Nitrosocystis	Rittmann and McCarty, 2001; Henze et al., 2002; Szewczyk, 2005
2	Nitratation	$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$	Nitrobacter, e.g. N. agilis, Nitrospira, Nitrococcus	
3	Aerobic denitrification	$C_{18}H_{19}O_9N + 0.74\ NH_3 + 8.8\ O_2 \rightarrow 1.74\ C_5H_7NO_2 + 9.3\ CO_2 + 4.25\ H_2O$	Pseudomonas, Bacillus, Alcaligenes, Paracoccus	
4	Anammox	$NH_4^+ + 1.32\ NO_2^- + 0.066HCO_3^- + 0.13\ H^+ \rightarrow 0.26\ NO_3^- + 1.02\ N_2 + 0.066\ CH_2O_{0.5}\ N_{0.15} + 2.03\ H_2O$	Planctomycetales, e.g. Candidatus *Brocadia anammoxidans*, Candidatus *Kuenenia stuttgartiensis*	van Dongen et al., 2001

Other studies (Mulder et al., 1995; Kuai and Verstraete, 1980) revealed that ammonia oxidizers could also denitrify using ammonia as electron donor under oxygen-limited conditions. Moreover, Schmidt et al. (2002b) demonstrated that *Nitrosomonas eutropha* could oxidise ammonia under anoxic conditions if NO_2 was present. Therefore, in the oxygen-limited environment many groups of bacteria can co-exist performing a variety of reactions simultaneously, like in the CANON system (Sliekers et al., 2002). Ammonium oxidizers can by oxidizing ammonia to nitrite keep the dissolved oxygen (DO) concentration very low, while Anammox bacteria utilise the remaining part of ammonium with nitrite to produce nitrogen gas.

Interestingly, it has been shown that nitrite oxidizers are outcompeted by ammonium oxidizers as they have lower affinity for oxygen (Hellinga et al., 1998). Great flexibility of the aerobic oxidizers should not be underestimated, as aerobic and anaerobic bacteria can be partners in performing overall efficient nitrogen elimination (Schmidt et al., 2002b). In gaining cooperation between the bacteria instead of competition, a composition of substrates is an important issue. Both ammonium- and nitrite-dependence of concurrent aerobic and anaerobic reactions should be taken into account. Schmidt et al. (2002a) provided strong indicators that even though two groups of ammonia oxidizers compete for the same substrate, it is possible to achieve their cooperation. Aerobic oxidizers can supply nitrite for the Anammox bacteria. These both groups might find suitable conditions for growth at the oxic-anoxic interface.

In Sweden, the system for nitrogen removal was designed with two stages, in which the preceding partial nitritation prepares a proper influent to the succeeding Anammox reactor for nitrogen removal (Gut et al., 2005a, b; Szatkowska, 2004; Trela et al., 2004; Płaza et al., 2003). This article is an attempt to recognize the distribution of the nitrifying bacteria and estimate their activity in a moving-bed deammonification system. Both suspended (activated sludge) and attached (biofilm) biomass is present in the reactors.

MATERIAL AND METHODS

Technical-scale pilot plant

The technical-scale pilot plant for studies of the deammonification system (Gut et al., 2005a; Szatkowska et al., 2004; Trela et al., 2004) is situated at the Himmerfjärden Wastewater Treatment Plant (WWTP) located in the southwestern part of Stockholm, Sweden. The pilot plant (Figure 1) consists of two reactors (2.1 m^3 each) that are divided into three zones. Each zone has a mechanical stirrer to assure proper mixing and diffusion of oxygen. Heaters that are installed in the first zone of each reactor keep the optimum temperature in reactors. The reactors are filled with Kaldnes rings (45-50% of volumetric filling) that provide a large protected surface for the biofilm bacterial culture. Kaldnes rings are the biofilm carrier elements made of polyethylene with a density slightly below that of water (Ødegaard et al., 1994). The activated sludge biomass is suspended by means of mixing. Under the investigated period the pilot plant was continuously supplied with a supernatant from dewatering of digested sludge, characterized by the high content of ammonium nitrogen varying from 260 to 880 mg NH_4-N/l. Complete characteristics of the influent supernatant was studied by Gut et al. (2005a, b) and Szatkowska et al. (2004).

