

Microbiological Community Structure of the Biofilm of a Methanol-Fed, Marine Denitrification System, and Identification of the Methanol-Utilizing Microorganisms

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Abstract

We demonstrated in a previous study that the biofilm of the methanol-fed fluidized marine denitrification reactor at the Montreal Biodome was composed of at least 15 bacterial phylotypes. Among those were 16S ribosomal RNA (rDNA) gene sequences affiliated to *Hyphomicrobium* spp., and *Methylophaga* spp.; the latter made up 70% of a clone library. By using fluorescent *in situ* hybridization (FISH), we investigated the structure of the biofilm during the colonization process in the denitrification reactor by targeting most of the bacterial families that the 16S rDNA gene library suggested would occur in the biofilm. Our results revealed that gamma-Proteobacteria (mostly *Methylophaga* spp.) accounted for up to 79% of the bacterial population, confirming the abundance of *Methylophaga* spp. within the biofilm. alpha-Proteobacteria represented 27–57% of the population, which included *Hyphomicrobium* spp. that appeared after 20 days of colonization and represented 7–8% of the population. We noticed a great abundance and diversity of eukaryotic cells, which made up 20% of the biomass at the beginning of the colonization but decreased to 3–5% in the mature biofilm. We then used FISH combined with microautoradiography (MAR–FISH) to identify the methylotrophs in the biofilm. The results showed that alpha-Proteobacteria used ¹⁴C methanol in the presence of nitrate, suggesting their involvement in denitrification. Despite their abundance, *Methylophaga* spp. did not assimilate methanol under those conditions.

Introduction

The concentration of nitrate rarely exceeds 1 mg/L in natural environments [44]. However, in closed circuit aquaria, nitrate can rapidly reach 50 mg NO_x-N/L [13, 32], a level that can be toxic to invertebrates and fishes. Based on various nitrate toxicity studies on aquatic animals, Camargo *et al.* [4] recommended that 20 mg N/L be the upper acceptable limit of nitrate in marine aquaria. In large marine aquaria such as the Montreal Biodome, maintaining that level requires large and frequent changes in artificial seawater, which is difficult and expensive.

Various methods such as reverse osmosis, resin, or electrochemistry are currently used to remove nitrate from freshwater [35, 39]. Those methods have little use in seawater and are replaced by biological denitrification. Denitrifying bacteria use nitrate as an electron acceptor when oxygen becomes a limiting factor. Various enzymes are used in this respiratory chain that reduces nitrate to nitrogen gas [48].

Few studies on denitrification in saltwater or seawater have been reported. Grgruric *et al.* [14] studied the performance of a commercial, methanol-fed denitrification reactor similar to the one installed at the Montreal Biodome. They presumed that *Pseudomonas* spp. were involved in the process. Catalan-Sakairi *et al.* [5, 6] achieved very high denitrification efficiency by treating artificial wastewater containing 30 g/L of NaCl with methanol and porous cellulose carriers, in which *Hyphomicrobium* spp. were detected. By using cultivation methods and denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)-amplified 16S ribosomal DNA (rDNA) genes, Yoshie *et al.* [45–47] found that *Halomonadaceae* spp. were the dominant bacterial group actively involved in an acetate-fed deni-

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trification process for saline metallurgic wastewater (more than 3.2% NaCl). Tal *et al.* [41] recently developed a denitrification process for closed maricultures using *Pseudomonas* sp. immobilized in a starch–alginate matrix that also served as a carbon source.

The functionality of microorganisms in an environmental sample can be investigated by using such techniques as microautoradiography combined with fluorescent *in situ* hybridization (MAR–FISH) and stable isotope probing (SIP). These techniques are based on assimilation by biologically active microorganisms of a substrate marked with a radioactive or stable isotope [12, 16, 25]. By using MAR–FISH analysis, Ginige *et al.* [12] suggested that members of Methylophilales were the active denitrifying population of a laboratory-scale methanol-fed system. Radajewski *et al.* [34] used SIP to investigate methanol-utilizing organisms in soil and found that *Acidobacterium* and *Beijerinckia* actively took up methanol in acidic soil.

The Montreal Biodome operates a large closed marine aquarium referred to as the St. Lawrence Mesocosm (SLM) [32]. Water salinity and temperature are 28 g/L and 10°C, respectively. A methanol-fed fluidized denitrification reactor was integrated into the SLM life support system in 1998. Methanol is the carbon source most frequently used in denitrification systems because it is inexpensive, easily available, and it produces less biomass than other carbon sources [3, 26]. In earlier investigations, we characterized the microbiota present into the biofilm on the fluidized carriers of the denitrification reactor by cultivable and molecular techniques. Three different isolates were isolated that were affiliated with alpha-Proteobacteria, including two known denitrifying bacteria—*Hyphomicrobium zavarzinii* and *Paracoccus denitrificans*—and a newly described bacterium—*Nitratireductor aquibiodomus* [21, 23]. Using a 16S rDNA gene library, we estimated at 15 the number of different phylotypes present into the biofilm [21]. For instance, sequences related to *Methylophaga* spp. (gamma-Proteobacteria) comprised more than two-thirds of clones found in this library, suggesting that they are in high abundance into the biofilm. However, these methylophags are not known to grow under denitrifying conditions. Bias introduced by DNA extraction, PCR amplification, and sequence cloning could explain the high percentage of clones of these unexpected bacteria.

