

Effect of the concentration of suspended solids on the enzymatic activities and biodiversity of a submerged membrane bioreactor for aerobic treatment of domestic wastewater

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Abstract A pilot-scale submerged membrane bioreactor was used for the treatment of domestic wastewater in order to study the influence of the variations in the concentration of volatile suspended solids (VSS) on the enzymatic activities (acid and alkaline phosphatases, glucosidase, protease, esterase, and dehydrogenase) and biodiversity of the bacterial community in the sludge. The influence of VSS concentration was evaluated in two separated experiments, which were carried out in two different seasons of the year (experiment 1 through spring–summer and experiment 2 through autumn–winter). Cluster analysis of the temperature gradient gel electrophoresis (TGGE) profiles demonstrated that the community composition was significantly different in both experiments. Within the same experiment, the bacterial community experienced sequential shifts as the biomass accumulated, as shown by the evolution of the population profiles through time as VSS concentration

increased. All enzymatic activities studied were significantly lower during experiment 2, except for glucosidase. Concentrations of VSS over 8 g/l induced a strong descent of all enzymatic activities, which overlapped with a significant modification of the community composition. Sequences of the major TGGE bands were identified as representatives of the Alpha-proteobacteria, filamentous bacteria (*Thiothrix*), and nitrite oxidizers (*Nitrospira*). Some sequences which were poorly related to any validated bacterial taxon were obtained.

Keywords Submerged membrane bioreactor · Domestic wastewater · Wastewater treatment · Biodiversity · Enzymatic activities · TGGE

Introduction

In the last decades, a fast development of the tertiary treatments of wastewater has been upraised. Macrofiltration systems (often followed by disinfection by ozonation or UV radiation), microfiltration, ultrafiltration, and reverse osmosis are the most commonly used methods (Hontoria et al. 2003), which provide an effluent of high quality that meets the parameters required for its direct reutilization. Submerged membrane bioreactors (SMBs) are based on the combination of the activated sludge process and the utilization of ultrafiltration or microfiltration membranes for the separation of the particulated material, avoiding the requirement of the secondary clarifier. Nowadays, there are over 1,000 SMBs currently in operation around the world, with approximately 66% in Japan and the remainder largely

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in Europe and North America. About 55% of the installations use membranes submerged inside the bioreactor, while the rest have external membrane modules (Cicek 2003).

SMBs have several interesting advantages over the conventional activated sludge technology (Ueda and Horan 2000; Stamper et al. 2003). One of the main benefits brought about by the SMB processes is the ability to work with high concentrations of volatile suspended solids (VSS) in the sludge, regardless of the organic load in the wastewater (Witzig et al. 2002). Elevated concentrations of VSS enable to work with high cell retention times, favoring the development of slow-growing bacteria, such as ammonia oxidizers and nitrite oxidizers, which are crucial for the transformation of the toxic nitrogen compounds ammonia and nitrite (Witzig et al. 2002). Microbial communities developed in SMB-based treatment systems are analogous to those present in conventional activated sludges. However, the microorganisms are exposed to completely different conditions, mainly due to the biomass recycling in SMB, which leads to changes in the biology of the system. Bacteria are subjected to a substrate-limited environment, which induces low growth rates and reduces the respiratory potential of the microorganisms, hence, influencing the activities and species composition of the sludge community (Konopka et al. 1996; Konopka 2000; Morgan et al. 2002).

Measuring biomass concentration and enzymatic activities is essential to achieve the biological characterization of a complex microbial microcosm, like the case of an activated sludge (Whiteley et al. 2002; Liwarska-Bizukojc and Ledakowicz 2003). During the formation of the activated sludge, microorganisms use their extracellular and intracellular enzymes to hydrolyze and degrade the organic matter (Cadoret et al. 2002; Gessesse et al. 2003). These enzymatic activities give an estimation of the active biomass, the elimination of organic matter, N and P, and are also used as indicators of the fate of specific populations. Some of the most important enzymatic activities during the biological treatment of wastewater are phosphatase, glucosidase, protease, and esterase (Goel et al. 1998; Boczar et al. 2001; Cadoret et al. 2002; Gessesse et al. 2003). Due to the organic matter fraction of wastewater, mainly composed of carbohydrates and proteins, glucosidases and proteases play an essential role in its hydrolysis. Variations of the enzymatic activities are thus an excellent indicator of the physiology of the mixed species sludge community and provide a way to evaluate the impact of the variation of conditions of operation, such as the concentration of VSS.

One of the major advances in the field of microbial ecology is the introduction of the molecular biology techniques based on the *in situ* detection of nucleic acids. These methods provide important information about the microorganisms in their natural habitats and have allowed

