Dynamics of a methanol-fed marine denitrifying biofilm: 2- Impact of environmental changes on the microbial community

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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-methylotrophic environments.

1 Dynamics of a methanol-fed marine denitrifying biofilm: 2- Impact of

2 environmental changes on the microbial community

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17   **Abstract**

19   **Background**. The biofilm of a methanol-fed, marine denitrification system is composed of a

20   multi-species microbial community, among which *Hyphomicrobium nitrativorans* and

21   *Methylophaga nitratireducenticrescens* are the principal bacteria involved in the denitrifying

22   activities. To assess its resilience to environmental changes, the biofilm was cultivated in

23   artificial seawater (ASW) under anoxic conditions and exposed to a range of specific

24   environmental conditions. We previously reported the impact of these changes on the

25   denitrifying activities and the co-occurrence of *H. nitrativorans* strain NL23 and *M.*

26   *nitratireducenticrescens* in the biofilm cultures. Here, we report the impact of these changes on

27   the dynamics of the overall microbial community of the denitrifying biofilm.

29   **Methods**. The original biofilm (OB) taken from the denitrification system was cultivated in

30   ASW under anoxic conditions with a range of NaCl concentrations, and with four combinations

31   of nitrate/methanol concentrations and temperatures. The OB was also cultivated in the

32   commercial Instant Ocean seawater (IO). The bacterial diversity of the biofilm cultures and the

33   OB was determined by 16S ribosomal RNA gene sequences. Culture approach was used to

34   isolate other denitrifying bacteria from the biofilm cultures. The metatranscriptomes of selected

35   biofilm cultures were derived, along with the transcriptomes of planktonic pure cultures of *H.*

36   *nitrativorans* strain NL23 and *M. nitratireducenticrescens* strain GP59.

38   **Results**. High proportions of *M. nitratireducenticrescens* occurred in the biofilm cultures. *H.*

39   *nitrativorans* strain NL23 was found in high proportion in the OB, but was absent in the biofilm

40   cultures cultivated in the ASW medium at 2.75% NaCl. It was found however in low proportions

41   in the biofilm cultures cultivated in the ASW medium at 0 to 1% NaCl and in the IO biofilm

42   cultures. Denitrifying bacterial isolates affiliated to *Marinobacter* spp. and *Paracoccus* spp. were

43   isolated. Up regulation of the denitrification genes of strains GP59 and NL23 occurred in the

44   biofilm cultures compared to the planktonic pure cultures. Denitrifying bacteria affiliated to the

45   *Stappia* spp. were metabolically active in the biofilm cultures.

47   **Conclusions.** These results illustrate the dynamics of the microbial community in the

48   denitrifying biofilm cultures in adapting to different environmental conditions. The NaCl

49   concentration is an important factor affecting the microbial community in the biofilm cultures.

50   Up regulation of the denitrification genes of *M. nitratireducenticrescens* strain GP59 and *H.*

51   *nitrativorans* strain NL23 in the biofilm cultures suggests different mechanisms of regulation of

52   the denitrification pathway in the biofilm. Other denitrifying heterotrophic bacteria are present in

53   low proportions, suggesting that the biofilm has the potential to adapt to heterotrophic, non-

54   methylotrophic environments.

Introduction

55

56   Most naturally-occurring microbial biofilms, such as those encountered in bioremediation

57   processes, are composed of multiple microbial species. Studying such complex biofilms is a

58   challenge, as each species can influence the biofilm development. The biofilm microbial

59   community inside a bioremediation process adapts to the prescribed operating conditions and

60   shapes the efficiency of the bioprocess to degrade the pollutant(s). Usually, the microbial

61   community in such bioprocesses is complex and composed of main degraders but also of

62   secondary microorganisms that could provide benefits to the degraders or could simply

63   contribute to the degradation intermediates or waste. It is recognized that a complex microbial

64   community is more resilient to "unexpected" changes in the operation of the bioprocesses than a

65   single species biofilm, as some of the minor degraders take over the main degraders affected by

66   the changes (Cabrol & Malhautier 2011; Roder et al. 2016; Salta et al. 2013; Tan et al. 2017).

67   The mechanisms of how a microbial population in a biofilm adapts to changes however are

68   poorly understood.

69   The Montreal Biodome, a natural science museum, operated a continuous fluidized-bed

70   methanol-fed denitrification reactor to remove nitrate (NO_3^-) that accumulated in the 3 million-L

71   seawater aquarium. The fluidized carriers in the denitrification reactor were colonized by

72   naturally-occurring multispecies microorganisms to generate a marine methylotrophic

73   denitrifying biofilm estimated to be composed of 15-20 bacterial species (Labbé et al. 2003). The

74   main bacteria responsible of the denitrifying activities belong to the alphaproteobacteria

75   *Hyphomicrobium nitrativorans* (strain representative NL23) and to the gammaproteobacteria

76   *Methylophaga nitratireducenticrescens* (strain representative JAM1), both methylotrophs, that

77   accounted for 60-80% of the biofilm (Labbé et al. 2003; Labbé et al. 2007; Martineau et al.

78   2013b; Villeneuve et al. 2013).

79   Denitrification takes place in bacterial cells where N oxides serve as terminal electron

80   acceptor instead of oxygen (O_2) for energy production when oxygen depletion occurs, leading to

81   the production of gaseous nitrogen (N_2). Four sequential reactions are strictly required for the

82   reduction of NO_3^- to gaseous nitrogen, via nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide

83   (N_2O), and each of these reactions is catalyzed by different enzymes, namely NO_3^- reductases

84   (Nar and Nap), NO_2^- reductases (NirS and NirK), NO reductases (Nor) and N_2O reductases (Nos)

85   (Kraft et al. 2011; Philippot & Hojberg 1999; Richardson et al. 2001). Whereas *H. nitrativorans*

86   strain NL23 possesses the four reductases for the complete denitrification pathway, *M.*

87   *nitratireducenticrescens* strain JAM1 performs incomplete denitrifying activities, as it lacks a

88   dissimilatory NO-forming nitrite reductase (Auclair et al. 2010; Martineau et al. 2013a; Mauffrey

89   et al. 2017; Mauffrey et al. 2015; Villeneuve et al. 2012). Using degenerated PCR primers for the

90   detection of denitrification genes, we showed that there are probably other denitrifying bacteria

91   in the biofilm, one to four orders of magnitude lower in proportions than *M.*

92   *nitratireducenticrescens* strain JAM1 and *H. nitrativorans* strain NL23 (Auclair et al. 2012).

93   These other bacteria may play a role if the bioprocess undergoes stress conditions or changes in

94   the operation mode.