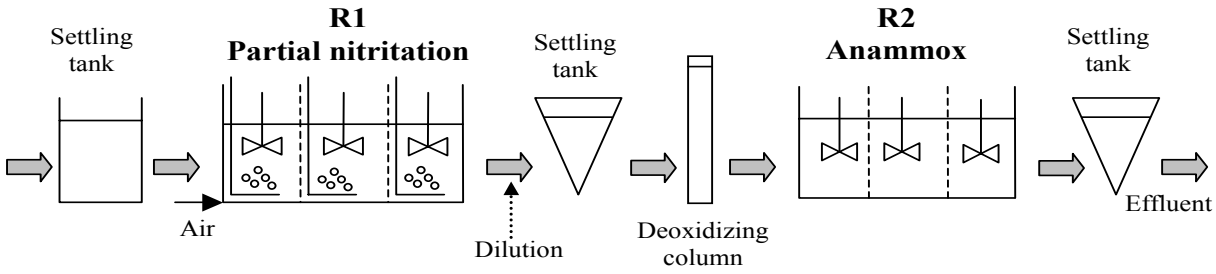

Figure 1. Scheme of the deammonification system.

Oxygen Uptake Rate (OUR) tests – performance description

The evaluation pattern for assessment of the nitrifying activity in both reactors of the pilot plant consisted in examining activated sludge (S) and Kaldnes biofilm (K) cultures separately as well as observing the activity of microorganisms in the mixture of Kaldnes and biofilm (K+S) from both reactors. The experiments had as a goal to check whether there is any nitrifying activity present in the Anammox reactor. In case of proving existence of nitrifiers, a qualitative and quantitative inspection of the nitrifying activity in the Anammox reactor was planned. The nitrifying activity (in biofilm and activated sludge) calculated in the partial nitritation reactor was used as a reference. Long time span of OUR test runs was applied with similar procedure and aimed at monitoring changes in the nitrifying activity of the pilot plant. Measurements of the dissolved oxygen concentration in the bulk liquid during OUR tests were carried out during a subsequent addition of selective inhibitors of two bacterial populations: ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (Surmacz-Górska et al., 1996) (Figure 2). The methodology was modified for the biofilm bacterial culture (Gut et al., 2005a). Sodium chlorate ($NaClO_3$) as an inhibitor of NO_2-N oxidation by NOB and Allylthiourea (ATU) as an inhibitor of NH_4-N oxidation by AOB were used. First, the total nitrification activity was measured. After some minutes (depending on the test progress), 5 ml of $NaClO_3$ (final concentration 17 mM) was added to the mixed liquor (a closed 600 ml respiration unit). Subsequently, after some time the 5 ml of ATU (final concentration 43 μM) was added. Data logger TESTO® 251 connected to a dissolved oxygen electrode recorded the values. The dissolved oxygen uptake rate was determined by linear regression from the slope of the oxygen utilization curve. The difference between total OUR and the one after $NaClO_3$ addition was considered as the oxygen uptake due to NO_2-N oxidation. Whereas the oxygen uptake due to NH_4-N oxidation was presented as a difference between the OUR with $NaClO_3$ and the OUR after

addition of two inhibitors. At the end, the OUR measured in the presence of two chemicals reflected the oxygen consumption of the heterotrophs (HT). By this method it is impossible to distinguish the oxygen consumption for substrate oxidation and endogenous respiration.

