Successful and unsuccessful bioaugmentation experiments monitored by fluorescent *in situ* hybridization

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Abstract Two nitrifying reactors were operated under the same conditions except that one was twice inoculated with the aerobic denitrifying bacteria *Microvirgula aerodenitrificans*. The first bioaugmentation induced a transient nitrogen loss. Fluorescent *in situ* hybridization revealed that the bioaugmented bacteria had been rapidly eaten by protozoa. The second massive inoculation unbalanced the ecosystem and resulted in an overgrowth of protozoa and perturbations of nitrification, whereas both parameters remained stable in the non bioaugmented reactor. To enhance the incorporation of the added bacteria to indigenous flocs, two strategies were then tested. First, coagulating and flocculating substances were added to the reactor just after bioaugmentation and second, the bacteria were embedded in alginate beads before inoculation. The latter strategy gave the best results. After break-up of the beads, alginate fragments, containing microcolonies of *M. aerodenitrificans*, were found to be incorporated into the existing flocs. Alginate beads offer a temporary protection against grazing and favor the adhesion of the exogenous bacterial microcolonies to the existing flocs. These beads therefore constitute a suitable bioaugmentation vector to incorporate a bacterial strain into activated sludge flocs.

Keywords Aerobic denitrification; alginate beads; bacterial augmentation; grazing; quantification of bacterial populations

Water Science and Technology **Vol 41 No 12 pp 61–68** © IWA Publishing 2000

Introduction

Bacterial augmentation or bioaugmentation are terms that describe the direct addition of a selected microbial biomass in order to improve certain biological properties of a particular ecosystem. This procedure has been used for decades in some ecosystems such as soils (van Veen *et al.*, 1997). In the field of wastewater treatment, the literature reports a few cases of successful bioaugmentation (Rittmann and Whiteman, 1994). Commercial products are even available today. However, there are numerous reports of bioaugmentation failure and the real efficiency of the procedure remains highly controversial (Stephenson and Stephenson, 1992). One reason for this is that the fate of the inoculated organisms and the reaction of the host ecosystem often remain unclear because of the lack of suitable microbiological monitoring techniques. Fluorescent *in situ* hybridization technique (FISH) now allows us to directly visualize a bacterial population in complex ecosystems and is thus particularly suited to the study of bioaugmentation experiments (Amann *et al.*, 1990).

We approached this problem of bioaugmentation in the field of biological nitrogen removal from wastewater. Conventional processes are composed of two steps: aerobic nitrification and anaerobic denitrification. Recently, bacteria able to denitrify under aerobic conditions have been isolated (Patureau *et al.*, 1994; Robertson *et al.*, 1989). Introducing and maintaining these bacteria and their activities in a complex nitrifying community could allow the combination of nitrification and denitrification in the same aerobic unit. In order to better characterize the biological phenomena involved during bioaugmentation, we monitored the introduction of the aerobic denitrifying bacterium *Microvirgula aerodenitrificans* (Patureau *et al.*, 1998) using the FISH technique. The first step of this study is based on the comparison of two reactors operated under the same conditions: a control and another

inoculated with the strain. In the second part of this work, the efficiency of two different strategies were evaluated in order to enhance the incorporation of the added strain to sludge flocs.

Methods

Reactors and culture conditions

Two litres Biolafitte reactors (HRT=3.1 d, 25ºC, 7.5<pH<8.5, between 1 g/l to 2.5 g/l of dry mass) were operated as Sequencing Batch Reactors (SBR) under strictly autotrophic nitrifying conditions as already described elsewhere (Bouchez *et al.*, 2000). Air saturated conditions were obtained by strong air sparging. Once stable nitrification was maintained, all reactors were fed with a sodium acetate solution once every two days to allow heterotrophic denitrification (day 1). The first reactor (SBR1) was operated without bioaugmentation. The second reactor (SBR2) was inoculated, at day 1, with an exponential phase pure culture of *M. aerodenitrificans* harvested by centrifugation. The quantity inoculated represented 6.6% of the Suspended Solids (SS) of the reactor at that time. It was inoculated once again, four days later, at 37.1% of SS. In the third reactor (SBR3), coagulating and flocculating agents, AP2 at 40 ppm and CS53 at 5 ppm (Degrémont-Erpack), were added immediately after bioaugmentation with the strain (at 10.6% of SS). In the fourth reactor (SBR4), before inoculation (at 6.6% of SS), *M. aerodenitrificans* was embedded in 1.6% alginate (SIGMA) beads polymerized during one hour with 0.1M $CaCl_2$.