Our research investigates the link between the phylotypes found in the methanol-fed marine denitrification system of the Montreal Biodome and their functions. In this report, we monitored the colonization of the reactor by using PCR-amplified 16S rDNA gene sequences migrated on PCR-denaturing gradient gel electrophoresis (DGGE), evaluated the abundance of the microorganisms in the biofilm by means of FISH, and determined which of the major biofilm constituents—*Methylophaga* spp., *Hyphomicrobium* spp., and alpha-

Proteobacteria—were methanol-utilizing bacteria in the presence of oxygen, nitrate, or nitrite as electron acceptors by MAR–FISH.

Methods

SLM Denitrification System. The SLM denitrification system operates in a circuit parallel to the main life support system. It comprises a 1-m³ deaeration tank, a 1-m³ denitrification reactor, and an overflow tank. The deaeration tank is a fixed-bed reactor filled with 9 cm NuPac[®] plastic packing. The reactor is a fluidized-bed type that contained 0.3 m³ of Bioflow[®] 9 mm carriers (1.020 density; Rauschert, Steinwiessen, Germany). The deaeration tank and reactor are both methanol-fed. Water temperature in the reactor varies between 16 and 18°C depending on the water flow. Twice a day, 370 mg FeSO₄·7H₂O, 130 mg MnSO₄·4H₂O, and 20 mg CuSO₄·5H₂O are added to the denitrification unit to enhance the denitrification rate [22]. The nitrate removing performance of the system during the colonization experiment varied from 65% to 90%.

Biofilms Sampling. Racks of microscope slides were suspended in the denitrification reactor. The slides were incubated between 7 and 37 days in order to monitor the colonization process and to preserve the biofilm integrity. A portion (1–2 cm²) of biofilm was left on each collected slide for the FISH analysis, and the rest of it was preserved for DNA extraction and DGGE analysis. The biofilm was fixed by soaking the slides for 2 h at 4°C in a freshly prepared 4% paraformaldehyde solution buffered at pH 7.2. The slides were then washed three times with PBS (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, pH 7.2) and kept at –20°C until further use.

DNA Extraction and PCR-DGGE. Each sample of biofilm collected for DNA extraction was mixed with 500 µL TEN (50 mM Tris–HCl pH 8.0; 100 mM EDTA pH 8.0 and 150 mM NaCl), 250 mg glass beads (0.45–0.50 mm), and 500 µL phenol/chloroform/isoamyl alcohol (25:24:1). The biofilm was broken twice for 20 s with FastPrep and then centrifuged at 16,000×g for 10 min. The supernatant was extracted once with chloroform/isoamyl alcohol (24:1). DNA was precipitated by adding 0.25 volume of 10 M ammonium acetate and 2 volumes of 95% ethanol. The resulting pellet was resuspended in 50 µL water.

PCR amplifications of 16 rDNA genes were performed as described by Labbé *et al.* [21] with 25 ng total DNA or 10 ng plasmid DNA corresponding to clones isolated from a 16S rDNA gene library derived from the denitrification reactor and representing *Methylophaga* spp., candidate OP11, and *Hyphomicrobium* spp. Primer

341f with a GC clamp (5'-CGCCCGCCGCGCGCGGC GGGCGGGGCGGGGGCACGGGGGGCCTACGG-GAGGCAGCAG-3') and primer 534r (5'-ATTACCGCG GCTGCTGG-3') were used and resulted in 250-bp DNA fragments. DGGE were done using the D-code system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) with PCR products (approximately 300 ng) electrophoresed onto 8% polyacrylamide gels (acrylamide/*N,N'*-methylene bisacrylamide 37.5:1) containing a linear denaturing gradient (100% denaturing gel contained 7 M urea and 40% deionized formamide). Electrophoresis was carried out using a TAE running buffer (40 mM Tris-HCl, 40 mM acetic acid, 1 mM EDTA, pH 8.0) for 16 h at 100 V and 60°C. The gels were stained with ethidium bromide (10 µg/mL).

FISH. The biofilm samples collected on slides for FISH analysis were permeabilized with 70% ethanol for 48 h at 4°C. They were then dehydrated with 95% ethanol. Before FISH, samples were acetylated to limit nonspecific hybridization: 12.5 µL acetic anhydride was added to 5 mL triethanolamine buffer and drops were left over the samples for 15 min before being washed out with water [24]. The fluorescent probes used are listed in Table 1. A probe concentration of 30 ng/µL was used for thick biofilm samples [43], whereas a probe concentration of 10 ng/µL was used for thin or dispersed biofilm samples (MAR-FISH). Samples were incubated with the appropriate hybridization buffer (Table 1) in small sealed chambers for 3 h at 46°C, then for 2 min at 48°C. Samples were then washed for 20 min with a

washing buffer having the appropriate NaCl concentration (Table 1). FISH samples were mounted with Vectashield™ antifading agent (Vector Laboratories, Burlingame, CA, USA) whereas MAR-FISH samples were mounted with Prolong™ Gold agent (Molecular Probes, Eugene, OR, USA). The antifading agent Vectashield™ remained aqueous throughout the microscopic observation period and maintained the hydration of the biofilm. It was very useful when unknown or uncultured organisms had to be observed. It also preserved biofilm integrity and cells size. The use of the antifading agent Prolong™ gold dried before the start of the microscopic observations, which allowed longer preservation of the slide samples and provided a better superposition of the MAR and FISH signals. A hybridization control with probe NON338 did not produce any nonspecific signal. An autofluorescent control resulted in a few autofluorescent signals in the green and red channels. Each FISH experiment was done with 10–15 samples.