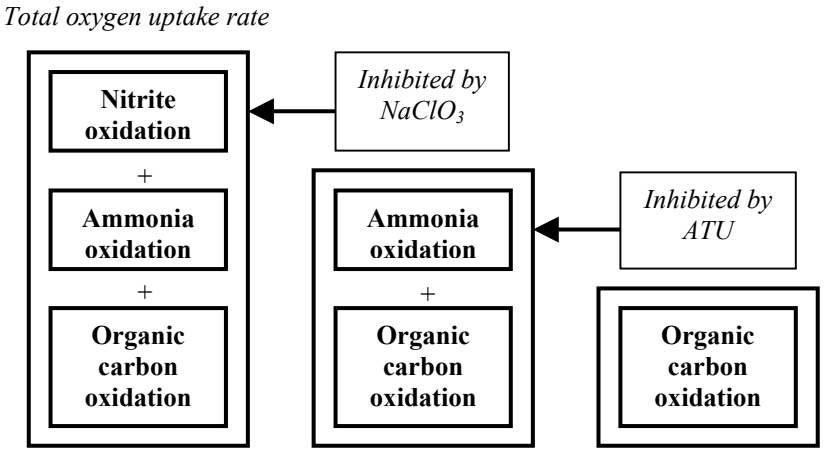

Figure 2. Schematic representation of the action of $NaClO_3$ and ATU on respiratory activity of the microorganisms (after Surmacz-Górska et al., 1996).

The OUR tests were performed for the samples with activated sludge (S), Kaldnes rings (K) with the filtrated supernatant and the combination of Kaldnes biofilm carries and activated sludge (K+S) from both reactors of the pilot plant. The samples were prepared purposefully by concentrating the activated sludge from the Anammox reactor and taking the sample of activated sludge from the partial nitritation step that represented its concentration in the reactor. Suspended Solids (SS) and Volatile Suspended Solids (VSS) were analysed at the beginning of each test. Inorganic nitrogen forms of ammonium (NH_4-N), nitrite (NO_2-N) and nitrate (NO_3-N) nitrogen were analysed as well using DrLange VIS Spectrophotometer XION 500 equipment. Aeration of the media was done before the test, which enabled to obtain the initial dissolved oxygen (DO) concentration of 7-8 mg O_2 l^{-1}. Temperature was kept at 32^{o}C and the pH was measured manually before the test.

RESULTS AND DISCUSSION

Technical-scale pilot plant

An example of two profiles for inorganic nitrogen forms is presented in Figure 3. The operational strategy of the deammonification system for nitrogen removal consisted in checking different reactor configurations. Two different periods for the period of OUR test performance can be naturally distinguished due to operational strategy for the system. Variable influent supernatant characteristics and application of internal and external recirculation caused divergences in the process performance, which was reflected in the profiles. Under the year 2004, although some nitrogen elimination was observed in the first reactor, whereas the bacterial culture in the second reactor was responsible for nitrogen removal. The dilution of the effluent from R1 was done before supplying to the second reactor. In the middle of 2005 much higher nitrogen elimination was possible in the R1. At the same time the nitrogen removal capacity was maintained in the Anammox reactor.

