Background. The biofilm of a methanol-fed, marine denitrification system is composed of a multi-species microbial community, among which *Hyphomicrobium nitrativorans* and *Methylophaga nitratireducenticrescens* are the principal bacteria involved in the denitrifying activities. To assess its resilience to environmental changes, the biofilm was cultivated in artificial seawater (ASW) under anoxic conditions and exposed to a range of specific environmental conditions. We previously reported the impact of these changes on the denitrifying activities and the co-occurrence of *H. nitrativorans* strain NL23 and *M. nitratireducenticrescens* in the biofilm cultures. Here, we report the impact of these changes on the dynamics of the overall microbial community of the denitrifying biofilm.

Methods. The original biofilm (OB) taken from the denitrification system was cultivated in ASW under anoxic conditions with a range of NaCl concentrations, and with four combinations of nitrate/methanol concentrations and temperatures. The OB was also cultivated in the commercial Instant Ocean seawater (IO). The bacterial diversity of the biofilm cultures and the OB was determined by 16S ribosomal RNA gene sequences. Culture approach was used to isolate other denitrifying bacteria from the biofilm cultures. The metatranscriptomes of selected biofilm cultures were derived, along with the transcriptomes of planktonic pure cultures of *H. nitrativorans* strain NL23 and *M. nitratireducenticrescens* strain GP59.

Results. High proportions of *M. nitratireducenticrescens* occurred in the biofilm cultures. *H. nitrativorans* strain NL23 was found in high proportion in the OB, but was absent in the biofilm cultures cultivated in the ASW medium at 2.75% NaCl. It was found however in low proportions in the biofilm cultures cultivated in the ASW medium at 0 to 1% NaCl and in the IO biofilm cultures. Denitrifying bacterial isolates affiliated to *Marinobacter* spp. and *Paracoccus* spp. were isolated. Up regulation of the denitrification genes of strains GP59 and NL23 occurred in the biofilm cultures compared to the planktonic pure cultures. Denitrifying bacteria affiliated to the *Stappia* spp. were metabolically active in the biofilm cultures.

Conclusions. These results illustrate the dynamics of the microbial community in the denitrifying biofilm cultures in adapting to different environmental conditions. The NaCl concentration is an important factor affecting the microbial community in the biofilm cultures. Up regulation of the denitrification genes of *M. nitratireducenticrescens* strain GP59 and *H. nitrativorans* strain NL23 in the biofilm cultures suggests different mechanisms of regulation of the denitrification pathway in the biofilm. Other denitrifying heterotrophic bacteria are present in low proportions, suggesting that the biofilm has the potential to
adapt to heterotrophic, non-methylo trophic environments.
Dynamics of a methanol-fed marine denitrifying biofilm: 2- Impact of environmental changes on the microbial community

Authors
Richard Villemur, Geneviève Payette, Valérie Geoffroy, Florian Mauffrey, Christine Martineau

Addresses
1 INRS-Centre Armand-Frappier Santé et Biotechnologie, Laval, QC, Canada
2 Present address: Lallemand, Montreal, QC, Canada
3 Present address: Université de Genève, Geneva, Switzerland
4 Present address: Laurentian Forestry Centre, Québec, QC, Canada

Corresponding author
Richard Villemur, richard.villemur@iaf.inrs.ca
Abstract

Background. The biofilm of a methanol-fed, marine denitrification system is composed of a multi-species microbial community, among which *Hyphomicrobium nitrativorans* and *Methylphaga nitratireducenticrescens* are the principal bacteria involved in the denitrifying activities. To assess its resilience to environmental changes, the biofilm was cultivated in artificial seawater (ASW) under anoxic conditions and exposed to a range of specific environmental conditions. We previously reported the impact of these changes on the denitrifying activities and the co-occurrence of *H. nitrativorans* strain NL23 and *M. nitratireducenticrescens* in the biofilm cultures. Here, we report the impact of these changes on the dynamics of the overall microbial community of the denitrifying biofilm.

Methods. The original biofilm (OB) taken from the denitrification system was cultivated in ASW under anoxic conditions with a range of NaCl concentrations, and with four combinations of nitrate/methanol concentrations and temperatures. The OB was also cultivated in the commercial Instant Ocean seawater (IO). The bacterial diversity of the biofilm cultures and the OB was determined by 16S ribosomal RNA gene sequences. Culture approach was used to isolate other denitrifying bacteria from the biofilm cultures. The metatranscriptomes of selected biofilm cultures were derived, along with the transcriptomes of planktonic pure cultures of *H. nitrativorans* strain NL23 and *M. nitratireducenticrescens* strain GP59.

Results. High proportions of *M. nitratireducenticrescens* occurred in the biofilm cultures. *H. nitrativorans* strain NL23 was found in high proportion in the OB, but was absent in the biofilm cultures cultivated in the ASW medium at 2.75% NaCl. It was found however in low proportions in the biofilm cultures cultivated in the ASW medium at 0 to 1% NaCl and in the IO biofilm cultures. Denitrifying bacterial isolates affiliated to *Marinobacter* spp. and *Paracoccus* spp. were isolated. Up regulation of the denitrification genes of strains GP59 and NL23 occurred in the biofilm cultures compared to the planktonic pure cultures. Denitrifying bacteria affiliated to the *Stappia* spp. were metabolically active in the biofilm cultures.

Conclusions. These results illustrate the dynamics of the microbial community in the
denitrifying biofilm cultures in adapting to different environmental conditions. The NaCl concentration is an important factor affecting the microbial community in the biofilm cultures. Up regulation of the denitrification genes of *M. nitratireducenticrescens* strain GP59 and *H. nitrativorans* strain NL23 in the biofilm cultures suggests different mechanisms of regulation of the denitrification pathway in the biofilm. Other denitrifying heterotrophic bacteria are present in low proportions, suggesting that the biofilm has the potential to adapt to heterotrophic, non-methylo trophic environments.
Introduction

Most naturally-occurring microbial biofilms, such as those encountered in bioremediation processes, are composed of multiple microbial species. Studying such complex biofilms is a challenge, as each species can influence the biofilm development. The biofilm microbial community inside a bioremediation process adapts to the prescribed operating conditions and shapes the efficiency of the bioprocess to degrade the pollutant(s). Usually, the microbial community in such bioprocesses is complex and composed of main degraders but also of secondary microorganisms that could provide benefits to the degraders or could simply contribute to the degradation intermediates or waste. It is recognized that a complex microbial community is more resilient to "unexpected" changes in the operation of the bioprocesses than a single species biofilm, as some of the minor degraders take over the main degraders affected by the changes (Cabrol & Malhautier 2011; Roder et al. 2016; Salta et al. 2013; Tan et al. 2017). The mechanisms of how a microbial population in a biofilm adapts to changes however are poorly understood.

The Montreal Biodome, a natural science museum, operated a continuous fluidized-bed methanol-fed denitrification reactor to remove nitrate (NO$_3^-$) that accumulated in the 3 million-L seawater aquarium. The fluidized carriers in the denitrification reactor were colonized by naturally-occurring multispecies microorganisms to generate a marine methylotrophic denitrifying biofilm estimated to be composed of 15-20 bacterial species (Labbé et al. 2003). The main bacteria responsible of the denitrifying activities belong to the alphaproteobacteria *Hyphomicrobium nitrativorans* (strain representative NL23) and to the gammaproteobacteria *Methylophaga nitratireducenticrescens* (strain representative JAM1), both methylotrophs, that accounted for 60-80% of the biofilm (Labbé et al. 2003; Labbé et al. 2007; Martineau et al. 2013b; Villeneuve et al. 2013).

Denitrification takes place in bacterial cells where N oxides serve as terminal electron acceptor instead of oxygen (O$_2$) for energy production when oxygen depletion occurs, leading to the production of gaseous nitrogen (N$_2$). Four sequential reactions are strictly required for the reduction of NO$_3^-$ to gaseous nitrogen, via nitrite (NO$_2^-$), nitric oxide (NO) and nitrous oxide (N$_2$O), and each of these reactions is catalyzed by different enzymes, namely NO$_3^-$ reductases (Nar and Nap), NO$_2^-$ reductases (NirS and NirK), NO reductases (Nor) and N$_2$O reductases (Nos) (Kraft et al. 2011; Philippot & Hojberg 1999; Richardson et al. 2001). Whereas *H. nitrativorans*...
strain NL23 possesses the four reductases for the complete denitrification pathway, *M. nitratireducenticrescens* strain JAM1 performs incomplete denitrifying activities, as it lacks a dissimilatory NO-forming nitrite reductase (Auclair et al. 2010; Martineau et al. 2013a; Mauffrey et al. 2017; Mauffrey et al. 2015; Villeneuve et al. 2012). Using degenerated PCR primers for the detection of denitrification genes, we showed that there are probably other denitrifying bacteria in the biofilm, one to four orders of magnitude lower in proportions than *M. nitratireducenticrescens* strain JAM1 and *H. nitrativorans* strain NL23 (Auclair et al. 2012). These other bacteria may play a role if the bioprocess undergoes stress conditions or changes in the operation mode.

We have initiated a study with the aim of assessing the performance of the Biodome denitrifying biofilm subjected to environmental changes. The original biofilm (OB) taken from the Biodome denitrification system was cultivated in an artificial seawater (ASW) under batch mode and anoxic conditions at laboratory scale and exposed to a range of specific physico-chemical parameters. Such parameters included a range of NaCl, NO$_3^-$, NO$_2^-$ and methanol concentrations, and varying pH and temperatures. These parameters were chosen as possible factors that could affect a denitrification reactor. Thus, the objectives of this study were to determine the impact of these changes: 1) on the denitrification performance of the biofilm; 2) on the dynamics of the co-occurrence of *H. nitrativorans* and *M. nitratireducenticrescens* in the biofilm; and 3) on the overall microbial community. The fourth objective of the study was to determine whether denitrifying bacteria other than *H. nitrativorans* strain NL23 and *M. nitratireducenticrescens* strain JAM1 are present in the biofilm.

Results for the first two objectives and partially the fourth objectives were reported by Geoffroy et al. (2018) and Payette et al. (2019). We showed that the denitrifying biofilm can sustain denitrifying activities in most of the tested conditions. Inhibition occurred when these biofilm cultures were exposed to high pH (10) or to high methanol concentration (1.5%). The highest specific denitrification rates occurred when the biofilm cultures were cultivated at 64.3 mM NO$_3^-$ and 0.45% methanol (C/N = 1.5), and at 30°C. Poor biofilm development occurred in biofilm cultures cultivated at 5% and 8% NaCl. We also showed that the NaCl concentrations in the ASW medium have significant impacts on the population of *H. nitrativorans* strain NL23, with its displacement by a subpopulation of the species *M. nitratireducenticrescens* (strain GP59 as representative), which can perform the complete denitrification pathway.
Results for the third and fourth objectives are presented here. The composition of the bacterial community of the different biofilm cultures was determined by sequencing the 16S ribosomal RNA (rRNA) genes. A culture dependent approach was used to recover new denitrifying bacterial isolates from the biofilm cultures. To complement these two objectives, we derived the metatranscriptome from selected biofilm cultures. These metatranscriptomes were analyzed to determine the composition of the active microbial community in the biofilm cultures but also to assess their metabolic contributions, such as those involved in denitrification.