Microscopy. Samples were examined under a confocal microscope Nikon E600 equipped with Bio-Rad laser Kr-Ar (line 488 and 568 nm) and a red diode (line 637 nm). Filter sets HQ515/30, HQ600/40, and HQ660LP were used for FITC, CY3, and CY5, respectively. Images were acquired by averaging eight Kalman frames taken with a 60× Plan Apo oil immersion objective.

Image Analysis. Hybridization signals were quantified with the MetaMorph software version 4.5r0 (Universal Imaging Corporation). Picture thresholds

Table 1. List of probes used in this study

Probe name	Usual name	Sequence	Fluorochrome	Target	% Formamide/NaCl concentration (mM)	References
S-Sc-aProt-0019-a-A-17	Alf1B	5'-CGTTCGYT CTGAGCCAG-3'	FITC	Alpha, some delta, and spirochetes	20/210	[29]
S*-Bact(D,V)-0338-a-A-18	Eub338-mix	5'-GCWGCCWC CCGTAGGWGT-3'	CY5	Almost all bacteria	0-35/900-70	[1, 8]
	Euk516	5'-ACCAGACT TGCCCTCC-3'	FITC	Most eukarya	35/70	[1]
L-C-gProt-1027-a-A-17	Gamma42a	5'-GCCTTCCC ACATCGTTT-3'	CY3	Gamma- Proteobacteria	35/70	[29]
	MPH-730	5'-CAGTAATGGCCC AGTGAGTCGCC-3'	CY3	Genus <i>Methylophaga</i> spp. except <i>natronica</i>	20 ^a /210	[19]
S*-OTU14-0129-a-A-20		5'-TCCGTACCG ATAGGAAGATT-3'	CY3	<i>Hyphomicrobium</i> spp.	20/210	[20]
L-C-bProt-1027-a-A-17	Bet42a	5'-GCCTTCCC ACTTCGTTT-3'	FITC	Beta-Proteobacteria	35/70	[29]
	NON338	5'-ACTCCTACGG GAGGCAGC-3'	CY3	Negative control	0–20	[1]
	CF319a	5'-TGGTCCGT GTCTCAGTAC-3'	CY3	<i>Cytophaga-Flexibacter</i> groups	35/70	[29]
	AMX368	5'-CCTTTCGGG CATTGCGAA-3'	FITC	Anammox bacteria	15/318	[36]

^aThe reported formamide concentration was 0%.

were manually adjusted to between 45- and 60-pixel intensity. Pictures were then transformed into binary images. A single cycle of dilate (3×3 pixels) and erode (3×3 pixels) filter was applied. Single pixels were then removed and pixel areas measured. Except for eukaryotes, the proportion of a given microbial group (e.g., *Methylophaga* spp.) was estimated by comparing the total pixel area produced by a specific probe with the total pixel area produced by probe Eub mix. The proportion of eukaryotes was estimated by comparing the total pixel area produced by probe Euk516 with the total area produced by both Euk516 and Eub mix. For each probe, at least 20 binary images from a representative sample were analyzed [24].

MAR-FISH. MAR biofilms were homogenized and suspended in artificial seawater at pH 8 (per liter: 27.5 g NaCl, 5 g MgCl₂, 2 g MgSO₄, 1 g KCl, 0.5 g CaCl₂, 1 mg FeSO₄, 256 mg KH₂PO₄, 170 mg Na₂HPO₄, plus trace metals). Incubations were done in 80-mL serum vials with radioactive 0.02% methanol v/v (50 mCi/mmol; American Radiochemical, St. Louis, MO, USA). An equivalent of 1.8 mg dry weight suspended solids (± 0.6 mL) and 3 mL artificial seawater were added to each vial. Four assays were done with or without a CO₂ trap, each in triplicate, with various electron acceptors: with oxygen (oxic assays), nitrate (NaNO₃), or nitrite (NaNO₂) (anoxic assays), or without oxygen/nitrate/nitrite (anaerobic assays). Two controls were also done once: one with live biomass, oxygen, and 0.02% ¹²C methanol; the other with oxygen, ¹⁴C methanol, and biomass fixed with paraformaldehyde. The anoxic assays were done by adding 200 mg N/L in each vial (C/N ratio of 1.5). A trace of nitrate (0.4 mg N-NO₃/L) was added to each anaerobic vial as a nitrogen source. CO₂ traps (1 mL KOH 1 M) were used to prevent bacteria from using ¹⁴CO₂ produced by mineralization. Nitrogen gas was injected in anoxic and anaerobic vials to flush oxygen and these vials were preincubated for 1 h before adding the ¹⁴C methanol to allow microorganisms to remove residual oxygen. At $t = 0$ h, 10 μ Ci of ¹⁴C methanol (0.02% methanol v/v) was added to each vial. All vials were incubated for 17 h at room temperature. Revelation of silver grain of autoradiographic emulsion LM1 (Amersham) was achieved with Kodak D19, as described by Lee *et al.* [25], and then fixed with 30% thiosulfate. The exposure time varied from 7 to 21 days depending on the electron acceptor source. Corresponding FISH analyses were also done in triplicate.