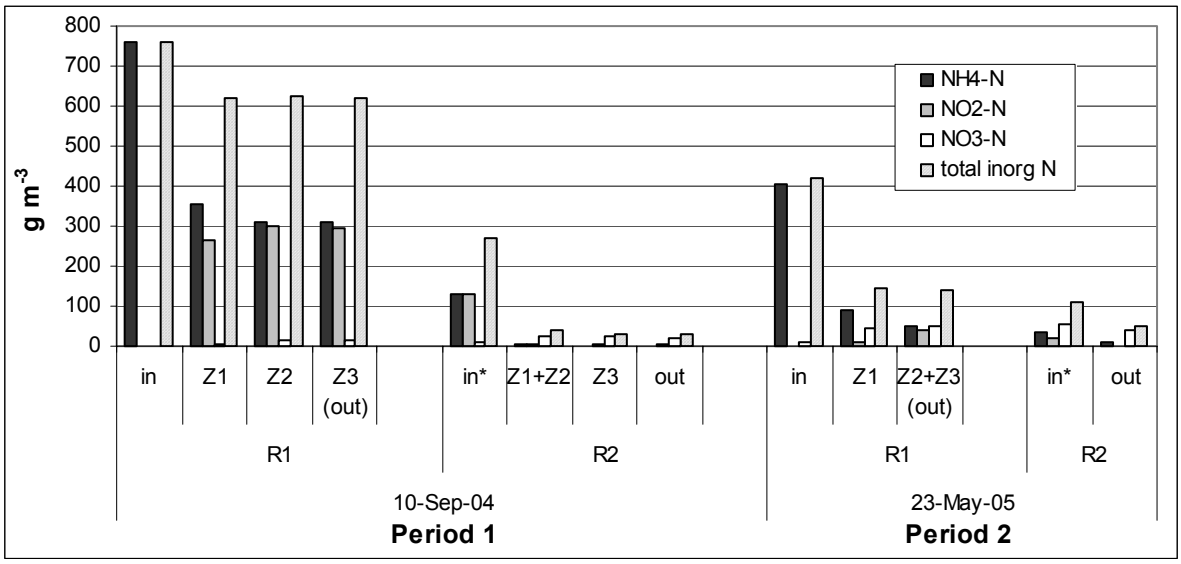

Figure 3. Two representative profiles of inorganic nitrogen forms in the deammonification system for periods 1 and 2 (*influent to R2 after dilution; Z=zone).

During the research period, the concentration of activated sludge in the Anammox reactor was very changeable. The dynamics of suspended biomass contribution was related to the changes in the operation of the pilot plant. The average values of SS for the studied periods 1 and 2 were 351±381 g m^{-3} and 1107±1151 g m^{-3} for reactor 1 and 2, respectively. The organic part (VSS) of suspension was estimated as average values of 81% for R1 and 74% for R2 of the total suspended solids under the period of investigations. It was obvious that high concentration of the suspended biomass in the system must have some impact on the performance of both steps. Owing to stable results for nitrogen removal efficiency, it seems probable that the activated sludge had rather positive influence on the process.

OUR tests

Thirteen series of OUR tests were conducted with the aim of examining nitrifying activity in the Anammox reactor of the deammonification system. The existence of nitrifying activity was detected mainly in the activated sludge in the Anammox reactor by performing routine OUR tests throughout one year of the pilot plant operation. The research was extended due to the fact that the phenomenon of seeding the nitrifying culture from the partial nitritation reactor into the Anammox reactor was presumed. Therefore the activities in the partial nitritation reactor were assessed as reference values.

Figure 4 presents the assessment of nitrifying activities in the concentrated activated sludge forming the biomass in the moving-bed Anammox reactor. Comparing the activities, a dissimilar pattern of the activities over time can be observed. The assessment of thirteen series of tests for the activated sludge provides information that both AOB and NOB activities were enhanced during one-year operation of the pilot plant (division into two periods in Figure 4) whereas HT activity was still insignificantly small despite doubling activity for period 2 (see also Table 2). Under the performance of initial tests (period 1) the NOB activity was occasionally higher than the AOB activity. It can be assumed that the aeration before the test results in enhanced NOB activity. The nitrite oxidizing bacteria have lower affinity for oxygen than ammonium oxidizers; therefore, when the dissolved oxygen concentration is increased higher NOB activity can be obtained. With time (period 2), the distribution bacterial activities revealed prevailing activity of AOB. On average AOB have 2.5 times larger activity than NOB in R2 for period 2 (Table 2).