Furthermore, metatranscriptomic analyses provided further indications on the dynamics of *H. nitrativorans* and *M. nitratireducenticrescens* in these cultures (second objective) by assessing changes in metabolic pathways of *H. nitrativorans* strain NL23 and *M. nitratireducenticrescens* strain GP59 between the planktonic pure cultures and the biofilm cultures. Our study is the first that give a comprehensive picture of the microbial community of a methylotrophic denitrifying biofilm and its adaptation to specific changes.

**Material and Methods**

**Cultivation of the original biofilm to different culture conditions**

The formulations of the artificial seawater (ASW) medium and the commercial Instant Ocean (IO) medium (Table S1), and the different conditions of the biofilm cultures were described by Payette *et al.* (2019). Briefly, the biomass of several carriers taken from the denitrification reactor of the Montreal Biodome was scraped, dispersed, then distributed to several vials containing twenty free carriers and 60 mL prescribed medium (Table 1; Fig. S1).

The vials were incubated under anoxic conditions at 23°C or 30°C (Table 1) and shaken at 100 rpm (orbital shaker). In average once a week, the twenty carriers were taken, gently washed to remove the excess medium and the planktonic bacteria, then transferred into fresh anoxic medium and incubated under the same conditions (Fig. S1). The Ref300N-23C biofilm cultures (for 300 mg NO$_3^-$-N/L, 23°C) were defined as the reference biofilm cultures. These cultures were used by Payette *et al.* (2019) as a reference to compare results between the different culture conditions. The protocols to measure NO$_3^-$ and NO$_2^-$ concentrations, and to extract DNA from the biofilm cultures or the planktonic pure cultures were described in Payette *et al.* (2019) and Geoffroy *et al.* (2018).
16S rRNA gene analysis

DNA extracted from triplicate biofilm cultures was pooled before sequencing. Total DNA samples from seven biofilm cultures (Table 1; Ref300N-23C, 300N-30C, 900N-23C, 900N-30C, 0%NaCl, 0.5%NaCl and 1.0%NaCl) were sent to the sequencing service at the Research and Testing Laboratory (RTL, Lubbock, Texas, USA). A region of the 16S rRNA gene was PCR amplified using the 28F-519R primers (5' GAGTTTGATCNTGGCTCAG 3' and 5' GTNTTACNGCGGCKGCTG 3', covering the V1-V2-V3 variable regions) and subjected to pyrosequencing using a Roche 454 FLX genome sequencer system. The sequencing service (RTL) performed denoising and chimera analyses (details provided in supplemental doc 1). The high-quality reads were then processed in the RDP pipeline at the Ribosomal data project (RDP) web site (Cole et al. 2014). Reads were clustered into operational taxonomic units (OTU) using a 97% identity threshold. DNA extracted from the OB (from frozen stock) and from fresh IO biofilm cultures (Table 1) were sent to the sequencing service of Genome Quebec Innovation Center (Montreal, QC, Canada). In these cases, the 16S rRNA sequences covering the V6-V7-V8 variable regions (5' ACACTGACGACATGGTTCTACA 3' and 5' TACGGTAGCAGAGACTTGGTCT 3') were PCR amplified and sequenced by Illumina MiSeq PE250 (250 bp paired-end sequencing reactions). The reads were processed based on Peck et al. (2016). Briefly, paired-end reads were merged with minimum and maximum overlap length between the two reads of 20 and 250 bases, respectively, with 30% mismatched bp tolerance in the overlap region. The merged reads were processed using the software UPARSE (Edgar 2013). Sequences were truncated to a uniformized length to 420 bp. Reads with a low-quality score were removed using 2.0 as the maximum expected error value. The high-quality reads were de-replicated, sorted by size and singletons were removed. The resulting reads were clustered into operational taxonomic units (OTU) with the UPARSE-OTU clustering method using a 97% identity threshold. Chimeric OTU were removed by UPARSE-REF algorithm and with the software UCHIME ran against ChimeraSlayer ‘gold’ reference database (Edgar et al. 2011). All representative sequences of the OTUs (from pyrosequencing and Illumina) were checked again for chimeras with the DECIPHER v 2.0 program (http://www2.decipher.codes/FindChimeras.html) (Wright et al. 2012). The affiliation of the OTUs to the most probable genus was determined by the CLASSIFIER program at the RDP web
Isolation of bacterial isolates

Biofilm of the Ref300C-23C biofilm cultures was scraped from the carriers and dispersed in saline solution (3% NaCl, 34.2 mM phosphate buffer pH 7.4), and serial dilutions were made and inoculated onto these agar plate media: (1) R2A medium (complex organic carbons; EMD Chemicals Inc., Gibbstown, NJ, USA), (2) Marine Agar 2216 (marine medium with yeast extract and peptone as carbon source; Becton, Dickinson and Co., Sparks, MD, USA), (3) Methylophaga medium 1403 (American Type Culture Collection [ATCC], Manassas, VA, USA) and (4) the ASW medium; these two latter media were supplemented with 1.5% agar and 0.3% v/v methanol. The isolation procedure, the taxonomic affiliation of the isolates and the measurement of their denitrifying activities were carried out as described by Geoffroy et al. (2018). The 16S rRNA gene sequences were deposited in GenBank under the accession numbers MK571459 to MK571476.

Transcriptomes

Planktonic pure cultures of M. nitratireducenticrescens strains JAM1 and GP59 were performed in the Methylophaga 1403 medium and of H. nitrativorans strain NL23 in the 337a medium as described by Martineau et al. (2015) and Mauffrey et al. (2015). These cultures were carried out in triplicate with methanol (0.3%) and NO$_3^-$ (21.4 mM [300 mg-N/L]) under anoxic conditions at 30°C. The biomass was collected by centrifugation when the NO$_3^-$ reduction was near completion, and total RNA was extracted as described by Mauffrey et al. (2015). For the biofilm cultures, at the end of the the fifth transfer cultures, the biomass of each replicate was scrapped from carriers and used to extract total RNA. The RNA samples were sent to the sequencing service for RNA sequencing (RNAseq) by Illumina (Genome Quebec Innovation Center, Montreal QC, Canada). Because of limited amount of biofilm available, total RNA from the triplicate biofilm samples were pooled before sending to the sequencing service. For the planktonic pure cultures, RNAseq was performed on each replicate. The Ribo-Zero™ rRNA
Removal Kit (Meta-Bacteria; Epicentre, Madison, WI, USA) was used to deplete total RNA of
the ribosomal RNA. The RNA was then treated with the TruSeq Stranded mRNA Sample Prep
Kit (Illumina Inc, San Diego, CA, USA).

All computations were made on the supercomputer Briarée from the Université de
Montréal, managed by Calcul Québec and Compute Canada. Raw reads were filtered to remove
low quality reads using FASTX toolkit (http://hannonlab.cshl.edu/ fastx_toolkit/) by discarding
any reads with more than 10% nucleotides with a PHRED score <20. The resulting reads from
each sample/replicate were aligned respectively to the genome of *M. nitratireducenticrescens*
strain JAM1 (GenBank accession number CP003390.3), to the genome and plasmids of *M.*
*nitratireducenticrescens* strain GP59 (CP021973.1, CP021974.1, CP021975.1) and to the
genome of *H. nitrativorans* strain NL23 (CP006912.1) using Bowtie (v 2.2.3) with default
parameters. SAMtools (v 0.1.18) and BEDtools (v 2.20.1) were used for the generation of sam
and bam files, respectively. Significance for difference in the relative transcript levels of a gene
(defined as transcript per million: TPM) between planktonic pure cultures and biofilm cultures
was performed with the R Bioconductor NOIseq package v2.14.0 (NOIseqBio) (Tarazona et al.
2011) and run with the R software v3.2.3 (Team 2015). Because the RNAseq from the biofilm
samples were derived from one pooled RNA preparation, the "no replicate parameter" was set
\((pmr=0.2, nss=5 \text{ and } v=0.02; \text{ pseudoreplicate generated})\) in NOIseq as described by Tarazona *et
al.* (2011) under the NOISeq-sim section. Briefly, NOISeq1-sim assumes (quoting) "that read
counts follow a multinomial distribution, where probabilities for each gene in the multinomial
distribution are the probability of a read to map to that gene". Results from this statistical
analysis showed that genes that had at least >2-fold higher transcript levels from one type of
cultures to the other showed significant differences. RNAseq reads from the planktonic pure
cultures and the biofilm cultures were deposited in the SRA under the accession number
PRJNA525230. Annotations were based on services provided by GenBank
(https://www.ncbi.nlm.nih.gov/genbank), RAST (Rapid Annotation using Subsystem
Technology; http://rast.nmpdr.org) and KEGG (Kyoto Encyclopedia of Genes and Genomes;
https://www.genome.jp/kegg) (Supplemental doc 3).

To derive transcript reads not associated to the *M. nitratireducenticrescens* and *H.*
nitrativorans*, reads were aligned to a concatenated sequence consisting of the three reference
genomes (JAM1+GP59+NL23) and the two plasmids (from strain GP59). The reads that did not
align were kept. Unaligned reads were de novo assembled at the National Center for Genome Analysis web site (https://galaxy.ncgas-trinity.indiana.edu) by Trinity v. 2.4.0 (Grabherr et al. 2011). These transcripts were deposited in SRA under the accession number PRJNA525230. Estimation of the transcript abundance of the de novo assembled sequences was performed by RSEM (Li & Dewey 2011). The resulting assembled sequences were annotated at the Joint Genomic Institute (https://img.jgi.doe.gov/cgi-bin/m/main.cgi) to find open reading frames with their putative function and affiliation (GOLD Analysis Project Id: Ga0307915, Ga0307877, Ga0307760). The annotations were then verified manually for discrepancies within the assembled sequences (Supplemental doc 4, 5, 6 and 7).

Results

**Bacterial composition of the biofilm cultures by 16S rRNA gene sequencing**

As reported by Payette et al. (2019), the original biofilm (OB) collected from the Biodome denitrification system was used as inoculum to colonize new carriers in a series of anoxic biofilm cultures cultivated under different conditions (Table 1; Fig. S1). We selected eight of these biofilm cultures for our present analysis for the following reasons. The Ref300N-23C biofilm cultures were used as reference for comparison analysis. The 300N-30C, 900N-23C and 900N-30C biofilm cultures had higher specific denitrification rates compared to the Ref300N-23C biofilm cultures. The 0%, 0.5% and 1.0% NaCl ASW biofilm cultures and the IO biofilm cultures were chosen because of the persistence of *H. nitrativorans* NL23 in these cultures as opposed to the other cultures. The composition of the bacterial community of these biofilm cultures and the OB was determined by sequencing the 16S rRNA genes to assess the impact of these specific conditions on the bacteria community (Fig. 1A; Table 2). The bacterial profiles of the OB and the IO biofilm cultures were distinct to each other, and to the seven biofilm cultures cultivated with different formulations in the ASW medium (Fig. 1B). The bacterial profiles of these latter cultures were however not very different because of the high proportions of *Methylophaga* spp. (>85%).