Results

Monitoring Biofilm Colonization. Microscope slides were incubated in the denitrification reactor to allow the development of the denitrifying biofilm. To monitor

the dynamics of biofilm colonization on the slides, total DNA was extracted from the biofilm, followed by PCR amplification of 16S rDNA sequences and resolution of the PCR products by DGGE. From day 7 to day 37, the DGGE migrating profiles revealed at least 18 different bands (Fig. 1). A maximum diversity of 22 bands was observed on day 22 (Fig. 1). Most of the bands were found throughout the colonization period, except for the pair corresponding to *Hyphomicrobium* spp. (Fig. 1, band e), which were detected once only on day 22. Bands corresponding to *Methylophaga* spp. (Fig. 1, bands a and b) were intense throughout the colonization process. The colonization profile was stable from day 30. A previous study [21] showed a similar DGGE profile even though plastic carriers or microscope slides were used for the microbial colonization.

Oxygen concentration in the Biodome denitrification reactor was recorded at 0.5 ppm. To determine if the

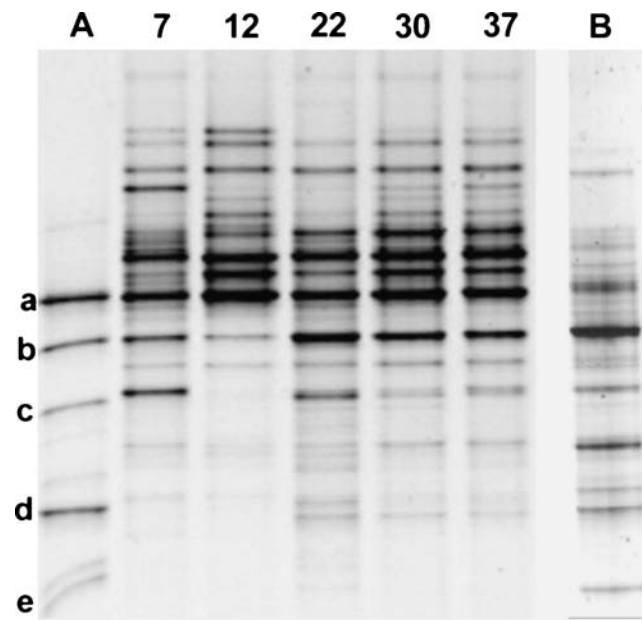


Figure 1. Monitoring of biofilm colonization by PCR-DGGE. Microscope slides were incubated for 37 days in the Biodome's denitrification reactor, and total DNA was extracted from the slides on days 7, 12, 22, 30, and 37. Lane B shows the DGGE profile of a biofilm from denitrification carriers incubated for 5 days in completely anoxic media (batch culture). The V3 region of the 16S rDNA genes was PCR-amplified. PCR products were segregated by DGGE with a 25 to 70% denaturant gradient. Lane A was made of plasmidic clones representing *Methylophaga* spp. (bands a and b), *E. coli* (band c), candidate OP11 (band d), and *Hyphomicrobium* spp. (band e) derived from a 16S rDNA gene library of the denitrifying biofilm [21] (except *E. coli*) that were PCR-amplified in the same 16S region. Lane B was generated from another DGGE with the same markers as lane A, and bands were aligned based on this marker and the migration pattern on the other lanes.

absence of oxygen (refer to MAR–FISH experiments described below) would affect bacterial diversity, we batch-cultured several carriers with the denitrifying biofilm under strict denitrifying conditions (no oxygen detected) for 5 days, as described by Labbé *et al.* [21]. The PCR-DGGE migration profile of that biomass was very similar to the biomass on the microscope slides (Fig. 1G), although the band representing the *Hyphomicrobium* spp. was more prominent.

Biofilm Structure. The biofilm structure was monitored during the colonization of the microscope slides. Observation by phase contrast microscopy or by fluorescent microscopy with DAPI coloration or with fluorescent probes (Fig. 2) revealed no apparent classic biofilm structure (channels, associations, regularities) although the integrity of the biofilm was well preserved during sampling and processing. The biofilm surface was randomly uneven, with bumps and depressions as well as thick and thin areas. The constant erosion/rejuvenation/remodeling of the biofilm surface by the fluidized bed and protozoan grazing probably prevented the biofilm from being macrostructurally organized.

Colonization of different bacterial families was monitored by means of FISH with representative probes selected according to the sequences obtained from the 16S rDNA gene library [21] (Table 1). No *Archaea* or anammox bacteria were detected in the biofilm with probes Arc915 and AMX368. beta-Proteobacteria and Cytophagales were detected with probes Beta42a and CF319a but at very low levels. The most abundant bacterial populations detected during the colonization process were affiliated to the gamma (Gamma42a) and alpha (Alf1B) subclasses of Proteobacteria, with percentages varying from 39% to 79% and 27% to 54%, respectively (Table 2). *Methylophaga* spp. (MPH-730) made up the vast majority of the gamma-Proteobacteria, representing 36–79% of the total bacterial population. *Hyphomicrobium* spp. (S*-OTU14-0129-a-A-20), which are affiliated to alpha-Proteobacteria, were detected in the biofilm only after day 22 and represented 7–8% of the bacterial population. Bacteria detected with probes Gamma42a and MPH730 were mostly rods and filaments, whereas those detected with probe Alf1B were mostly rods and large cocci (up to 5 µm). Microcolonies of *Methylophaga* spp. and *Hyphomicrobium* spp. were dispersed throughout the biofilm (Fig. 2).