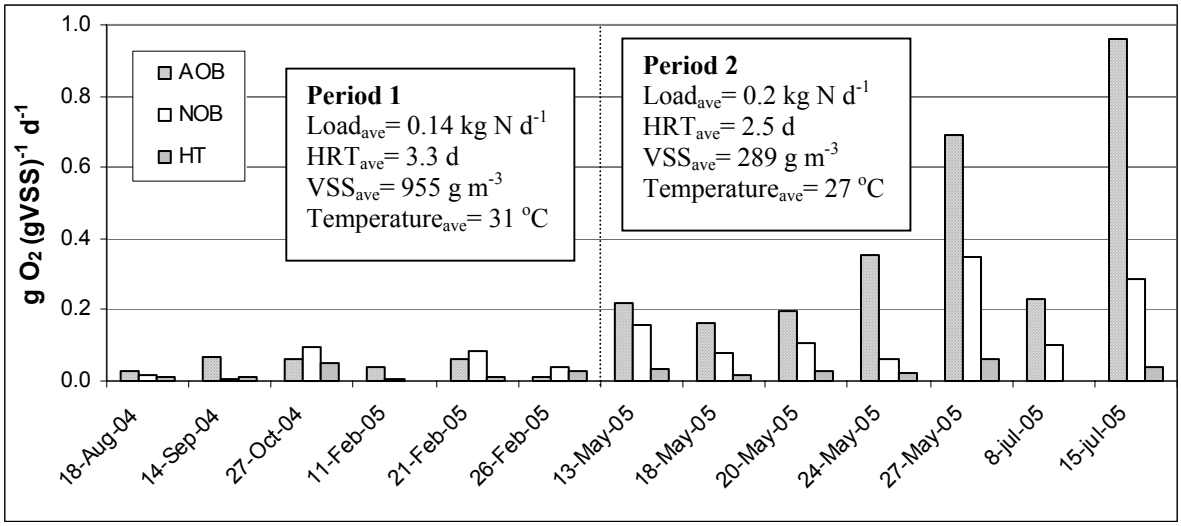

Figure 4. Nitrifying activities in the concentrated activated sludge from the Anammox reactor for thirteen series of OUR tests (pilot-plant operational values for period 1 and 2 are given in text boxes).

Table 2. Statistics for thirteen OUR tests for the concentrated activated sludge from R2.

R2 Concentrated sludge		Average±S.D.	
		Period 1	Period 2
g O_2 (g VSS)$^{-1}$ d^{-1}	AOB[1]	0.0446±0.0216	0.3939±0.3121
	NOB[2]	0.0409±0.0400	0.1596±0.1140
	HT[3]	0.0184±0.0179	0.0421±0.0252
g m^{-3}	VSS[4]	1693±785	1585±797

[1]AOB – ammonia oxidizing bacteria; [2]NOB – nitrite oxidising bacteria; [3]HT – heterotrophic activity (oxygen consumption for substrate oxidation and endogenous respiration); [4]VSS – volatile suspended solids

Figure 5 demonstrates the distribution of activities in the deammonification system by comparing average activities in both reactors for seven tests performed during period 2. The extension of routine tests by adding samples from the partial nitritation process was done with the aim of understanding a significance of the nitrifying capacity in the Anammox reactor. For both reactors the values were recalculated for two units (g O_2 (g VSS)$^{-1}$ d^{-1} and g O_2 m^{-2} d^{-1}) in order to differentiate between the contribution of the activated sludge and the biofilm culture to the nitrifying capacity.

Figures 4 and 5 demonstrate general trends in the distribution of the activities in the system. The nitrifying activity of the activated sludge is much lower in R2. Irrespective of the increase of the nitrifying activity in R2 during period 2, the nitrifying culture in R2 exhibits 11 times lower activity than the culture in R1 (Figure 5 a,b). Moreover, Figure 5a reveals that in R1 there is a substantial increase of activity for the samples with the combination of sludge and Kaldnes compared to the sludge only. In the sample of sludge enriched by the biofilm culture, the AOB and NOB have 2.4 times higher activities whereas HT 4.4 times higher. Moreover, Figure 5c confirms that the nitrifying activity in R1 is mainly present on Kaldnes rings. Interestingly, for the samples combining Kaldnes and sludge from R1 (what can be assumed as an imitation of conditions inside the reactor) the activities are enhanced positively only for ammonia-oxidizing bacteria (1.7-fold increase). It validates favourable conditions in R1 to obtain oxidation from ammonia to nitrite and suppressing the second phase of nitrification.

In R2, however, the contribution of biofilm in oxidation of nitrogen forms is minor (Figure 5b and d). The main responsibility for the nitrifying activity is put on the activated sludge, which can be seen in Figure 5b. The sample of sludge with the addition of the Kaldnes biofilm bacteria did not influence the overall oxidation capacity. Only a very small increase of the AOB activity is calculated in the sample for both Kaldnes and sludge. When comparing samples of Kaldnes with the combination of Kaldnes and concentrated sludge from R2 (Figure 5d), one can clearly notice the enhancement of activities in the combined sample. Figures 5b and 5d complement each other in understanding the distribution of nitrifying activity in R2. In general, the activated sludge is responsible for the oxidation of nitrogen forms in R2. The indirect conclusion could be that the total inorganic nitrogen removal in R2 (see Figure 3) is due to the Anammox bacterial culture that finds suitable conditions on Kaldnes rings and develops biofilm.