95   We have initiated a study with the aim of assessing the performance of the Biodome

96   denitrifying biofilm subjected to environmental changes. The original biofilm (OB) taken from

97   the Biodome denitrification system was cultivated in an artificial seawater (ASW) under batch

98   mode and anoxic conditions at laboratory scale and exposed to a range of specific physico-

99   chemical parameters. Such parameters included a range of NaCl, NO_3^-, NO_2^- and methanol

100   concentrations, and varying pH and temperatures. These parameters were chosen as possible

101   factors that could affect a denitrification reactor. Thus, the objectives of this study were to

102   determine the impact of these changes: 1) on the denitrification performance of the biofilm; 2) on

103   the dynamics of the co-occurrence of *H. nitrativorans* and *M. nitratireducenticrescens* in the

104   biofilm; and 3) on the overall microbial community. The fourth objective of the study was to

105   determine whether denitrifying bacteria other than *H. nitrativorans* strain NL23 and *M.*

106   *nitratireducenticrescens* strain JAM1 are present in the biofilm.

107   Results for the first two objectives and partially the fourth objectives were reported by

108   Geoffroy *et al.* (2018) and Payette *et al.* (2019). We showed that the denitrifying biofilm can

109   sustain denitrifying activities in most of the tested conditions. Inhibition occurred when these

110   biofilm cultures were exposed to high pH (10) or to high methanol concentration (1.5%). The

111   highest specific denitrification rates occurred when the biofilm cultures were cultivated at 64.3

112   mM NO_3^- and 0.45% methanol (C/N = 1.5), and at 30°C. Poor biofilm development occurred in

113   biofilm cultures cultivated at 5% and 8% NaCl. We also showed that the NaCl concentrations in

114   the ASW medium have significant impacts on the population of *H. nitrativorans* strain NL23,

115   with its displacement by a subpopulation of the species *M. nitratireducenticrescens* (strain GP59

116   as representative), which can perform the complete denitrification pathway.

117   Results for the third and fourth objectives are presented here. The composition of the

118   bacterial community of the different biofilm cultures was determined by sequencing the 16S

119   ribosomal RNA (rRNA) genes. A culture dependent approach was used to recover new

120   denitrifying bacterial isolates from the biofilm cultures. To complement these two objectives, we

121   derived the metatranscriptome from selected biofilm cultures. These metatranscriptomes were

122   analyzed to determine the composition of the active microbial community in the biofilm cultures

123   but also to assess their metabolic contributions, such as those involved in denitrification.

124   Furthermore, metatranscriptomic analyses provided further indications on the dynamics of *H.*

125   *nitrativorans* and *M. nitratireducenticrescens* in these cultures (second objective) by assessing

126   changes in metabolic pathways of *H. nitrativorans* strain NL23 and *M. nitratireducenticrescens*

127   strain GP59 between the planktonic pure cultures and the biofilm cultures. Our study is the first

128   that give a comprehensive picture of the microbial community of a methylotrophic denitrifying

129   biofilm and its adaptation to specific changes.

130

131   **Material and Methods**

132

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

134   The formulations of the artificial seawater (ASW) medium and the commercial Instant

135   Ocean (IO) medium (Table S1), and the different conditions of the biofilm cultures were

136   described by Payette *et al.* (2019). Briefly, the biomass of several carriers taken from the

137   denitrification reactor of the Montreal Biodome was scraped, dispersed, then distributed to

138   several vials containing twenty free carriers and 60 mL prescribed medium (Table 1; Fig. S1).

139   The vials were incubated under anoxic conditions at 23°C or 30°C (Table 1) and shaken at 100

140   rpm (orbital shaker). In average once a week, the twenty carriers were taken, gently washed to

141   remove the excess medium and the planktonic bacteria, then transferred into fresh anoxic

142   medium and incubated under the same conditions (Fig. S1). The Ref300N-23C biofilm cultures

143   (for 300 mg NO_3^--N/L, 23°C) were defined as the *reference biofilm cultures*. These cultures were

144   used by Payette *et al.* (2019) as a reference to compare results between the different culture

145   conditions. The protocols to measure NO_3^- and NO_2^- concentrations, and to extract DNA from

146   the biofilm cultures or the planktonic pure cultures were described in Payette *et al.* (2019) and

147   Geoffroy *et al.* (2018).

148

16S rRNA gene analysis

150   DNA extracted from triplicate biofilm cultures was pooled before sequencing. Total DNA

151   samples from seven biofilm cultures (Table 1; Ref300N-23C, 300N-30C, 900N-23C, 900N-30C,

152   0%NaCl, 0.5%NaCl and 1.0%NaCl) were sent to the sequencing service at the Research and

153   Testing Laboratory (RTL, Lubbock, Texas, USA). A region of the 16S rRNA gene was PCR

154   amplified using the 28F-519R primers (5' GAGTTTGATCNTGGCTCAG 3' and 5'

155   GTNTTACNGCGGCKGCTG 3', covering the V1-V2-V3 variable regions) and subjected to

156   pyrosequencing using a Roche 454 FLX genome sequencer system. The sequencing service

157   (RTL) performed denoising and chimera analyses (details provided in supplemental doc 1). The

158   high-quality reads were then processed in the RDP pipeline at the Ribosomal data project (RDP)

159   web site (Cole et al. 2014). Reads were clustered into operational taxonomic units (OTU) using a

160   97% identity threshold. DNA extracted from the OB (from frozen stock) and from fresh IO

161   biofilm cultures (Table 1) were sent to the sequencing service of Genome Quebec Innovation

162   Center (Montreal, QC, Canada). In these cases, the 16S rRNA sequences covering the V6-V7-V8

163   variable regions (5' ACACTGACGACATGGTTCTACA 3' and 5'

164   TACGGTAGCAGAGACTTGGTCT 3') were PCR amplified and sequenced by Illumina MiSeq

165   PE250 (250 bp paired-end sequencing reactions). The reads were processed based on Peck *et al.*

166   (2016). Briefly, paired-end reads were merged with minimum and maximum overlap length

167   between the two reads of 20 and 250 bases, respectively, with 30% mismatched bp tolerance in

168   the overlap region. The merged reads were processed using the software UPARSE (Edgar 2013).