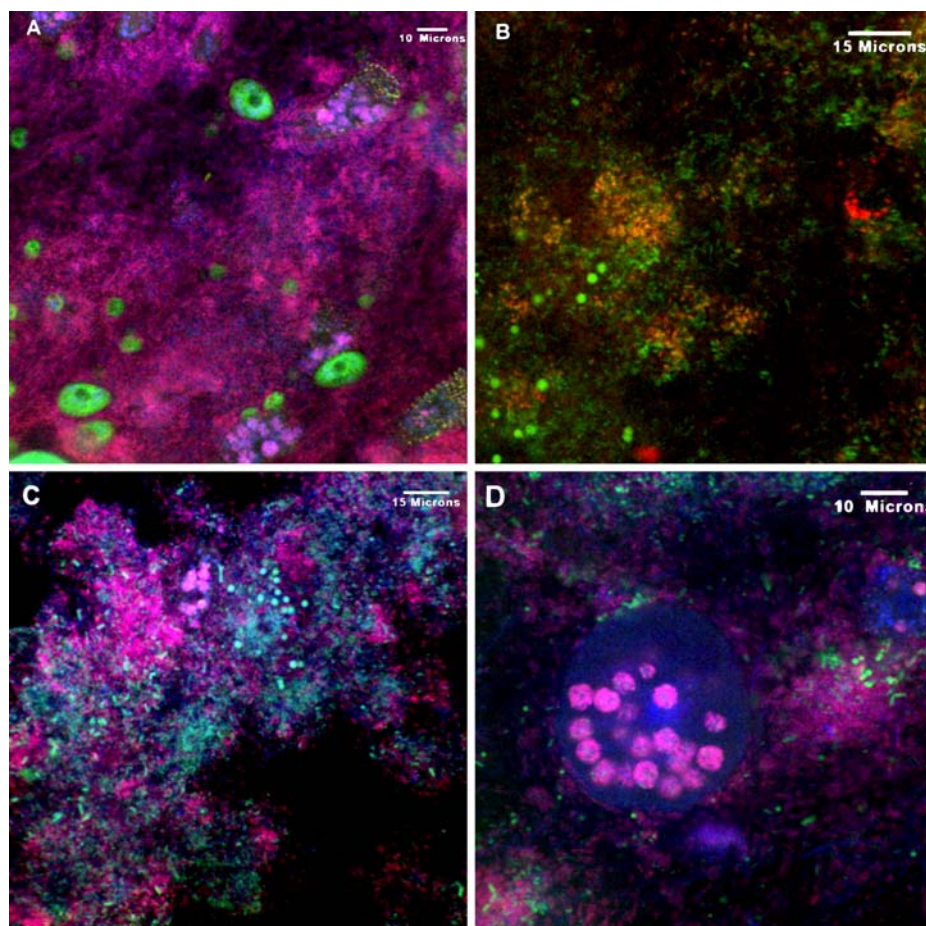


Figure 2. FISH images of a 30-day-old biofilm. (A) Hybridization with probes Euk516-FITC (green), Gam42a-CY3 (red), and Eub-mix-CY5 (blue). Magenta signals resulted from the merging of signals from Gamma42a and Eub-mix. (B) Hybridization with probes Alf1B-FITC (green) and S*-OTU14-0129-a-A-20-CY3 (*Hyphomicrobium*) (red). (C) Hybridization with probes Alf1B-FITC (green), MPH-730-CY3 (red), and Eub-mix-CY5 (blue). Cyan and magenta signals resulted from the merging of Eub-mix-CY5 with the Alf1B-FITC or MPH-730-CY3 probes. (D) Hybridization with probes Alf1B-FITC (green), MPH-730-CY3 (red), and Eub-mix-CY5 (blue). Magenta signals resulted from the merging of signals from MPH-730 and Eub-mix.

Table 2. Quantification of FISH signals expressed as a percent of Eub338 mix, except for Euk516, which is expressed as a percent of Eub338 mix plus Euk516 (mean \pm SE, $n \geq 20$ binary images)

Colonization (days)	Probes				
	<i>Gamma42a</i>	<i>MPH-730</i>	<i>Alf1B</i>	<i>S⁻-OTU14-0129-a-A-20 (Hyphomicrobium)</i>	<i>Euk516</i>
7	62 \pm 19	50 \pm 15	54 \pm 16	– ^a	20 \pm 10
12	79 \pm 13	79 \pm 12	29 \pm 9	–	11 \pm 4
22	39 \pm 11	36 \pm 9	44 \pm 14	7 \pm 3	10 \pm 5
30	53 \pm 13	51 \pm 14	44 \pm 11	8 \pm 4	3 \pm 2
37	67 \pm 13	57 \pm 14	27 \pm 9	7 \pm 5	5 \pm 2

^aNo hybridization signal.

FISH analysis also revealed a great abundance of eukaryotes, mostly on the biofilm surface. They represented up to 20% of the total pixel area of binary images at the beginning of the colonization process, but decreased in relative abundance as the bacterial biofilm grew (Table 2). Microscope examination of slides freshly collected from the denitrification reactor showed that most eukaryotes were protists such as Choanoflagellates, *Vorticella*, *Uronema*, *Cyclidium*, and *Bodo*. FISH signals were observed with probes MPH-730 and Gamma42a

inside a *Vorticella*-like cell, suggesting ingestion of *Methylophaga* spp. and gamma-Proteobacteria by the protists (Fig. 2D).

