Impact of bioaugmentation with nitrifying biomass in a CAS and in an MBR pilot plants

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Abstract

A Conventional Activated Sludge (CAS) system and a Membrane Bioreactor (MBR) were bioaugmented with a same nitrifying biomass selected in a side-stream MBR fed with a high nitrogen-loaded influent. Microbial communities evolution was monitored and comparatively analysed through bio-molecular investigation (FISH and T-RFLP techniques) followed by statistical analyses. The bioaugmentation process caused, indeed, a conspicuous increase of nitrifying bacteria of the genera Nitrosomonas and Nitrobacter in both the inoculated CAS and MBR reactors. The overall structure of the microbial community was actually changed in these reactors, although not evolving toward an overlapping with that of the side-stream community. Finally, the effect of bioaugmentation was significantly more pronounced on the biomass of the MBR reactor with respect to the CAS.

Keywords
Activated sludge, Bioaugmentation, Conventional Activated Sludge Systems, Membrane Bio-Reactors, Microbial community shift, nitrification

INTRODUCTION

Nitrogen removal is a key step in wastewater treatment, however, nitrifiers activity is negatively affected by several environmental and operational factors, including low temperature, extreme pH, low dissolved oxygen concentration, and chemical inhibitors (Satoh et al., 2003; Siripong and Rittman 2007).

Bioaugmentation of the activated sludge by the addition of indigenous or allochthonous strains or consortia of microorganisms could be a powerful instrument to enhance biological nitrification (Bartolì et al., 2011) and, in recent years, several bioaugmentation strategies and configurations have been investigated to improve nitrification, however, most of previous works refer to systems where settling was the biomass separation technology. Even though in situ bioaugmentation from a side stream reactor has been proven effective also at full scale (Krhutkovi et al., 2006; Podmirseg et al., 2010; Szoke et al., 2011), its efficiency as a function of selective conditions still need to be quantified (Munz et al., 2012; Yu et al., 2012).

Bioaugmentation in membrane bioreactors (MBRs) has been seldom investigated (Zhang et al., 2009) nor compared with a control conventional activated sludge system (CAS), where settling may diversely affect nitrifiers selection respect to other bacteria and not yet compared with a control conventional activated sludge system, while a differential selection of settling either on the seeded or on indigenous biomass has been hypothesized (Yu et al., 2012). Since the success of bioaugmentation primarily depends on the effective metabolic adaptation and growth of the seeded biomass in the new system (Satoh et al., 2003), a better understanding of the microbial dynamics in bioaugmentation would potentially improve the nitrification stability (Wang et al., 2010) and lead the process to an optimal development.

In particular, the knowledge of the mechanisms of adaptation of the biomass, once seeded, would allow to select for biomass with the desired characteristics through the application of selective pressure in the source reactor.

While Fluorescence in Situ Hybridization (FISH) is a well-established technique for detection and monitoring only of specific and pre-identified microorganisms, metagenomic DNA extraction and its analysis through

Terminal Restriction Fragment Length Polymorphism (T-RFLP) offers a fast, complete and low-cost quantitative assessment of the entire microbial community.
This work is aimed at the evaluation of the impact of bioaugmentation with nitrifying sludge in a CAS and in an MBR pilot plants subjected to identical operational regime. The shift in bacterial community due to the presence of the bioaugmentation have been assessed through FISH and T-RFLP analysis.

MATERIALS AND METHODS

The schematic of the experimental set used during the experiment is reported in Figure 1.1.