169   Sequences were truncated to a uniformized length to 420 bp. Reads with a low-quality score

170   were removed using 2.0 as the maximum expected error value. The high-quality reads were de-

171   replicated, sorted by size and singletons were removed. The resulting reads were clustered into

172   operational taxonomic units (OTU) with the UPARSE-OTU clustering method using a 97%

173   identity threshold. Chimeric OTU were removed by UPARSE-REF algorithm

174   and with the software UCHIME ran against ChimeraSlayer 'gold' reference database (Edgar et

175   al. 2011). All representative sequences of the OTUs (from pyrosequencing and Illumina) were

176   checked again for chimeras with the DECIPHER v 2.0 program

177   (http://www2.decipher.codes/FindChimeras.html) (Wright et al. 2012). The affiliation of the

178   OTUs to the most probable genus was determined by the CLASSIFIER program at the RDP web

179   site (Supplemental doc 2). 16S rRNA sequence reads were deposited in the GenBank Sequence

180   Read Archive (SRA) under the accession number PRJNA524642. Principal component analysis

181   of the proportion of reads associated to the bacterial profiles were performed at ClustVis web site

182   (https://biit.cs.ut.ee/clustvis/) (Metsalu & Vilo 2015).

183

184   **Isolation of bacterial isolates**

185   Biofilm of the Ref300C-23C biofilm cultures was scraped from the carriers and dispersed

186   in saline solution (3% NaCl, 34.2 mM phosphate buffer pH 7.4), and serial dilutions were made

187   and inoculated onto these agar plate media: (1) R2A medium (complex organic carbons; EMD

188   Chemicals Inc., Gibbstown, NJ, USA), (2) Marine Agar 2216 (marine medium with yeast extract

189   and peptone as carbon source; Becton, Dickinson and Co., Sparks, MD, USA), (3) *Methylophaga*

190   medium 1403 (American Type Culture Collection [ATCC], Manassas, VA, USA) and (4) the

191   ASW medium; these two latter media were supplemented with 1.5% agar and 0.3% v/v

192   methanol. The isolation procedure, the taxonomic affiliation of the isolates and the measurement

193   of their denitrifying activities were carried out as described by Geoffroy *et al*. (2018). The 16S

194   rRNA gene sequences were deposited in GenBank under the accession numbers MK571459 to

195   MK571476.

196

197   **Transcriptomes**

198   Planktonic pure cultures of *M. nitratireducenticrescens* strains JAM1 and GP59 were

199   performed in the *Methylophaga* 1403 medium and of *H. nitrativorans* strain NL23 in the 337a

200   medium as described by Martineau *et al*. (2015) and Mauffrey *et al*. (2015). These cultures were

201   carried out in triplicate with methanol (0.3%) and NO_3^- (21.4 mM [300 mg-N/L]) under anoxic

202   conditions at 30°C. The biomass was collected by centrifugation when the NO_3^- reduction was

203   near completion, and total RNA was extracted as described by Mauffrey *et al*. (2015). For the

204   biofilm cultures, at the end of the the fifth transfer cultures, the biomass of each replicate was

205   scrapped from carriers and used to extract total RNA. The RNA samples were sent to the

206   sequencing service for RNA sequencing (RNAseq) by Illumina (Genome Quebec Innovation

207   Center, Montreal QC, Canada). Because of limited amount of biofilm available, total RNA from

208   the triplicate biofilm samples were pooled before sending to the sequencing service. For the

209   planktonic pure cultures, RNAseq was performed on each replicate. The Ribo-Zero™ rRNA

210   Removal Kit (Meta-Bacteria; Epicentre, Madison, WI, USA) was used to deplete total RNA of

211   the ribosomal RNA. The RNA was then treated with the TruSeq Stranded mRNA Sample Prep

212   Kit (Illumina Inc, San Diego, CA, USA).

213   All computations were made on the supercomputer Briarée from the Université de

214   Montréal, managed by Calcul Québec and Compute Canada. Raw reads were filtered to remove

215   low quality reads using FASTX toolkit (http://hannonlab.cshl.edu/ fastx_toolkit/) by discarding

216   any reads with more than 10% nucleotides with a PHRED score <20. The resulting reads from

217   each sample/replicate were aligned respectively to the genome of *M. nitratireducenticrescens*

218   strain JAM1 (GenBank accession number CP003390.3), to the genome and plasmids of *M.*

219   *nitratireducenticrescens* strain GP59 (CP021973.1, CP021974.1, CP021975.1) and to the

220   genome of *H. nitrativorans* strain NL23 (CP006912.1) using Bowtie (v 2.2.3) with default

221   parameters. SAMtools (v 0.1.18) and BEDtools (v 2.20.1) were used for the generation of sam

222   and bam files, respectively. Significance for difference in the relative transcript levels of a gene

223   (defined as transcript per million: TPM) between planktonic pure cultures and biofilm cultures

224   was performed with the R Bioconductor NOIseq package v2.14.0 (NOIseqBio) (Tarazona et al.

225   2011) and run with the R software v3.2.3 (Team 2015). Because the RNAseq from the biofilm

226   samples were derived from one pooled RNA preparation, the "no replicate parameter" was set

227   (*pnr*=0.2, *nss*=5 and *v*=0.02; pseudoreplicate generated) in NOIseq as described by Tarazona *et*

228   *al.* (2011) under the NOISeq-sim section. Briefly, NOISe1-sim assumes (quoting) "that read

229   counts follow a multinomial distribution, where probabilities for each gene in the multinomial

230   distribution are the probability of a read to map to that gene". Results from this statistical

231   analysis showed that genes that had at least >2-fold higher transcript levels from one type of

232   cultures to the other showed significant differences. RNAseq reads from the planktonic pure

233   cultures and the biofilm cultures were deposited in the SRA under the accession number

234   PRJNA525230. Annotations were based on services provided by GenBank

235   (https://www.ncbi.nlm.nih.gov/genbank), RAST (Rapid Annotation using Subsystem

236   Technology; http://rast.nmpdr.org) and KEGG (Kyoto Encyclopedia of Genes and Genomes;

237   https://www.genome.jp/kegg) (Supplemental doc 3).

238   To derive transcript reads not associated to the *M. nitratireducenticrescens* and *H.*

239   *nitrativorans*, reads were aligned to a concatenated sequence consisting of the three reference

240   genomes (JAM1+GP59+NL23) and the two plasmids (from strain GP59). The reads that did not

241 align were kept. Unaligned reads were *de novo* assembled at the National Center for Genome

242 Analysis web site (https://galaxy.ncgas-trinity.indiana.edu) by Trinity v. 2.4.0 (Grabherr et al.

243 2011). These transcripts were deposited in SRA under the accession number PRJNA525230.

244 Estimation of the transcript abundance of the *de novo* assembled sequences was performed by

245 RSEM (Li & Dewey 2011). The resulting assembled sequences were annotated at the Joint

246 Genomic Institute (https://img.jgi.doe.gov/cgi-bin/m/main.cgi) to find open reading frames with

247 their putative function and affiliation (GOLD Analysis Project Id: Ga0307915, Ga0307877,

248 Ga0307760). The annotations were then verified manually for discrepancies within the

249 assembled sequences (Supplemental doc 4, 5, 6 and 7).