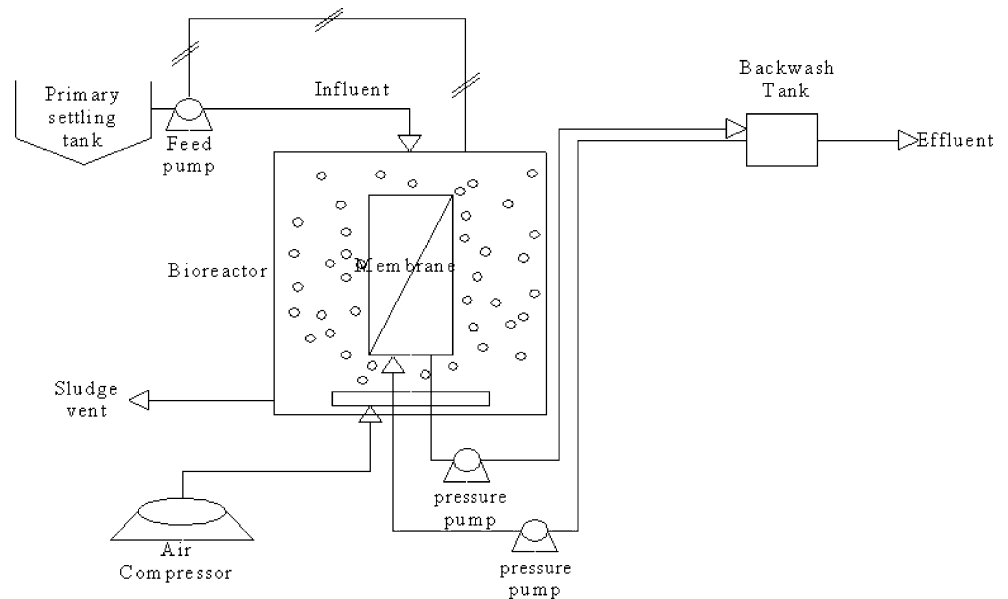
for the discovery of a wide number of microbial species that were previously unknown, due to the difficulties for their culture and preservation under laboratory conditions. In this context, denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis (DGGE/TGGE) yield extensive information about the diversity of microorganisms in their habitats (Muyzer 1999), also allowing for the taxonomic identification of community members (Cortés-Lorenzo et al. 2006). These data make possible to monitor variations in the community profiles due to external factors. Both techniques were often used in recent studies on the ecology of biological processes in wastewater treatment plants (WWTPs), providing interesting new data in this area (Wagner et al. 2002; Cortés-Lorenzo et al. 2006).

In this paper, we aimed to study the changes in the enzymatic activities and structure of the microbial community in a pilot-scale SMB setup for the treatment of real domestic wastewater, as influenced by variations in the concentration of VSS. For this purpose, we monitored through time the evolution of the enzymatic activities in the sludge and the number of total heterotrophic cultivable microorganisms, and compared the community composition profiles based on TGGE separation of partial 16S rRNA genes (V3 hypervariable region), amplified by PCR from total DNA extracted weekly from the sludge. Predominant TGGE bands were further reamplified and identified by sequence comparison to get a further insight of the main changes experienced by the sludge community. The influence of the concentration of VSS was evaluated in two separate experiments, which were carried out in two different seasons of the year (experiment 1 through spring–summer, and experiment 2 through autumn–winter).

Materials and methods

Description of the pilot-scale SMB experimental plant The experimental plant (ZeeWeed-10; Fig. 1) was purchased from ZENON S.A. The main elements of the system were the cylindrical bioreactor (0.8 m height and 0.6 m diameter, 224 L operating volume), the control panel, pumps, and rotameter. An ultrafiltration membrane of 0.04- μ m pore size was submerged inside the aerated bioreactor, and the extraction of the effluent water takes place by mechanical suction. Control dispositives to monitor the membrane pressure and temperature of the activated sludge were available. To avoid clogging of the membrane, the activated sludge was kept in agitation below the membrane by means of an aeration system, which was independent of the process air. A second tank (25 L) was needed to keep the treated water, which is also used for the backflushing of the membrane when required. Influent water was pumped in, keeping the flow rate required by the suction pump. The

Fig. 1 Diagram of the SMB pilot-scale plant used in the study



running of the plant consisted of 10-min cycles: a cycle includes 9 min 45 s of filtration and a 15-s backflush cycle. A system for the purge of excess sludge is also provided.

Plant working conditions The system worked with real domestic wastewater, provided by Estación depuradora de “Los Vados” (Granada), run by EMASAGRA, S.A. (Spain). Average composition of wastewater was determined by standard methods (APHA 2001) and was as follows: chemical oxygen demand (COD), 450 mg/l; biological oxygen demand at 5 days (BOD₅), 300 mg/l; NO₃⁻, 5 mg/l; NH₄⁺, 75 mg/l; total suspended solids (TSS), 0.10 g/l; volatile suspended solids (VSS), 0.78 g/l. The reactor was started up with no previous inoculation; thus, the microorganisms in the sludge came from the influent wastewater.

The experimental work in the plant was divided in two periods. The first experiment was conducted for 106 days (April to July 2004), during which the influent water inflow was kept constant at 0.34 l/min, reaching a maximum concentration of TSS of 7 g/l. The second experiment was conducted during 113 days (September to December 2004), during which the water inflow was kept constant at 0.5 l/min, reaching a maximum concentration of TSS of 15 g/l. COD and BOD₅ of the influent water through the experiments are shown in Fig. 2. The evolution of temperature, TSS, and VSS concentrations of the sludge in both experiments are also displayed in Fig. 2. The pH of the sludge was monitored daily and always remained constant between 6 and 8 in all experiments.

Monitoring of the concentration of mixed liquor suspended solids in the activated sludge The concentrations of TSS and VSS were monitored daily by a gravimetric method (APHA 2001).

Activated sludge sampling In all experiments, weekly sampling of the activated sludge was done to measure the total heterotrophic plateable microbiota and microbial enzymatic activities. These samples were also used for DNA extraction. Activated sludge samples were collected from the biological reactor in sterile plastic containers (1 L), transported to the laboratory in isothermic boxes, and analyzed within 24 h of sampling.

Total heterotrophic plateable microbiota Total heterotrophic plateable microorganisms (THPM) counts were conducted during six consecutive weeks in both experiments, through the intervals shown in Fig. 2c. The number of THPM was measured by standard serial dilution and plating on tryptic soy agar (TSA; Difco). Plates were incubated at both 22°C (48 h) and 37°C (24 h) before colonies were counted.

