Microbial community dynamics in a chemolithotrophic denitrification reactor inoculated with methanogenic granular sludge

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Abstract

Denitrification is applied in the tertiary treatment of wastewater to reduce nitrogen pollution. Fluorescence *in situ* hybridization (FISH), catalyzed reporter deposition (CARD)-FISH, cloning, and scanning electron microscopy (SEM) were applied to follow the evolution of the microbial composition and structure of granular sludge in chemolithotrophic denitrifying bioreactors fed with nitrate and thiosulfate. FISH oligonucleotide probes for the chemolitoautotrophic denitrifiers *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* were designed and their utility tested. CARD-FISH and cloning data showed that bacterial diversity in the biofilms changed during the reactor operation. Chemoorganotrophic fermentative Gram-positive strains in the phyla, *Actinobacteria* and *Firmicutes*, were dominant in the methanogenic inoculum, both in terms of biodiversity and in number. Other significant phyla were *Bacteroidetes* and *Chloroflexi*. After 6 months of operation, *Proteobacteria* became dominant (83% of the clones). The diversity of Gram-positive bacteria was partially maintained although their abundance decreased notably. After 110 d of operation, the abundance of *Tb. denitrificans* cells increased considerably, from 1 to 35% of total DAPI-stained cells and from no isolated clones to 15% of the total clones. *Tm. denitrificans* only represented a minor fraction of the microorganisms in the sludge (1-4% of the DAPI-stained cells). These findings confirm that *Tb. denitrificans* was the dominant chemolitoautotrophic denitrifying microorganism in the bioreactors. The Archaeal diversity remained almost unchanged and it was represented mostly by *Methanosaeta soehngenii*. SEM results indicated a considerable loss in the integrity of the sludge granules during the operation, with risk of sludge buoyancy.

Keywords: Autotrophic denitrification; denitrifying bacteria; granular sludge; molecular ecology; *Thiobacillus denitrificans*; *Thiomicrospira denitrificans*, UASB reactor.

1. Introduction

Inorganic nitrogen compounds, *i.e.*, ammonium, nitrate and nitrite, are common wastewater contaminants. Nitrogen removal is important to prevent a wide array of public-health and environmental impacts. Upon wastewater discharge, ammonium can be oxidized by nitrifying microorganisms, leading to a decrease of the dissolved oxygen content in the receiving waters. Inorganic N-compounds contribute to eutrophication of rivers and lakes. Ammonium is toxic to aquatic organisms. Nitrate formed from nitrification can cause the disease methemoglobinemia in infants. Nitrate, originating from natural or anthropogenic sources, can limit the utilization of groundwater for drinking water purposes. Lastly, incomplete denitrification releases N_2O, a greenhouse gas. Therefore, there is a need to treat N-compounds in effluents. Biological processes combining sequential nitrification and denitrification can be used for this purpose.

The process of denitrification involves the reduction of nitrate to dinitrogen gas by anaerobic facultative bacteria that utilize nitrate as electron acceptor. Denitrifying bacteria are generally heterotrophic and utilize organic matter as electron donor. A limited number of bacteria are capable of chemolithotrophic denitrification, and they can utilize inorganic compounds such as reduced sulfur compounds, hydrogen, ammonium, nitrite, ferrous iron or uranium (IV) as electron donors for the reduction of nitrate, and inorganic carbon (CO_2 or HCO_3^-) as carbon source for microbial cell synthesis (Zumft, 1992; Straub et al., 1996; Beller, 2005).

Denitrification combined with the process of nitrification is the most common approach for the removal of N in the tertiary treatment of wastewaters. Denitrification processes can also be applied to remediate nitrate in drinking water. Potential problems associated with residual organics in heterotrophic denitrification processes can be avoided if inorganic substances are used as electron donors for chemolithoautotrophic denitrification. Chemolithotrophic denitrification with reduced sulfur compounds offers a great biotechnological potential. The process can attain the simultaneous removal of N and S contamination in a single-phase system and transform these contaminants into environmentally acceptable forms (N_2 gas and sulfate or S^0). Sulfur-based denitrification has been studied for the treatment of drinking water (Sierra-Alvarez et al., 2007), for the simultaneous

removal of S and N from petrochemical industries (Cardoso et al., 2006), for the removal of N from metal plating wastewaters (Flores et al., 2006), municipal wastewater (Am et al., 2005), and highly-concentrated wastewaters such as baker's yeast effluent, in a new process known as DEAMOX (DEnitrifying AMmonium OXidation) (Kalyuzhnyi et al., 2006).

In spite of the scientific and technologic interest of chemolithotrophic denitrification with reduced sulfur compounds, little is known about the structure of the microbial communities implicated in the process. Only two obligate chemolithoautotrophs species that grow with S-reduced compounds as electron donors at neutral pH values have been described, *Tb. denitrificans* and *Tm. denitrificans* (Kuenen et al., 1992). Two novel chemolithoautotrophic nitrate-reducing bacteria capable of sulfur or thiosulfate oxidization have been recently published, *Sulfurimonas paralvinellae* (Takai et al., 2006) and a *Tm. denitrificans*-like (Brettar et al., 2006). However, they are marine bacteria, isolated from a nest of hydrothermal vent polychaetes, *Paralvinella sp*., and from water samples obtained from an anoxic basin in the central Baltic Sea, respectively, so that their presence is not expected in engineered bioreactor systems.

Traditionally, approaches for the identification and characterization bacteria in complex ecosystems involved isolation of the microorganisms and their subsequent morphological, biochemical and metabolic characterization. Culture-independent, molecular biology techniques based on 16S rRNA gene such as fluorescence *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and genetic libraries have become essential tools to study structure and biodiversity of both natural and engineered complex microbial ecosystems (Amann et al., 1995). Molecular ecology techniques have been applied in a number of studies concerned with the heterotrophic denitrifying communities in wastewater treatment systems (Sanz and Köchling, 2007). In contrast, our current knowledge of the microbial diversity of autotrophic denitrifying biofilms is very limited and existing studies have only considered microbial communities in laboratory-scale sulfur-based denitrification reactors (Ahn et al., 2004; Koening et al., 2005).

This study investigated the structure of the microbial community in the chemolithotrophic denitrifying granular biofilms developed in UASB reactors, originally inoculated with methanogenic granular biofilms, fed with nitrate and thiosulfate. FISH, catalyzed reporter deposition (CARD)-FISH and clone library analysis were utilized to investigate the evolution of the microbial communities resulting from the shift from methanogenic to chemolithotrophic denitrifying conditions. Novel specific oligonucleotide probes targeting *Tb. denitrificans* and *Tm. denitrificans* were designed and probe hybridization conditions were optimized for their application in the monitoring of these chemolithotrophic denitrifiers by FISH and CARD-FISH. To the best of our knowledge, this is the first molecular ecology study of chemolithotrophic denitrifying communities in bioreactors removing nitrate and thiosulfate simultaneously.

2. Materials and methods

2.1 Microorganisms

The methanogenic sludge used as inoculum was obtained from a full-scale UASB bioreactor treating recycle paper factory (Eerbeek, The Netherlands). The volatile suspended solids (VSS) concentration of the sludge was 12.9%. Pure cultures of the target microorganisms (*Tb. denitrificans* DSM12475, *Tm. denitrificans* DSM1251 and *Tb. thioparus* DSM505) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

2.2 Continuous bioreactor experiments

Continuous experiments were conducted in two UASB reactors (2 L volume) placed in a temperature-controlled room at 30°C. Reactor 1 (R1) was inoculated with Eerbeek granular sludge (15 g VSS L^{-1}). Reactor 2 (R2) was inoculated with Eerbeek sludge (15 g VSS L^{-1}) and then supplied with granular activated carbon (Filtrasorb 400F, Calgon carbon Corp., Pittsburg) (116.3 g

oven dry weight L^{-1}). Both reactors were fed with a synthetic wastewater consisting of the basal medium supplemented with nitrate (37.5 mM) and thiosulfate (20 mM). The basal medium was composed of (g L^{-1}): K_2HPO_4, 0.8; KH_2PO_4, 0.3; NH_4Cl, 0.4; $MgSO_4 \cdot 7H_2O$, 0.4; $NaHCO_3$, 2.0 and trace element solution at a rate of 2 ml L^{-1}. The trace element solution was composed of (mg L^{-1}): EDTA, 500.0; $ZnSO_4$, 22.0; $CaCl_2$, 55.0; $MnCl_2 \cdot 4H_2O$, 50.6; $(NH4)_6MoO_{24} \cdot 4H_2O$, 11.0; $CuSO_4 \cdot 5H_2O$, 15.7; and $CoCl_2 \cdot 6H_2O$, 16.1. A constant molar $NO_3^-/S_2O_3^{-2}$ ratio of 1.875 was maintained through the experiment. The bioreactors were operated at a hydraulic retention time (HRT) varying from 6 to 24 h, depending on the experimental period.