Biofilm Functionality. We fed the biofilm with ¹⁴C methanol and tried to identify the active methylotroph microorganisms by means of MAR-FISH. The biomass from the marine denitrification system was incubated for 17 h in the presence of one of the following electron acceptors: O₂, NO₂, and NO₃, or with none of these (a-

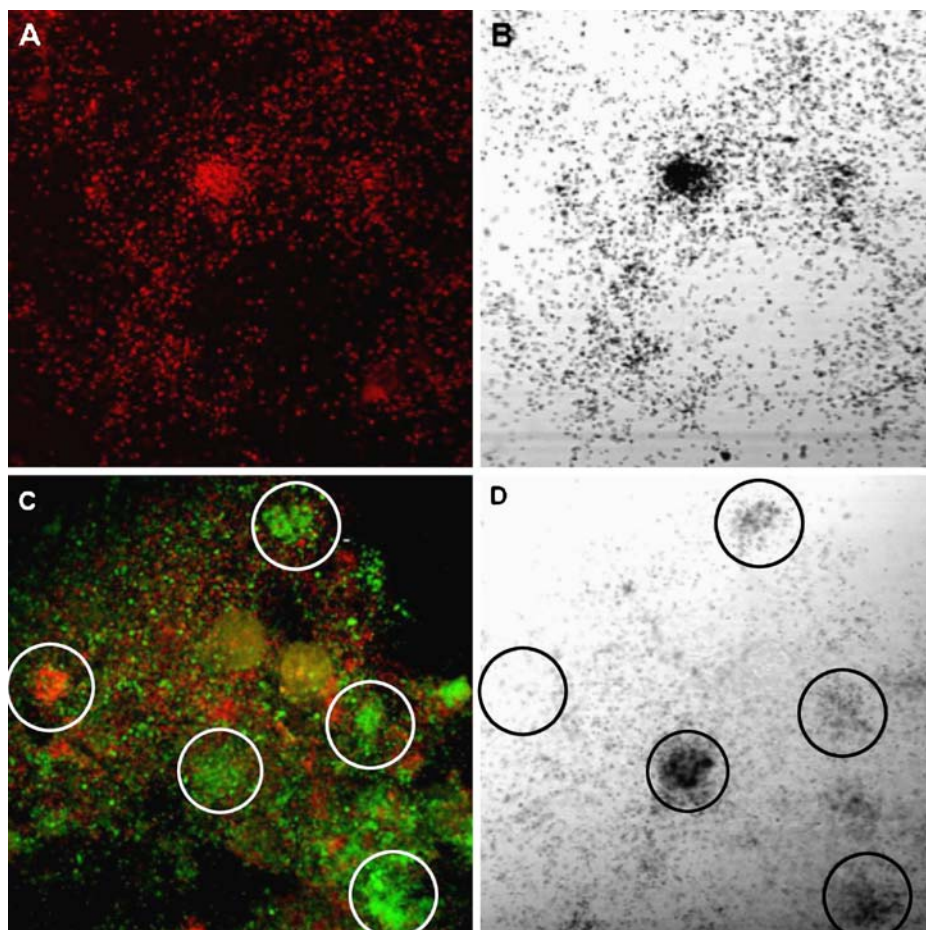


Figure 3. MAR-FISH images of a 30-day-old biofilm. The biofilm was incubated for 17 h in oxic conditions (O₂) (A and B) or in anoxic conditions with nitrate (C and D). (A) Hybridization with probe Gamma42a-CY3; (B) MAR image of the same field; (C) hybridization with probes Alf1b-FITC (green) and MPH-730-CY3 (red); (D) MAR image of the same field.

naerobic conditions). Half of the assays were performed with a CO₂ trap to limit the bias introduced by the ¹⁴C₂ assimilation after substrate mineralization. For each condition, gamma-Proteobacteria, *Methylophaga* spp., alpha-Proteobacteria, *Hyphomicrobium* spp., and Eukarya were monitored because they represent most of the microbiota in the denitrification reactor, as described earlier.

Under oxic conditions (O₂), most *Methylophaga* cells revealed by the MPH-730 or Gamma42a probes produced MAR signals (silver grains) (Fig. 3A–B). alpha-Proteobacteria also produced positive MAR signals. However, few MAR signals corresponding to *Hyphomicrobium* cells were detected. Under anoxic conditions with nitrite and under anaerobic conditions, no FISH signals and very few MAR signals were observed, suggesting that the biomass was not active in taking up methanol under those conditions.

Under anoxic conditions with nitrate, MAR signals corresponded to the bacteria cells that hybridized with the probe Alf1B (alpha-Proteobacteria) (Fig. 3C–D). Weak positive FISH signals showed the presence of *Hyphomicrobium*, but a clear correspondence with the MAR signals was not possible. When superimposed, very few or none of the MAR signals matched the FISH signals of probe MPH-730 (Fig. 3C–D), suggesting that *Methylophaga* spp. did not take up methanol under anoxic conditions with nitrate.