250

251 **Results**

252

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

254 As reported by Payette *et al.* (2019), the original biofilm (OB) collected from the Biodome

255 denitrification system was used as inoculum to colonize new carriers in a series of anoxic biofilm

256 cultures cultivated under different conditions (Table 1; Fig. S1). We selected eight of these

257 biofilm cultures for our present analysis for the following reasons. The Ref300N-23C biofilm

258 cultures were used as reference for comparison analysis. The 300N-30C, 900N-23C and 900N-

259 30C biofilm cultures had higher specific denitrification rates compared to the Ref300N-23C

260 biofilm cultures. The 0%, 0.5% and 1.0% NaCl ASW biofilm cultures and the IO biofilm

261 cultures were chosen because of the persistence of *H. nitrativorans* NL23 in these cultures as

262 opposed to the other cultures. The composition of the bacterial community of these biofilm

263 cultures and the OB was determined by sequencing the 16S rRNA genes to assess the impact of

264 these specific conditions on the bacteria community (Fig. 1A; Table 2). The bacterial profiles of

265 the OB and the IO biofilm cultures were distinct to each other, and to the seven biofilm cultures

266 cultivated with different formulations in the ASW medium (Fig. 1B). The bacterial profiles of

267 these latter cultures were however not very different because of the high proportions of

268 *Methylophaga* spp. (>85%).

269 In the OB, high proportions of the 16S rRNA gene sequences were related to

270 *Hyphomicrobium* spp. (45.8%) followed by *Oceanibaculum* spp. (12.3%), *Aquamicrobium* spp.

271 (11.6%); *Methylophaga* spp. accounted for 3.5% (Table 2). In the IO biofilm cultures, the

272 proportion of *Methylophaga* spp. was 12 times higher (42.8%) than in the OB, whereas it was 5.5

273 times lower for *Hyphomicrobium* spp. (8.4%). Higher proportions of *Marinicella* spp. (7.2%)

274 and *Winogradskyella* spp. (4.3%) along with a much lower proportion of *Aquamicrobium* spp.

275 (0.44%) were observed in the IO biofilm cultures compared to the OB (Table 2).

276   In the biofilm cultures cultivated with the four combinations of NO_3^- / methanol

277 concentrations and temperatures in ASW medium containing 2.75% NaCl (Ref300N-23C, 300N-

278 30C, 900N-23C, 900N-30C), *Methylophaga* spp. accounted for >90% of the 16S rRNA gene

279 sequences followed by *Marinicella* spp. with proportions ranging from 1.5% to 5.0% (Fig. 1;

280 Table 2). No sequences were found affiliated to *Hyphomicrobium* spp. under these conditions. In

281 the biofilm cultures cultivated at low NaCl concentrations (0% NaCl, 0.5% NaCl, 1% NaCl),

282 *Hyphomicrobium* spp. accounted for 11.8%, 6.8% and 0.25%, respectively of the 16S rRNA

283 gene sequences (Fig. 1; Table 2). *Methylophaga* spp. was still the dominant genus with more

284 than 85% of the 16S rRNA gene sequences; 16S rRNA gene sequences affiliated to *Marinicella*

285 spp. were also found in significant proportions (Fig. 1; Table 2). Finally, 16S rRNA gene

286 sequences affiliated to *Stappia* spp. were found in all biofilm cultures and in the OB.

287   The 16S rRNA gene sequences from the OB and the IO biofilm cultures that were derived

288 by Illumina sequencing generated several thousands of reads affiliated to *Hyphomicrobium* spp.

289 and *Methylophaga* spp. (Table 3). This tremendous amounts of sequences allowed assessing the

290 presence of species other than *H. nitrativorans* and *M. nitratireducenticrescens* in these two

291 biofilms. The phylogenic analyses performed on these sequences allowed regrouping the OTUs

292 in three clusters for *Hyphomicrobium* spp., and also three clusters for *Methylophaga* spp. (Fig.

293 S2A and S2B). The vast majority (>90%) of the 16S rRNA gene sequences associated to these

294 OTUs were affiliated to *H. nitrativorans* or *M. nitratireducenticrescens,* respectively, in the OB

295 and the IO biofilm cultures (Clusters 1, Table 3). A small proportion of the OTUs (clusters 2 and

296 3) was affiliated to other *Hyphomicrobium* or *Methylophaga,* which suggests that other members

297 of these genera were present in these biomasses in very low proportions.

298

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

300   The biomass of the Ref300N-23C biofilm cultures was dispersed on different nutrient agar

301 plates to isolate denitrifying bacteria other than *H. nitrativorans* strain NL23 and *M.*

302 *nitratireducenticrescens* strain GP59. Isolates affiliated to the genera *Marinobacter,*

303 *Pseudomonas, Paracoccus, Roseovarius, Thalassobius, Winogradskyella, Aequorivita* and

304 *Exiguobacterium* (Table 4) were recovered from the Marine medium 2216 plates, which contains

305 yeast extract and peptone (Atlas 1993). Only isolates affiliated to the genera *Marinobacter* and

306 *Paracoccus* showed consumption of NO_3^- and NO_2^- and production of gas, suggesting that they

307 possess the complete denitrification pathway. The three isolates affiliated to the *Paracoccus* spp.

308 have identical 16S rRNA sequences with the one of *Paracoccus* sp. strain NL8 that was isolated

309 from the Biodome denitrification system (Labbé et al. 2003). This result suggests that strain NL8

310 persisted in the biofilm cultures. One representative of *Paracoccus* isolates (GP3) could grow

311 with methanol as sole source of carbon; *Marinobacter* sp. GP2 could not.

312

313 **Metatranscriptomic analysis of the biofilm cultures**

314   The metatranscriptomic approach has allowed assessing the contributions of the microbial

315 community to the metabolic processes in the biofilm cultures. We have chosen to focus on three

316 biofilm cultures, which were the Ref300N-23C (the reference biofilm cultures), 900N-30C

317 (highest denitrification rates; Table 1) and 0% NaCl (persistence of *H. nitrativorans* strain NL23)

318 biofilm cultures. Because the genomes of *H. nitrativorans* strain NL23 and *M.*

319 *nitratireducenticrescens* strain JAM1 and strain GP59 were available, we first determined

320 changes in the transcript levels of genes associated to these genomes between the biofilm

321 cultures and the planktonic pure cultures of the respective strains. To assess the contribution of

322 other microorganisms in biofilm cultures, the reads from the metatranscriptomes that did not

323 align with the three reference genomes were used to derive *de novo* assembled transcripts. These

324 transcripts were annotated for function and bacterial affiliation.