Biomass was sampled periodically and immediately stored at –20°C until DNA extraction was performed. Samples for FISH analysis were immediately fixed with 4% paraformaldehyde and ethanol as described previously (Amann, 1995).

2.3 Specific metabolic activities

The maximum specific autotrophic denitrifying activity of sludge samples was determined in glass serum flasks (125 ml volume) supplied with 50 ml of basal medium (described above) and with 1 g VSS L^{-1} of the inoculum. The assays were amended with 37.5 mM nitrate and 20 mM thiosulfate. To prevent oxygen contamination, the bottles were sealed with thick butyl rubber stoppers and aluminum crimp caps and, then flushed thoroughly with N_2/CO_2 gas (80/20, v/v) to establish anaerobic conditions. Abiotic medium controls and killed sludge controls were run in parallel. Abiotic controls and killed-sludge controls were sterilized by autoclaving at 120 °C for 20 min. All assays were incubated in an orbital shaker in the dark in a climatized room at 30 °C. Liquid samples were taken periodically to determine substrate or/an electron acceptor utilization and product formation. All batch bioassays were conducted in triplicate.

2.4 Probe design, FISH and CARD-FISH

Design of oligonucleotide probes (ARB software, http://www.mikro.biologie.tu-muenchen.de/), FISH (Amann, 1995, 2001) and CARD-FISH (Pernthaler et al., 2002) were performed as previously described. Accessibility of 16S rRNA for the designed probes was checked as described elsewhere (Fuchs et al., 1998). The intensity of the fluorescence was analyzed using Metamorph® Imaging System (Universal Imaging Corporation, West Chester, USA). The intensity was determined as the average of the integrated optical density of the cells, where:

$$\text{Integrated OD} = \sum\left(GV - \log\frac{MaxGV}{GV}\right)$$

GV means Grey Value which is defined as the brightness of pixels in a digitized image.

Cell counting and sample preparation were performed as described by Díaz et al. (2003). Hybridized cells were quantified by image analysis using the freeware program ImageJ (http://rsb.info.nih.gov/ij/). To quantify the hybridized and DAPI-stained cells an epifluorescenct microscope Zeiss Axioskop equipped with filters for Cy3 (G-2A, λ = 550–570), DAPI (UV-2A, λ = 359–461), FITC (B-2A, λ = 459–519) was used. The oligonucleotide probes were supplied by Genotek (Barcelona, Spain). All probes were labeled at the 5'-end with Cy3. Universal probes were also labeled with fluorescein. The probes tested in this work, with indication of the hybridization and washing conditions, are summarized in the Table 1.

2.5 DNA extraction, amplification, and genetic libraries

DNA was extracted using FastDNA Spin kit for Soil (BIO101 Inc, Q-Biogene) according to the manufacturer protocol. Purified DNA samples were stored at -20 °C. The 16S rRNA genes from mixed microbial DNA were amplified by the polymerase chain reaction (PCR) using standard conditions, using 2 mM Mg^{2+} for both domains. For 100 µl reaction, 0.5 units of Taq DNA polymerase (Promega, Madison, WI, USA) were added. To obtain almost complete 16S rRNA gene sequences, two oligonucleotide primer pairs were used: 27F and 1492R for the *Bacteria* domain

and 25F and 1492R for the *Archaea* domain (Lane, 1991). The thermal profile for amplification included 10 min at 94 ºC for denaturing, 30 cycles at 94 ºC for 1 min, primer annealing for 1 min at 56 ºC (*Bacteria*) or at 52ºC (*Archaea*), 3 min of elongation at 72 °C, and, finally, 10 min at 72 °C to complete the elongation. The 16S rRNA gene amplificates (length 1465-1467 bp) were cloned using TOPO Cloning Kit (Invitrogen, San Diego, CA). Competent *E. coli* cells were transformed with these plasmids. A total of 188 clones (96 for *Bacteria* and 92 for *Archaea*) for the methanogenic inoculum and 181 (96 for *Bacteria* and 85 for *Archaea*) for the denitrifiying sludge were analyzed. Plasmid inserts were screened by Amplified Ribosomal DNA Restriction Analysis (ARDRA) using the enzyme Sau3AI (New England BioLabs Inc., USA). Fragments were separated by 2% (w/v) agarose (Pronadisa, Madrid, Spain) gel electrophoresis and visualized by ethidium bromide staining. Clones were grouped according to their restriction patterns defining different Operational Taxonomic Units (OTUs). An OTU was defined as a set of clones with the same restriction band pattern in the ARDRA analysis. Subsequently, the inserts of two clones of each OTU were amplified by PCR using the M13 primer set (Invitrogen) and sequenced with an ABI model 377 sequencer (Applied Biosystems). The clones with a similarity of sequence equal or higher than 97% were assigned to the same OTU.

2.6 Phylogenetic analysis

Sequences were analysed with CHIMERA CHECK program to avoid sequences from chimeric origin (Cole et al., 2003). 16S rRNA sequence similarity searches were performed in the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST) algorithm Sequences were aligned with the program. Alignment of the 16S rRNA sequences was performed by using the FastAligner V1.03 tool of the ARB program package. The resulting alignments were manually checked and manually corrected when necessary. Phylogenetic trees were constructed using Parsimony method.

206 *2.7 Nucleotide sequence accession numbers*

207 The sequences of clones reported in this paper have been deposited in the GenBank (NCBI)
208 database under accession numbers EF063609-EF063624 (clones from methanogenic inoculum) and
209 EF063625-EF063636 (clones from chemolithotrophic denitrifying sludge).

210

211 *2.8 Scanning electron microscopy (SEM)*

212 Sludge granules were studied using a SEM Philips XL30 microscope as described elsewhere
213 (Alphenaar et al., 1994).

214

215 *2.9 Chemical analysis*

216 Nitrate, nitrite, sulfate and thiosulfate were determined by ion chromatography with suppressed
217 conductivity detection using a Dionex DX-500 system equipped with an AS11-HC Dionex column
218 (Dionex, Sunnydale, CA). The eluent gradient of KOH utilized was: 1 mM at 0 min; 2 mM at 5.5
219 min and 8.5 mM at 12 min, and the flow rate 1.2 ml min^{-1}. Liquid samples were membrane filtered
220 (0.20 μm) prior to chromatographic analysis. Sulfide in liquid samples was analyzed immediately
221 after sampling to prevent compound losses by volatilization and/or abiotic oxidation. Sulfide was
222 quantified colorimetrically by the methylene blue method (Trüper and Schlegel, 1964). N_2 and N_2O
223 in gaseous samples were analyzed by gas chromatography using an HP5290 Series II system
224 (Agilent Technologies, Palo Alto, CA) fitted with a Carboxen^{TM} 1010 Plot capillary column (30 m
225 x 0.32 mm, Supelco, St. Louis, MO) and with a thermal conductivity detector. Gas samples (100 μl)
226 were collected using a pressure-lock gas syringe. The pH was determined immediately after
227 sampling with an Orion model 310 PerpHecT pH-meter with a PerpHecT ROSS glass combination
228 electrode. VSS content was determined according to standard methods APHA (1998).

229

230 **3. Results and discussion**

231

232 *3.1 Reactor Performance*

233 The bioreactors were operated under chemolithotrophic denitrifying conditions at HRT ranging
234 from 7 to 24 h. Table 2 summarizes the average performance data determined for the mature
235 chemolithotrophic denitrifying bioreactors. R1 and R2 could accommodate thiosulfate loading rates
236 of 47.4 and 38.5 mM d^{-1}, respectively, with high thiosulfate removal efficiencies.. The maximum
237 volumetric conversion rates of nitrate were estimated to be 88.9 and 72.4 mmol NO_3^- $L^{-1}d^{-1}$ for R1
238 and R2, respectively. Sulfate recovery paralleled the nitrate removal efficiencies very closely.