In the OB, high proportions of the 16S rRNA gene sequences were related to *Hyphomicrobium* spp. (45.8%) followed by *Oceanibaculum* spp. (12.3%), *Aquamicrobium* spp. (11.6%); *Methylophaga* spp. accounted for 3.5% (Table 2). In the IO biofilm cultures, the
proportion of *Methylophaga* spp. was 12 times higher (42.8%) than in the OB, whereas it was 5.5 times lower for *Hyphomicrobium* spp. (8.4%). Higher proportions of *Marinicella* spp. (7.2%) and *Winogradskyella* spp. (4.3%) along with a much lower proportion of *Aquamicrobium* spp. (0.44%) were observed in the IO biofilm cultures compared to the OB (Table 2).

In the biofilm cultures cultivated with the four combinations of NO$_3^-$ / methanol concentrations and temperatures in ASW medium containing 2.75% NaCl (Ref300N-23C, 300N-30C, 900N-23C, 900N-30C), *Methylophaga* spp. accounted for >90% of the 16S rRNA gene sequences followed by *Marinicella* spp. with proportions ranging from 1.5% to 5.0% (Fig. 1; Table 2). No sequences were found affiliated to *Hyphomicrobium* spp. under these conditions. In the biofilm cultures cultivated at low NaCl concentrations (0% NaCl, 0.5% NaCl, 1% NaCl), *Hyphomicrobium* spp. accounted for 11.8%, 6.8% and 0.25%, respectively of the 16S rRNA gene sequences (Fig. 1; Table 2). *Methylophaga* spp. was still the dominant genus with more than 85% of the 16S rRNA gene sequences; 16S rRNA gene sequences affiliated to *Marinicella* spp. were also found in significant proportions (Fig. 1; Table 2). Finally, 16S rRNA gene sequences affiliated to *Stappia* spp. were found in all biofilm cultures and in the OB.

The 16S rRNA gene sequences from the OB and the IO biofilm cultures that were derived by Illumina sequencing generated several thousands of reads affiliated to *Hyphomicrobium* spp. and *Methylophaga* spp. (Table 3). This tremendous amounts of sequences allowed assessing the presence of species other than *H. nitrativorans* and *M. nitratireducenticrescens* in these two biofilms. The phylogenic analyses performed on these sequences allowed regrouping the OTUs in three clusters for *Hyphomicrobium* spp., and also three clusters for *Methylophaga* spp. (Fig. S2A and S2B). The vast majority (>90%) of the 16S rRNA gene sequences associated to these OTUs were affiliated to *H. nitrativorans* or *M. nitratireducenticrescens*, respectively, in the OB and the IO biofilm cultures (Clusters 1, Table 3). A small proportion of the OTUs (clusters 2 and 3) was affiliated to other *Hyphomicrobium* or *Methylophaga*, which suggests that other members of these genera were present in these biomasses in very low proportions.

**Isolation of denitrifying bacterial isolates from the biofilm cultures**

The biomass of the Ref300N-23C biofilm cultures was dispersed on different nutrient agar plates to isolate denitrifying bacteria other than *H. nitrativorans* strain NL23 and *M. nitratireducenticrescens* strain GP59. Isolates affiliated to the genera *Marinobacter*,...
Pseudomonas, Paracoccus, Roseovarius, Thalassobius, Winogradskyella, Aequorivita and Exiguobacterium (Table 4) were recovered from the Marine medium 2216 plates, which contains yeast extract and peptone (Atlas 1993). Only isolates affiliated to the genera Marinobacter and Paracoccus showed consumption of NO$_3^-$ and NO$_2^-$ and production of gas, suggesting that they possess the complete denitrification pathway. The three isolates affiliated to the Paracoccus spp. have identical 16S rRNA sequences with the one of Paracoccus sp. strain NL8 that was isolated from the Biodome denitrification system (Labbé et al. 2003). This result suggests that strain NL8 persisted in the biofilm cultures. One representative of Paracoccus isolates (GP3) could grow with methanol as sole source of carbon; Marinobacter sp. GP2 could not.

**Metatranscriptomic analysis of the biofilm cultures**

The metatranscriptomic approach has allowed assessing the contributions of the microbial community to the metabolic processes in the biofilm cultures. We have chosen to focus on three biofilm cultures, which were the Ref300N-23C (the reference biofilm cultures), 900N-30C (highest denitrification rates; Table 1) and 0% NaCl (persistence of H. nitrativorans strain NL23) biofilm cultures. Because the genomes of H. nitrativorans strain NL23 and M. nitratireducencrescens strain JAM1 and strain GP59 were available, we first determined changes in the transcript levels of genes associated to these genomes between the biofilm cultures and the planktonic pure cultures of the respective strains. To assess the contribution of other microorganisms in biofilm cultures, the reads from the metatranscriptomes that did not align with the three reference genomes were used to derive *de novo* assembled transcripts. These transcripts were annotated for function and bacterial affiliation.

**Gene expression profiles of M. nitratireducencrescens strain GP59 in the biofilm cultures**

Because >80% of the genomes of strains JAM1 and GP59 are identical, high proportions of reads from the biofilm metatranscriptomes can align to both genomes. Geoffroy *et al.* (2018) showed that the gene expression profiles of the common genes between both strains in planktonic pure cultures were similar. In Payette *et al.* (2019), the concentrations of strain GP59 and strain JAM1 in the biofilm cultures were determined by qPCR. In the three selected biofilm cultures for the metatranscriptomic analysis, the concentrations of strain GP59 (copies of *nirK* by ng biofilm DNA) were one to three orders of magnitude higher than those of strain JAM1 (copies...
of tagH by ng biofilm DNA). Because of these differences, it was assumed that most of the
transcript reads associated to *M. nitratireducenticrescens* in the biofilm cultures were from strain
GP59. Metatranscriptomic analysis in relation with strain JAM1 is described in supplemental doc
8 and 11. The transcriptomes of strain GP59 were also derived from planktonic pure cultures
cultivated under anoxic conditions in the *Methylophaga* 1403 medium (Fig. S1). The choice of
this medium was because suboptimal growth occurred with strain GP59 in ASW medium. The
relative transcript levels of the corresponding genes in the biofilm cultures and the planktonic
pure cultures were compared to assess changes in the metabolisms of the strain that occurred
between the two environments. All quantitative changes described below of the transcript levels
in the biofilm cultures are expressed relative to the transcript levels in the planktonic pure
cultures.

Among all genes of strain GP59, between 11% and 21% of them had higher relative
transcript levels in the biofilm cultures. At the opposite, 6 to 17% of all genes of strain GP59
were expressed at higher relative transcript levels in planktonic pure cultures (Fig. 2). Strain
GP59 contains two plasmids, and most of the genes encoded by these plasmids had much lower
relative transcript levels in the biofilm cultures (Fig. 2). Genes involved in the nitrogen
metabolism and iron transport were globally at higher relative transcript levels in the biofilm
cultures (Table 5; Fig. 2 and 3).

For the denitrification genes, *narXL* encoding the regulatory factors of the *nar* systems
showed no differences between the biofilm cultures and the planktonic pure cultures in the
relative transcript levels (Table 5). Small upregulation of the *nar2* operon with about 3-fold
increases in relative transcript levels occurred in the biofilm cultures. These levels were lower in
the 900N-30C biofilm cultures for the *nar1* operon and were about the same levels in the two
other biofilm cultures and the planktonic pure cultures. The *nor1* operon had the same relative
transcript levels in the 300N-23C and 900N-30C biofilm cultures and the planktonic pure
cultures, and a 3-fold decrease was noticed in these levels in the 0%NaCl biofilm cultures (Table
5). No significant changes in the expression of the *nos* operon occurred between both types of
cultures. The relative transcript levels of *nirK* were 5 to 10-times higher in the 300N-23C and
900N-30C biofilm cultures, whereas these levels were similar in the 0%NaCl biofilm cultures
and the planktonic pure cultures (Table 5). Higher relative transcript levels of genes involved in
the ammonium transport and the assimilatory $\text{NO}_3^-$/NO$_2^-$ reduction pathway were observed in the
biofilm cultures (Table 5; Fig. 3). Absence of nitrogen source other than NO$_3^-$ in the ASW medium and presence of 37 mM NH$_4^+$ in the medium used for the planktonic pure cultures (Methylophaga 1403) could explain these differences in the assimilatory pathway.

Figure 3 illustrates changes in the relative expression profiles in the biofilm cultures of major pathways in strain GP59. The relative transcript levels of $mxaFJGI$ encoding the small and large subunits of the methanol dehydrogenase (MDH) and the cytochrome c-L increased by 2-6 fold in biofilm cultures. Two out of the four $mxaF$-related products ($xoxF$) showed 2- to 9-fold decreases in their relative transcript levels in the biofilm cultures. As observed in Methylorubrum extorquens, the genome of M. nitratireducenticrescens strain GP59 encodes three formate dehydrogenases with the same gene arrangement (Chistoserdova et al. 2004). The $fdhCBAD$ operon that encodes the NAD-linked, Mo-formate dehydrogenases showed ca. 60-fold increases in the relative transcript levels in the biofilm cultures, whereas the two other $fdh$ operons stayed at the same levels of those of the planktonic pure cultures. Gene encoding NAD-dependent formate dehydrogenase was upregulated in biofilms formed by Desulfovibrio vulgaris compared to planktonic cultures (Clark et al. 2012). This was also the case in biofilms formed by Staphylococcus aureus where the NAD-dependent formate dehydrogenase gene was among the highest upregulated genes (Resch et al. 2005). In both studies, this upregulation correlated with increase in formate dehydrogenase activity. Contrary to planktonic cultures, accumulation of formate could occur in cell vicinity in the biofilm that would be toxic for the cells (Resch et al. 2005). Therefore, upregulation of $fdhCBAD$ operon could be related to detoxification. The relative transcript levels of the gene encoding the cytochrome c555 were 40 to 70 times higher in the biofilm cultures. Genes encoding the other cytochromes, pseudoazurins and azurin were expressed at similar levels in both types of cultures. Genes involved in the formaldehyde metabolism to formate and CO$_2$, glycolysis, the ribulose monophosphate pathway, the Entner Doudorof pathway, the tricarboxylic acid cycle, and the pentose pathway showed their relative transcript levels in general unchanged. Few genes in these pathways had 2-11 fold differences between the planktonic pure cultures and the biofilm cultures. The genome of strain GP59 encodes the major enzymes involved in the Calvin-Benson-Bassham cycle: the ribulose-bisphosphate carboxylase (Rubisco) and the phosphoribulokinase (Prk). In the 0% NaCl biofilm cultures, the relative transcript levels of the Rubisco gene operon ($rbcSL$) jumped by 66 times. This upregulation was less pronounced in the Ref300N-23C biofilm cultures (3-fold increase).
For the prk gene, the relative transcript levels were 3 to 4 times higher in the 0% NaCl biofilm cultures. The nature of this upregulation is unknown. Except for the cytochrome c555, the relative transcript levels of genes encoding for the oxidative phosphorylation metabolism were unchanged. Several genes involved in iron transport showed higher relative transcript levels in the biofilm cultures (2 to >50-fold increases). The nature of this upregulation in the biofilm cultures is unknown, as the biofilm and planktonic pure cultures were cultivated with trace elements containing iron (Payette et al. 2019).