325

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

327   Because >80% of the genomes of strains JAM1 and GP59 are identical, high proportions

328 of reads from the biofilm metatranscriptomes can align to both genomes. Geoffroy *et al.* (2018)

329 showed that the gene expression profiles of the common genes between both strains in

330 planktonic pure cultures were similar. In Payette *et al.* (2019), the concentrations of strain GP59

331 and strain JAM1 in the biofilm cultures were determined by qPCR. In the three selected biofilm

332 cultures for the metatranscriptomic analysis, the concentrations of strain GP59 (copies of *nirK* by

333 ng biofilm DNA) were one to three orders of magnitude higher than those of strain JAM1 (copies

334   of *tagH* by ng biofilm DNA). Because of these differences, it was assumed that most of the

335   transcript reads associated to *M. nitratireducenticrescens* in the biofilm cultures were from strain

336   GP59. Metatranscriptomic analysis in relation with strain JAM1 is described in supplemental doc

337   8 and 11. The transcriptomes of strain GP59 were also derived from planktonic pure cultures

338   cultivated under anoxic conditions in the *Methylophaga* 1403 medium (Fig. S1). The choice of

339   this medium was because suboptimal growth occurred with strain GP59 in ASW medium. The

340   relative transcript levels of the corresponding genes in the biofilm cultures and the planktonic

341   pure cultures were compared to assess changes in the metabolisms of the strain that occurred

342   between the two environments. All quantitative changes described below of the transcript levels

343   in the biofilm cultures are expressed relative to the transcript levels in the planktonic pure

344   cultures.

345   Among all genes of strain GP59, between 11% and 21% of them had higher relative

346   transcript levels in the biofilm cultures. At the opposite, 6 to 17% of all genes of strain GP59

347   were expressed at higher relative transcript levels in planktonic pure cultures (Fig. 2). Strain

348   GP59 contains two plasmids, and most of the genes encoded by these plasmids had much lower

349   relative transcript levels in the biofilm cultures (Fig. 2). Genes involved in the nitrogen

350   metabolism and iron transport were globally at higher relative transcript levels in the biofilm

351   cultures (Table 5; Fig. 2 and 3).

352   For the denitrification genes, *narXL* encoding the regulatory factors of the *nar* systems

353   showed no differences between the biofilm cultures and the planktonic pure cultures in the

354   relative transcript levels (Table 5). Small upregulation of the *nar2* operon with about 3-fold

355   increases in relative transcript levels occurred in the biofilm cultures. These levels were lower in

356   the 900N-30C biofilm cultures for the *nar1* operon and were about the same levels in the two

357   other biofilm cultures and the planktonic pure cultures. The *nor1* operon had the same relative

358   transcript levels in the 300N-23C and 900N-30C biofilm cultures and the planktonic pure

359   cultures, and a 3-fold decrease was noticed in these levels in the 0%NaCl biofilm cultures (Table

360   5). No significant changes in the expression of the *nos* operon occurred between both types of

361   cultures. The relative transcript levels of *nirK* were 5 to 10-times higher in the 300N-23C and

362   900N-30C biofilm cultures, whereas these levels were similar in the 0%NaCl biofilm cultures

363   and the planktonic pure cultures (Table 5). Higher relative transcript levels of genes involved in

364   the ammonium transport and the assimilatory NO_3^-/NO_2^- reduction pathway were observed in the

365   biofilm cultures (Table 5; Fig. 3). Absence of nitrogen source other than NO_3^- in the ASW

366   medium and presence of 37 mM NH_4^+ in the medium used for the planktonic pure cultures

367   (*Methylophaga* 1403) could explain these differences in the assimilatory pathway.

368   Figure 3 illustrates changes in the relative expression profiles in the biofilm cultures of

369   major pathways in strain GP59. The relative transcript levels of *mxaFJGI* encoding the small and

370   large subunits of the methanol dehydrogenase (MDH) and the cytochrome c-L increased by 2-6

371   fold in biofilm cultures. Two out of the four *mxaF*-related products (*xoxF*) showed 2- to 9-fold

372   decreases in their relative transcript levels in the biofilm cultures. As observed in *Methylorubrum*

373   *extorquens*, the genome of *M. nitratireducenticrescens* strain GP59 encodes three formate

374   dehydrogenases with the same gene arrangement (Chistoserdova et al. 2004). The *fdhCBAD*

375   operon that encodes the NAD-linked, Mo-formate dehydrogenases showed ca. 60-fold increases

376   in the relative transcript levels in the biofilm cultures, whereas the two other *fdh* operons stayed

377   at the same levels of those of the planktonic pure cultures. Gene encoding NAD-dependent

378   formate dehydrogenase was upregulated in biofilms formed by *Desulfovibrio vulgaris* compared

379   to planktonic cultures (Clark et al. 2012). This was also the case in biofilms formed by

380   *Staphylococcus aureus* where the NAD-dependent formate dehydrogenase gene was among the

381   highest upregulated genes (Resch et al. 2005). In both studies, this upregulation correlated with

382   increase in formate dehydrogenase activity. Contrary to planktonic cultures, accumulation of

383   formate could occur in cell vicinity in the biofilm that would be toxic for the cells (Resch et al.

384   2005). Therefore, upregulation of *fdhCBAD* operon could be related to detoxification. The

385   relative transcript levels of the gene encoding the cytochrome c555 were 40 to 70 times higher in

386   the biofilm cultures. Genes encoding the other cytochromes, pseudoazurins and azurin were

387   expressed at similar levels in both types of cultures. Genes involved in the formaldehyde

388   metabolism to formate and CO_2, glycolysis, the ribulose monophosphate pathway, the Entner

389   Doudorof pathway, the tricarboxylic acid cycle, and the pentose pathway showed their relative

390   transcript levels in general unchanged. Few genes in these pathways had 2-11 fold differences

391   between the planktonic pure cultures and the biofilm cultures. The genome of strain GP59

392   encodes the major enzymes involved in the Calvin-Benson-Bassham cycle: the ribulose-

393   bisphosphate carboxylase (Rubisco) and the phosphoribulokinase (Prk). In the 0% NaCl biofilm

394   cultures, the relative transcript levels of the Rubisco gene operon (*rbcSL*) jumped by 66 times.