239 The specific activities of the reactor sludge were determined in batch assays. The activities
240 were 35.3 and 25.6 mmol NO_3^- g VSS^{-1} d^{-1} or 47.2 and 38.3 mmol SO_4^{2}-formed g VSS^{-1} d^{-1} for
241 sludge sampled from R1 and R2, respectively. These values were 8 to 11-fold higher than the
242 original Eerbeek sludge, clearly indicating enrichment of anoxic sulfoxidizing microorganisms. The
243 molar ratio between the sulfate formation and nitrate consumption rates was 1.37 to 1.41, which are
244 close to the theoretical stoichiometry of 1.25.

245

246 *3.2 Oligonucleotide design*

247 Two new oligonucleotide probes, TBD1419 and TMD131 (Table 1), were initially designed for
248 the specific detection of *Tb. denitrificans* and *Tm. denitrificans,* respectively, by FISH. Species-
249 specific probes targeting these well-known chemolithotrophic denitrifying microorganisms were
250 not available when the research was conducted. The brightness of cells hybridized with the *Tb.*
251 *denitrificans*-specific probe TBD1419 was satisfactory for CARD-FISH and FISH using confocal
252 laser scanning microscopy, but insufficient to be useful in FISH using a conventional
253 epifluorescence microscope. For that reason, TBD121, a new probe targeting *Tb. denitrificans* was
254 designed. Probes were selected based on their specificity and accessibility to the rRNA target in the
255 ribosome (brightness classes I and II according to Fuchs et al., 1998).

256 The formamide concentration required for optimal probe hybridization was determined in
257 experiments utilizing pure cultures of the target microorganisms (*Tb. denitrificans* strain

DSM12475 or *Tm. denitrificans* strain DSM1251). The *Tb. denitrificans* specific probe, TBD131 also hybridized with *Tb. thioparus* strain DSM505, a microorganism that presents a single miss-match in its rRNA for these probes. *Tb. thioparus* is an aerobic chemolitoautotrophic microorganism that oxides reduced sulfur compounds but that is not able to use nitrate as electron donor (Vlasceanu et al., 1997). Therefore, it is not to be expected that *Tb. thioparus* will be a source of error in this study. The specificity of the TMD131 probe should be expected to be high since the 16S rRNA gene sequences of the closest relatives, *Mycoplasma pulmonis* and *Staphylococcus caprae,* present two mismatches for the probe and only 70 and 69% similarity with *Tm. denitrificans,* respectively.

The optimum formamide doses determined for the various specific probes are listed TBD121 in Table 1. As an example, Fig. 1 shows the intensity of the fluorescent signal as a function of the formamide concentration, both for the bacterial samples hybridized and non-hybridized with TBD1419. The optimum concentration of formamide, allowing the best discrimination between *Tb. denitrificans* and *Tb. thioparus,* was 50%.

3.3 FISH and CARD-FISH

The biomass samples were analyzed by FISH and CARD-FISH to investigate the evolution of the microbial communities with operation time. Specific probes for different groups of Bacteria, Archaea, and for the chemolithotrophic denitrifiers, *Tb. denitrificans* and *Tm. denitrificans,* were utilized (Table 1). The higher signal intensity in the hybridization using CARD-FISH allowed a better cell detection and quantification by means of image analysis and, therefore, CARD-FISH was utilized to assess the abundance of different microbial communities in the reactor biofilms. A sharp change in the microbial composition was observed in biomass samples obtained from both reactors after 4 months of operation as compared to the inoculum. Bacteria was the most abundant domain, accounting for 40 to 60% of the total DAPI-stained cells (Fig. 2 and 3). Cells hybridizing with the Gram-positive bacteria (*Firmicutes* and *Actinobacteria*) and α-*Proteobacteria* specific probes,

284 which were dominant in the original inoculum, nearly disappeared, and microorganisms closely
285 related to the γ-*Proteobacteria* became dominant (25-45% of total count of stained cells). γ-
286 *Proteobacteria*, in combination with α- and β-*Proteobacteria*, represented the Bacteria almost in its
287 entirety. γ- and β-Proteobacteria were also found to be dominant in whole-cell hybridization studies
288 of biofilms obtained from sulfur-based bioreactors (Ahn et al., 2004). The microbiota of both
289 reactors was found to be very similar and few changes in the microbial composition of the biofilms
290 were detected in samples obtained periodically after 4 months of operation.

291 The specific oligonucleotide probes developed in this study showed the presence of *Tm.*
292 *denitrificans* and *Tb. denitrificans* in the biomass samples obtained from the chemolithotrophic
293 denitrifying bioreactors (Fig. 2). Negative, or less than 1%, hybridization was observed with all
294 other bacteria-specific probes utilized in this study, including probe SRB385 (sulfate-reducing
295 bacteria), DSS658 (*Desulfosarcina* spp, *Desulfococcus* spp), DSV698 (*Desulfovibrio* spp) and
296 SYN835 (*Syntrophobacter*). Throughout the continuous bioreactor experiments, *Tm. denitrificans*
297 only represented a minor fraction of the microorganisms in the sludge (1-4% of the DAPI-stained
298 cells). In contrast, *Tb. denitrificans* became the dominant species and its relative abundance
299 increased from 1% to 35% of the total DAPI-stained cells after 110 d of reactor operation. These
300 results suggest that *Tb. denitrificans* is responsible of the effective autotrophic denitrification in our
301 reactors. Phylogenetically, *Thiobacillus* is grouped in the β-*Proteobacteria*. Surprisingly, the
302 number of *Tb. denitrificans* cells detected was close to the number of γ-*Proteobacteria* cells.
303 Further tests with pure cultures showed that *Tb. denitrificans* hybridized with the γ-*Proteobacteria*
304 probe, GAM42a, but not with the β-*Proteobacteria* probe, BET42a. This apparent incongruity can
305 be explained considering that the probes BET42a and GAM42a, which were designed against 23S
306 rRNA, differ by only one nucleotide (T instead of A in position 52 according to *E. coli* position).
307 Comparison of the sequence of both probes with the recently published genome of *Tb. denitrificans*
308 (Beller et al. 2006; http://gib.genes.nig.ac.jp/single/main.php?spid=Tden_ATCC25259) confirms
309 that *Tb. denitrificans* presents, as the γ-proteobacteria, an A in the target for the probe. This fact can

310 explain why enrichment of the biofilms with *Tb. denitrificans* during the reactor operation was
311 accompanied by an increase in the relative abundance of cells that hybridized with the γ-
312 *Proteobacteria* probe.

313 Microorganisms in the *Archaea* domain were less abundant and did represent about 10% of the
314 total DAPI-stained cells in the inoculum (Fig. 3). Only cells from the orders *Methanosarcinales*
315 (8% of the total DAPI-stained cells) and, to a lesser extent, *Methanobacteriales* (1%) were detected.
316 As expected, the relative abundance of *Methanosarcinales* and *Methanobacteriales* in biomass
317 samples obtained from the chemolithotrophic denitrifying reactors decreased significantly after
318 several months of operation. However, the total number of Archaea appeared to increase with time
319 during the reactor operation (Fig. 3). Unspecific hybridization of *Thiobacillus* cells with the
320 archaeal probe, ARC915, (González-Toril, personal comm.) could explain the unexpected increase
321 in the number of cells that hybridized with that probe. The intensity of the hybridization signal of
322 *Thiobacillus* with the probe ARC915, although lower compared to TBD121, is sufficient to cause
323 the detection of false positives.

324

325 *3.4 Microbial biodiversity by cloning*

326 Analysis of 16S rRNA gene clone libraries was used to assess the diversity of microbial
327 communities in the methanogenic inoculum and in chemolithotrophic denitrifying biofilms obtained
328 after 186 d of reactor operation. In total, 16 OTUs were identified for the methanogenic inoculum
329 (labeled Eb∗), and 12 for the chemolithotrophic denitrifying biofilm (labeled R1Cb∗). Figures 4 and
330 5 summarize the phylogenetic affiliations of 16S clones in the Gram-negative and Gram-positive
331 bacteria, respectively, corresponding to the methanogenic inoculum and to autotrophic denitrifying
332 biomass obtained at the end of the operation of R1.