Gene expression profiles of H. nitrativorans strain NL23 in the 0% NaCl biofilm cultures

As with M. nitratireducenticrescens strain GP59, the transcript levels of genes associated to H. nitrativorans strain NL23 were compared between the biofilm cultures and the planktonic pure cultures to assess changes in the metabolisms that occurred between the two environments. The planktonic pure cultures of strain NL23 were cultivated under anoxic conditions in the 337a medium (Fig. S1). The choice of this medium was because strain NL23 could not grow in ASW (with 2.75% NaCl).

The overall analysis of the three metatranscriptomes confirmed the results obtained by qPCR assays (Payette et al. 2019) and the 16S rRNA gene analysis (Table 2). High number of reads (40 x 10^6) derived from the metatranscriptome of the 0% NaCl biofilm cultures aligned with the NL23 genome, but <20 000 reads derived from the Ref300N-23C and 900N-30C metatranscriptomes did. In the 0% NaCl biofilm cultures, <10% of all NL23 genes had a higher relative transcript levels in the 0% NaCl biofilm cultures, whereas this was the case for >40% genes in planktonic pure cultures (Fig. 2). These results suggest important changes had occurred in the regulation of gene expression between the planktonic pure cultures and the biofilm cultures. Genes involved in the energy (Fig. S4) and nitrogen metabolisms (Fig. 2; Table 5) had globally higher relative transcript levels in the 0%NaCl biofilm cultures (Fig. 2).

Higher relative transcript levels (5 to 8-times) for the nap, nor and nos operons were observed in the 0% NaCl biofilm cultures (Table 5). nirK was highly upregulated in the biofilm cultures with 49-fold increase in the relative transcript levels (Table 5). The napGH operon however had a 9.4-fold decrease in the relative transcript levels in the 0% NaCl biofilm cultures. As observed with strain GP59, substantial changes in the relative transcript levels of genes involved in the ammonium transport and the assimilatory NO₃⁻/NO₂⁻ reductase were observed.
with 3- to 22-fold increases in these levels (Table 5, Fig. 4). These results correlate with the absence of NH$_4^+$ in the ASW medium, and thus NO$_3^-$ the only source of N, compared to the 337a medium used for the planktonic pure cultures, which contains 3.8 mM NH$_4^+$.

Figure 4 illustrates changes in relative expression profiles of major pathways in the 0% NaCl biofilm cultures of strain NL23. The relative transcript levels of *mxaFJGI* increased by 2-fold in the biofilm cultures. The relative transcript levels of the *mau* operon (methylamine dehydrogenase) showed a 15-fold decrease in the biofilm cultures. The nature of such decrease is unknown as strain NL23 was not fed with methylamine in any of our cultures. The three xoxF genes did not show substantial changes in their transcript levels in both types of cultures. Genes involved in the formaldehyde metabolism to formate and CO$_2$, glycolysis, the tricarboxylic acid cycle, and the pentose pathway showed their transcript levels in general unchanged between the planktonic pure cultures and the biofilm cultures. Few genes in these pathways had 2-5 fold differences in their relative transcript levels. The two genes encoding the key enzymes in the serine pathway (alanine-glyoxylate transaminase, glycine hydroxymethyltransferase) had 3- to 7-fold increases in their relative transcript levels in the biofilm cultures. As *Methylocaldum extorquens*, the NL23 genome encodes the ethymalonyl-CoA pathway (Chistoserdova et al. 2003; Peyraud et al. 2009), which did not show changes overall in the transcript levels of the corresponding genes between the two types of cultures. Contrary to *M. extorquens* however, a gene encoding the isocitrate lyase is present in strain NL23 and showed a 25-fold upregulation in the biofilm cultures. The isocitrate lyase is one of the key enzymes of the glyoxylate bypass that catalyzes the transformation of isocitrate to succinate and glyoxylate. Gene encoding isocitrate lyase is also present in other available *Hyphomicrobium* genomes. All these results suggest that in the 0% NaCl biofilm cultures, the carbon metabolism increased in activity and that the glycine regeneration for the serine pathway by the glyoxylate was upregulated. Among genes involved in the oxidative phosphorylation, the relative transcript levels were higher (2 to 13 times) in the biofilm cultures with those encoding the NADH dehydrogenase, the cytochrome c reductase, with one of the cytochromes c and the F-type ATPase. Combined with increases in the relative transcript levels of the denitrification and the carbon pathways, these results suggest that increases in electron donor activities correlates with the need of electron for the nitrogen dissimilatory metabolism in the biofilm cultures. Strain NL23 possesses four types of cytochrome oxidase (aa$_3$, bo, bd-I and cbb$_3$) (reduction of O$_2$ in H$_2$O) that are in general
expressed at the same levels in both types of cultures. Figure 4 also illustrates the dynamic changes of transporters and two component systems. Several of these transporters had lower relative transcript levels in the 0% NaCl biofilm cultures. Contrary to strain GP59, genes involved in iron transport were not strongly affected in their gene expression in the 0% NaCl biofilm cultures (Fig. 2 and 4).

The composition of the active microbial community in the biofilm cultures

As mentioned above, the reads from the three metatranscriptomes that did not align with the genomes of H. nitrativorans strain NL23 and M. nitratireducenticrescens strain GP59 and strain JAM1 were de novo assembled. These reads were subsequently aligned to the de novo assembled transcripts to derive the relative levels of these transcripts in the biofilm cultures. The de novo assembled sequences were then annotated for function and affiliation. Finally, these sequences were grouped by microbial affiliation to determine the active populations in the biofilm cultures and to assess their level of involvement in these biofilm cultures (Table 6).

It was estimated that between 5 to 10% reads of the three metatranscriptomes were derived from other microorganisms than H. nitrativorans strain NL23 and M. nitratireducenticrescens strain GP59 and strain JAM1 (Supplemental doc 4). The proportions of transcripts affiliated to Archaea and Eukarya accounted together for <0.1% (Table 6), which suggests very low abundance of these microorganisms in the biofilm cultures. The proportions of transcripts affiliated to viruses, phages and plasmids in the de novo assembled transcripts represented between 0.6 and 21.7% (Table 6).

Twenty-seven bacterial taxa were selected for their overall transcript levels in at least one of the three biofilm cultures (Table 6). All the taxa detected by the 16S rRNA gene sequencing are present in this list (Table 2 and supplemental doc 2). These 27 taxa represented between 22% and 35% of the de novo assembled transcripts. Among these taxa, genes encoding the four denitrification reductases were present in the de novo transcripts affiliated to Marinobacter spp., Stappia spp. and Pseudomonas spp. However, only de novo assembled transcripts affiliated to the Stappia spp. showed the complete set of denitrification genes in the three biofilm cultures and organized in operons (napABC, napADFE, norCBQD, nosRZDF).

Further analysis of the expression profiles of the 27 bacterial taxa was performed to assess whether some taxa were influenced in their global metabolic activities by the specific conditions
of the biofilm cultures. The overall transcript levels of the 27 bacterial taxa (Table 6) were compared between each biofilm culture by clustering analysis (Fig. 5). NaCl concentration was the main factor of clustering as two distinct clusters were derived. The low salt cluster consisting of nine bacterial taxa showed higher relative transcript levels in the 0% NaCl biofilm cultures, whereas the marine cluster of 13 bacterial taxa had higher relative transcript levels in the Ref300N-23C and 900-30C biofilms cultures. A third cluster showed five bacterial taxa with lower relative transcript levels in the 900N-30C biofilm cultures compared to the 0% NaCl and Ref300N-23C biofilm cultures. In these cases, higher temperature (30°C vs 23°C) and higher NO$_3^-$ and methanol concentration (64.3 mM vs 21.4 mM NO$_3^-$; 0.45% vs 0.15% methanol) may have negatively affected these populations.

Discussion

In the environment, numerous bacteria belonging to different taxa can accomplish denitrifying activities, and many of them were encountered in different types of denitrification processes (Lu et al. 2014). Very few studies describing the microbial community of methanol-fed denitrification systems have been reported so far. Most of these studies are based on cloned 16S rRNA gene sequences of around 100 clones or based on fluorescence in situ hybridization (Baytshtok et al. 2008; Ginige et al. 2004; Hallin et al. 2006; Neef et al. 1996; Osaka et al. 2008; Osaka et al. 2006; Rissanen et al. 2016; Rissanen et al. 2017; Sun et al. 2016; Yoshie et al. 2006). In all these studies, high proportions of *Hyphomicrobium* spp. were found in combination with high proportions of other methylotrophs such as *Methyloversatilis* spp., *Methylophilus* spp., *Methylotenera* spp. or *Paracoccus* spp. The Biodome marine denitrification system showed no exception to this trend with co-occurrence of *Hyphomicrobium* spp. and the marine methylotroph *Methylphaga* spp. This co-occurrence was also observed in two other denitrification systems treating saline effluents (Osaka et al. 2006; Rissanen et al. 2016) (see Discussion by Payette et al. 2019). The bacterial diversity of the biofilm taken from the Biodome denitrification system was assessed before when the reactor was operational in 2002 by deriving a 16S rRNA gene library and by culture approach (Labbé et al. 2003; Labbé et al. 2004). Beside *Hyphomicrobium* sp. and *Methylphaga* sp., *Paracoccus* sp., *Sulfitobacter* sp., *Nitratireductor aquibiodomus*, and *Delftia* sp. among others were identified. In the present report, a more complete determination of the composition of the bacterial community of the denitrifying biofilm that was frozen in 2006
The composition of the bacterial community of the OB and the IO biofilm cultures was derived from a different region of the 16S rRNA genes (V6 to V8) than that used for the other biofilm cultures (V1 to V3), and each region was sequenced by a different technology (Illumina and pyrosequencing, respectively). Pyrosequencing technology was no longer available at the time of the sampling of the OB and the IO biofilm cultures, which were carried two years later than the other biofilm cultures. Despite these differences, we believe that the results generated by these two approaches were comparable because they are consistent with the results obtained by qPCR assays (Table 2) that have determined the concentrations of *M. nitratireducenticrescens* (copies *narG1* per ng biofilm DNA) and *H. nitrativorans* strain NL23 (copies *napA* per ng biofilm DNA) in the biofilm cultures (Payette et al. 2019). For instance, qPCR showed very low levels of *H. nitrativorans* strain NL23 in the Ref300C-23C biofilm cultures and high level in the OB. These results concur with the absence of 16S rRNA sequences associated to *Hyphomicrobium* spp. in the Ref300C-23C biofilm cultures (pyrosequencing) and high number of these sequences in the OB (Illumina sequencing).