395   This upregulation was less pronounced in the Ref300N-23C biofilm cultures (3-fold increase).

396   For the *prk* gene, the relative transcript levels were 3 to 4 times higher in the 0% NaCl biofilm

397   cultures. The nature of this upregulation is unknown. Except for the cytochrome c555, the

398   relative transcript levels of genes encoding for the oxidative phosphorylation metabolism were

399   unchanged. Several genes involved in iron transport showed higher relative transcript levels in

400   the biofilm cultures (2 to >50-fold increases). The nature of this upregulation in the biofilm

401   cultures is unknown, as the biofilm and planktonic pure cultures were cultivated with trace

402   elements containing iron (Payette et al. 2019).

403

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

405   As with *M. nitratireducenticrescens* strain GP59, the transcript levels of genes associated

406   to *H. nitrativorans* strain NL23 were compared between the biofilm cultures and the planktonic

407   pure cultures to assess changes in the metabolisms that occurred between the two environments.

408   The planktonic pure cultures of strain NL23 were cultivated under anoxic conditions in the 337a

409   medium (Fig. S1). The choice of this medium was because strain NL23 could not grow in ASW

410   (with 2.75% NaCl).

411   The overall analysis of the three metatranscriptomes confirmed the results obtained by

412   qPCR assays (Payette et al. 2019) and the 16S rRNA gene analysis (Table 2). High number of

413   reads (40×10^6) derived from the metatranscriptome of the 0% NaCl biofilm cultures aligned

414   with the NL23 genome, but <20 000 reads derived from the Ref300N-23C and 900N-30C

415   metatranscriptomes did. In the 0% NaCl biofilm cultures, <10% of all NL23 genes had a higher

416   relative transcript levels in the 0% NaCl biofilm cultures, whereas this was the case for >40%

417   genes in planktonic pure cultures (Fig. 2). These results suggest important changes had occurred

418   in the regulation of gene expression between the planktonic pure cultures and the biofilm

419   cultures. Genes involved in the energy (Fig. S4) and nitrogen metabolisms (Fig. 2; Table 5) had

420   globally higher relative transcript levels in the 0%NaCl biofilm cultures (Fig. 2).

421   Higher relative transcript levels (5 to 8-times) for the *nap*, *nor* and *nos* operons were

422   observed in the 0% NaCl biofilm cultures (Table 5). *nirK* was highly upregulated in the biofilm

423   cultures with 49-fold increase in the relative transcript levels (Table 5). The *napGH* operon

424   however had a 9.4-fold decrease in the relative transcript levels in the 0% NaCl biofilm cultures.

425   As observed with strain GP59, substantial changes in the relative transcript levels of genes

426   involved in the ammonium transport and the assimilatory NO_3^-/NO_2^- reductase were observed

427   with 3- to 22-fold increases in these levels (Table 5, Fig. 4). These results correlate with the

428   absence of NH_4^+ in the ASW medium, and thus NO_3^- the only source of N, compared to the 337a

429   medium used for the planktonic pure cultures, which contains 3.8 mM NH_4^+.

430   Figure 4 illustrates changes in relative expression profiles of major pathways in the 0%

431   NaCl biofilm cultures of strain NL23. The relative transcript levels of *mxaFJGI* increased by 2-

432   fold in the biofilm cultures. The relative transcript levels of the *mau* operon (methylamine

433   dehydrogenase) showed a 15-fold decrease in the biofilm cultures. The nature of such decrease is

434   unknown as strain NL23 was not fed with methylamine in any of our cultures. The three *xoxF*

435   genes did not show substantial changes in their transcript levels in both types of cultures. Genes

436   involved in the formaldehyde metabolism to formate and CO_2, glycolysis, the tricarboxylic acid

437   cycle, and the pentose pathway showed their transcript levels in general unchanged between the

438   planktonic pure cultures and the biofilm cultures. Few genes in these pathways had 2-5 fold

439   differences in their relative transcript levels. The two genes encoding the key enzymes in the

440   serine pathway (alanine-glyoxylate transaminase, glycine hydroxymethyltransferase) had 3- to 7-

441   fold increases in their relative transcript levels in the biofilm cultures. As *Methylorubrum*

442   *extorquens*, the NL23 genome encodes the ethymalonyl-CoA pathway (Chistoserdova et al.

443   2003; Peyraud et al. 2009), which did not show changes overall in the transcript levels of the

444   corresponding genes between the two types of cultures. Contrary to *M. extorquens* however, a

445   gene encoding the isocitrate lyase is present in strain NL23 and showed a 25-fold upregulation in

446   the biofilm cultures. The isocitrate lyase is one of the key enzymes of the glyoxylate bypass that

447   catalyzes the transformation of isocitrate to succinate and glyoxylate. Gene encoding isocitrate

448   lyase is also present in other available *Hyphomicrobium* genomes. All these results suggest that

449   in the 0% NaCl biofilm cultures, the carbon metabolism increased in activity and that the glycine

450   regeneration for the serine pathway by the glyoxylate was upregulated. Among genes involved in

451   the oxidative phosphorylation, the relative transcript levels were higher (2 to 13 times) in the

452   biofilm cultures with those encoding the NADH dehydrogenase, the cytochrome c reductase,

453   with one of the cytochromes c and the F-type ATPase. Combined with increases in the relative

454   transcript levels of the denitrification and the carbon pathways, these results suggest that

455   increases in electron donor activities correlates with the need of electron for the nitrogen

456   dissimilatory metabolism in the biofilm cultures. Strain NL23 possesses four types of

457   cytochrome oxidase (aa3, bo, bd-I and cbb3) (reduction of O_2 in H_2O) that are in general

458   expressed at the same levels in both types of cultures. Figure 4 also illustrates the dynamic

459   changes of transporters and two component systems. Several of these transporters had lower

460   relative transcript levels in the 0% NaCl biofilm cultures. Contrary to strain GP59, genes

461   involved in iron transport were not strongly affected in their gene expression in the 0% NaCl

462   biofilm cultures (Fig. 2 and 4).

463

464   **The composition of the active microbial community in the biofilm cultures**

465   As mentioned above, the reads from the three metatranscriptomes that did not align with

466   the genomes of *H. nitrativorans* strain NL23 and *M. nitratireducenticrescens* strain GP59 and

467   strain JAM1 were *de novo* assembled. These reads were subsequently aligned to the *de novo*

468   assembled transcripts to derive the relative levels of these transcripts in the biofilm cultures. The

469   *de novo* assembled sequences were then annotated for function and affiliation. Finally, these

470   sequences were grouped by microbial affiliation to determine the active populations in the

471   biofilm cultures and to assess their level of involvement in these biofilm cultures (Table 6).