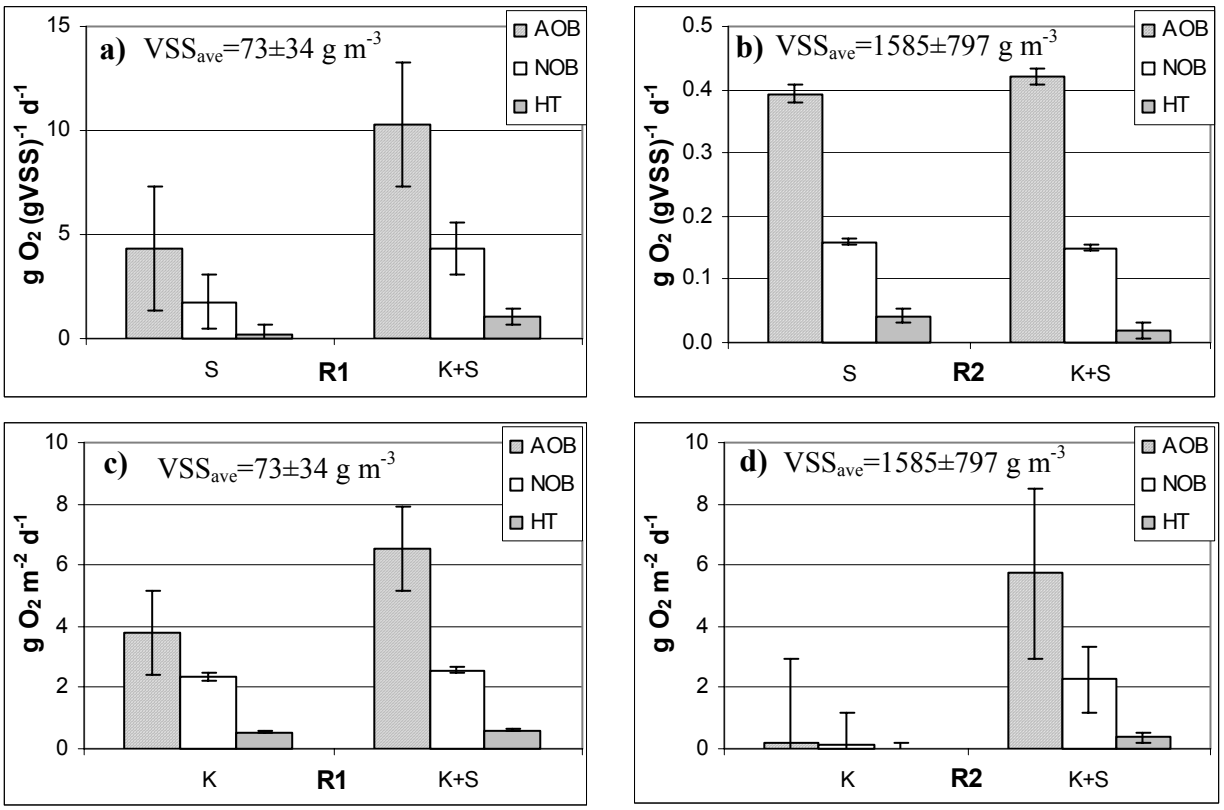

Figure 5. Overview of the average activities in both reactors of the deammonification system for seven OUR tests during period 2 (bars indicate standard errors showing standard deviation in the process between the samples; K - Kaldnes, S - sludge, K+S - Kaldnes and sludge).

The order of magnitude in the results of the nitrifying activities reveals differences between reactors (Figure 5a and b). The comparison of activities for the samples with activated sludge only demonstrates much higher activities in R1. On average, AOB and NOB have 11 times higher activities in R1 and HT show a 5.4-fold increase. The difference in activities on Kaldnes rings between reactors (Figure 5c and d) shows that AOB and NOB have 22 times higher activity in R1 whereas HT exhibit a 17-fold increase of activity in R1. Similar average activities of nitrifiers for samples combining Kaldnes and sludge from both reactors (Figure 5c, d) are related with over 20 times higher concentration of organic suspended material in the tests for R2 (see test boxes).