Analytical measurements

Ammonium was determined using a Büchi 320 apparatus, according to the method recommended by Rodier (1975). Nitrate and nitrite were measured by an exchange ion chromatography system using conductivity detection (DIONEX-100). Acetate was assayed by gas chromatography using nitrogen as carrier gas and a flame ionization detector (Chrompack CP9000). Suspended Solids (SS) measurements were performed by filtering the sample through a 0.2 µm nylaflor (Gelman Sciences) nylon filter and then drying the filtered biomass for 24 hours in an oven held at 105ºC.

In situ hybridization

Fluorescent *in situ* hybridization with 16S rRNA targeted probes and washes was performed on slides according to conventional protocols (Manz *et al.*, 1992) except that the sample centrifugation steps were replaced by gentle filtration on nylon 0.2 µm filters to preserve floc structures. The following set of fluorescent-labeled probes was used in the conditions recommended by the different authors: EUB338 for almost all bacteria (Amann *et al.*, 1990), Alf968 for alpha subclass of *Proteobacteria* (Neef, 1997), BET42a for beta subclass of Proteobacteria (Manz *et al.*, 1992), GAM42a for gamma subclass of *Proteobacteria* (Manz *et al.*, 1992), HGC for Gram positive bacteria with High DNA G+C content (Roller *et al.*, 1994), BONE23a for beta one subclass of *Proteobacteria* (Amann *et al.*, 1996), BTWO23a (used as a competitor for BONE23a), CF319a for *Cytophaga-Flavobacterium* cluster (Wagner *et al.*, 1994), ACA for genus *Acinetobacter* (Wagner *et al.*, 1994), Nso190 for ammonia-oxidizing bacteria of the beta subclass of *Proteobacteria* (Mobarry *et al.*, 1996), NEU23a for halophilic and halotolerant members of the genus *Nitrosomonas* (Wagner *et al.*, 1995), CTE (Used as a competitor for NEU23a), S-*-Ntspa-1026-a-A-18 for activated sludge clones affiliated with the genus *Nitrospira*; *Nitrospira moscoviensis* (Juretschko *et al.*, 1998), NIT3 for genus *Nitrobacter* (Wagner *et al.*, 1996), S-S-Mae-1414-a-A-18 for *M. aerodenitrificans* (Bouchez *et al.*, 2000).

Image analysis and quantification procedure

Quantification of bacterial populations in SBR1 and SBR2 was performed using the area-ratio method after FISH and confocal laser scanning microscopy. A combination of one

probe of interest labeled with Cy3 and probe EUB338 labeled with Cy5 was used (Bouchez *et al.*, 2000). For samples from SBR3 and SBR4, a dispersion procedure was applied before hybridization. In the first step of this procedure the sample was centrifuged and the resulting pellets were resuspended in v/v PBS X1/EDTA 0.5 M and placed in a bead beater at maximum speed for 15 min. This treatment was repeated twice. It resulted in the complete dissolution of alginate fragments and sludge macroflocs (100–150 μm) had been disrupted into microflocs (<15 μm). The proportion of S-S-Mae-1414-a-A-18-positive cells after *in situ* hybridization was then quantified against the total biomass stained with DAPI using the area-ratio method. In this case, images were numerized with an epifluorescence microscope (Olympus BX60 fitted with 100X and 40X Uplan Apo objectives and with U-MWU and U-MMF filter sets) coupled with camera and image analysis software (Optimas 6.2, Imasys). At least twenty randomly selected microscopic fields were examined.

Dot blot hybridization

The eukaryotic fraction of the biomass was followed by radioactive dot-blot hybridization on rRNA extracts as previously described (Doré *et al.*, 1998). The two synthetic HPLC purified oligonucleotidic probes S-*-UNIV-1390-a-A-18 (Zheng *et al.*, 1996) and S-D-Euca-502-a-A-16 (Amann *et al.*, 1990) (Cybergène SA, Saint-Malo, France) were used with prehybridization temperature of 42ºC and subsequent washing step at 44ºC and 52ºC, respectively. After hybridization the rate of particle emission from those parts of the membrane spotted with extract was counted with an instant-imager (Packard-Instrument, SA, Rungis, France).