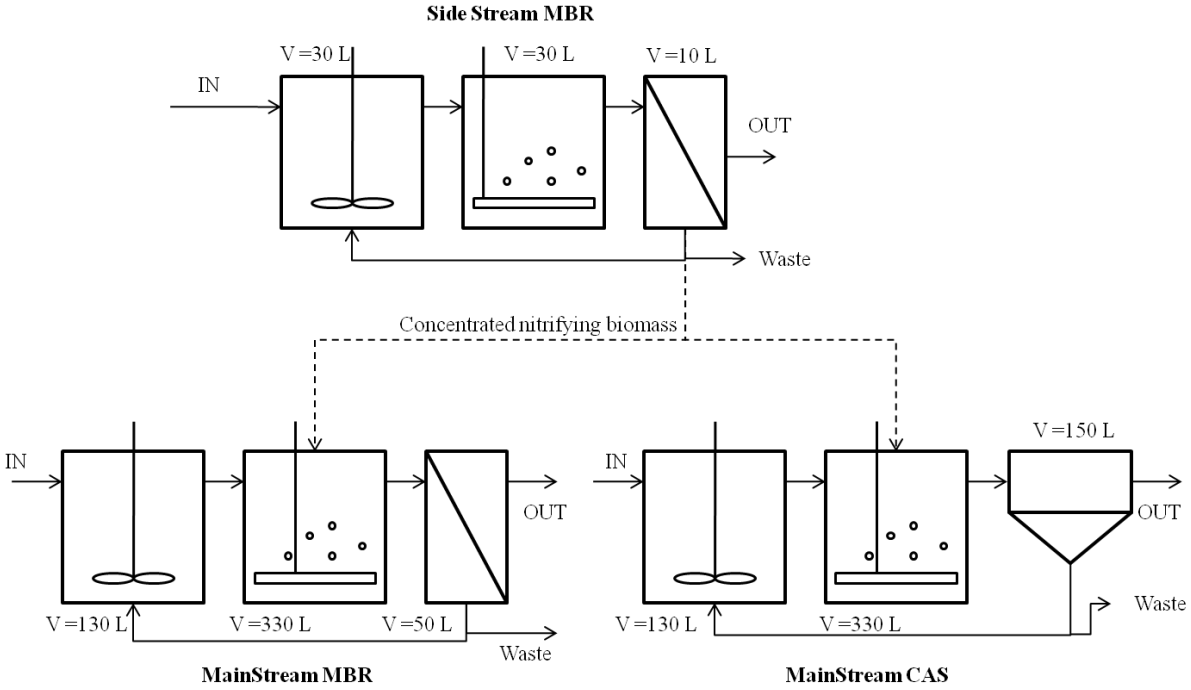

Figure 1.1 Scheme of the experimental setup.

An experimental set composed by three activated sludge pilot plants have been used in the experiment: a concentrated nitrifying biomass was selected in a side-stream MBR treating high strength ammonia wastewater, while biological treatment of domestic wastewater was carried out using MBR and CAS systems (Figure 1).
The side-stream MBR reactor consisted of a pre-denitrification (30 L), a nitrification (30 L) and a filtration tank equipped with three flat sheet membranes (DF-10 Kubota, Japan) with a nominal cut-off of 0.1 µm for an effective membrane area of 0.3 m2. The system was inoculated with biological sludge collected from a waste water treatment plant (WWTP) treating domestic wastewater (Pistoia, Italy) and was fed with domestic wastewater where ammonia and alkalinity were added in order to simulate the nitrogen load (650 mg N-NH4+) of a digester supernatant (Table 1).
After a start-up phase, the reactor operated for more than 120 days with a solid retention time (SRT) of 20 d during which the ammonia loading rate (ALR) was maintained close to 40 g N-NH4+ L-1d-1.
An MBR and a CAS pilot plant constituted the main stream treatment trains of domestic wastewater. Both consisted of a pre-denitrification (130 L) and of an oxidation-nitrification (330 L) while differed for the liquid-solid separation system: CAS was equipped with a 150 L settler, while MBR with two hollow fiber ultra-filtration membranes (Zenon Environmental Inc., Canada). Dissolved Oxygen (DO) in the aeration tank and pH varied in the range 6-7.5 mg L-1 and 7.8-8.2, respectively in both CAS and MBR. The temperature in the side-stream MBR was maintained at 20±0.5 °C while in main-stream systems varied according to seasonal room temperature.
Both reactors have been inoculated with biological sludge collected from a tannery WWTP (Cuoiodepur Spa, San Miniato, Pisa, Italy) and operated in steady state conditions with an SRT and an hydraulic retention time (HRT) of 2.5 d and 16 h respectively for more than 400 d before bioaugmentation started (non-bioaugmented period, from 30th September 2010 till 20 December 2011); domestic wastewater, continuously collected from the sewer at the Cuoiodepur WWTP, have been used as feeding (Table 1.1).