333

334 *Methanogenic inoculum:* The Gram-positive phyla, *Actinobacteria* and *Firmicutes*, were
335 dominant in the inoculum, both in terms of biodiversity and in number (Table 3 and Fig. 5). A total

of 4 OTUs, including 52.3% of the clones found in the inoculum, were most closely related to *Actinobacteria,* while 5 OTUs, representing 25% of the clones, were closest to *Firmicutes*. All genera detected include strict or facultative anaerobes, which is in agreement with the source of the microbial culture, an anaerobic reactor treating a carbohydrate-rich paper mill wastewater. Most of the OTUs are relatives to clones found in anaerobic digesters (AB195904, AB195906, AF275916, AB267031, U81750) and/or close to the fermentative microorganisms, *Propionimicrobium* (former *Propionibacterium*) *lymphophilum, Propionicimonas paludicola,* and *Cellulomonas fermentans*. The actinobacterium, *P. lymphophilum,* and the cellulose-hydrolysing *Cellulomonas* were also found in sludge from a methanogenic reactor treating paper mill wastewater (Roest et al., 2005).

In the same fashion, the presence of three OTUs (9% of the clones) included in the *Bacteroidetes* phylum (Eb36, Eb89 and Eb6, Table 3 and Fig. 4) could be interpreted based on the origin of the sludge. Bacteroids, which are strict anaerobic, fermentative microorganisms, are common in ecosystems characterized by high levels of organic matter. They are polysaccharilytic bacteria that produce acetate and succinate as the major metabolic end products. The OTUs Eb36 and Eb89 are relative to the classes *Flavobacteria-Sphingobacteria*. Cytophagas, included in this last class, are often detected in anaerobic granular sludge (Sekiguchi et al., 1998; Chan et al., 2001) but their physiological role remains unclear. Many *Cytophaga* spp. digest polysaccharides (cellulose, agar or chitin) but these well-known cellulose decomposers are obligately aerobic.

Other significant phylum present in the inoculum was *Chloroflexi* (green non-sulfur bacteria), with three OTUs (11.3% of the clones, Table 3), one of which, OTU Eb24, was also found in the sludge at the end of the continuous bioreactor experiment (OTU R1C10). The presence of photosynthetic bacteria in an anaerobic bioreactor would be surprising were not that other members of the *Chloroflexi*, most of which have not been isolated to date, have been detected repeatedly in anaerobic reactors (Sekiguchi et al., 1998, 2001; Roest et al., 2005; Díaz et al., 2006). Some of the known microorganisms in this group are filamentous chemoorganotrophic bacteria, including aerobes (*Herpetosiphon*), facultative (*Caldilinea*), and strictly anaerobic microorganisms

(*Anaerolinea*). These microorganisms must play an important role in the degradation of organic matter since they are routinely detected in granular sludge. Sekiguchi et al. (2001, 2003) isolated filamentous green non-sulfur bacteria from thermophilic granular sludge and hypothesized that the strain was a carbohydrate-fermenting bacterium. Roest et al. (2005) speculated that the clones detected in granular sludge close to the deeply branching *Chloroflexi* were "directly or indirectly involved in butyrate degradation".

In the inoculum, surprisingly, the phylum *Proteobacteria* was represented by only one OTU (2.3% of the clones), which was included in the class δ–*Proteobacteria*. The OTU Eb8 is closely related to a *Desulfovibrio* sp clone (AY340826) identified in a sulfate-reducing fluidized reactor. According with the hybridization results, 20% of the total cells are affiliated to *Proteobacteria*. We have not explanation for this disagreement, although it must be kept in mind that cloning is not a quantitative technique.

The inoculum was obtained from a full-scale UASB reactor treating recycle paper wastewater. Based on the physiology of the microorganisms identified in the methanogenic inoculum, the existente of a trophic chain can be hypothesized in which *Cellulomonas* (OTU Eb30), Bacteroids (OTUs Eb6, Eb36 and Eb89) and, possibly, the green non-sulfur bacteria (OTUs Eb24, Eb59, Eb85) initiate the hydrolysis of the complex carbohydrates present in the recycle paper wastewater. It is possible that a *Clostridium* sp. (OTU Eb26, Eb64) might excrete extracellular proteases. The fermentative bacteria, *Propionimicrobium* (OTU Eb76), *Propionicimonas* (OTU Eb37), *Cellulomonas fermentans* (OTU Eb30) and *Clostridium bowmanii* (OTU R1Cb79) would generate C1-C5 volatile fatty acids (VFA) succinate, lactate and ethanol. Although we failed to found syntrophobacteria or other acetogens, most species of *Desulfovibrio* oxidize organic compounds such VFA and lactate incompletely to acetate. Moreover, the OTU Eb76 is closed to a clone (AB195904) found in an anaerobic reactor fed VFA, and the OTU Eb33 is closed to an uncultured propionate-oxidizing soil clone (AY607140). *Methanosaeta soehgenii*, the only methanogenic archaea detected, is at the end of the trophic chain, transforming acetate to methane.

388

389 *Chemolithotrophic denitrifying biofilms:* Analysis of the R1 clone library after six months of
390 reactor operation showed that sequences affiliated with species most closely related to the
391 *Proteobacteria* became dominant (Table 3 and Fig. 4). Sequences grouping with the α, β and γ-
392 *Proteobacteria* were the most abundant, accounting for 39.2, 21.7 and 21.7% of the clones,
393 respectively, while Gram-positive bacteria corresponded to only 15.3 of the clones. *Proteobacteria*
394 were also dominant (83% of the total clones) in a sulfur-based denitrification bioreactor (Koenig et
395 al., 2005), although the biodiversity was lower than in the present study (only six OTUs detected).
396 In our chemolithotrophic biolfilms, a considerable number of OTUSs (R1Cb1, R1Cb63, R1Cb86,
397 R1Cb90, accounting for 28.2% of total clones) corresponded to former "*Pseudomonas*", currently
398 subdivided in the orders *Burkholderiales*, *Xhantomonadales* and *Pseudomonadales*. The dominant
399 OTU, R1Cb20, (37% of the clones) was 99% similar to *Ochrobactrum* sp. (DQ337573). Sequences
400 most closely related to *Tb. denitrificans* (OTU R1Cb91), the only chemolithoautotrophic denitrifier
401 detected, accounted for 15.2% of the clones.

402 The diversity of Gram-positive bacteria was partially maintained (Table 3 and Fig. 5) although
403 their abundance decreased notably (Table 3). The OTUs clustering with the *Firmicutes* included,
404 R1Cb41, with a 98% of similarity with the fermentor *Clostridium bowmanii* (AJ506120); R1Cb79,
405 with a 98% of similarity with *Exiguobacterium aestuarii* (AY594264), and R1Cb8 (probably the
406 same as the inoculum OTU Eb25). The only *Actinobacteria* OTU detected, R1Cb30 (2.2% of the
407 clones), corresponded to OTU Eb76, which accounted for 36.4% of the clones in the inoculum.
408 OTUs affiliated with the Gram-positive bacteria accounted for only 15.3% of the total number of
409 clones in R1, but they represented 77.3% of the clones in the methanogenic inoculum.

410 Many of the OTUs detected in the chemolithotrophic biofilm are affiliated with heterotrophic
411 microorganisms. While some of the sequences were closely related to those of heterotrophic clones
412 in the methanogenic inoculum (eg. OTUs R1Cb30 and Eb76; OTUs R1Cb8 and Eb25), the
413 dominant OTU in the mature chemolithotrophic biofilm (R1Cb20, most closely related to

414 *Ochrobactrum* sp. CHNTR29, DQ337573) was not observed in the initial inoculum.
415 Microorganisms in the genus *Ochrobactrum* (Lebuhn et al., 2000) as well as various species in the
416 orders *Burkholderiales*, *Xhantomonadales* and *Pseudomonadales* (Bergey´s Manual, Brenner et al.,
417 2005) are able to respire nitrate using organic products from cell lysis as electron donors, which
418 would explain their presence in the denitrifying bioreactor. Koenig et al. (2005) has also
419 hypothesized that endogeneous decay of chemolithotrophic bacterial cells can contribute to the
420 development of heterotrophic microorganisms in denitrification bioreactors fed with inorganic
421 substrates. The author observed a significant contribution of chemoheterotrophic bacteria (34% of
422 the clones) in a chemolithotrophic denitrification bioreactor utilizing elemental sulfur as electron
423 donor. The OTU R1C10, belonging to the *Chloroflexi phylum*, was also found in the methanogenic
424 inocule (OTU Eb24).