Evolution of microbial enzymatic activities in the bioreactor The analysis of the enzymatic activities was done directly on sludge samples. Phosphatase determination was done by the method of Goel et al. (1998), using *p*-nitrophenyl phosphate (0.1%), which is converted by the enzyme to *p*-nitrophenol. Different buffers were used for estimating acid and alkaline phosphatases (acetate–acetic, pH 4.8, or carbonate–bicarbonate, pH 9.6, respectively). Glucosidases were measured by the colorimetric method of Goel et al. (1998), with Tris–HCl buffer and 1% *p*-nitrophenil α -D-glucopyranoside as substrate for the reaction. For both phosphatases and glucosidases, standard curves were made with known concentrations of *p*-nitrophenol (Sigma). Protease activity was calculated by the colorimetric method of Cadoret et al. (2002). Total esterase activity was measured by the methods of Boczar et al. (2001). Substrates used in

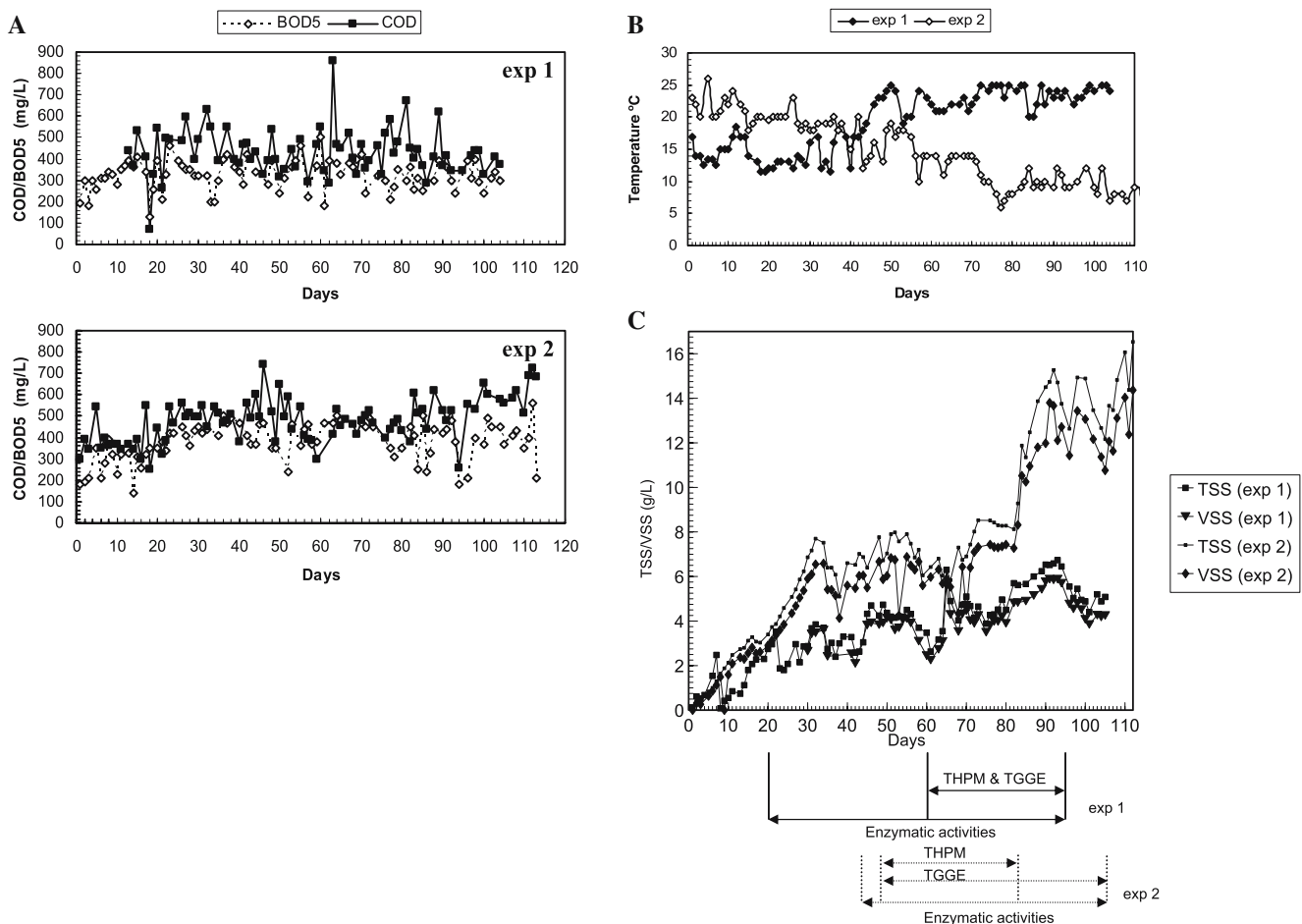


Fig. 2 Time evolution of BOD₅ and COD in the influent water (a), temperature of the sludge (b), and concentration of TSS and VSS in the sludge (c) in the two experiments conducted in the SMB. The intervals

during which THPM counts, enzymatic activities, and TGGE studies were conducted are indicated in c

the assays were *p*-nitrophenyl esters of acetate, butyrate, caproate, caprilate, laurate, and palmitate. Dehydrogenase activity was estimated by the method of Liwarska-Bizukojc and Ledakowicz (2003), based on the conversion of (*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) to formazan.

PCR amplification of partial 16S rRNA gene, TGGE, and DNA sequencing TGGE profiling of the community in the activated sludge was conducted in both experiments on a weekly basis during the periods indicated in Fig. 2c. DNA was extracted from the activated sludge samples using the Ultra Clean Soil DNA Isolation kit (MoBio, USA). A nested approach was used for PCR amplification, as this strategy has been previously reported to avoid nonspecific priming, improve amplification of species present in low numbers, and yield richer band patterns in DGGE (Nicolaisen and Ramsing 2002; Ward and O'Mullan 2002). One microliter (2–5 ng) of DNA extracted from

sludge was used as template for PCR using universal primers fD1 and rD1 to amplify the 16S rRNA gene in almost its full length (Weisburg et al. 1991). Subsequently, 1 μl of the first PCR product was used as a template for a nested PCR using universal primers GC-P1 and C-P2, targeting the V3 hypervariable region (Muyzer et al. 1993). Conditions for each of the PCR reactions were kept as previously described (Vinuesa et al. 1998; Watanabe et al. 1998). HPLC-purified oligonucleotides were purchased from Sigma, and AmpliTaq Gold polymerase (Applied Biosystems) was used for all PCRs. Final PCR products were cleaned and/or concentrated (when required) using Microcon YM cartridges. Two to five microliters (60–100 ng DNA) were loaded in each well for TGGE, using a TGGE Maxi system (Whatman-Biometra).