Results and discussion

SBR1 and SBR2 before start of bioaugmentation experiment

Before the start of the experiment, nitrification was maintained stable in all SBR. 100% of the ammonia fed during each cycle was consumed and almost fully converted to nitrate. Nitrogen loss per cycle caused by stripping, assimilation or internal aerobic denitrifying activity was always inferior to 8%. An ecological study was performed for SBR1 and SBR2

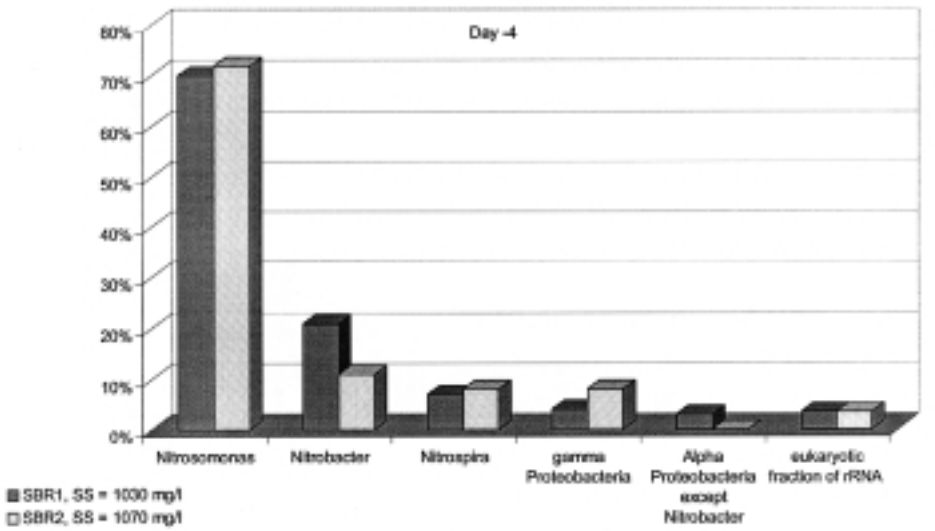

Figure 1 Sludge communities present in SBR1 and SBR2 at day –4, before the start of the bioaugmentation experiment. Bacterial proportions were measured with the area-ratio method after FISH. Eukaryotic fraction of rRNA was obtained by radioactive dot-blot hybridization on rRNA extracts

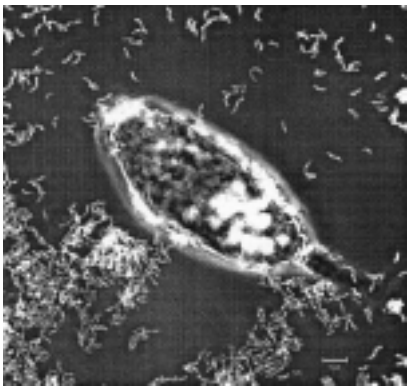

Figure 2 FISH on the bioaugmented reactor on day 5. Bacteria hybridized with probe S-S-Mae-1414-a-A-18 are labeled white

using FISH combined with the area-ratio quantification method. On day –4, before bioaugmentation of SBR2, bacterial communities were dominated by nitrifying bacteria belonging to halophilic and halotolerant members of the genus *Nitrosomonas* (NH_4^+-oxidizers) (Figure 1). Interestingly, nitrite-oxidizing bacteria of the genera *Nitrobacter* (alpha *Proteobacteria*) and *Nitrospira* (*Nitrospira*-phylum) were simultaneously present in the reactors. *Proteobacteria* from the gamma and alpha subclasses were also present in significant numbers but no members of the *Cytophaga-Flavobacterium* cluster or from the Gram-positive bacteria with high DNA G+C content were detectable before bioaugmentation and acetate feeding of the reactors. It should also be noted that the eukaryotic fraction of the biomass was determined to be 3.5% in both reactors by radioactive dot-blot hybridization.

Fate of the bioaugmented bacteria

Acetate was fed and *M. aerodenitrificans* was twice added to SBR2. Only a slight increase in nitrogen loss (16% during the SBR cycle of inoculation) was recorded after the first introduction of the strain. No aerobic denitrification was detected thereafter. FISH experiments revealed that for both inoculations, the bioaugmented bacteria almost completely disappeared from the reactor within two days. Washout alone could not explain the rapid disappearance of the bacterium. Since *M. aerodenitrificans* was able to grow in filter-sterilized samples removed from the reactor, it was not starved or lysed by a chemical substance present in the ecosystem. Examination under the microscope of the protozoa in SBR2 showed that their digestive vacuoles were full of bacteria giving a positive signal with the *M.aerodenitrificans*-specific probe (Figure 2), which would indicate that this bacterium had been ravenously grazed.