425 For the Archaea domain, 85 clones were obtained which after restriction digestion with the
426 enzyme Sau3A1 could be grouped into 7 different restriction patterns. Surprisingly, all clone
427 sequences were closely related to the methanogen *Methanosaeta soehngenii* (97 to 99% similarity).
428 In the autotrophic reactors, the methanogens must be likely resting forms from the methanogenic
429 inoculum.

430

431 *3.5 Analysis of the granular structure by SEM*

432 The structure of representative granules from the methanogenic inoculum and denitrifying
433 sludge obtained from both reactors at the end of the continuous experiment was studied by SEM.
434 The methanogenic inoculum had a compact structure with a fluffy surface (Figs 6b and 6c). These
435 granules are made up for a wide variety of microorganisms (Fig 6d). With time, the structure of
436 granules from the denitrifying bioreactors became looser, showing increased voids (Fig. 7). Some
437 granules were broken (Fig. 7a) and hollow (Fig. 7b). Possibly the growth of microorganisms was
438 restricted to the outer layers of the granule, giving the granule surface a continuous, dense, smooth
439 aspect (Figs. 7b and 7c), and to sheets perpendicular to the surface (Fig. 7c and 7d). These effects

could cause operation problems in the reactor by increasing the buoyancy of the sludge, which may result in sludge flotation and/or wash-out problems. Nonetheless, such problems were not observed during the 186 d of reactor operation.

4. Conclusions

Specific probes for *Tb. denitrificans* and *Tm. denitrificans* were designed, probe hybridization conditions were optimized, and their utility in the detection of the target microorganisms in reactor biofilms by FISH and CARD-FISH was tested. Results from cloning and CARD-FISH showed that bacterial diversity in the chemolithotrophic denitrifying bioreactor operated with thiosulfate as electron donating substrate and bicarbonate as carbon source changed considerably during the initial period of operation (days 0-112) and later became nearly constant. Bacterial sequences most closely related to Gram-positive microorganisms (*Actinobacteria* and *Firmicutes*) were dominant in the methanogenic inoculum, while α, β and γ-*Proteobacteria* were dominant in the mature denitrifying reactors. The presence of a high number of fermentative bacteria in the chemolithotrophic denitrifying biofilms is remarkable. The abundance of *Tb. denitrificans* cells was found to increase from 1% to 35% of total DAPI-stained cells, suggesting that *Tb. denitrificans* was responsible for the majority of the chemolithotrophic denitrifying activity in the reactor. Archaeal diversity remained almost unchanged and was represented by *Methanosaeta soehngenii*. SEM results showed that the anaerobic granules for the most part lost their structural integrity during the operation, turning into a shell probably due to the superficial growth of microorganisms.

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Table 1

rDNA oligonucleotide probes used in this study. Specificity and hybridization/washing conditions are shown.

Probe name	Target organisms	Probe sequence (5'-3')	FA (%)/NaCl (mM)	Reference
EUB338	Bacteria	GCTGGCTCCCGTAGGAGT	35/70	Amann et al, 1990
NON338	Negative control	ACTCCTACGGGAGGCAGC	35/70	Wallner et al., 1993
ALF968	α-*Proteobacteria*	GGTAAGGTTCTGCGCGTT	20/215	Neef, 1997
BET42a	β-*Proteobacteria*	GCCTTCCCACTTCGTTT	35/70	Manz et al, 1992
GAM42a	γ-*Proteobacteria*	GCCTTCCCACATCGTTT	35/70	Manz et al, 1992
SRB385	Sulfate-reducing bacteria	CGGCGTCGCTGCGTCAGG	35/70	Amann et al, 1990
DSS658	*Desulfosarcina, Desulfococcus*	TCCACTTCCCTCTCCCAT	60/4	Manz et al, 1998
DSV698	*Desulfovibrio spp.*	GTTCCTCCAGATATCTACGG	35/70	Manz et al, 1998
SYN835	*Syntrophobacter*	GCGGGTACTCATTCCTG	35/70	Harmsem et al, 1996
BAC1080	*Bacteroidetes*	GCACTTAAGCCGACACCT	20/215	Doré et al, 1998
LGC354A	*Firmicutes*	TGGAAGATTCCCTACTGC	20/215	Meier et al, 1999
HGC69A	*Actinobacteria*	TATAGTTACCACCGCGT	25/149	Roller et al, 1994
ARC915	Archaea	GTGCTCCCCCGCCAATTCCT	20/215	Stahl and Amann, 1991
MEB859	*Methanobacteriales* (except *Methanothermaceae*)	GGACTTAACAGCTTCCCT	25/149	Boetius et al, 2000
MC1109	*Methanococcales*	GCAACATAGGGCACGGGTCT	35/70	Raskin et al, 1994
MG1200	*Methanomicrobiales*	CGGATAATTCGGGGCATGCTG	5/630	Raskin et al, 1994
MSSH859	*Methanosarcinales*	CTCACCCATACCTCACTCGGG	35/70	Boetius et al, 2000
MS1414	*Methanosarcinales* (except *Methanosaeta*)	CTCACCCATACCTCACTCGGG	35/70	Raskin et al, 1994
MX825	*Methanosaeta*	TCGCACCGTGGCCGACACCTAGC	20/215	Raskin et al, 1994
TBD1419	*Tb. denitrificans*	ACTTCTGCCAGATTCCAC	50/18	This work
TBD121	*Tb. denitrificans; Tb. thioparus*	CTCGGTACGTTCCGACGC	20/215	This work
TMD131	*Tm. denitrificans*	TCCCAGTCTTTGAGGTAC	35/70	This work

Table 2

Bioreactor performance at steady-state. Nitrate and thiosulfate loadings rate and removal efficiencies of nitrate and thiosulfate as well as net recovery of sulfate

	Reactor 1	Reactor 2
Days of performance	130-186	130-186
HRT (h)	10.0± 0.5	12.2±2.1
pH influent	7.0-7.3	7.0-7.3
pH effluent	6.9-7.3	6.9-7.3
NO_3^- Load (mmol/$L_{reactor}$*d)	86.4±7.8	72.4±12.6
$S_2O_3^{2-}$ Load (mmol/$L_{reactor}$*d)	46.0±4.2	38.5±6.1
NO_3^- Removal (% $NO_{3\ in}^-$)	85.8±4.4	79.0±6.0
$S_2O_3^{2-}$ Removal (% $S_2O_{3\ in}^{2-}$)	99.7±1.2	97.8±3.6
SO_4^{2-}-S as a % of $S_2O_3^{2-}$-S	73.2±9.3	70.0±15.2

622 **Table 3**
623 Number of OTUs and clone percentages distributed in phylogenetic groups.
624

Phylogenetic group	Methanogenic inoculum		Chemolitothrophic sludge	
	OTUs	% clones	OTUs	% clones
Proteobacteria				
α-proteobacteria	-	-	2	39.2
β-proteobacteria	-	-	3	21.7
γ-proteobacteria	-	-	2	21.7
δ-proteobacteria	1	2.3	-	-
Bacteroidetes	3	9.1	-	-
Chloroflexi	3	11.3	1	2.2
Firmicutes	5	25.0	3	13.1
Actinobacteria	4	52.3	1	2.2
Total	16	100	12	100

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627 **Figure captions**

628 Fig. 1. Intensity of the fluorescent signal determined for *Tb. denitrificans* and *Tb thioparus*
629 before (autofluorescence, empty symbols) and after hybridization with the Tb1419 probe
630 (filled symbols) as a function of the formamide concentration.

631

632 Fig. 2. Relative abundance of total bacteria (EUB338), *Alpha* (ALF968), *Beta* (BET42a),
633 *Gamma* (Gam42a) classes of *Proteobacteria*, Gram-positives with low content of G+C,
634 phylum *Firmicutes* (LGC354A), Gram-positives with high content of G+C, phylum
635 *Actinobacteria* (HGC69A), and the sulphur-oxidizing bacteria (SOB) *Tb. denitrificans*
636 (TBD121) and *Tm. denitrificans* (TMD131) present in the methanogenic inoculum (day 0)
637 and in sludge samples obtained from the chemolithotrophic denitrifying reactors after 112,
638 148 and 186 d of operation. Hybridizations were performed using the specific probes and
639 conditions listed in Table 1. The relative abundance is referred to the total number of
640 microorganisms present in each sludge sample as determined by staining with DAPI. The data
641 plotted are the average of the data obtained for both reactors.