472   It was estimated that between 5 to 10% reads of the three metatranscriptomes were derived

473   from other microorganisms than *H. nitrativorans* strain NL23 and *M. nitratireducenticrescens*

474   strain GP59 and strain JAM1 (Supplemental doc 4). The proportions of transcripts affiliated to

475   Archaea and Eukarya accounted together for <0.1% (Table 6), which suggests very low

476   abundance of these microorganisms in the biofilm cultures. The proportions of transcripts

477   affiliated to viruses, phages and plasmids in the *de novo* assembled transcripts represented

478   between 0.6 and 21.7% (Table 6).

479   Twenty-seven bacterial taxa were selected for their overall transcript levels in at least one

480   of the three biofilm cultures (Table 6). All the taxa detected by the 16S rRNA gene sequencing

481   are present in this list (Table 2 and supplemental doc 2). These 27 taxa represented between 22%

482   and 35% of the *de novo* assembled transcripts. Among these taxa, genes encoding the four

483   denitrification reductases were present in the *de novo* transcripts affiliated to *Marinobacter* spp.,

484   *Stappia* spp. and *Pseudomonas* spp. However, only *de novo* assembled transcripts affiliated to

485   the *Stappia* spp. showed the complete set of denitrification genes in the three biofilm cultures

486   and organized in operons (*napABC, napADFE, norCBQD, nosRZDF)*.

487   Further analysis of the expression profiles of the 27 bacterial taxa was performed to assess

488   whether some taxa were influenced in their global metabolic activities by the specific conditions

489   of the biofilm cultures. The overall transcript levels of the 27 bacterial taxa (Table 6) were

490   compared between each biofilm culture by clustering analysis (Fig. 5). NaCl concentration was

491   the main factor of clustering as two distinct clusters were derived. The low salt cluster consisting

492   of nine bacterial taxa showed higher relative transcript levels in the 0% NaCl biofilm cultures,

493   whereas the marine cluster of 13 bacterial taxa had higher relative transcript levels in the

494   Ref300N-23C and 900-30C biofilms cultures. A third cluster showed five bacterial taxa with

495   lower relative transcript levels in the 900N-30C biofilm cultures compared to the 0% NaCl and

496   Ref300N-23C biofilm cultures. In these cases, higher temperature (30°C vs 23°C) and higher

497   NO$_3^-$ and methanol concentration (64.3 mM vs 21.4 mM NO$_3^-$; 0.45% vs 0.15% methanol) may

498   have negatively affected these populations.

499

500   **Discussion**

501   In the environment, numerous bacteria belonging to different taxa can accomplish

502   denitrifying activities, and many of them were encountered in different types of denitrification

503   processes (Lu et al. 2014). Very few studies describing the microbial community of methanol-

504   fed denitrification systems have been reported so far. Most of these studies are based on cloned

505   16S rRNA gene sequences of around 100 clones or based on fluorescence *in situ* hybridization

506   (Baytshtok et al. 2008; Ginige et al. 2004; Hallin et al. 2006; Neef et al. 1996; Osaka et al. 2008;

507   Osaka et al. 2006; Rissanen et al. 2016; Rissanen et al. 2017; Sun et al. 2016; Yoshie et al.

508   2006). In all these studies, high proportions of *Hyphomicrobium* spp. were found in combination

509   with high proportions of other methylotrophs such as *Methyloversatilis* spp., *Methylophilus* spp.,

510   *Methylotenera* spp. or *Paracoccus* spp. The Biodome marine denitrification system showed no

511   exception to this trend with co-occurrence of *Hyphomicrobium* spp. and the marine methylotroph

512   *Methylophaga* spp. This co-occurrence was also observed in two other denitrification systems

513   treating saline effluents (Osaka et al. 2006; Rissanen et al. 2016) (see Discussion by Payette *et*

514   *al.* (2019). The bacterial diversity of the biofilm taken from the Biodome denitrification system

515   was assessed before when the reactor was operational in 2002 by deriving a 16S rRNA gene

516   library and by culture approach (Labbé et al. 2003; Labbé et al. 2004). Beside *Hyphomicrobium*

517   sp. and *Methylophaga* sp., *Paracoccus* sp., *Sulfitobacter* sp., *Nitratireductor aquibiodomus*, and

518   *Delftia* sp. among others were identified. In the present report, a more complete determination of

519   the composition of the bacterial community of the denitrifying biofilm that was frozen in 2006

520   (when the denitrification system was dismantle by the Biodome) was possible with the new

521   sequencing technology.

522   The composition of the bacterial community of the OB and the IO biofilm cultures was

523   derived from a different region of the 16S rRNA genes (V6 to V8) than that used for the other

524   biofilm cultures (V1 to V3), and each region was sequenced by a different technology (Illumina

525   and pyrosequencing, respectively). Pyrosequencing technology was no longer available at the

526   time of the sampling of the OB and the IO biofilm cultures, which were carried two years later

527   than the other biofilm cultures. Despites these differences, we believe that the results generated

528   by these two approaches were comparable because they are consistent with the results obtained

529   by qPCR assays (Table 2) that have determined the concentrations of *M. nitratireducenticrescens*

530   (copies *narG1* per ng biofilm DNA) and *H. nitrativorans* strain NL23 (copies *napA* per ng

531   biofilm DNA) in the biofilm cultures (Payette et al. 2019). For instance, qPCR showed very low

532   levels of *H. nitrativorans* strain NL23 in the Ref300C-23C biofilm cultures and high level in the

533   OB. These results concur with the absence of 16S rRNA sequences associated to

534   *Hyphomicrobium* spp. in the Ref300C-23C biofilm cultures (pyrosequencing) and high number

535   of these sequences in the OB (Illumina sequencing).

536   The OB cultivated under the different conditions in the ASW medium showed important

537   changes in the *Methylophaga* and the *Hyphomicrobium* populations. The proportion of 16S

538   rRNA gene sequences associated to *Methylophaga* spp. was 3.5% in the OB but was very high,

539   between 85% and 97%, in these cultures. On the contrary, the proportion of 16S rRNA gene

540   sequences associated to *Hyphomicrobium* spp. was high (46%) in the OB, but was very low (0%

541   to 11%) in these cultures. The NaCl concentrations in the ASW had an impact on the

542   *Hyphomicrobium* populations. 16S rRNA gene sequences associated to *Hyphomicrobium* spp.

543   were absent in the biofilm cultures cultivated in ASW at 2.75% NaCl (Ref300N-23C, 300N-30C,

544   900N-23C, 900N-30C), but were present in the biofilm cultures cultivated at low NaCl

545   concentrations (0%, 0.5% and 1%). These results concur with those obtained by qPCR for the

546   concentrations of *M. nitratireducenticrescens* and *H. nitrativorans* strain NL23 (Payette et al.