Denaturing gels (6% polyacrylamide gel electrophoresis with 20% deionized formamide, 2% glycerol and 8 M urea) were made and run with 2× Tris–acetate–EDTA buffer. Temperature gradient was optimized previously for efficient

separation of bands at 43 to 63°C (Cortés-Lorenzo et al. 2006). The gels were run at 125 V for 18 h. Bands were visualized by silver staining using the Gel Code Silver Staining kit (Pierce). A six-species marker was included, made by amplification of V3 region from DNA of the following culture collection strains: *Staphylococcus aureus* ATCC 25923, *Pseudomonas putida* ATCC 8750, *Acinetobacter calcoaceticus* ATCC 15308, *Escherichia coli* DH5 α , *Nocardia corynebacterioides* ATCC 21253, *Micrococcus luteus* ATCC 9341. The bands corresponding to the six markers are seen from right to left in the same order in the gel lane marked as M in Fig. 6.

Portions of individual bands on silver-stained TGGE gels were picked up with sterile pipette tips, placed in 10 μ l of filtered and autoclaved water, and directly used for reamplification with the appropriate primers. PCR products were purified by gel running and extraction with the Quiaex-II kit (Quiagen). DNA recovered was directly used for automated sequencing in an ABI PRISM 3100 Avant Genetic Analyzer.

Cluster analysis of TGGE fingerprints Band patterns generated by TGGE were compared and clustered using the Quantity One image analysis software, version 4.6.1 (Biorad). Bands were automatically detected and matched, and further corrections were applied manually. Cluster analysis was used to investigate the relationships between TGGE profiles with the software program MEGA2 (Kumar et al. 2001), as reported previously (Hoefel et al. 2005). The TGGE banding patterns were converted to a binary matrix by scoring bands as present (G) or absent (A). The number of differences between profiles allowed for the construction of a p-distance similarity matrix, used to conduct a Neighbor-joining cluster analysis. Bootstrap values were calculated for each dichotomy.

Statistics Data were analyzed by either one-way or multi-factor analysis of variance (ANOVA), using the software package STATGRAPHICS 5.0 (STSC, Rockville, MD, USA) to identify significant differences between measurements. A significance level of 95% ($P < 0.05$) was selected.

Biocomputing DNA sequences were analyzed using the biocomputing tools provided online by the European Bioinformatics Institute (<http://www.ebi.ac.uk>). The BLASTn program (Altschul et al. 1997) was used for comparing and sequencing similarity analysis.

Results

Total heterotrophic plateable microorganisms THPM were determined in both experiments for six consecutive weeks, selecting a period of time during which the concentration of

VSS was 2.6–6.2 g/l in experiment 1 (days 60 to 95) and 6.5–8.2 g/l in experiment 2 (days 48 to 83; see Fig. 2c). The temperatures during experiment 1 were 19–24°C, while remained between 5 and 17°C during experiment 2 (see Fig. 2b).

The numbers of THPM at both 22°C and 37°C were slightly lower during experiment 1, but statistically significant differences were only observed for counts of plates incubated at 22°C (Fig. 3). In experiment 1, plate counts at 22°C showed a tendency to reduce through time, while there was a remarkable increment in the number of plateable microorganisms in experiment 2 after week 3, correlating with the increase of VSS concentration.

Enzymatic activities The evolution through time of the enzymatic activities alkaline phosphatase, acid phosphatase, glucosidase, protease, esterase, and dehydrogenase (experiment 2 only), is shown in Figs. 4 and 5. During experiment 1, enzymatic activities were measured when the concentration of VSS ranged from 1.5 to 6 g/l (days 22 to 99 of plant operation), while during experiment 2 the activities were measured in the VSS range of 6–14 g/l (days 41 to 104 of plant operation). Noticeably, the overall values of the