Changes among microbial communities after bioaugmentation

Our assumption that grazing was responsible for the disappearance of the introduced strain was further corroborated by the increase of the eukaryotic fraction of rRNA in SBR2. This fraction increased to 22.3% in SBR2 on day 15 (Figure 3). Over the same period it had been almost stable in SBR1, indicating that acetate feeding alone did not notably affect this parameter. After bioaugmentation, the high number of bactivors in SBR2 probably resulted in a strong increase of the grazing pressure exerted on the indigenous bacteria. It is known that nitrifying bacteria are particularly sensitive to grazing (Lee and Welander, 1994). The fact that perturbations in nitrifying activity were noticed only in SBR2 could therefore be a consequence of this high grazing pressure. Several other observations could also be related

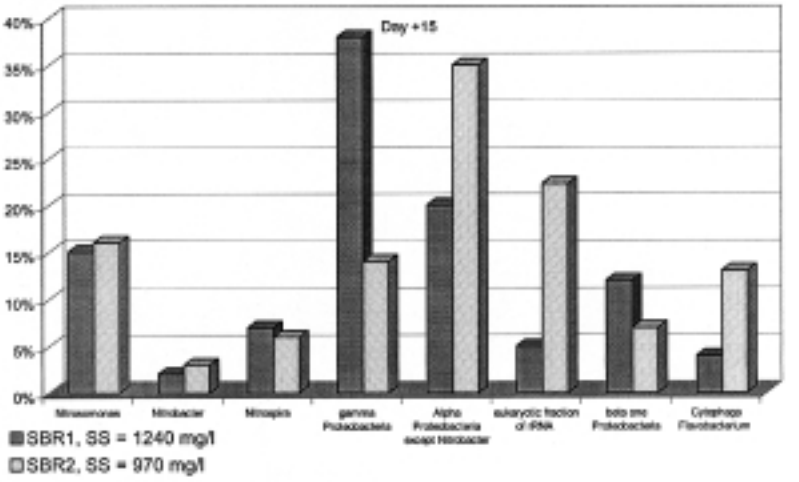

Figure 3 Sludge communities present in SBR1 and SBR2 at day 15

to this increased grazing activity. First, free-living bacteria almost disappeared from SBR2. Second, between day –4 and day +15, SS decreased by 9.3% of the initial value in SBR2, despite biomass being added during bioaugmentation. At the same time, it increased by 20% in SBR1, due to growth of heterotrophic bacteria. It could therefore be concluded that massive bioaugmentation resulted in undesirable effects (increase in population of bactivors) that unbalanced the ecosystem equilibrium. Moreover, sludge bacterial communities had been modified in both SBR. On one hand, *Proteobacteria* from the alpha subclass had become dominant in the flocs of SBR2 on day 15. They seemed to have developed a strategy for overcoming the grazing pressure. On the other hand, *Proteobacteria* from the gamma subclass, especially from the genus *Acinetobacter*, dominated the flocs of SBR1 on day 15. This observation could be linked to the high affinity of this genus to acetate, used as the carbon source during this experiment (Belia and Smith, 1997).

Enhancing the incorporation of the added bacteria to sludge flocs

In SBR3, coagulating and flocculating agents were added just after bioaugmentation of the strain. *M. aerodenitrificans* then aggregated around the nitrifying flocs. However, this adhesion was partially reversed under the combined effects of agitation and aeration. During the cycle of bioaugmentation, a fraction of *M. aerodenitrificans* was progressively released into the liquid phase and was grazed as observed by in situ hybridization. In total, one day after the bioaugmentation, the percentage of *M. aerodenitrificans* in the total biomass had fallen from 33.4% to 10.0%. It then decreased more progressively to reach less than 2% after seven days. Another bioaugmentation experiment was attempted on SBR4 with inoculation of *M. aerodenitrificans* embedded in alginate beads. Growth of *M. aerodenitrificans* was observed within the beads by *in situ* hybridization. Fragmentation of the beads occurred three days after their introduction. However, microscopic observation of the sludge revealed that pieces of alginate were remaining and were mixed within the indigenous flocs. The presence of cationic residues in the alginate matrix could play an important role for the first adhesion step of alginate fragments to sludge flocs. Indeed, it had previously been shown that calcium ions play an important role in maintaining the floc cohesion, creating links between negatively charged bacteria and exopolymers (Frolund *et al.*, 1996; Urbain *et al.*, 1993). Colonization of the alginate matrix by indigenous bacteria was also observed. These alginate-fragments contained microcolonies of *M. aerodenitrificans* that