The OB cultivated under the different conditions in the ASW medium showed important changes in the *Methylophaga* and the *Hyphomicrobium* populations. The proportion of 16S rRNA gene sequences associated to *Methylophaga* spp. was 3.5% in the OB but was very high, between 85% and 97%, in these cultures. On the contrary, the proportion of 16S rRNA gene sequences associated to *Hyphomicrobium* spp. was high (46%) in the OB, but was very low (0% to 11%) in these cultures. The NaCl concentrations in the ASW had an impact on the *Hyphomicrobium* populations. 16S rRNA gene sequences associated to *Hyphomicrobium* spp. were absent in the biofilm cultures cultivated in ASW at 2.75% NaCl (Ref300N-23C, 300N-30C, 900N-23C, 900N-30C), but were present in the biofilm cultures cultivated at low NaCl concentrations (0%, 0.5% and 1%). These results concur with those obtained by qPCR for the concentrations of *M. nitratireducenticrescens* and *H. nitrativorans* strain NL23 (Payette et al. 2019). Cultivating the OB in the IO medium had a different impact on the *Hyphomicrobium* populations. Although the concentrations of salts in the IO medium and in the ASW with 2.75% NaCl were similar (around 3.5%), persistence of *Hyphomicrobium* spp. occurred in the IO biofilm cultures. In fact, the concentrations of *H. nitrativorans* strain NL23 determined by qPCR
between the OB and the IO biofilm cultures was in the same level of magnitude. The lower proportion of 16S rRNA gene sequences associated to *Hyphomicrobium* spp. in the IO biofilm cultures compared to OB was a consequence of the substantial growth of *M. nitratireducenticrescens* in these cultures, with a 10-fold increase in concentration as revealed by qPCR (Table 2) (see Payette et al., 2019 for further discussion). Grob et al. (2015) also observed strong growth of *Methylophaga* spp. in their seawater samples that were fed with 100 µM methanol with relative proportions of 16S rRNA gene sequences raising from <0.5% at T=0 to 84% after 3 days.

Cultivating the OB in higher concentrations of NO$_3^-$ and methanol (64.3 mM/0.45%; C/N 1.5) and/or at 30°C (300N-30C, 900N-23C and 900N-30C biofilm cultures) resulted in increases of 20% to 85% in the specific denitrification rates compared to the Ref300N-23C biofilm cultures (Table 1) (Payette et al. 2019). Temperature was shown to be the main factor of these increases. However, raising the NO$_3^-$ and methanol concentrations or/and temperature in these cultures did not have an important impact on the bacterial community when compared to the Ref300N-23C biofilm cultures. Metatranscriptomic analysis of the Ref300N-23C and 900-30C biofilm cultures did not reveal either substantial changes in the gene expression profiles between these two cultures. The higher specific denitrification rates of these biofilm cultures could be related to higher metabolisms at the protein level such as the processing of NO$_3^-$ by the reductases and transporters.

Results from the 16S rRNA gene sequences showed that *Marinicella* spp. were present in the OB and all the biofilm cultures, and were the second most abundant bacterial population in the biofilm culture cultivated in ASW at 2.75% NaCl. These results concur with the metatranscriptomes of the biofilm cultures where *Marinicella* spp. had the relative transcript levels among the highest in the *de novo* assembled transcripts. *Marinicella* spp. are considered strict aerobic bacteria with no indication of NO$_3^-$ reduction (Romanenko et al. 2010) although a previous study reported high relative abundances of *Marinicella* spp. in anoxic sulfide oxidizing reactors in which nitrate was used as the electron acceptor (Huang et al. 2015). Genome annotations of two *Marinicella* strains (GenBank: *Marinicella* sp. F2 - assembly number ASM200005v1 and *M. litoralis* KMM 3900 - ASM259191v1) (Wang et al. 2018) did not reveal complete denitrification pathway, beside a nitric oxide reductase gene cluster also detected here in our metatranscriptomes. Together with the presence of *nirS* gene (Table 6), these results...
indicated that *Marinicella* spp. might have the capacity to use intermediates of the denitrification cycle to support their growth.

The 16S rRNA gene sequences provided evidence of the presence of *Pseudomonas* spp., *Marinobacter* spp., *Stappia* spp., *Paracoccus* spp. and *Aquamicrobium* spp. in the OB and in the biofilm cultures. Some species belonging to these genera were reported to carry denitrification. Isolates affiliated to the genera *Pseudomonas*, *Marinobacter* and *Paracoccus* were recovered from the Ref300N-23C biofilm cultures. The *Marinobacter* and *Paracoccus* isolates could perform denitrifying activities and grow under anoxic conditions, whereas the *Pseudomonas* isolate could only consume NO$_3^-$.

Although denitrification genes were found in several of the bacterial populations identified by the metatranscriptomic approach, only transcripts encoding the four denitrification reductases affiliated to *Stappia* spp. were found in the three examined biofilm cultures. The proportions of *Stappia* spp. in the 16S rRNA gene sequences of these biofilm cultures ranged between 0.09% and 0.42% (Table 2). *Stappia* spp. are chemoorganotrophic bacteria found in marine environments (Weber & King 2007) that can oxidize carbon monoxide. They possess the form I *coxL* gene encoding the large subunit of carbon monoxide (CO) dehydrogenase. Some also contain a gene for the large subunit of ribulose-1,5-bisphosphate carboxylase (RuBisCO) and may be able to couple CO utilization to CO$_2$ fixation (King 2003). A *coxL* gene was found in the *de novo* assembled transcripts affiliated to *Stappia* spp., but not RuBisCO. Sequences analogue to transporters for simple and multiple sugars such as xylose and fructose, and acetate were found (Supplemental doc 5, 6, 7), which suggest that the *Stappia* bacteria fed on the biofilm material for carbon sources. Combined with the isolation of denitrifying isolates affiliated to *Marinobacter* spp. and *Paracoccus* spp., these results suggest that the biofilm has the potential to adapt to heterotrophic non-methylotrophic environments.

The proportion of 16S rRNA gene sequences associated to *Bacteroidetes* in the OB and in the eight biofilm cultures ranged from 0.2% to 4.9%, and several genera of this phylum were identified in the three metatranscriptomes. Significant proportions of bacteria affiliated to the *Bacteroidetes* phylum were also found in other methanol-fed denitrification systems. For instance, 29% of cloned 16S rRNA gene sequences were affiliated to *Bacteroidetes* in an acclimated activated sludge in a methanol-fed anoxic denitrification process treating a synthetic wastewater with 4% NaCl (Osaka et al. 2008). Isolates affiliated to the *Bacteroidetes Aequorivita*
spp. and *Winogradskyella* spp. were isolated from the Ref300N-23C biofilm cultures. None of these two isolates, however, could sustain growth under denitrifying conditions. Although denitrification genes affiliated to *Bacteroidetes* genera were found in *de novo* assembled transcripts, genes encoding all four denitrification reductases were not found to any of them. These results suggest that *Bacteroidetes* are not involved in denitrification, although they may be involved in some steps of the denitrification pathway.

The metatranscriptomic data provided some insights of specific metabolisms in *H. nitrativorans* strain NL23 and *M. nitratireducenticrescens* strain GP59 that were regulated in the biofilm environment. In absence of strain NL23 in the Ref300C-23C and the 900N-30C biofilm cultures, the *nirK* gene of strain GP59 was upregulated by 5 to 10 times compared to the planktonic pure cultures that was also cultivated under denitrifying conditions. On the contrary, the relative transcript levels of this gene did not change between the 0% NaCl biofilm cultures and the planktonic pure cultures, while the relative transcript levels of the NL23 *nirK* were 49 times higher in the 0% NaCl biofilm cultures. These results suggest coordination in the expression of *nirK* between these two strains in the 0% NaCl biofilm cultures.

The gene clusters encoding the three other denitrification reductases (*nap, nor, nos*) in strain NL23 showed higher relative transcript levels in the 0% NaCl biofilm cultures. *napGH* was however down regulated in these biofilm cultures. *napGH* is located in a separate chromosomal region than the *napABCDEF* operon. NapGH and NapC have redundant function of transferring electrons to NapB across the membrane. It was proposed that NapC transfers electrons from the menaquinol, whereas NapGH do it from ubiquinol (Simon 2011). The physiological consequence of *napGH* transcript decrease in the biofilm is unknown.

Observations on *napEDABC* found in the denitrifier *Shewanella denitrificans* OS217, and *napDAGHB* in the respiratory NO\textsubscript{3}\textsuperscript{-} ammonifier *Shewanella oneidensis* MR-1 suggest that NapGH is more involved in the ammonification system (Simpson et al. 2010). Despite the denitrifying conditions applied in both types of cultures, the biofilm environment has induced strong up regulation of denitrification genes in *H. nitrativorans* strain NL23. This may be in response to the rapid processing of NO\textsubscript{3}\textsuperscript{-} by *M. nitratireducenticrescens* strain JAM1/GP59 (Mauffrey et al. 2015) that could generate rapidly high level of NO\textsubscript{2}\textsuperscript{-}, which is toxic for strain NL23.
Conclusion

The OB taken from the Biodome denitrification system underwent substantial changes in its bacterial community when subjected to environmental changes. Cultivating the OB in the homemade ASW medium with different formulations (varying NaCl, NO\textsubscript{3}\textsuperscript{-} and methanol concentrations, and temperature) or in the commercial IO medium showed much higher proportions of *Methylophaga* spp. in these biofilm cultures compared to the OB. These results concur with the growth of *M. nitratireducenticrescens* strain GP59 in these cultures. The population of *Hyphomicrobium* spp showed a more complex trend. It was not detected in the biofilm cultures cultivated in ASW at 2.75% NaCl, but persisted in the biofilm cultures cultivated in ASW at low NaCl concentration, and also cultivated in the IO medium. Other denitrifiers affiliated to *Marinobacter* spp. and *Paracoccus* spp. were isolated from the biofilm cultures. Moreover, metatranscriptomic analysis revealed that denitrifying bacteria affiliated to *Stappia* spp. were metabolically active in the biofilm cultures. The biofilm environment has favored the upregulation of the denitrification pathway in *M. nitratireducenticrescens* strain GP59 and *H. nitrativorans* strain NL23 compared to planktonic pure cultures, despite the facts that these two types of cultures were grown under denitrifying conditions. All these results demonstrated the dynamics and the plasticity of the denitrifying biofilm to sustain environmental changes and illustrate a comprehensive picture of the microbial community of the biofilm and its adaptation to these changes. This could benefit in the development of optimal denitrifying bioprocess under marine conditions. For instance, the fact that non-methylotrophic, denitrifying bacteria are present in the biofilm could suggest adaptation of denitrification process to another source of carbon such as ethanol or acetate.

Acknowledgements

We thank Karla Vasquez for her technical assistance.

References

Atlas RM. 1993. *Handbook of microbiological media. Ed. Parks L. C. CRC press Boca Raton, Fl, USA. pp 543.*
Auclair J, Lepine F, Parent S, and Villemur R. 2010. Dissimilatory reduction of nitrate in seawater by a *Methylophaga* strain containing two highly divergent *narG* sequences. *ISME Journal* 4:1302-1313. doi: 10.1038/ismej.2010.47

Auclair J, Parent S, and Villemur R. 2012. Functional diversity in the denitrifying biofilm of the methanol-fed marine denitrification system at the Montreal Biodome. *Microb Ecol* 63:726–735. doi: 10.1007/s00248-011-9960-2

Baytshtok V, Kim S, Yu R, Park H, and Chandran K. 2008. Molecular and biokinetic characterization of methylotrophic denitrification using nitrate and nitrite as terminal electron acceptors. *Water Science and Technology* 58:359-365. doi: 10.2166/wst.2008.391

Cabrol L, and Malhautier L. 2011. Integrating microbial ecology in bioprocess understanding: the case of gas biofiltration. *Appl Microbiol Biotechnol* 90:837-849. doi: 10.1007/s00253-011-3191-9

Chistoserdova L, Chen SW, Lapidus A, and Lidstrom ME. 2003. Methylotrophy in *Methylobacterium extorquens* AM1 from a genomic point of view. *J Bacteriol* 185:2980-2987. doi: 10.1128/Jb.185.10.2980-2987.2003

Chistoserdova L, Laukel M, Portais JC, Vorholt JA, and Lidstrom ME. 2004. Multiple formate dehydrogenase enzymes in the facultative methylotroph *Methylobacterium extorquens* AM1 are dispensable for growth on methanol. *J Bacteriol* 186:22-28.