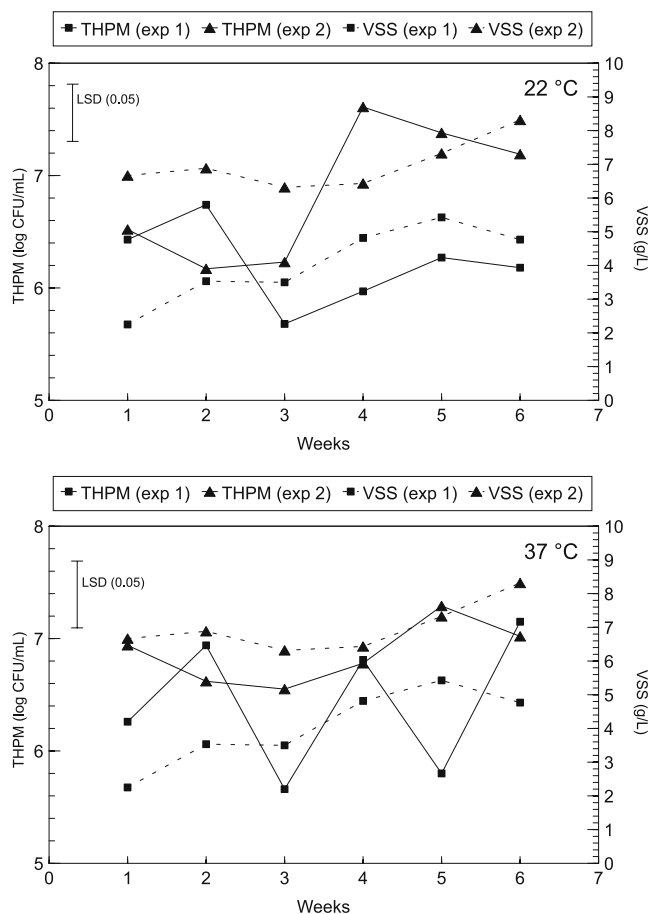
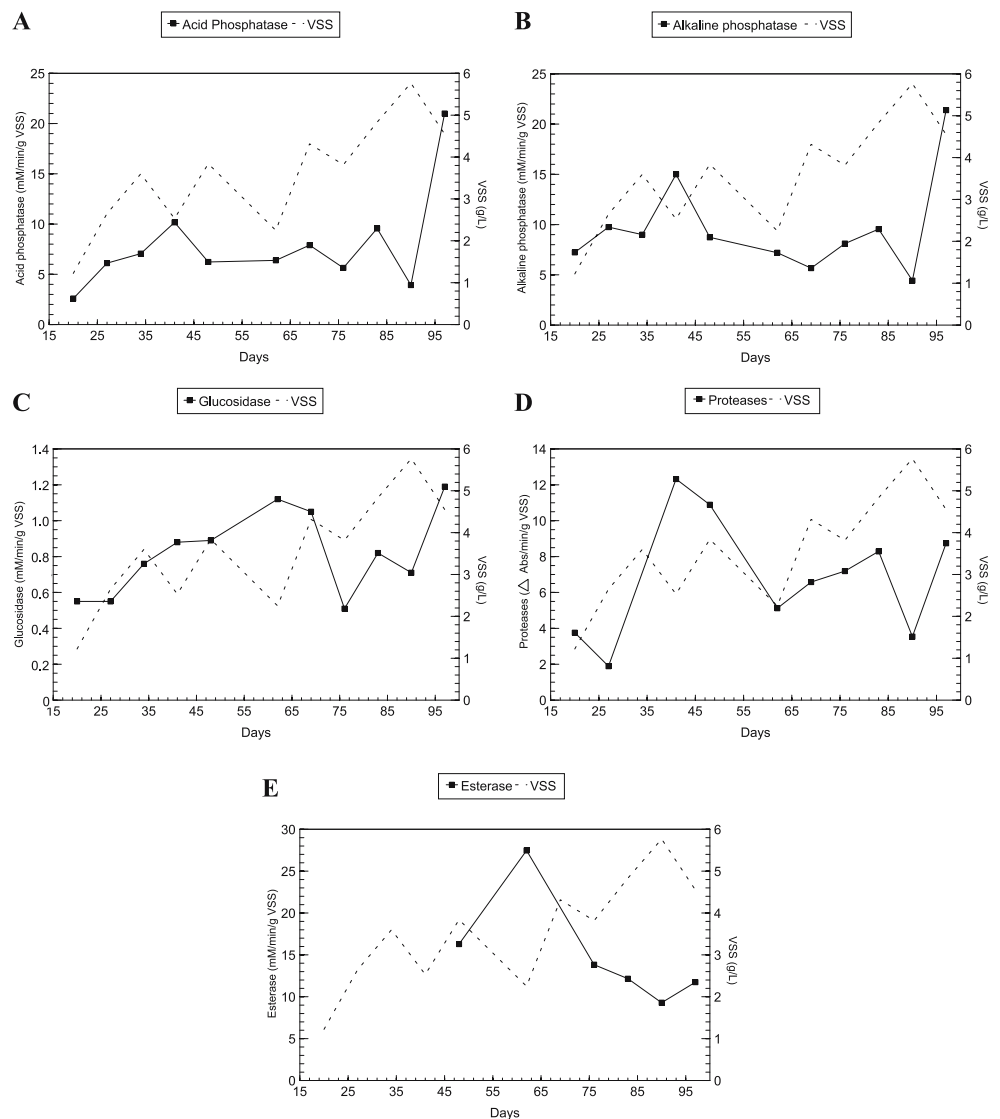


Fig. 3 Total heterotrophic plateable microorganisms at 22°C and 37°C. LSD Least significant difference ($P < 0.05$)

Fig. 4 Enzymatic activities in the SMB during experiment 1. **a** Acid phosphatase (LSD=0.24), **b** alkaline phosphatase (LSD=0.37), **c** glucosidase (LSD=0.11), **d** protease (LSD=1.01), **e** esterase (LSD=2.02)



enzymatic activities measured during experiment 1 were significantly higher than those recorded during experiment 2, except for the glucosidase activity. The average temperature was considerably lower during experiment 2 (Fig. 2b), which was carried out during the autumn–winter season, thus influencing enzymatic activity.

In experiment 1, alkaline phosphatase activity was slightly higher than acid phosphatase. The increase of the phosphatase activities correlated with the increase of the VSS concentration, but only during the first 4 weeks of the experiment (Fig. 4a,b). Glucosidase, protease, and esterase activities also increased with the rise of the VSS concentration, but as shown on Fig. 4c–e, the three activities experienced a tendency to decay after a certain VSS concentration was reached (4 g/l for glucosidase and esterase, and 3 g/l for protease).