No MAR signals were observed with both controls (¹²C and killed biomass). In all assays, no MAR signals corresponded to Eukarya hybridized with probe Euk516. In the anoxic assays, the use of a CO₂ trap did not produce significantly different results than those without a trap. However, in the oxic assays, the CO₂ trap significantly reduced the incorporation of ¹⁴C into the biomass. Incorporation of ¹⁴C methanol was 12% with the CO₂ trap and 30% without the trap. The intensity of the MAR signals was slightly weaker with the CO₂ trap than without, but there was no difference in the bacteria species that produced signals.

Discussion

In this study, we showed that the bacterial population on the microscope slides and the dynamics of the colonization process were similar to those in the carriers of the methanol-fed fluidized denitrification reactor at the Montreal Biodome. The late colonization of the biofilm by *Hyphomicrobium* spp. is not surprising because they are slow-growing bacteria with an optimal division time close to 10 h [17]. The presence of fast-growing bacteria did not prevent *Hyphomicrobium* spp. from colonizing the denitrification reactor.

The FISH analyses revealed that gamma-Proteobacteria were abundant in the reactor and most of them were affiliated to *Methylophaga* spp. This finding confirmed our previous study that showed that two thirds of

the 16S rDNA gene library clones were affiliated to *Methylophaga* spp. This finding also agreed with the results of the PCR-DGGE analyses where strong bands corresponding to *Methylophaga* spp. were present throughout the biofilm colonization process. The presence of *Methylophaga* spp. in the denitrification reactor was puzzling because they are considered strictly aerobic [10, 18]. The usual explanation is the continuous presence of residual dissolved oxygen (±0.5 ppm) during the colonization period. However, this explanation is not sufficient for two reasons. First, *Methylophaga* spp. were still present when the carriers were batch-cultured under strict anoxic conditions with nitrate and no oxygen for 5–7 days (Fig. 1B). Second, residual dissolved oxygen could not diffuse deep enough into the biofilm to reach the *Methylophaga* populations where they were found. Assuming a zero-order reaction rate for the oxygen consumption and knowing that the carbonaceous substrate (methanol) is not limiting, this maximum diffusion depth (beta) can be estimated by the following equation [15]:

$$\beta = [(2DS)/k]^{1/2} \quad (1)$$

where D is the diffusion coefficient of the target substrate (here, the oxygen), S is its concentration at the biofilm surface, and k is the first-order consumption rate in the biofilm. In the case of the Biodome's denitrification reactor, using mean values generally accepted for biofilters ($D_{O_2} = 1.7 \times 10^{-4}$ m²/day, $k_{O_2} = 100,000$ g/m³ J), for $S_{O_2} = 0.5$ mg/L, we calculated that oxygen would not penetrate more than 40 μm deep into the biofilm. Denitrification is known to be inhibited when there is more than 0.2 mg/L of dissolved oxygen. Using the same model, we can estimate that these conditions are met only in the upper 20 μm of the biofilm. On the other hand, during the same period nitrate concentration (S_{NO_3}) in the reactor was 50 mg/L. Filling in Eq. (1) with values for nitrate ($D_{NO_3} = 0.7 \times 10^{-4}$ m²/day, $k_{NO_3} = 30,000$ g/m³ J), we calculated a penetration depth of 500 μm, which correspond to the biofilm thickness as it was observed on microscope slides. The abundance of *Methylophaga* spp. in the deeper parts of the biofilm therefore suggests that those species can survive without oxygen. What is known about *Methylophaga* spp. is based on a limited number of studies performed on a few strains. The genus is widely distributed and is found in diverse marine environments. They were described as strict aerobes by Doronina *et al.* [10, 11], Janvier *et al.* [18], and de Zwart *et al.* [9]. In natural habitats, *Methylophaga* spp. are associated with benthic and planctonic algae. Algae can produce methanol through demethylation of organic compounds [18].

Sequences of proven *Methylophaga* species in GenBank showed similarity ranging from 94% to 98%, which suggests high intrageneric diversity. Further characterization of the genus *Methylophaga* is definitely needed.

Alpha-Proteobacteria were also found in high percentages during the colonization of the slides. Potential denitrifiers affiliated to that class of bacteria were previously isolated from the same reactor and identified as *Paracoccus* sp., *N. aquibiodomus*, and *Hyphomicrobium* sp. [21, 23]. After 22 days of incubation, *Hyphomicrobium* spp. made up 7–8% of the total bacteria. Those slow-growing species have been found in almost all methanol-fed denitrification systems at ratios varying from 1% to 35% of total bacteria [12, 20, 26, 31]. They are also found in marine environments [30]. It was therefore not surprising to find *Hyphomicrobium* spp. in such abundance in the Biodome's denitrification reactor. *Paracoccus* sp. and *N. aquibiodomus* were not examined by FISH because they were expected to be present in less than 1% into the biofilm. Labbé *et al.* [21] showed that these two bacteria were respectively 200 and 25 times less concentrated than *Hyphomicrobium* sp. Finally, numerous alpha-Proteobacteria are known to be denitrifiers; among them some are able to use methanol such as Rhizobiaceae and Rhodobacteriales [27, 38].

Although some *Archaea* are known denitrifiers [33], we did not detect any of them in the Biodome's denitrification reactor. No *Archaea* or anammox bacteria have ever been found in a denitrification system. Tal *et al.* [42] found anammox bacteria in a marine denitrification reactor after enrichment under anammox conditions. These suggest that *Archaea* and anammox bacteria probably play a minor role in methanol-fed marine denitrification systems or cannot be targeted yet.