Parameter	Avg	Dev. St.

pH	8.0	0.2
T (°C)	16.1	4.3
Total COD (mg L^{-1})	286.7	75.7
Soluble COD (mg L^{-1})	194.8	54.6
SST (mg L^{-1})	117	87
N-NH_4^+ (mg L^{-1})	32.3	5.0
Total N (mg L^{-1})	38	24
Total P (mg L^{-1})	0.7	0.4

Table 1.1 Main stream reactor influent characterization

No significant variations of the influent characteristics were identified if not as a result of strong rain events.
The SRT of the main streams was maintained low to increase both the efficiency of an hypothetic anaerobic treatment of the biological sludge and to work close to limit conditions for nitrifiers washout to better understand the effect of bioaugmentation on nitrification process.
After a non bioaugmentd period that lasted more than 200 d for both CAS and MBR, main stream reactor have been continuously inoculated with the biomass collected from the side stream MBR nitrification tank with a constant flow of 2.5 L d-1. Bioaugmentation phase lasted more than 150 days.
NH_4^+-N, NO_2^--N, NO_3^--N concentrations both in the influent and in the effluent of the plants have been analyzed three times a week through colorimetric tests (Xion 500, Hach-Lange GMBH, Germany).
Samples collected from the three plants in steady state conditions both in non-bioaugmented and bioaugmented period have been used for FISH analyses using specific probes for the genus *Nitrosomonas, Nitrobacter, Nitrospira* and *Nitrosospira*.
Fixed samples of day 0 and day 30 of the bioaugmentation phase for the three reactors were mechanically homogenized by a potter bender and a syringe needle for 30 minutes to disrupt the flocs. 15 µl of each samples were fixed on a slide and dehydrated with passages in 50-70-100% ethanol. In Situ Hybridization was performed according to conventional protocol (Manz et al., 1992) utilizing the eubacterial universal probe EUB_338 I (Amann et al., 1990), probe b-AO233 (Stephen et al., 1998), specific for betaproteobacterial ammonia-oxidizing bacteria, Nsm_156 (Mobarry et al., 1996), specific for the genus Nitrosomonas, NIT3 (Wagner et al., 1996) for the genus Nitrobacter, NTSPA714 (Loy et al., 2002) specific for the phylum Nitrospira and probe Nsv443 (Mobarry et al., 1996) specific for the genus Nitrosospira. Hybridization conditions followed author's suggestions or the information available from ProbeBase (Loy et al., 2007). Slides were mounted with Slow Fade Light Antifade Kit (Molecular Probe, Invitrogen) containing 4,6-diamidino-2-phenylindole (DAPI). The samples were observed with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany), equipped for epifluorescence and a digital camera. Quantification of bacterial population was performed randomly choosing 5 fields on each slides and counting the total number of cells with DAPI staining and the number of positive cells labeled by the group-specific probe. Relative abundance of each specific group of bacteria was then expressed as a percentage on the total number of cells evidenced by DAPI staining.
T-RFLP analysis was performed on all the samples collected twice a week during the entire experiment.
Genomic DNA for T-RFLP analysis was amplified using primer 8F (see above) labelled with the fluorophores 6FAM or NED on the 5′ end and R1492. PCR reaction was performed with previously described conditions. 26.4 µl of purified PCR product were then digested with the restriction endonuclease AluI (0.2 µ/µl final concentration, Fermentas, Canada) and BsuRI (0.2 µ/µl final concentration, Fermentas, Canada) at 37° C for at least three hours. After digestion, DNA was precipitated with 9 µl of 3 M sodium acetate and 100 µl of 100% ethanol and centrifugated at 10,000*g* for 15 min at 4°C. DNA pellets were air dried and suspended in 20 µl of sterile H2O. An appropriate volume of DNA solution was mixed with 0.5 µl of Genescan 500 LIZ Size Standard (Applied Biosystems, USA) and deionized formamide (Applied Biosystems, USA) for a total volume of 20 µl. The volume of DNA solution was calculated on the basis of its final concentration evaluated by electrophoresis on 2% agarose gels and subsequent ethidium bromide staining. After DNA denaturation at 94°C for 2 min, the fluorescently labelled terminal restriction fragments (T-RFs) were run on an Abi Prism 310 Genetic Analyzer (Applied Biosystems, USA). T-RFLP analysis was performed on all the samples collected during the entire experimentation.
The T-RFLP profiles were analyzed using GeneScan™ software (Applied Biosystems, USA) and species diversities were studied through non-metric Multi Dimensional Scaling (nmMDS). Plots were created using the Bray-Curtis similarity distance. An analysis of similarity (ANOSIM) was used for inferential statistical analyses. This ANOVA-like test is based on the rank similarities in bacterial community among predefined groups and is free from any assumption of normality.

RESULTS AND DISCUSSIONS

After the start-up phase, a constant ALR (40 g $N\text{-}NH_4^+$ $L^{-1}d^{-1}$) and an ammonia removal efficiency (RE_N) higher than 98% were maintained in the side stream.