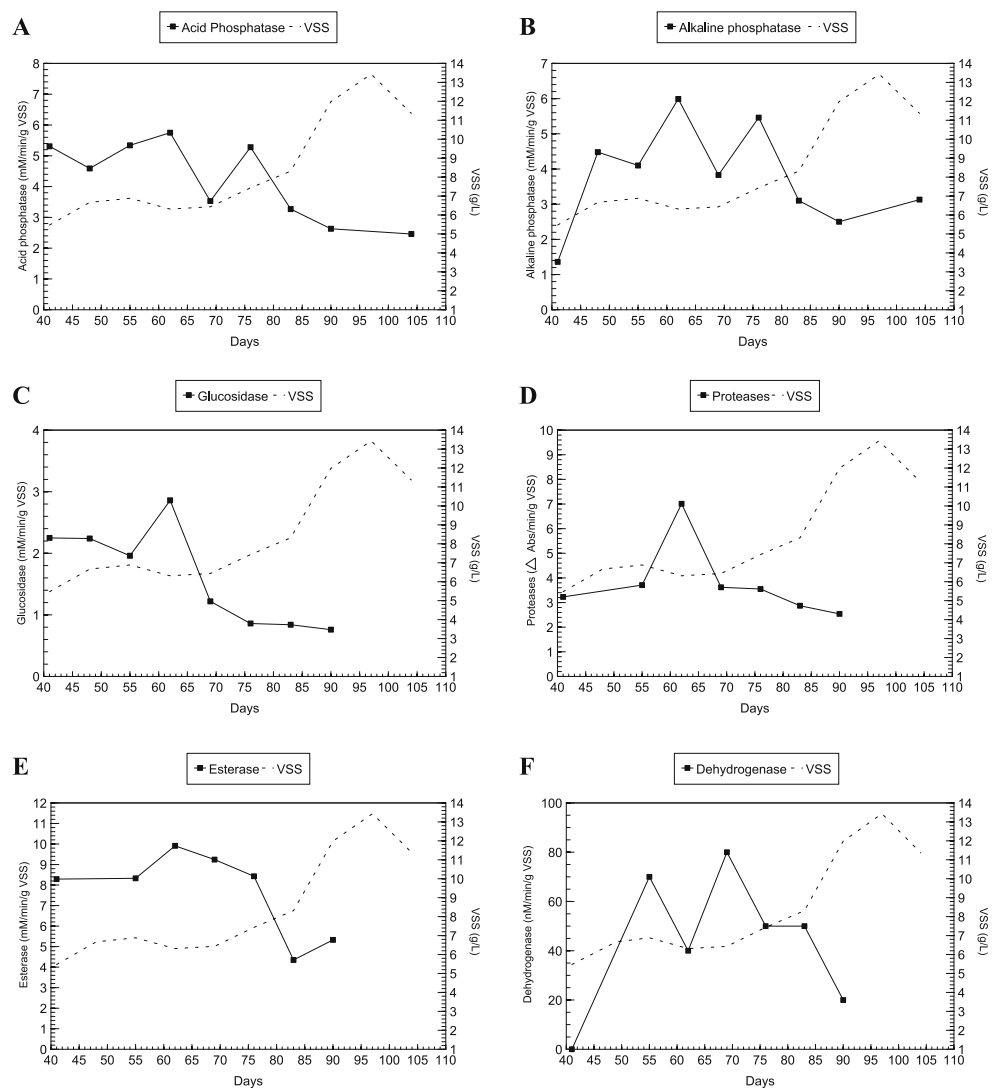
During experiment 2, a strong descent of glucosidase, protease, esterase, and dehydrogenase activities was observed

during the last 4 weeks, when average temperature was around 10°C and VSS concentration was set at 7–11.4 g/l (Fig. 5). Phosphatase activities were more regular through time, but also experienced a decrease during the last 3 weeks. Alkaline phosphatases gave higher values than acid phosphatases, as demonstrated during experiment 1.

The maximum values of each activity, as influenced by temperature and VSS concentration, are summarized in Table 1. The highest activities of all enzymes were recorded with an VSS concentration ranging from 2 to 6 g/l and at temperatures from 13 to 23°C.

Community fingerprinting Figure 6 shows the TGGE community profiles of the microbiota in the SMB system during experiments 1 (lanes 1 to 6) and 2 (lanes 7 to 14), as well as the cluster analysis of the TGGE fingerprints. Clustering of the TGGE profiles demonstrated that the community composition in experiment 1 was significantly

Fig. 5 Enzymatic activities in the SMB during experiment 2. **a** Acid phosphatase (LSD=0.81), **b** alkaline phosphatase (LSD=0.56), **c** glucosidase (LSD=0.09), **d** protease (LSD=0.92), **e** esterase (LSD=2.31), **f** dehydrogenase (LSD=0.086)



different from that of experiment 2. The fingerprints of both experiments clearly clustered away, showing only a 47% similarity. Within the same experiment, the bacterial community experienced sequential shifts as the biomass accumulated, as shown by the evolution of the population profiles through time as VSS concentration increased, with

Table 1 Optimal parameters for maximum enzymatic activities in the bioreactor

Enzymatic activity	mM min ⁻¹ g ⁻¹ VSS	VSS (g/l)	T (°C)	pH
Acid phosphatase	21.00	4.8	23	6.4
Alkaline phosphatase	21.38	4.8	23	6.4
Glucosidase	2.86	5.6	13	7.3
Protease	12.32 ^a	2.1	17	7.3
Esterase	9.91	6.0	13	7.3
Dehydrogenase	0.24	6.0	13	7.3

^a Protease activity is expressed as ΔAbsorbance min⁻¹ g⁻¹ VSS.

samplings from early and late operation stages of the SMB tending to group apart.

Table 2 shows the closest phylogenetic affiliations of seven of the TGGE bands that experienced shifting through SMB operation, which were successfully reamplified from the gel and further sequenced. Representatives of the Alpha-proteobacteria (bands M8 and M9), *Thiothrix* filamentous Gamma-proteobacteria (band M13), photoheterotrophic Beta-proteobacteria (band M12), and nitrite oxidizers of the genus *Nitrospira* (band M19) were identified, as well as some sequences poorly related to any validated bacterial taxons (bands M9, M22, M26).