642

643 Fig. 3. Relative abundance of total archaea (ARC915) and the methanobacterial orders
644 *Methanobacteriales* (MEB859) and *Methanosarcinales* (MSSH859) present in the
645 methanogenic inoculum (day 0) and in sludge samples obtained from the autotrophic
646 denitrifying bioreactors after 112, 148 and 186 d of operation. Hybridizations were performed
647 using the specific probes and conditions listed in Table 1. Less than 1 cell/field hybridized
648 with the probes specific for *Methanococcales* and *Methanomicrobiales* (abundance<0.2%).
649 The relative abundance is referred to the total number of microorganisms present in each
650 sludge sample as determined by staining with DAPI. The data plotted are the average of the
651 data obtained for both reactors.

652

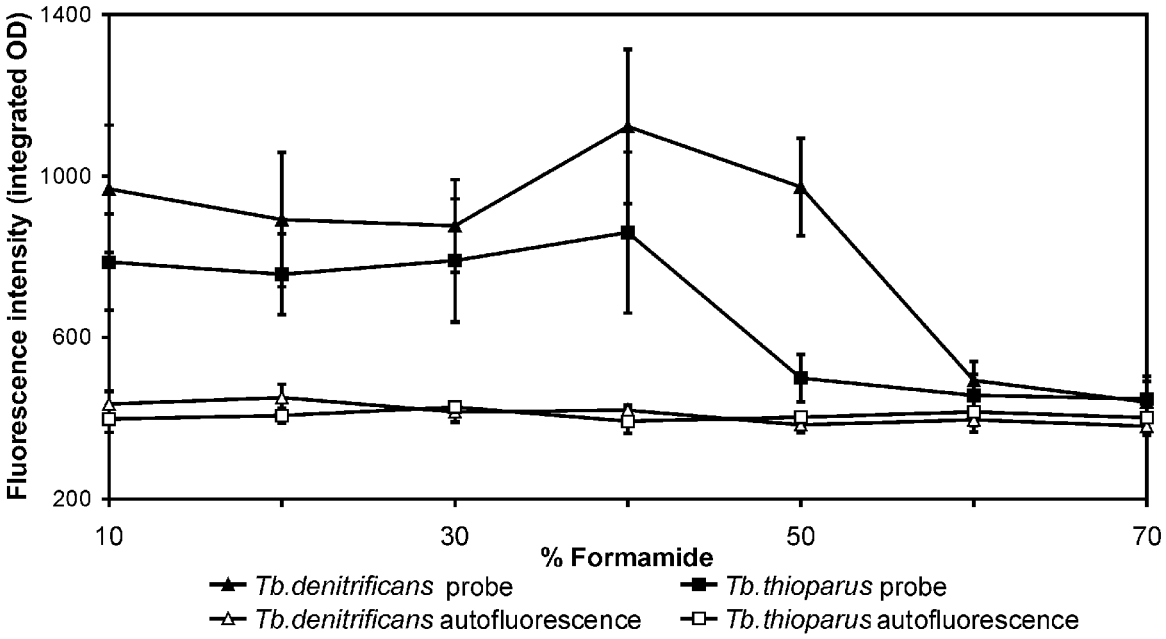
Fig. 4. Phylogenetic tree of the 16S rRNA sequences cloned (corresponding to the nucleotide positions 25 to 1492) for *Proteobacteria*, *Bacteroidetes* and *Chloroflexi phyla*. The percentage that each OTU represents on the total number of clones is indicated. Accession numbers are also shown in parenthesis. The scale bar indicates 0.1 substitutions per site. Eb: clones from the inoculum; R1Cb: clones from the R1 after six months of operation.

Fig. 5. Phylogenetic tree of the 16S rRNA sequences cloned (corresponding to the nucleotide positions 25 to 1492) for *Actinobacteria* and *Firmicutes phyla*. The percentage that each OTU represents on the total number of clones is indicated. Accession numbers are also shown in parenthesis. The scale bar indicates 0.1 substitutions per site. Eb: clones from the inoculum; R1Cb: clones from the R1 after six months of operation.

Fig. 6. Structure of representative granules from the inoculum. All photographs, except A were taken with a SEM. A) View of the granules under magnifying glass. B) Section of a 2x1 mm granule (amplification 50x). C) Surface of the granule (250x). D) Detail of the biodiversity in the inner of a methanogenic granule (6500x).

Fig. 7. Structure of representative granules from the sludge obtained from both reactors after six months of operation. All photographs, except 7a, were taken with a SEM. a) View of the granules under magnifying glass. b) Section of a granule from the R2 and the end of the experiment (130x). Note the smooth and compact surface and the holes and void areas in the inner. c) Detail of the surface and the sheet-like structures of a granule from the reactor R1 (400x). d) Amplification of one of these sheets covered with *Thiobacillus*-like microorganisms (2500x).

679 Figure 1



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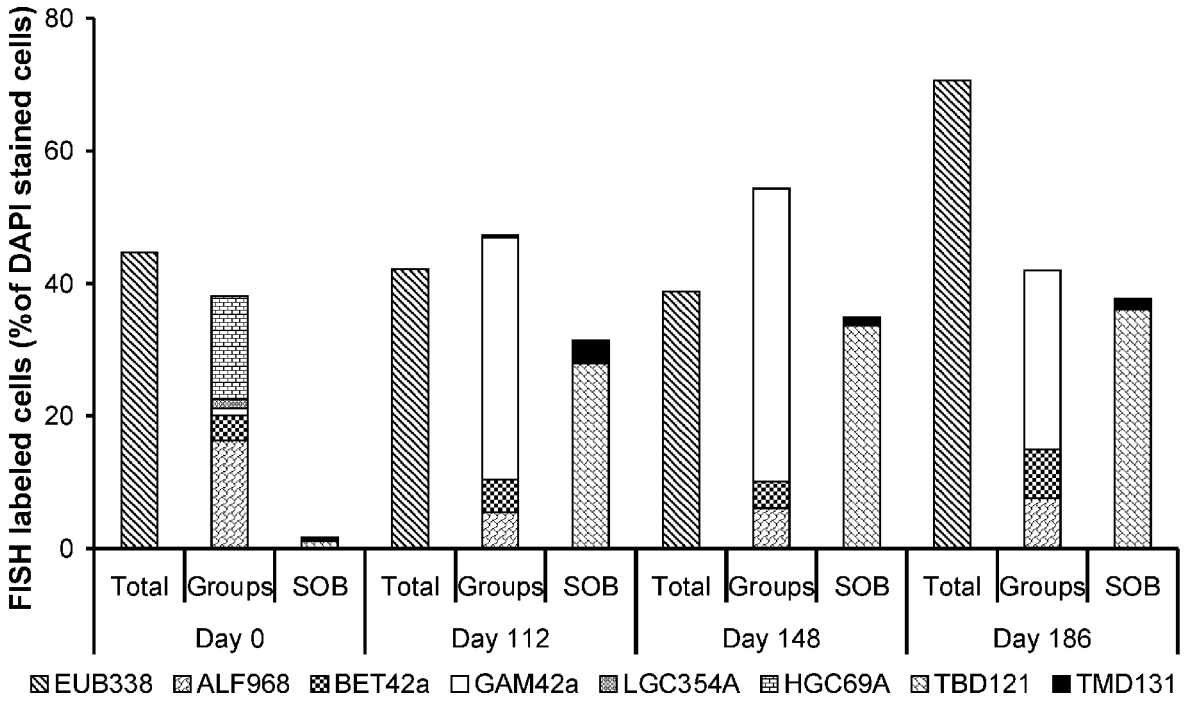
696 Figure 2