Both for the test with the Kaldnes rings only and for the activated sludge in both reactors ammonia-oxidizing bacteria show higher activity. For seven consecutive tests, the average activity ratio

AOB/NOB for the activated sludge amounts to 2.5 for both reactors. The ratio AOB/NOB for the Kaldnes biofilm culture is equal to 1.6 also for both reactors. It gives implication that both in the biofilm and the activated sludge AOB prevail at the same ratio, which demonstrates comparable enhancement of AOB activity in every reactor.

To sum up, despite the application of different pilot plant configurations throughout the period of OUR test performance, two periods of the test performance (Figure 4 and Figure 5a, c) give similar patterns in the distribution of nitrifying activity. For period 1 the discrepancy over time in nitrifying activity is on average much smaller than for period 2 (Table 2). Nevertheless, small contribution of nitrifiers in R2 to the system performance is confirmed.

Due to the fact that the activity of AOB is depended on the substrate concentration (Anthonisen et al., 1976), the correlation curves for the test with Kaldnes biofilm culture and the combination of biofilm and activated sludge from reactor 1 were obtained (Figure 6). Correlation coefficients show high dependence of the AOB activity on the substrate concentration.

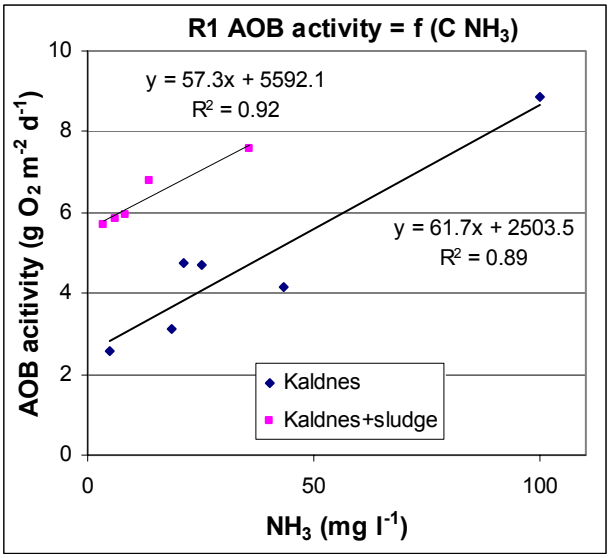

Figure 6. Substrate concentration influence on the AOB activity in R1.

Conclusions

1. In the partial nitritation reactor, the biofilm bacterial culture mainly exhibits nitrifying activity. In comparison with the biofilm nitrifying activity on the Kaldnes rings in the Anammox reactor, the activities in reactor 1 are substantially higher. Activities are on average 22 times higher in reactor 1 when comparing activities in the biofilm.
2. In the Anammox reactor, the activated sludge culture is responsible for the nitrifying capacity. This activity is however small as it is on average 11 times lower than the activity in reactor 1 in the activated sludge.
3. In both reactors, the AOB culture is more dynamic than the NOB culture. It sustains the initial principle of suppressing the nitrification process in reactor 1 at the level of nitrite formation. Tests proved that the operation conditions in reactor 1 were preferable for establishing the ammonia-oxidizing culture. Such predominance of AOB in the bacterial community is advantageous for the Anammox reactor as well. Ammonium-oxidizing bacteria improve the Anammox reaction by supplying additional nitrite nitrogen and concomitantly removing dissolved oxygen in the bulk liquid and therefore sustaining oxygen-limited conditions for the Anammox bacteria.

4. A substantial variety in bacterial activities is related to changes in the influent characteristics to the Anammox reactor in relation to the system configuration strategy.
5. The moving-bed system is adequate to gain a cooperation of many bacterial cultures in removing nitrogen. It can be suspected that the cooperation of activated sludge and biofilm positively contributed to the efficient Anammox process performance. The removal of oxygen by nitrifiers provides conditions for the Anammox bacteria to develop on the Kaldnes rings.

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