Clark ME, He ZL, Redding AM, Joachimiak MP, Keasling JD, Zhou JZZ, Arkin AP, Mukhopadhyay A, and Fields MW. 2012. Transcriptomic and proteomic analyses of *Desulfovibrio vulgaris* biofilms: Carbon and energy flow contribute to the distinct biofilm growth state. *Bmc Genomics* 13. doi: 10.1186/1471-2164-13-138

Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, and Tiedje JM. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res* 42:D633-642. doi: 10.1093/nar/gkt1244

Edgar RC. 2013. Uparse: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 10:996+. doi: 10.1038/Nmeth.2604

Edgar RC, Haas BJ, Clemente JC, Quince C, and Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27:2194-2200. doi: 10.1093/bioinformatics/btr381
Geoffroy V, Payette G, Mauffrey F, Lestin L, Constant P, and Villemur R. 2018. Strain-level genetic diversity of *Methylophaga nitratireducenticrescens* confers plasticity to denitrification capacity in a methylotrophic marine denitrifying biofilm. *PeerJ* 6:e4679. doi: 10.7717/peerj.4679

Ginige MP, Hugenholtz P, Daims H, Wagner M, Keller J, and Blackall LL. 2004. Use of stable-isotope probing, full-cycle rRNA analysis, and fluorescence in situ hybridization-microautoradiography to study a methanol-fed denitrifying microbial community. *Appl Environ Microbiol* 70:588-596. doi: 10.1128/Aem.70.1.588-596.2004

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, and Regev A. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol* 29:644-652. doi: 10.1038/nbt.1883

Grob C, Taubert M, Howat AM, Burns OJ, Dixon JL, Richnow HH, Jehmlich N, von Bergen M, Chen Y, and Murrell JC. 2015. Combining metagenomics with metaproteomics and stable isotope probing reveals metabolic pathways used by a naturally occurring marine methylotroph. *Environ Microbiol* 17:4007-4018. doi: 10.1111/1462-2920.12935

Hallin S, Throback IN, Dicksved J, and Pell M. 2006. Metabolic profiles and genetic diversity of denitrifying communities in activated sludge after addition of methanol or ethanol. *Appl Environ Microbiol* 72:5445-5452. doi: 10.1128/AEM.00809-06

Huang C, Zhao YK, Li ZL, Yuan Y, Chen C, Tan WB, Gao S, Gao LF, Zhou JZ, and Wang AJ. 2015. Enhanced elementary sulfur recovery with sequential sulfate-reducing, denitrifying sulfide-oxidizing processes in a cylindrical-type anaerobic baffled reactor. *Bioresource Technology* 192:478-485. doi: 10.1016/j.biortech.2015.04.103

King GM. 2003. Molecular and culture-based analyses of aerobic carbon monoxide oxidizer diversity. *Appl Environ Microbiol* 69:7257-7265.

Kraft B, Strous M, and Tegetmeyer HE. 2011. Microbial nitrate respiration - Genes, enzymes and environmental distribution. *Journal of Biotechnology* 155:104-117. doi: 10.1016/j.jbiotec.2010.12.025
Labbé N, Juteau P, Parent S, and Villemur R. 2003. Bacterial diversity in a marine methanol-fed denitrification reactor at the Montreal Biodome, Canada. Microb Ecol 46:12-21. doi: 10.1007/s00248-002-1056-6

Labbé N, Laurin V, Juteau P, Parent S, and Villemur R. 2007. Microbiological community structure of the biofilm of a methanol-fed, marine denitrification system, and identification of the methanol-utilizing microorganisms. Microb Ecol 53:621-630. doi: 10.1007/s00248-006-9168-z

Labbé N, Parent S, and Villemur R. 2004. Nitratireductor aquibiodomus gen. nov., sp nov., a novel alpha-proteobacterium from the marine denitrification system of the Montreal Biodome (Canada). International Journal of Systematic and Evolutionary Microbiology 54:269-273. doi: 10.1099/ijs.0.02793-0

Li B, and Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12:323. doi: 10.1186/1471-2105-12-323

Lu HJ, Chandran K, and Stensel D. 2014. Microbial ecology of denitrification in biological wastewater treatment. Water Research 64:237-254. doi: 10.1016/j.watres.2014.06.42

Martineau C, Mauffrey F, and Villemur R. 2015. Comparative analysis of denitrifying activities of Hyphomicrobium nitrativorans, Hyphomicrobium denitrificans, and Hyphomicrobium zavarzinii. Appl Environ Microbiol 81:5003-5014. doi: 10.1128/Aem.00848-15

Martineau C, Villeneuve C, Mauffrey F, and Villemur R. 2013a. Complete genome sequence of Hyphomicrobium nitrativorans strain NL23, a denitrifying bacterium isolated from biofilm of a methanol-fed denitrification system treating seawater at the Montreal Biodome. Genome Announc 2: e01165-13. doi: 10.1128/genomeA.01165-13

Martineau C, Villeneuve C, Mauffrey F, and Villemur R. 2013b. Hyphomicrobium nitrativorans sp. nov., isolated from the biofilm of a methanol-fed denitrification system treating seawater at the Montreal Biodome. Int J Syst Evol Microbiol 63:3777-3781. doi: 10.1099/ijs.0.048124-0

Mauffrey F, Cucaita A, Constant P, and Villemur R. 2017. Denitrifying metabolism of the methylotrophic marine bacterium Methylophaga nitratireducentircrescens strain JAM1. Peerj 5:e4098. doi: 10.7717/Peerj.4098
Mauffrey F, Martineau C, and Villemur R. 2015. Importance of the two dissimilatory (Nar) nitrate reductases in the growth and nitrate reduction of the methylotrophic marine bacterium *Methylophaga nitratireducenticrescens* JAM1. *Front Microbiol* 6:1475.

Metsalu T, and Vilo J. 2015. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res* 43:W566-570. doi: 10.1093/nar/gkv468

Neef A, Zaglauer A, Meier H, Amann R, Lemmer H, and Schleifer KH. 1996. Population analysis in a denitrifying sand filter: Conventional and in situ identification of *Paracoccus* spp. in methanol-fed biofilms. *Appl Environ Microbiol* 62:4329-4339.

Osaka T, Shirotani K, Yoshie S, and Tsuneda S. 2008. Effects of carbon source on denitrification efficiency and microbial community structure in a saline wastewater treatment process. *Water Research* 42:3709-3718. doi: 10.1016/j.watres.2008.06.007

Osaka T, Yoshie S, Tsuneda S, Hirata A, Iwami N, and Inamori Y. 2006. Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing. *Microb Ecology* 52:253-266. doi: 10.1007/s00248-006-9071-7

Payette G, Geoffroy V, Martineau C, and Villemur R. 2019. Dynamics of a methanol-fed marine denitrifying biofilm: 1-Impact of environmental changes on the denitrification and the co-occurrence of *Methylophaga nitratireducenticrescens* and *Hyphomicrobium nitrativorans*. *PeerJ* submitted.

Peck V, Quiza L, Buffet JP, Khdhiri M, Durand AA, Paquette A, Thiffault N, Messier C, Beaulieu N, Guertin C, and Constant P. 2016. Towards the development of multifunctional molecular indicators combining soil biogeochemical and microbiological variables to predict the ecological integrity of silvicultural practices. *Microbial Biotechnology* 9:316-329. doi: 10.1111/1751-7915.12348

Peyraud R, Kiefer P, Christen P, Massou S, Portais JC, and Vorholt JA. 2009. Demonstration of the ethylmalonyl-CoA pathway by using C-13 metabolomics. *Proceedings of the National Academy of Sciences of the United States of America* 106:4846-4851. doi: 10.1073/pnas.0810932106

Philippot L, and Hojberg O. 1999. Dissimilatory nitrate reductases in bacteria. *Biochimica Et Biophysica Acta-Gene Structure and Expression* 1446:1-23. doi: 10.1016/S0167-4781(99)00072-X
Resch A, Rosenstein R, Nerz C, and Gotz F. 2005. Differential gene expression profiling of
Staphylococcus aureus cultivated under biofilm and planktonic conditions. Appl Environ
Microbiol 71:2663-2676. doi: 10.1128/Aem.71.5.2663-2676.2005

Richardson D, Berks B, Russell D, Spiro S, and Taylor C. 2001. Functional, biochemical and
genetic diversity of prokaryotic nitrate reductases. Cellular and Molecular Life Sciences
CMLS 58:165-178.

Rissanen AJ, Ojala A, Dernjatin M, Jaakkola J, and Tiirola M. 2016. Methylophaga and
Hyphomicrobium can be used as target genera in monitoring saline water methanol-
utilizing denitrification. Journal of Industrial Microbiology and Biotechnology 43:1647-
1657. doi: 10.1007/s10295-016-1839-2

Rissanen AJ, Ojala A, Fred T, Toivonen J, and Tiirola M. 2017. Methylophilaceae and
Hyphomicrobium as target taxonomic groups in monitoring the function of methanol-fed
denitrification biofilters in municipal wastewater treatment plants. Journal of Industrial
Microbiology and Biotechnology 44:35-47. doi: 10.1007/s10295-016-1860-5

Roder HL, Sorensen SJ, and Burmolle M. 2016. Studying Bacterial Multispecies Biofilms:
Where to Start? Trends Microbiol 24:503-513. doi: 10.1016/j.tim.2016.02.019

Romanenko LA, Tanaka N, Frolova GM, and Mikhailov VV. 2010. Marinicella litoralis gen.
nov., sp. nov., a gammaproteobacterium isolated from coastal seawater. Int J Syst Evol
Microbiol 60:1613-1619. doi: 10.1099/ijs.0.016147-0

Salta M, Wharton JA, Blache Y, Stokes KR, and Briand JF. 2013. Marine biofilms on artificial
surfaces: structure and dynamics. Environ Microbiol 15:2879-2893. doi: 10.1111/1462-
2920.12186

Simon J. 2011. Organization of respiratory electron transport chains in nitrate-reducing and
nitrifying bacteria. In: Moir JWB, ed. Nitrogen cycling in bacteria Molecular analysis
Caister academic press, Norfolk, UK. pp 39-58.