The bioaugmentation efficiency, expressed as the increase of RE_N due to the presence of bioaugmentation, depended on the temperature difference between seeding and seeded reactor (Mannucci et al., 2014). Same temperature was mainteined in bioaugmented and non bioaugmented conditions.

To explain the influence of temperature on the bioaugmentation performance, temperature range was divided into three intervals. Temperatures higher than 17°C allowed complete nitrification also without bioaugmentation: RE_N higher than 95% and 80% were observed without bioaugmentation in the MBR and the CAS, respectively. In the MBR, an average RE_N increase of 4 ± 0.4% was obtained due to bioaugmentation, while, in the same conditions RE_N in the CAS increased by 8 ± 1%. Within the temperature range 12-15 °C, low temperature in main stream plants and high ΔT between side and main stream lowered the effect of bioaugmentation and RE_N increased only by 1 ± 0.2% and 5 ± 0.6% due to bioaugmentation in MBR and CAS, respectively. The highest impact was obtained with the temperature in the main stream in the range 15 to17°C, that is, a RE_N increase of 19 ± 3% in the MBR and 10 ± 3% in the CAS.

According to FISH results (Table 2), AOB (Ammonia Oxidizing Bacteria) represented more than 46% of the active biomass in the side stream.

The initial AOB relative abundance in the CAS system is lower than in the MBR and this reflects the differences between ammonia RE in main stream plants before bioaugmentation starts (77 ± 7.5 % and 63 ± 8.2 % for MBR and CAS, respectively).

The bioaugmentation caused *Nitrosomonas* concentration to double and *Nitrobacter* concentration to increase by a factor three.

Sample	*Nitrosomonas sp.*	*Nitrobacter sp.*	*Nitrospira sp.*	*Nitrosospira sp.*
	46.8 ± 18.4	19.6 ± 7.2	8.5 ± 2.1	0.1 ± 0.1
Main-stream CAS without bioaugmentation	13.6 ± 4.1	1.9 ± 0.9	7.1 ± 3.7	0.5 ± 0.7
Main-stream CAS with bioaugmentation	30.5 ± 8.6	5.9 ± 1.1	6.2 ± 1.2	0.3 ± 0.2
Main-stream MBR without bioaugmentation	16.0 ± 3.5	1.4 ± 0.6	9.6 ± 7.4	0.6 ± 0.7
Main-stream MBR with bioaugmentation	33.3 ± 6.1	4.1 ± 2.0	7.2 ± 4.8	0.8 ± 0.4

Table 1.2 Percentages of probe positive cells relative to DAPI staining in samples collected in steady state condition with and without bioaugmentation.

The species richness of the microbial communities and the differences between the reactors were also assessed by comparing the T-RFLP profiles. A nm-MDS analysis was undertaken to compare the microbial communities of the three reactors both during both the non-bioaugmented and the bioaugmented periods. During the non-bioaugmented phase communities of CAS and MBR main stream reactors showed a remarkable distance from the community of the side stream reactor (Figure 2A). This distance was maintained during the bioaugmentation period (Figure 2B), despite the changes in nitrifying biomass confirmed by FISH. This means that the biomass of the main stream reactor always remained significantly different from that of the side stream. Moreover, nm-MDS results demonstrated a variation in the microbial composition of both the main-stream reactors due to bioaugmentation (Figure 2 C, D).

ANOSIM results show a clearer effect of the bioaugmentation in the MBR reactor rather than in CAS. Indeed, only in MBR main stream reactor the microbial community composition shows statistically significant differences ($p<0.05$) between the non-bioaugmented and the bioaugmented period. This result could be probably ascribed to the plant structure. In membrane bioreactors the activated sludge is separated from the liquid as it passes through the membrane rather than for conventional sedimentation. As the short SRT of 2.5 days was the same in the two main stream reactors, the different separation technology probably represented the main differential selecting parameter (Fenu et al., 2010). The biomass inoculated during bioaugmentation was actually selected in a MBR reactor (the side stream) and could have been characterized by a limited sedimentability with respect to the CAS autoctonous microbial community. In this case, the bioaugmentation inoculum could be more likely washed out in CAS than in MBR. This could explain why the effect of bioaugmentation on the microbial community is less pronounced in the CAS main stream reactor.