Discussion

In activated sludge from conventional WWTPs, an increase of VSS concentration is commonly correlated to an increase

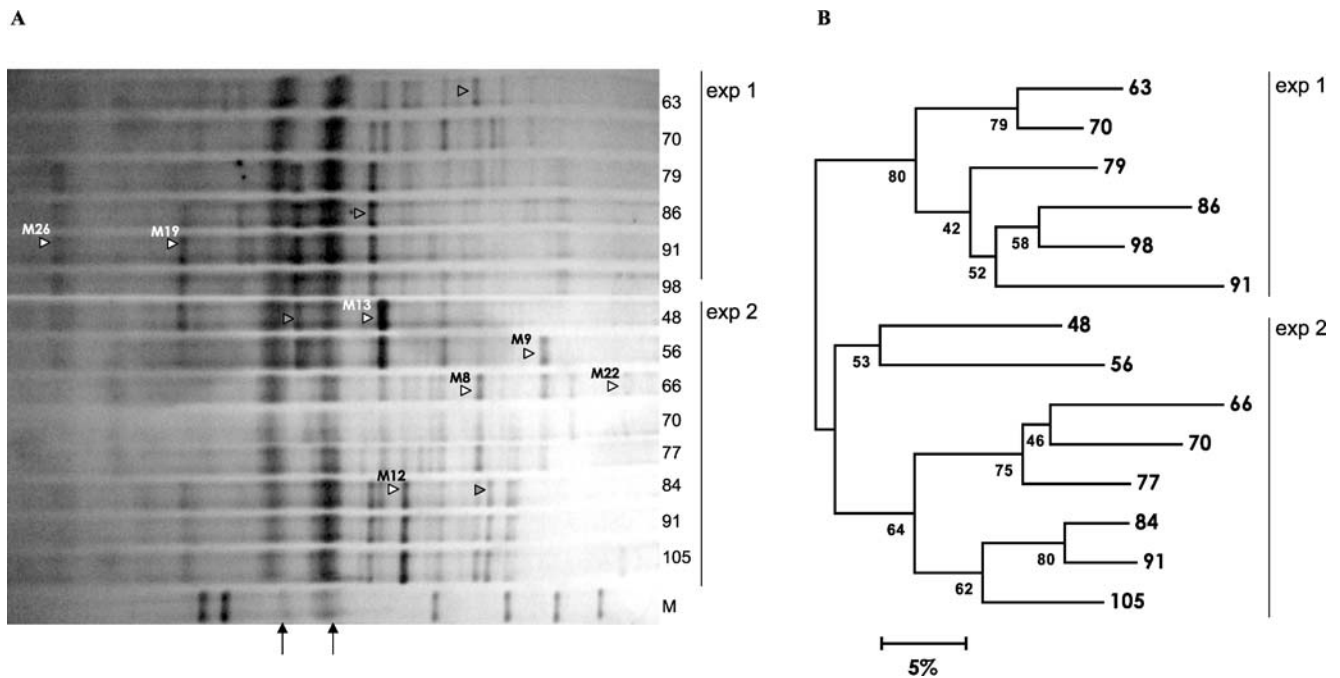


Fig. 6 a TGGE fingerprinting of activated sludge samples taken weekly from the SMB. Profiles are based on amplification and separation of the V3 hypervariable region of the 16S rRNA gene. Bands that shifted, which were successfully amplified and sequenced (see Table 2), are marked with *white arrowheads* and labeled. Bands

that yielded ambiguous sequences after re-amplification are marked with *gray arrowheads*. *Arrows* point to artifact bands. *M* six-species marker. **b** Neighbor-joining cluster analysis of TGGE banding profiles. Similarity is based in the presence or absence of individual bands. Bootstrap values are indicated for each dichotomy

of the number of microorganisms. However, THPM counts conducted in this study showed that such correlation only took place during experiment 2, and for the cultivable microbiota incubated at 22°C. Witzig et al. (2002) reported that high biomass retention rates modify the biology of the reactor, lowering growth rates due to the limitation of available substrates. The number of THPM found in the activated sludge in the SMB are slightly lower compared to

the values reported by other authors in activated sludge from a conventional, non-biomass recycling system (Güde 1982; Kämpfer 1988). Microbial populations from SMB are also reported to contain less bacterial cells detectable by fluorescence in situ hybridization (FISH) than conventional activated sludge samples (Witzig et al. 2002).

The diversity of bacteria in the activated sludge from the SMB was also studied by a culture-independent approach

Table 2 Identification of bands in the TGGE community fingerprint of the activated sludge

Band	Closest taxonomic affiliation (class/family)	Overlap (nt)	Most similar organisms	Access no.	Percent identity
M- 08	Alpha-proteobacteria/Sphingomonadaceae	123	<i>Blastomonas</i> sp. <i>Sphingomonas</i> sp.	AB242676 AJ812013	99.1 99.1
M- 09	Alpha-proteobacteria	123	<i>Sinorickettsia chlamys</i> ^a <i>Roseobacter</i> sp.	AY174894 AY136130	90.2 88.6
M- 12	Beta-proteobacteria/Comamonadaceae	149	<i>Rhodoferax</i> sp. <i>Rhodoferax fermentans</i>	AY788978 AJ289110	96.6 96
M- 13	Gamma-proteobacteria/Thiotrichaceae	112	<i>Thiothrix eikelboomii</i> <i>Thiothrix nivea</i>	L79965 L40993	100 96
M- 19	Bacteria/Nitrospiraceae	125	Candidatus <i>Nitrospira defluvii</i> <i>Nitrospira moscoviensis</i>	DQ059545 X82558	100 96
M- 22	Eubacteria	123	Bacterium TSA-5.4 (grassland soil isolate) <i>Eubacterium formicigenerans</i>	AF240150 L34619	96.7 82.5
M- 26	Gemmatimonadetes	159	Bacterium Ellin 5290 <i>Gemmatimonas aurantiaca</i>	AY234641 AB072735	89.3 86.2