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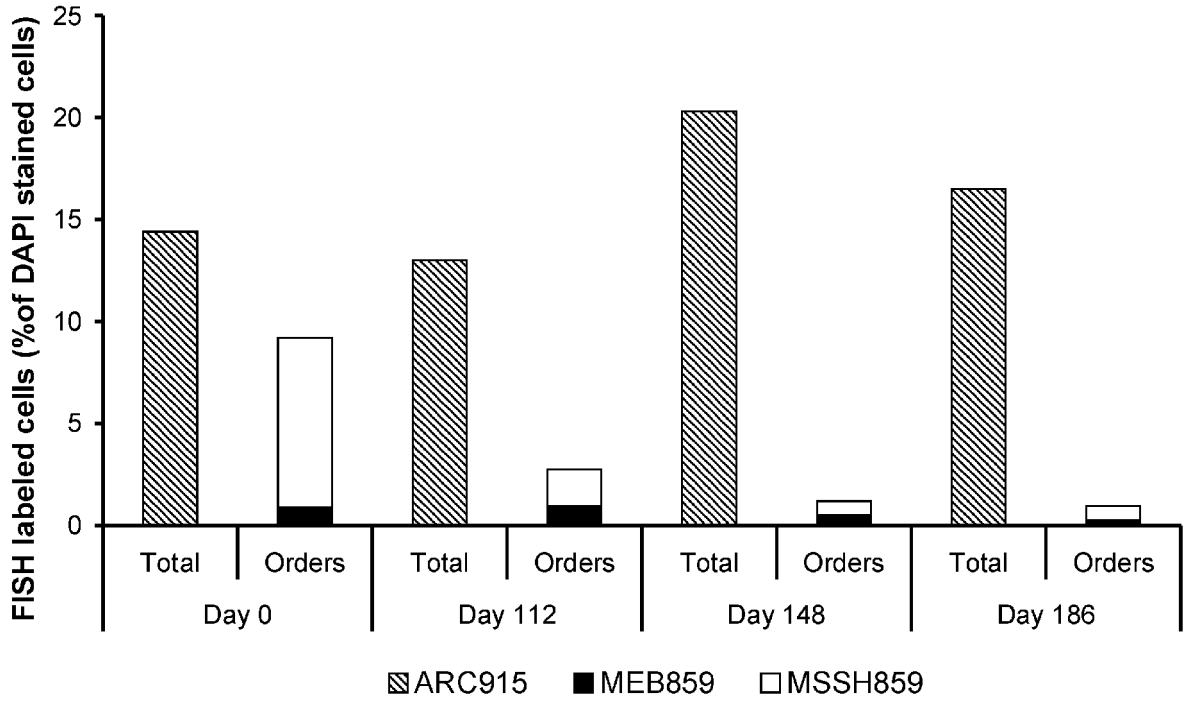
712 Figure 3



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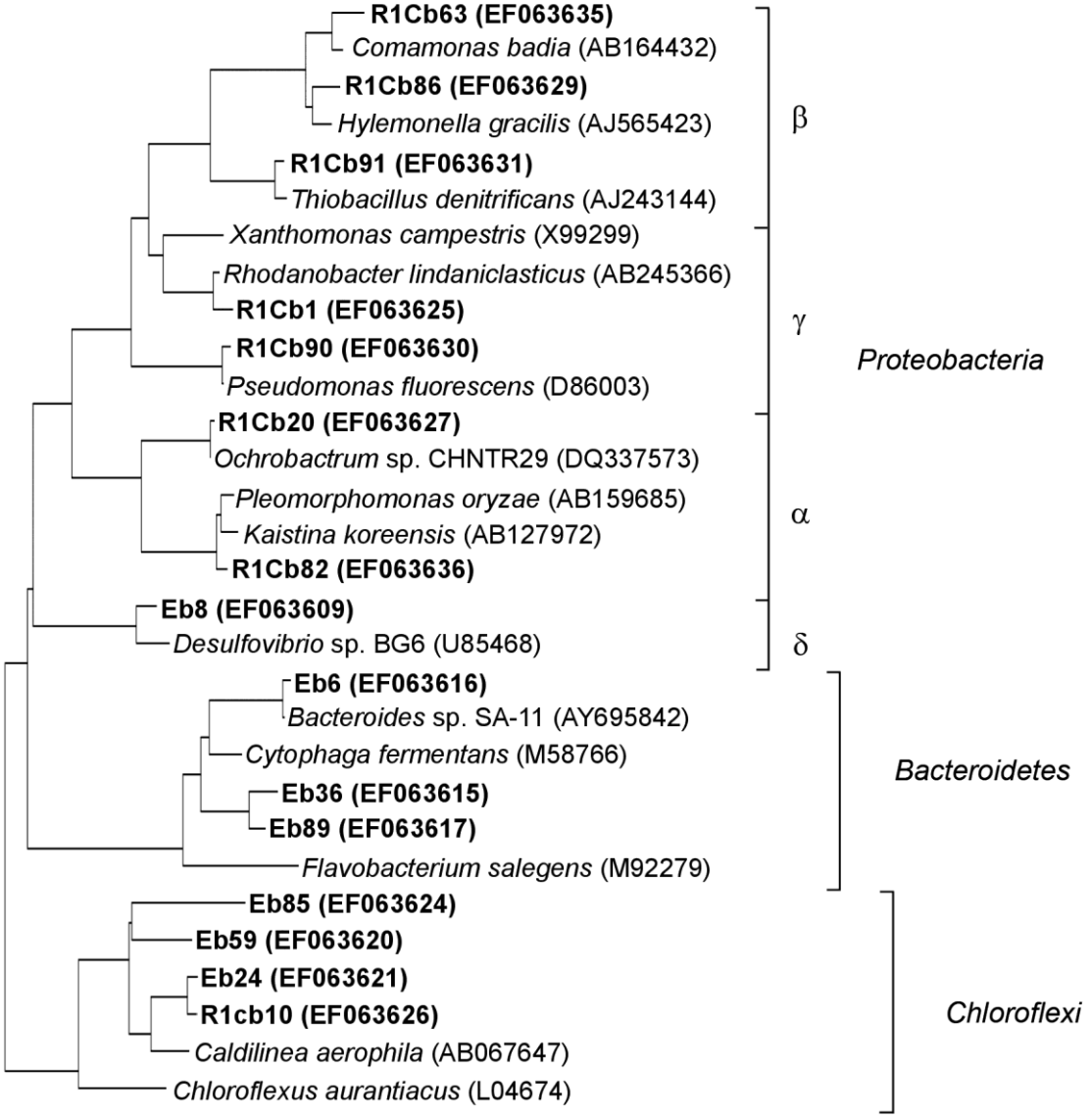
728 Figure 4



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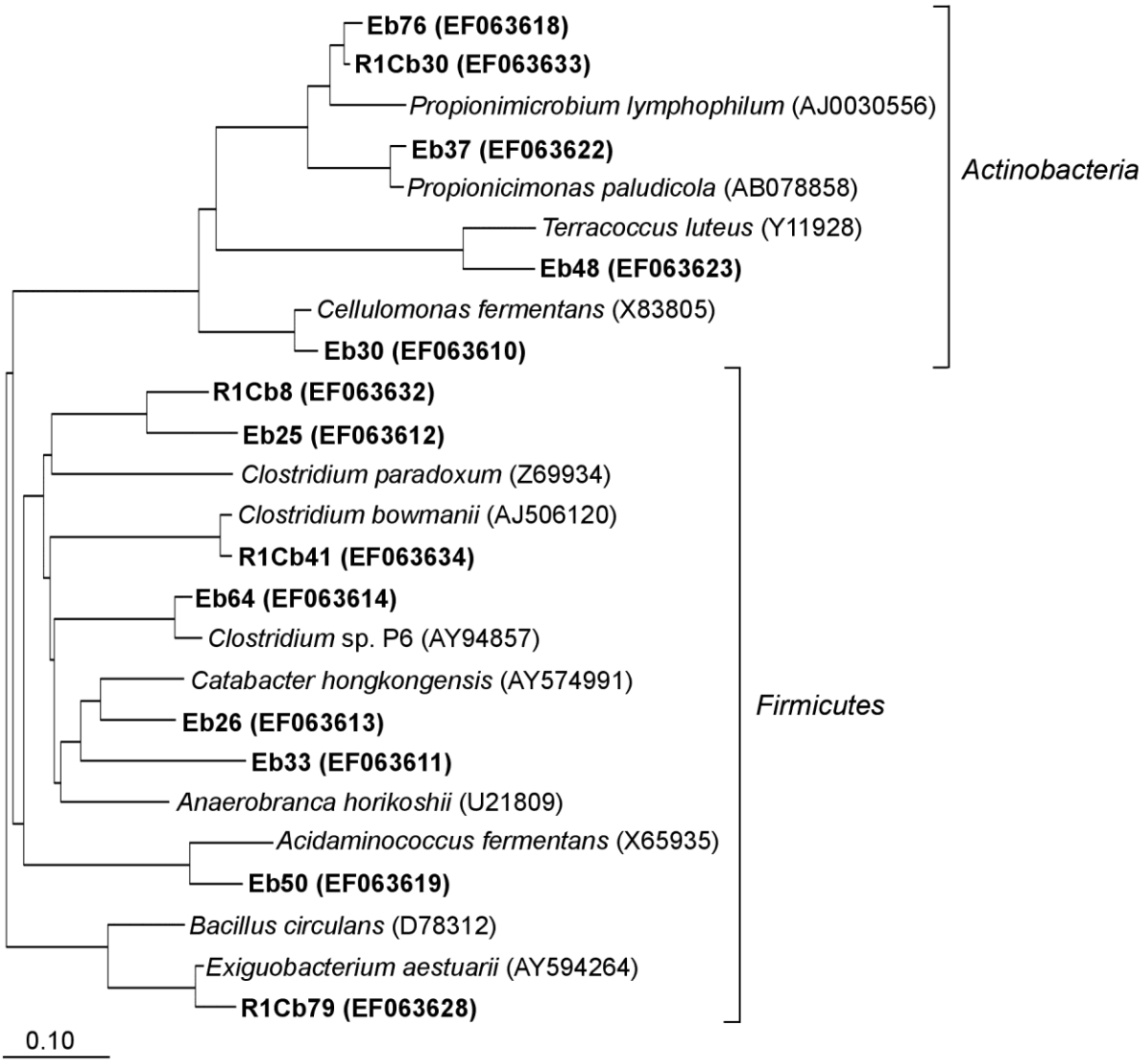
738 Figure 5