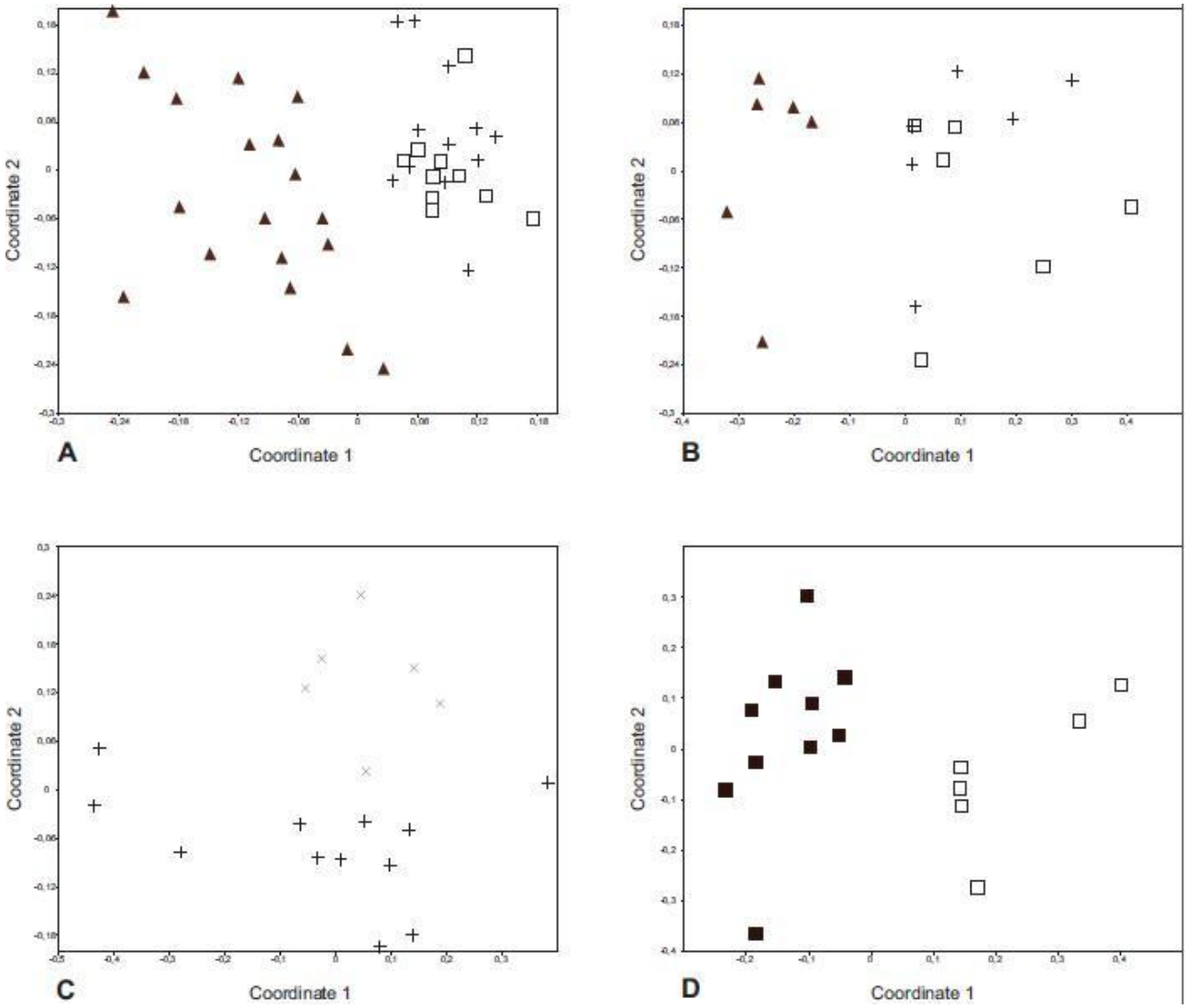

Figure 1.2 nm-MDS ordination plot of T-RFLP profiles before (A) and during the bioaugmentation (B). Side-stream samples are represented by black triangles, main-stream MBR ones by squares and main-stream CAS ones by crosses. (C) CAS samples distribution during without (+) and with bioaugmentation (x). (D) main stream MBR samples distribution without (black squares) and with bioaugmentation (white squares).

CONCLUSIONS

Bioaugmentation efficiency depends on plant configuration and on temperature difference between side and main stream systems.

The bioaugmentation process caused a significant increase of nitrifying bacteria of genera *Nitrosomonas* and *Nitrobacter* in both the inoculated reactors.

The overall structure of the microbial community was actually changed in these reactors, although not evolving toward an overlapping with that of the side-stream community.

The effect of bioaugmentation was clearly more pronounced on the MBR than on CAS microbial community.

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