Simpson PJ, Richardson DJ, and Codd R. 2010. The periplasmic nitrate reductase in Shewanella:
the resolution, distribution and functional implications of two NAP isoforms,
NapEDABC and NapDAGHB. Microbiology 156:302-312. doi: 10.1099/mic.0.034421-0

Sun Y, Shen D, Zhou X, Shi N, and Tian Y. 2016. Microbial diversity and community structure
of denitrifying biological filters operated with different carbon sources. Springerplus
5:1752. doi: 10.1186/s40064-016-3451-3
Tan CH, Lee KW, Burmoller M, Kjelleberg S, and Rice SA. 2017. All together now: experimental multispecies biofilm model systems. *Environ Microbiol* 19:42-53. doi: 10.1111/1462-2920.13594

Tarazona S, Garcia-Alcalde F, Dopazo J, Ferrer A, and Conesa A. 2011. Differential expression in RNA-seq: A matter of depth. *Genome Research* 21:2213-2223. doi: 10.1101/gr.124321.111

Team RC. 2015. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Villeneuve C, Martineau C, Mauffrey F, and Villemur R. 2012. Complete genome sequences of *Methylophaga* sp. strain JAM1 and *Methylophaga* sp. strain JAM7. *J Bacteriol* 194:4126-4127. doi: 10.1128/Jb.00726-12

Villeneuve C, Martineau C, Mauffrey F, and Villemur R. 2013. *Methylophaga nitratireducenti crescens* sp. nov. and *Methylophaga frappieri* sp. nov., isolated from the biofilm of the methanol-fed denitrification system treating the seawater at the Montreal Biodome. *Int J Syst Evol Microbiol* 63:2216-2222. doi: 10.1099/ijsem.0.044545-0

Wang XQ, Li CM, Dunlap CA, Rooney AP, and Du ZJ. 2018. *Marinicella sediminis* sp. nov., isolated from marine sediment. *International Journal of Systematic and Evolutionary Microbiology* 68:2335-2339. doi: 10.1099/ijsem.0.002839

Weber CF, and King GM. 2007. Physiological, ecological, and phylogenetic characterization of *Stappia*, a marine CO-oxidizing bacterial genus. *Appl Environ Microbiol* 73:1266-1276. doi: 10.1128/AEM.01724-06

Wright ES, Yilmaz LS, and Noguera DR. 2012. DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. *Appl Environ Microbiol* 78:717-725. doi: 10.1128/AEM.06516-11

Yoshie S, Ogawa T, Makino H, Hirosawa H, Tsuneda S, and Hirata A. 2006. Characteristics of bacteria showing high denitrification activity in saline wastewater. *Lett Appl Microbiol* 42:277-283. doi: 10.1111/j.1472-765X.2005.01839.x
Figure 1

Proportion of affiliated OTUs in the biofilm cultures

Panel A. Bacterial composition of OB and the IO biofilm cultures was determined by sequencing the V6-V7-V8 variable regions of the 16S rRNA gene by Illumina, whereas the other samples were determined by sequencing the V1-V2-V3 variable regions by pyrosequencing. Panel B. Principal component analysis of the bacterial profiles of the biofilm cultures and the OB listed in Table 2.
Relative expression profiles of *M. nitratireducenticrescens* GP59 and *H. nitrativorans* NL23 in biofilm cultures

All the deduced amino acid sequences associated to the genome and plasmids of strain GP59 and the genome of strain NL23 were submitted to the BlastKOALA (genome annotation and KEGG mapping) at the Kyoto encyclopedia of genes and genomes (KEGG). Genes associated to specific metabolisms were sorted out and the corresponding ratio of the Biofilm Transcripts Per Million (TPM) versus the pure culture TPM was derived. When the ratios were <1, the negative inverse value (-1/ratio) was calculated. Data are expressed as the percentage of genes in each category that are more expressed in the biofilm cultures (right, 2 to 5 times, and > 5 times) or in pure cultures (left, -2 to -5 times and > -5 times). Number within parentheses is the number of genes involved in the selected pathways. Other metabolic profiles are detailed in Figures S3 and S4.
Figure 3

Relative gene expression profiles of selected metabolic pathways of *M. nitratireducencrescens* GP59 in the biofilm cultures

The pathways are based on functions deduced by the annotations (provided by KEGG BlastKoala, RAST and GenBank). The arrow thickness is proportional to the value of the ratio of the Biofilm TPM divided by the planktonic pure-culture TPM. The blue arrows represent genes with at least 2-fold lower relative transcript levels in the biofilm cultures. The red arrows represent genes with at least 2-fold higher relative transcript levels in the biofilm cultures. The black arrows represent no changes between both types of cultures in the relative transcript levels. The two-component systems and the transporters that are illustrated in blue are encoded by strains GP59 and NL23. See Supplemental doc 9 and 10 for gene description.
Figure 4

Relative gene expression profiles of selected metabolic pathways of *H. nitrativorans* strain NL23 in the 0% NaCl biofilm cultures

See Figure 3 legend.
Figure 5

Hierarchical clustering of the selected bacterial taxa in biofilm culture metatranscriptomes

Heatmap represents differences in the overall gene expression patterns (expressed as TPM; from Table 6) (log$_{10}$ [TPM by geometric average of TPM]) between the three biofilm cultures for the respective bacterial taxa. Analysis was performed at ClustVis web site (https://biit.cs.ut.ee/clustvis/) (Metsalu and Vilo, 2015).
Manuscript to be reviewed
**Table 1** (on next page)

Biofilm culture conditions
| Name       | Medium | NO₃⁻ (%) | Methanol | NaCl (%) | Temp | Specific denitrification rates |
|------------|--------|----------|----------|----------|------|-------------------------------|
| Ref300N-23C | ASW    | 21.4 (300) | 0.15     | 2.75     | 23   | 0.0530                        |
| 300N-30C    | ASW    | 21.4 (300) | 0.15     | 2.75     | 30   | 0.0946                        |
| 900N-23C    | ASW    | 64.3 (900) | 0.45     | 2.75     | 23   | 0.0637                        |
| 900N-30C    | ASW    | 64.3 (900) | 0.45     | 2.75     | 30   | 0.0979                        |
| 0%NaCl     | ASW    | 21.4 (300) | 0.15     | 0        | 23   | 0.0911                        |
| 0.5%NaCl   | ASW    | 21.4 (300) | 0.15     | 0.5      | 23   | 0.0712                        |
| 1%NaCl     | ASW    | 21.4 (300) | 0.15     | 1.0      | 23   | 0.0357                        |
| IO         | IO     | 21.4 (300) | 0.15     | 3.0d     | 23   | 0.0611                        |

The original biofilm was cultured in triplicates in these conditions at pH 8.0. The carriers were transferred 5 times in fresh medium around each week before measuring the denitrifying activities.

a From Payette et al. (2019).
b Reference biofilm cultures.
c The C/N ratio was 1.5 in all biofilm cultures.
d The exact amount of NaCl added in the IO medium is not known. See Payette et al. (2019) for the IO composition. For comparison, the amount of Na⁺ and Cl⁻ in the ASW medium is 3.2%.
In gray are changed parameters from the reference biofilm cultures.

IO: Instant Ocean medium.
Table 2 (on next page)

Most probable affiliation of 16S rRNA gene sequences in the biofilm cultures and the original biofilm
Table 2. Most probable affiliation of 16S rRNA gene sequences in the biofilm cultures and the original biofilm

| Affiliation | Biofilm cultures (proportion %) | OB | IO | Ref300N-23C | 900N-23C | 300N-30C | 900N-30C | 0%NaCl | 0.5%NaCl | 1.0%NaCl |
|------------|---------------------------------|----|----|-------------|----------|----------|----------|--------|---------|---------|
| Ignavibacteriae; Igavibacteriales; Ignavibacterium | 0.37 | 0.13 | 0.01 | 0.19 | 0.06 | | | | | |
| Tenericutes; Acholeplasmatales; Acholeplasma | 0.05 | 0.04 | 0.05 | 0.04 | 0.52 | 0.02 | | | | |
| Bacteroidetes; Flavobacteriales; Aequorivita | 0.01 | 0.18 | 0.10 | 0.14 | 0.04 | 0.01 | 0.11 | 0.22 | | |
| " " Muricauda | 0.06 | 0.08 | 0.01 | 0.01 | 0.10 | | | | | |
| " " Winogradskyella | 0.28 | 4.28 | 0.10 | 0.11 | 0.15 | 0.11 | 0.20 | 0.11 | | |
| Proteobacteria; Alphaproteobacteria; Rhizobiales; Aminobacter | 0.33 | 0.01 | 0.05 | 0.22 | | | | | | |
| " " Aquamicrobium* | 11.6 | 0.46 | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
| " " Hoeflea | 0.30 | 0.76 | 0.03 | 0.01 | 0.12 | 0.06 | | | | |
| " " Nitratireductor | 1.40 | 0.03 | | | | | | | | |
| " " Hyphomicrobiu* | 45.8 | 8.40 | 10.8 | 6.78 | 0.25 | | | | | |
| H. nitrativorans NL23, qPCR (cp napA/ng) | 8.7(7.2)*10^4 | 7.0(4.3)*10^4 | 1.8(1.8)*10^4 | 6.0(3.2)*10^4 | 1.3(0.5)*10^4 | 1.3(0.9)*10^4 | 2.8(1.0)*10^4 | 5.3(6.5)*10^4 | 1.1(0.9)*10^4 | | |
| Alphaproteobacteria; Rhodobacterales; Litorisediminico | 0.38 | 1.43 | | | | | | | | |
| " " Lutimarinibacter | 1.71 | 8.93 | | | | | | | | |
| " " Marinovum | 1.28 | 1.62 | | | | | | | | |
| " " Maritimibacter | 2.50 | 2.44 | 0.01 | 0.01 | | | | | | |
| " " Oceanicola | 0.06 | 0.01 | 0.08 | 0.03 | 0.01 | | | | | |
| " " Paracoccus* | 0.01 | 0.07 | 0.01 | 0.02 | | | | | | |
| " " Roseovarius | 7.32 | 8.18 | | | | | | | | |
| " " Stappia* | 0.02 | 1.26 | 0.09 | 0.43 | 0.27 | 0.42 | 0.27 | 0.35 | 0.17 | | |
| Alphaproteobacteria; Rhodospirillales Oceanibaculum | 12.3 | 7.36 | 0.03 | 0.01 | 0.01 | 0.20 | 0.04 | | | | |
| Proteobacteria; Gammaproteobacteria; Alteromonadales Marinobacter* | 0.15 | 0.05 | 0.06 | 0.02 | 0.01 | 0.01 | | | | |
| " " Oceanospirillales Marinicella | 0.78 | 7.24 | 1.46 | 1.50 | 3.40 | 5.02 | 0.23 | 1.55 | 0.94 | | |
| " " Pseudomonadales Pseudomonas* | 0.03 | 0.11 | 0.04 | 0.13 | 0.32 | 0.64 | 0.80 | 0.05 | | | |
| " " Thiotrichales Methylophaga | 3.50 | 42.8 | 97.7 | 97.1 | 95.1 | 91.8 | 85.7 | 88.3 | 97.9 | | |
| M. nitratireducenticrescens, qPCR (cp narG1/ng) | 5.6(3.2)*10^4 | 5.8(4.6)*10^4 | 2.3(0.6)*10^4 | 1.9(0.5)*10^4 | 2.2(1.4)*10^4 | 2.1(1.4)*10^4 | 4.7(2.0)*10^4 | 5.3(6.5)*10^4 | 1.1(0.9)*10^4 | | |
| Others | 9.96 | 4.77 | 0.25 | 0.37 | 0.53 | 2.00 | 2.28 | 0.69 | 0.25 | | |
| Total of reads | 348358 | 312965 | 9810 | 14048 | 8532 | 13815 | 13800 | 9375 | 12560 | | |

The V1-V3 regions of the 16S rRNA gene sequences were amplified and sequenced by pyrosequencing except for the OB and IO samples from which the V6-V8 regions were sequenced by Illumina. (see supplemental document 2 for complete analysis and sequences). OB: Original biofilm.