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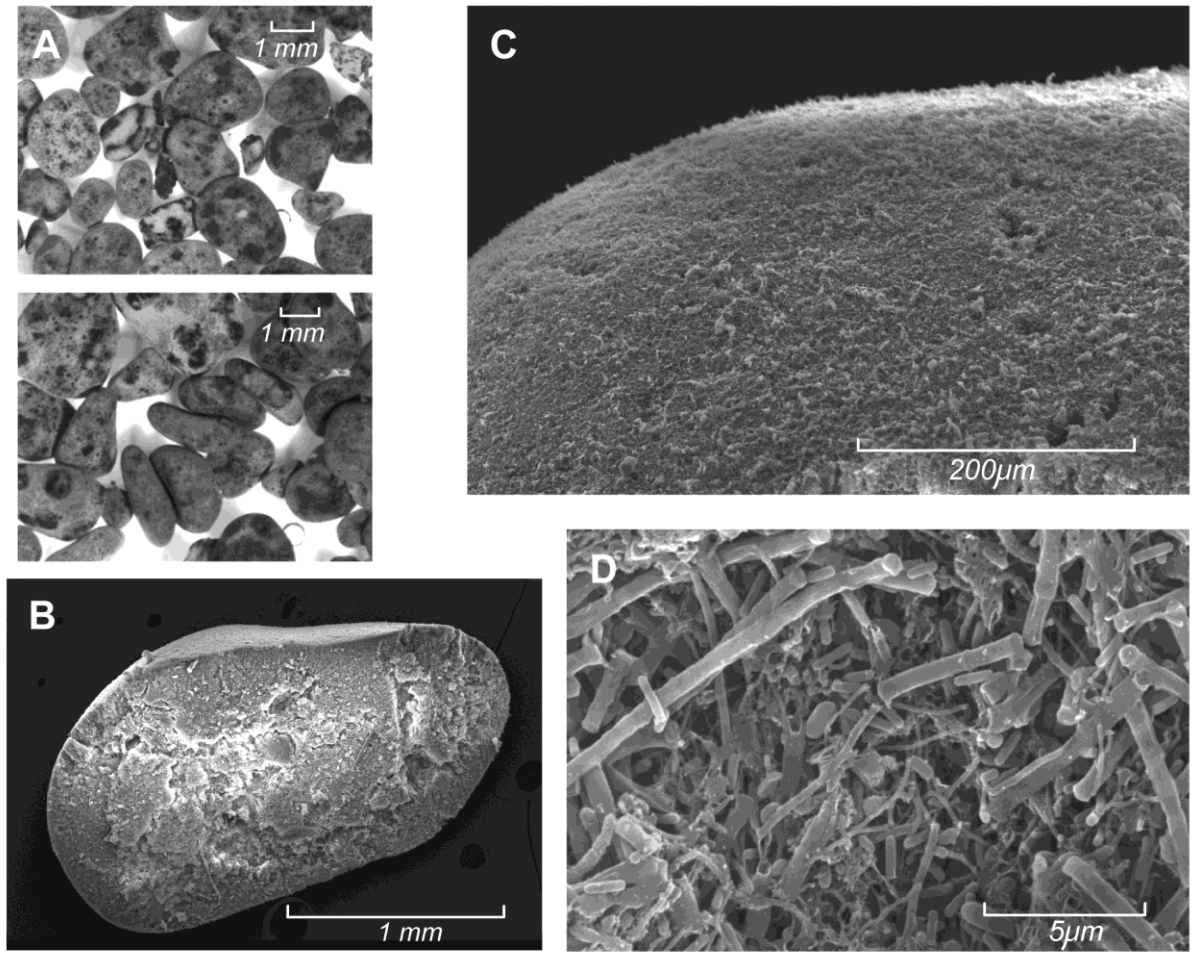
749 Figure 6



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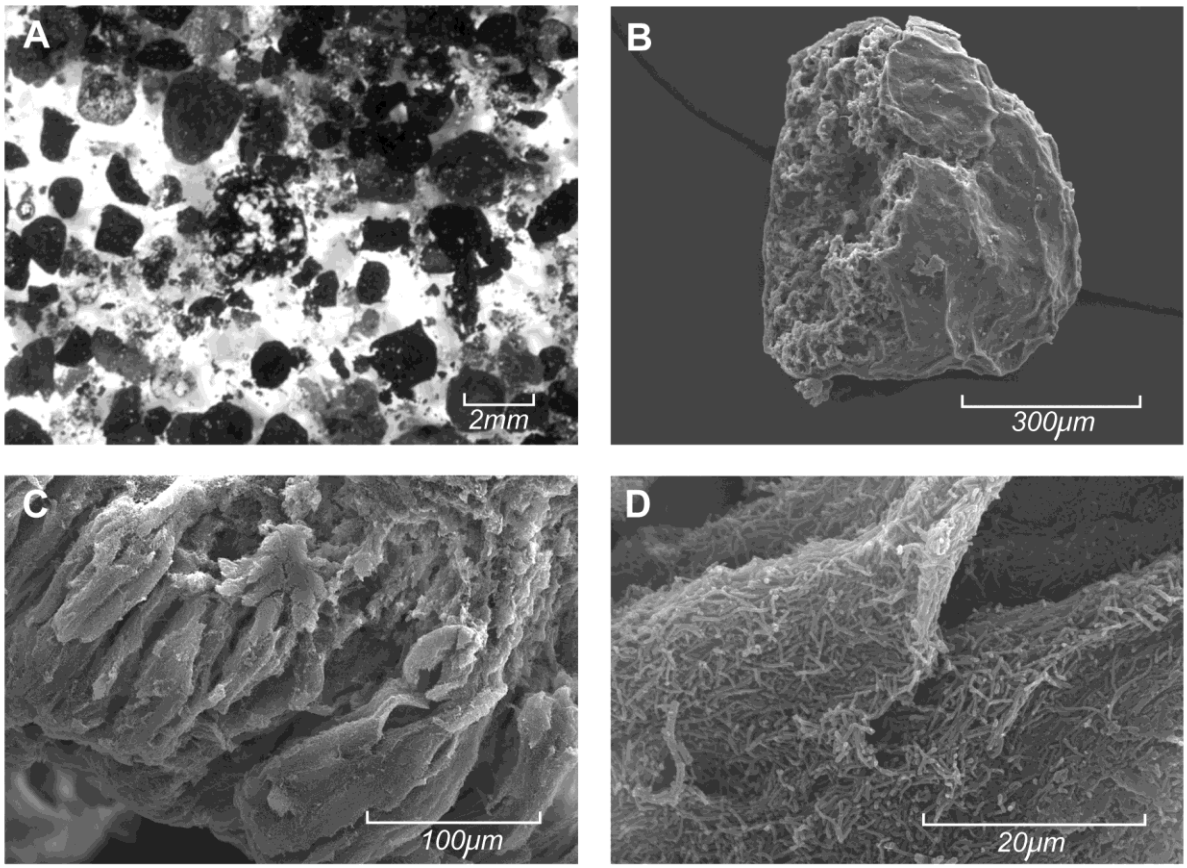
762 Figure 7



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