^a Nonvalidated organism name

based on TGGE, which generates profiles of the dominant populations in complex communities in the environment. This technique has been extensively applied in recent years to the study of a variety of different bacterial ecosystems, including sludge and biofilms from WWTPs (Wagner et al. 2002; Cortés-Lorenzo et al. 2006). We found that the behavior of the community responsible for the treatment of real domestic wastewater was analogous to what has been described for bacterial consortia growing under stringent nutrient limitation on synthetic wastes (LaPara et al. 2001; Morgan et al. 2002) and gray water (Stamper et al. 2003). TGGE fingerprints generally clustered following the time course, displaying the effect of the accumulation of VSS in the bioreactor on the species composition of the sludge (Fig. 6), which induced a gradual shifting of the dominant members of the community. Several bands were detected in the TGGE profiles, which were specific of certain stages of a particular experiment (Fig. 6). Also, the TGGE fingerprints showed a connection between the concentration of VSS and the community changes, especially during experiment 2. The clustering change between days 66–77 and 84–105 (Fig. 6) is clearly linked to a sharp increase of VSS concentration from 4.5–7.3 to 10.5–13.8 g/l (Fig 2c). These alterations of community composition overlapped with the drop of all the enzymatic activities recorded during the last 4 weeks of experiment 2, pointing to the operational importance of certain populations affected by the community shifting.

Stamper et al. (2003) analyzed the biological diversity of the content of an SBM bioreactor treating gray water and proposed that major changes of the community structure were correlated to occasional peak increases of BOD₅ values and of the content of oils and grease in the inflow. Interestingly, the data of Stamper et al. (2003) showed also the separated clustering of DGGE profiles from samples taken within a period of operation when the VSS concentration was over 10 g/ml. In contrast, we could not find in our experiments a clear correlation between COD or BOD₅ changes of the influent domestic wastewater (Fig. 2a) and the variations experienced by the bacterial community (Fig. 6). It must be taken into consideration that domestic wastewater and gray water have different qualitative and quantitative compositions (Stamper et al. 2003).

Although TGGE is not a quantitative method due to the bias introduced by the PCR reaction, some bands clearly changed their intensity through the experiment, which can be interpreted as a consequence of a change in their relative abundance in the community composition (Brüggemann et al. 2000; Stamper et al. 2003). Identification of bands by reamplification and sequencing demonstrated the significant occurrence (at least 1% of total population, according to the estimated average TGGE detection threshold) of

nitrite oxidizers of the *Nitrospira* genus. *Nitrospira* populations prevail over *Nitrobacter* in SMBs previously investigated by the FISH technique (Witzig et al. 2002). Remarkably, some of the dominant bands displayed very low sequence identity with validated bacterial taxons, suggesting the possible importance of unknown bacterial groups in SMB operation.

Enzymatic activities are essential for the hydrolysis and mineralization of the proteins, carbohydrates, and lipids, which are part of the organic matter removed from the wastewater (LaPara et al. 2002). When the activities measured in both experiments are compared, significantly lower values are measured during experiment 2. External factors such as temperature and pH of the sludge have a strong influence on the enzymatic activities and solubility of sludge in the bioreactor (Banister and Pretorius 1998). Whiteley et al. (2002) reported a positive correlation between temperature and proteolytic activity, which achieved maximum values at temperatures ranging 20–22°C. In experiment 2, carried out during autumn and winter, the temperature of the sludge was 5–15°C and seldom raised over 10°C during days 75–113, while maximum enzymatic activities were recorded at temperatures of 13–23°C (Table 1).

Temporal variations of the hydrolysis rates are explained by two major factors: the changes of the active biomass and the shifting of the dominant populations in the bioreactor (Cadoret et al. 2002). The ability of SMB to catabolize substrates is proportional to the level of active biomass (LaPara et al. 2001). VSS gives an estimation of the active biomass; thus, an increment of the activity is expected when VSS concentrations rise. However, in our experiments we found a tendency of all enzymatic activities tested to decline as VSS concentration increased through time. One explanation is the existence of a feedback inhibition of the activities due to the accumulation of end products in the biomass recycling system. For instance, it is well-documented that phosphatase activity is repressed in aquatic environments by high concentrations of dissolved inorganic P (Wright and Reddy 2001). The efficiency of oxygen transfer decreases at high VSS concentrations, leading to an increase in viscosity and a change of the rheology of the sludge (Rosenberger et al. 2002). Limited oxygen availability can have a strong impact on the survival and biological activity of particular groups of microorganisms, especially the nitrifying bacteria. Nitrite oxidation is particularly sensitive to oxygen availability (Wyffels et al. 2003). Besides, the inhibition of the enzymatic activities at high VSS concentration was more evident in experiment 2, which was carried out in winter. Low temperatures (<8°C) also have a negative effect on the nitrifying bacteria, mainly the ammonia oxidizers (Martin et al. 2005; Egli et al. 2003). A failure of the nitrification process in the SMB reactor can lead to the accumulation of toxic nitrogen species such as

ammonia and nitrite, which may affect community composition of the sludge and change the overall performance of the enzymatic activities. Further experiments are in progress to study the influence of high VSS concentration and low temperature on the nitrifying process and the diversity of nitrifying bacteria in the SBM reactor.

In conclusion, the evolution of the enzymatic activities and community fingerprints generated by TGGE demonstrated that the bacteria adapted progressively to the conditions inflicted by the biomass retention in the bioreactor, due to the variation of the VSS concentration. High concentrations of VSS induced a decay of the enzymatic activities, although the threshold concentration of VSS that generates such effect varied in the two experiments, done under different average temperatures of operation and inflow rates. Our results suggest the need for careful monitoring of the concentrations of VSS reached in the SMB, in order to choose and maintain those most appropriated for the proper operation of the biomass recycling system. In the bioreactor tested in this study, VSS concentrations over 8.0 g/l generated very low enzymatic hydrolysis rates, potentially affecting the features of the biological treatment.

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