In grey: qPCR results are from Payette et al. (2019), with standard deviation values under parentheses of triplicate cultures. narG1 used in qPCR for M. nitratireducenticrescens cannot discriminate strain JAM1 and strain GP59 (identical sequences between the two).

* Identified genus with species that were reported involved in denitrification.
Table 3 (on next page)

16S Operational Taxonomic Units (OTUs) affiliated to *Hyphomicrobium* spp. and *Methylophaga* spp. in the OB and the IO biofilm cultures
Table 3: 16S Operational Taxonomic Units (OTUs) affiliated to *Hyphomicrobium* spp. and *Methylophaga* spp. in the OB and the IO biofilm cultures

| Sequence identity with               | Number of OTUs | Number reads | Proportion of reads % |
|-------------------------------------|----------------|--------------|-----------------------|
|                                     |                | OB           | IO                    |
| **H. nitrativorans**                |                |              |                       |
| Cluster 1 95-100%                   | 12             | 147209       | 26239                 | 92.3 | 97.8 |
| Cluster 2 91-97%                    | 5              | 10779        | 530                   | 6.8  | 2.0  |
| Cluster 3 89-92%                    | 3              | 1537         | 51                    | 0.96 | 0.19 |
| **M. nitratireducenticrescens**     |                |              |                       |
| Cluster 1 94-100%                   | 18             | 11809        | 136197                | 96.4 | 99.7 |
| Cluster 2 92.9-93.6%                | 3              | 321          | 284                   | 2.62 | 0.21 |
| Cluster 3 93.4-94.1%                | 3              | 115          | 65                    | 0.94 | 0.05 |

Cluster classification is based on phylogenic analyses of the 16S rRNA gene sequences affiliated to *H. nitrativorans* and *M. nitratireducenticrescens* (see Tables S1 and S2, and Figures S2A and S2b).
**Table 4**

Affiliation of the isolates isolated from the reference biofilm cultures (Ref300N-23C)
| Isolates                          | Denitrifying activities* |
|---------------------------------|--------------------------|
| *Alphaproteobacteria, Rhodobacterales* |                          |
| *Rhodobacteraceae* GP11          | no                       |
| *Paracoccus* sp. GP3, GP8, GP20   | Full                     |
| *Roseovarius* sp. GP9, GP10, GP13, GP14 | no                   |
| *Roseovarius* sp. GP12           | no                       |
| *Thalassobius* sp. GP19          | no                       |
| *Gammaproteobacteria*            |                          |
| *Marinobacter* sp. GP1, GP2, GP24 | Full                     |
| *Pseudomonas* GP41               | Nitrate                  |
| *Bacteroidetes, Flavobacterales* |                          |
| *Aequorivita* sp. GP15           | no                       |
| *Winogradskyella* sp. GP16, GP18 | no                       |
| *Bacillales*                     |                          |
| *Exiguobacterium* sp. GP46       | no                       |

* Full: nitrate and nitrite are consumed. Nitrate: only nitrate was consumed
Table 5 (on next page)

Changes in the relative transcript levels of genes involved in the nitrogen metabolism in strain GP59 and strain NL23
Table 5: Changes in the relative transcript levels of genes involved in the nitrogen metabolism in strain GP59 and strain NL23

| Genes       | Ref300N-23C | 900N-30C | 0%NaCl | 0%NaCl | Genes       |
|-------------|-------------|----------|--------|--------|-------------|
| NarXL       | ns          | ns       | ns     | -9.4   | napGH       |
| NarK1K2GHJI-1 | ns         | -7.4     | 2.1    | 8.2    | napEFDABC   |
| NarK12      | ns          | ns       | 2.0    |        |             |
| NarGHJI-2   | 2.7         | 2.8      | 3.0    |        |             |
| NirK        | 11          | 4.7      | ns     | 49     | nirKV       |
| NorCBDQ-1   | ns          | ns       | -2.7   | 5.9    | norCBQDE    |
| NorRE       | ns          | ns       | -3.1   |        |             |
| NorCBQD-2   | 2.4         | 3.8      | 2.8    |        |             |
| NosRZDFYLYL | ns          | ns       | ns     | 5.2    | nosRZDFYLYL |
| MnrS (3)    | ns          | ns       | ns     |        | MnrS (2)    |
| NsrR (2)    | 2.1         | 2.5      | ns     |        | NsrR        |
| DnrN        | 2.3         | 2.8      | -3.0   | ns     | MnrU        |
| Nodiox (2)  | ns          | ns       | ns     | 4.0    | MnrR        |
| Nitrogen assimilatory pathway | ns | nasTS |
| NO3/NO2 trp | 6.0         | 7.2      | 17.9   | 7.8    | ntrABC      |
| NasAnirBD   | 12          | 24       | 42     | 3.3    | nirBAnasA   |
| NtrYX       | ns          | ns       | ns     | ntrYX  |
| GlnA        | 2.0         | 3.1      | 5.1    | 19     | GlnA        |
|             | -2.1        |          |        |        | GlnA (3)    |
| GltBD: GOGAT| ns          | ns       | ns     |        | GltBD       |
| GlnB        | ns          | ns       | ns     | 14     | GlnB        |
| Gdh         | ns          | ns       | ns     |        | Gdh         |
| GlnD        | ns          | ns       | ns     | -2.9   | GlnD        |
| GlnE        | ns          | ns       | ns     | -3.2   | GlnE        |
| GlnK        | 18          | 24       | 25     | 22     | GlnK        |
| NHa trp     | 11          | 11       | 11     | 20     | NHa-trp     |
| GlnLG       | ns          | 2.1      | 5.9    |        | GlnLG       |

Data values are the ratio of the Biofilm TPM divided by the planktonic pure-culture TPM. When the ratios were <1, the negative inverse value (-1/ratio) was calculated. Compared to each other, the positive ratio means higher relative transcript levels in the biofilm, and negative ratio higher relative transcript level in the planktonic pure cultures. ns: no significant changes.

GlnA: glutamine synthetase; GltBD: glutamate synthase; GDH: glutamate dehydrogenase; GlnB: nitrogen regulatory protein P-II 1; GlnK: nitrogen regulatory protein P-II 2; GlnD: uridylyltransferase; GlnLG: nitrogen regulation sensor histidine kinase and response regulator; trp: transporter; Diox: dioxygenase; NnrS: involved in response to NO; NsrR: NO-sensitive transcriptional repressor; DnrN: NO-dependent regulator; nasAnasBD: assimilatory nitrate and nitrite reductase; ntrXY: Nitrogen regulation proteins; NarXL: nitrate/nitrite sensor histidine kinase and response regulator; NarK: nitrate/nitrite transporter; NosRE: NO- reductase transcription regulator and activation protein.
Table 6 (on next page)

Microbial diversity and the associated denitrification genes in the biofilm cultures from the *de novo* transcript assembly
### Table 6. Microbial diversity and the associated denitrification genes in the biofilm cultures from the de novo transcript assembly

| Affiliation           | Biofilm cultures (TPM) | Denitrification genes                  |
|-----------------------|------------------------|----------------------------------------|
|                       | Ref300N-23C  | 900N-30C  | 0%NaCl |                       |
| Actinobacteria        |                       |           |        | norB                   |
| Streptomyces          | 6103                  | 729       | 2499   |                       |
| Bacteroidetes         |                       |           |        |                       |
| Sunxiuqinia            | 212                   | 504       | 2639   |                       |
| Geofilum              | 77                    | 109       | 11870  |                       |
| Acquorivita            | 18218                 | 25569     | 1398   | nirK, norB;C;D;Q, nosZ;DFY |
| Winogradskyella        | 8182                  | 7917      | 169    | nirK, norC, nosZ;L;D  |
| Lentimicrobiun         | 3032                  | 3370      | 1671   |                       |
| Xanthomarina           | 190                   | 4555      | 9598   | nirK, norB;D;C;Q, nosZ;L;D |
| Ignavibacterales       | 5513                  | 14206     | 171    | napC;H                 |
| Tenericutes            | 6367                  | 5003      | 13409  |                       |
| Alphaproteobacteria    |                       |           |        |                       |
| Aminobacter            | 18                    | 438       | 5385   | napA                   |
| Aquamicrobium          | 23                    | 456       | 22835  | napA                   |
| Hoeflea                | 3882                  | 87        | 1365   | narG;H;I, nosZ;R;D, nirK |
| Hyphomicrobium         | 0                     | 1679      | 30057  | narH;I, norB;C;D;E, nosZ;R;F |
| Mesorhizobium          | 411                   | 1050      | 12505  | napA;D;E, nirK, nosZ  |
| Paracoccus             | 1427                  | 1219      | 1204   | nirS, narC;D;Q, nosR  |
| Roseovarius            | 12367                 | 16479     | 686    | nirS, nirK, norB;C;D;E, nosZ;D;R |
| Stappia               | 39926                 | 30885     | 39745  | napABC, napADFE-nirS, nirK, norCBQD, nosRZDF;E |
| Maritimibacter         | 6363                  | 8360      | 10000  | narI                   |
| Oceanibaculum          | 18115                 | 9071      | 25544  | narG;H;J;I             |
| Betaproteobacteria     |                       |           |        |                       |
| Azoarcus              | 48                    | 120       | 2854   | napA, nosZ             |
| Deltaproteobacteria    |                       |           |        |                       |
| Bradymonas            | 73                    | 9652      | 11178  |                       |
| Gammaproteobacteria    |                       |           |        |                       |
| Marinicella            | 36554                 | 125466    | 14924  | nirS, norB;C;Q;D      |
| Marinobacter           | 14545                 | 1122      | 264    | narG;H;J;I, norB;C;Q, nirK, nirS, nosZ;F;D;R |
| Methylphaga            | 64802                 | 78566     | 80482  | narG;H;J;I, norB;C;D;Q;E, nosD;R |
| Idiomarina             | 5147                  | 1027      | 513    | narG;H;J nirK, nirS, norB |
| Pseudomonas            | 6175                  | 1333      | 18198  | narG;H;J, nirK, nirS, norB;C;D;Q, nosZ;R, nosRZDF;E |
| Wenzhouxiangella       | 132                   | 2857      | 83     |                       |
| Other bacteria         | 113200                | 243523    | 155554 |                       |
| Archaea                | 113                   | 297       | 303    |                       |
| Eukaryota              | 181                   | 352       | 416    |                       |
| Phages, viruses, plasmids | 99623              | 5740     | 217095 |                       |
| Unclassified           | 281015                | 207759    | 157515 |                       |
| Transcripts with no genes | 248782            | 196062    | 148792 |                       |

Reads that did not align to the three reference genomes were de novo assembled. These reads were then aligned to these assembled sequences. The relative transcript levels of the assembled sequences in a metatranscriptome were expressed as transcripts per million (TPM). Putative genes from the assembled
sequences were annotated for function and affiliation. The TPM of the genes affiliated to specific bacteria taxa were then summed. Denitrification genes identified by annotations from respective affiliated bacterial taxa were sorted out. * Denitrification genes were found scattered in other bacterial taxa.