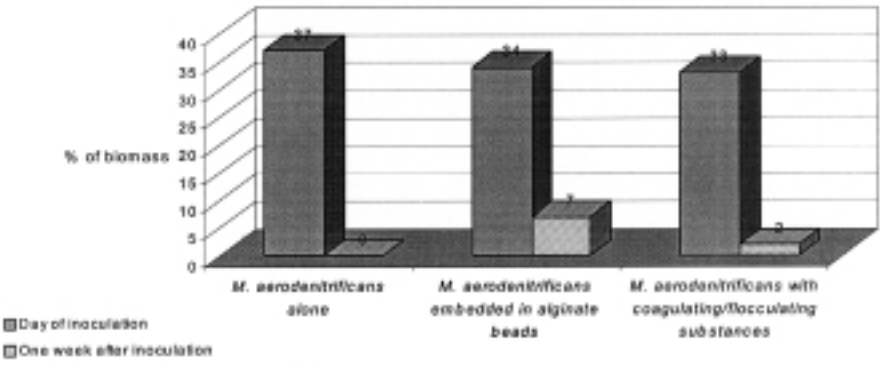

Figure 4 Effects of different inoculation strategies on persistence of M. aerodenitrificans. Bacterial proportion was measured against the total biomass stained with DAPI after hybridization with the *M. aerodenitrificans*-specific probe and dispersion of the sample (see methods section)

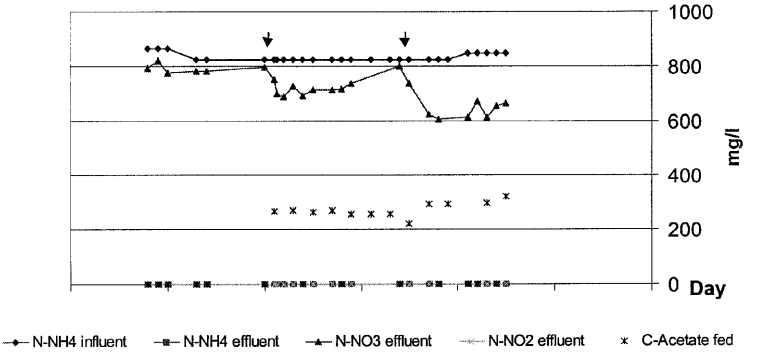

Figure 5 Evolution of the concentrations of dissolved nitrogen compounds in the reactors bioaugmented with aliginate-embedded *M. aerodenitrificans*: Arrows indicate days of bioaugmentation

were emitting strong signals after FISH, indicating high ribosomal content of these cells. This adhesion/colonization of the alginate fragments finally resulted in the incorporation of exogenous bacterial microcolonies within the indigenous flocs. A quantification performed seven days after bioaugmentation, showed that *M. aerodenitrificans* represented 7% of the total biomass stained with DAPI at that time (Figure 4).

This persistence of *M. aerodenitrificans* was accompanied by a significant decrease of nitrate concentration in the outflow of the reactor (Figure 5). However, in the second week following the introduction, this incorporated fraction of exogenous bacteria slowly decreased. In order to improve the ecosystem's colonization by *M. aerodenitrificans*, a second more massive inoculation was attempted using beads with higher gel strength. Similar results were obtained. After incorporation within the indigenous flocs of a fraction of *M. aerodenitrificans*, the proportion of the introduced bacterium among the total bacterial population again progressively decreased.

This suggested that, even if the first floc-colonization step was successful, the ecological conditions prevailing in the reactors did not allow a long term proliferation of the introduced strain. This long-term proliferation probably depends on the intrinsic ecological selectivity of the inoculum for its host ecosystem. However, we observed, for at least six weeks after the last introduction of the strain, that a small and rather constant fraction of *M. aerodenitrificans* remained visible after *in situ* hybridization, which was not the case in

the other reactors. This observation could indicate that the use of alginate as a bioaugmentation vector had allowed the selection of an adapted fraction of the introduced population. This adapted fraction may have colonized the reactor's ecosystem. However, these assumptions underlie fundamental biological mechanisms that still remains to be verified.

Conclusions

The technique of FISH gives direct experimental evidence that grazing is the main reason for failure of bioaugmentation. In our case, we even showed that massive bioaugmentation was correlated with an increase in the population of bactivors and disturbances of the ecosystem's equilibrium. To overcome these limitations, we propose the use of a bioaugmentation vector such as alginate beads. They offer a temporary protection against grazing. Moreover, the chemical properties of this biopolymer favor the adhesion of exogenous bacterial microcolonies to the existing flocs and finally result in the incorporation of a fraction of the introduced population within the indigenous flocs. This may enable the development in the host ecosystem of an adapted fraction of the introduced population.

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