Microscope observation of freshly mounted slides revealed a high diversity of Eukarya, mainly protists. More than 30 types of protists were estimated to coexist with the biofilm bacteria in the Biodome's denitrification system (Villemur, unpublished data). Our results showed that Eukarya represented up to 20% of the biomass and were most abundant during the early days of the colonization process. The quantification of protists determined here by FISH analysis was probably underestimated because unattached cells were flushed during the FISH washing steps. The protists found on the FISH slides were mostly stalked and were members of the *Vorticella*-like and Choanoflagellate-like groups. Interestingly, we were able to record a strong FISH signal with probes MPH-730 and Gamma42a from the digestive vacuoles of those protists.

MAR–FISH experiments under anoxic conditions with nitrate showed positive MAR signals for the Alf1B probe, suggesting that alpha-Proteobacteria were active in assimilating methanol during denitrification. Howev-

er, very few, if any, *Hyphomicrobium* spp. were detected with MAR signals. The low proportion of *Hyphomicrobium* in the biofilm (7–8%) might make detection difficult. Although they made up 35% of the bacterial population in a sequential batch reactor (SBR) denitrification system, Ginige *et al.* [12] observed that *Hyphomicrobium* spp. did not appear positive for assimilation of methanol as determined both by SIP and MAR. On the other hand, *Hyphomicrobium* spp. are known to be effective denitrifiers with methanol as the carbon source [7, 40]. The absence of a positive response from *Hyphomicrobium* spp. under anoxic conditions is probably linked to their slow growth rate that makes the use of SIP and MAR techniques problematic.

Most *Methylophaga* cells generated strong MAR signals in the presence of O₂, which is not surprising because those bacteria are well known as aerobic methylophages and make limited use of other carbon and energy sources. Alpha-Proteobacteria and *Hyphomicrobium* spp. produced positive MAR signals under oxic conditions. Those bacterial groups are known to assimilate methanol from the serine pathway. *Hyphomicrobium* spp. is also known to use a limited range of carbon sources, mostly C1 compounds such as methanol.

When superimposed, the MAR signals recorded under anoxic conditions with nitrate did not match the FISH signals for *Methylophaga* spp. or gamma-Proteobacteria, which suggests that *Methylophaga* spp. did not assimilate methanol under those conditions. On the other hand, this result suggests that *Methylophaga* spp. were active and could survive in anoxic environment. However, the short incubation time (17 h) could have been insufficient to decrease cell activities. Nevertheless, *Methylophaga* spp. were clearly present in the denitrifying biofilm attached to the carriers incubated in batch culture for more than 5 days under anoxic conditions with nitrate (Fig. 1). *Methylophaga* spp. were also present and abundant in a denitrification pilot unit that did not contain residual oxygen and that was filled with active carriers from the Biodome's denitrification reactor (Villemur, unpublished results). A possible explanation for their abundance under those conditions is that *Methylophaga* spp. had access to a carbon reserve, such as polyhydroxyalkanoate (PHA), exopolysaccharide (EPS) or protist waste, and used methanol as an energy source rather than as a carbon source. A good example is phosphorus accumulating organisms (PAO) that use this growing strategy when carbon source and electron acceptor were not occurring together [37]. Lueders *et al.* [28] showed that more than 90% of the methanol consumed by methylophages was converted into CO₂ in rice field soil and only 10% of carbon was incorporated into the biomass. In the Biodome's denitrification reactor, residual oxygen can support the accumulation

of an intracellular carbon reserve or EPS production. It is possible that under such conditions, *Methylophaga* spp. prefer to consume EPS rather than methanol, which would explain the lack of MAR signals from *Methylophaga* cells in our anoxic incubation tests. Finally, some *Methylophaga* strains, such as *M. alcalica*, *M. murata*, and *M. natronica*, are strictly aerobic but can convert nitrate to nitrite [10, 11]. This suggests a possible nitrate reduction activity for *Methylophaga* spp., although our results with MAR do not support that.

In each test, a CO₂ trap was added in half of our vials to see if it would reduce the bias caused by the assimilation of the ¹⁴CO₂ resulting from the substrate mineralization. As discussed by Hesselsoe *et al.* [16], most bacteria are able to assimilate CO₂ during growth. The addition of the trap should allow for longer incubation periods with limited bias. Little radioactivity was retained by the biomass under anoxic conditions even after 17 h of incubation. In this case, a bias from the re-assimilation of ¹⁴CO₂ produced by mineralization is not suspected. However, under oxic conditions, fewer amounts of methanol were consumed in the presence of the CO₂ trap and a lower proportion of methanol was incorporated into the biomass (12%) compared to the biomass without the trap (30%). In the absence of the CO₂ trap, the biomass may have assimilated ¹⁴CO₂ along with methanol in serine pathways for methanol or with the Calvin–Benson–Bassham cycle by other methylotrophs [2]. CO₂ traps should be useful in SIP tests that require an incubation period of more than 7 days. They should prove particularly useful when testing slow-growing organisms such as *Hyphomicrobium* spp.

In conclusion, we showed by means of FISH that *Methylophaga* spp. are very abundant in the biofilm of the Biodome's denitrification reactor, confirming our previous results with PCR-DGGE experiments and the 16S rDNA gene library. However, the MAR–FISH experiments suggest that *Methylophaga* spp. are not active under denitrification conditions and that denitrification is done mostly by alpha-Proteobacteria.

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