547   2019). Cultivating the OB in the IO medium had a different impact on the *Hyphomicrobium*

548   populations. Although the concentrations of salts in the IO medium and in the ASW with 2.75%

549   NaCl were similar (around 3.5%), persistence of *Hyphomicrobium* spp. occurred in the IO

550   biofilm cultures. In fact, the concentrations of *H. nitrativorans* strain NL23 determined by qPCR

551   between the OB and the IO biofilm cultures was in the same level of magnitude. The lower

552   proportion of 16S rRNA gene sequences associated to *Hyphomicrobium* spp. in the IO biofilm

553   cultures compared to OB was a consequence of the substantial growth of *M.*

554   *nitratireducenticrescens* in these cultures, with a 10-fold increase in concentration as revealed by

555   qPCR (Table 2) (see Payette *et al.*, 2019 for further discussion). Grob *et al.* (2015) also observed

556   strong growth of *Methylophaga* spp. in their seawater samples that were fed with 100 μM

557   methanol with relative proportions of 16S rRNA gene sequences raising from <0.5% at T=0 to

558   84% after 3 days.

559   Cultivating the OB in higher concentrations of NO_3^- and methanol (64.3 mM/0.45%; C/N

560   1.5) and/or at 30°C (300N-30C, 900N-23C and 900N-30C biofilm cultures) resulted in increases

561   of 20% to 85% in the specific denitrification rates compared to the Ref300N-23C biofilm

562   cultures (Table 1) (Payette et al. 2019). Temperature was shown to be the main factor of these

563   increases. However, raising the NO_3^- and methanol concentrations or/and temperature in these

564   cultures did not have an important impact on the bacterial community when compared to the

565   Ref300N-23C biofilm cultures. Metatranscriptomic analysis of the Ref300N-23C and 900-30C

566   biofilm cultures did not reveal either substantial changes in the gene expression profiles between

567   these two cultures. The higher specific denitrification rates of these biofilm cultures could be

568   related to higher metabolisms at the protein level such as the processing of NO_3^- by the

569   reductases and transporters.

570   Results from the 16S rRNA gene sequences showed that *Marinicella* spp. were present in

571   the OB and all the biofilm cultures, and were the second most abundant bacterial population in

572   the biofilm culture cultivated in ASW at 2.75% NaCl. These results concur with the

573   metatranscriptomes of the biofilm cultures where *Marinicella* spp. had the relative transcript

574   levels among the highest in the *de novo* assembled transcripts. *Marinicella* spp. are considered

575   strict aerobic bacteria with no indication of NO_3^- reduction (Romanenko et al. 2010) although a

576   previous study reported high relative abundances of *Marinicella* spp. in anoxic sulfide oxidizing

577   reactors in which nitrate was used as the electron acceptor (Huang et al. 2015). Genome

578   annotations of two *Marinicella* strains (GenBank: *Marinicella* sp. F2 - assembly number

579   ASM200005v1 and *M. litoralis* KMM 3900 - ASM259191v1) (Wang et al. 2018) did not reveal

580   complete denitrification pathway, beside a nitric oxide reductase gene cluster also detected here

581   in our metatranscriptomes. Together with the presence of *nirS* gene (Table 6), these results

582   indicated that *Marinicella* spp. might have the capacity to use intermediates of the denitrification

583   cycle to support their growth.

584   The 16S rRNA gene sequences provided evidence of the presence of *Pseudomonas* spp.,

585   *Marinobacter* spp., *Stappia* spp., *Paracoccus* spp. and *Aquamicrobium* spp. in the OB and in the

586   biofilm cultures. Some species belonging to these genera were reported to carry denitrification.

587   Isolates affiliated to the genera *Pseudomonas*, *Marinobacter* and *Paracoccus* were recovered

588   from the Ref300N-23C biofilm cultures. The *Marinobacter* and *Paracoccus* isolates could

589   perform denitrifying activities and grow under anoxic conditions, whereas the *Pseudomonas*

590   isolate could only consume NO_3^-.

591   Although denitrification genes were found in several of the bacterial populations identified

592   by the metatranscriptomic approach, only transcripts encoding the four denitrification reductases

593   affiliated to *Stappia* spp. were found in the three examined biofilm cultures. The proportions of

594   *Stappia* spp. in the 16S rRNA gene sequences of these biofilm cultures ranged between 0.09%

595   and 0.42% (Table 2). *Stappia* spp. are chemoorganotrophic bacteria found in marine

596   environments (Weber & King 2007) that can oxidize carbon monoxide. They possess the form I

597   *coxL* gene encoding the large subunit of carbon monoxide (CO) dehydrogenase. Some also

598   contain a gene for the large subunit of ribulose-1,5-bisphosphate carboxylase (RuBisCO) and

599   may be able to couple CO utilization to CO_2 fixation (King 2003). A *coxL* gene was found in the

600   *de novo* assembled transcripts affiliated to *Stappia* spp., but not RuBisCO. Sequences analogue

601   to transporters for simple and multiple sugars such as xylose and fructose, and acetate were

602   found (Supplemental doc 5, 6, 7), which suggest that the *Stappia* bacteria fed on the biofilm

603   material for carbon sources. Combined with the isolation of denitrifying isolates affiliated to

604   *Marinobacter* spp. and *Paracoccus* spp., these results suggest that the biofilm has the potential to

605   adapt to heterotrophic non-methylotrophic environments.

606   The proportion of 16S rRNA gene sequences associated to *Bacteroidetes* in the OB and in

607   the eight biofilm cultures ranged from 0.2% to 4.9%, and several genera of this phylum were

608   identified in the three metatranscriptomes. Significant proportions of bacteria affiliated to the

609   *Bacteroidetes* phylum were also found in other methanol-fed denitrification systems. For

610   instance, 29% of cloned 16S rRNA gene sequences were affiliated to *Bacteroidetes* in an

611   acclimated activated sludge in a methanol-fed anoxic denitrification process treating a synthetic

612   wastewater with 4% NaCl (Osaka et al. 2008). Isolates affiliated to the *Bacteroidetes Aequorivita*

613   spp. and *Winogradskyella* spp. were isolated from the Ref300N-23C biofilm cultures. None of

614   these two isolates, however, could sustain growth under denitrifying conditions. Although

615   denitrification genes affiliated to *Bacteroidetes* genera were found in *de novo* assembled

616   transcripts, genes encoding all four denitrification reductases were not found to any of them.

617   These results suggest that *Bacteroidetes* are not involved in denitrification, although they may be

618   involved in some steps of the denitrification pathway.

619   The metatranscriptomic data provided some insights of specific metabolisms in *H.*

620   *nitrativorans* strain NL23 and *M. nitratireducenticrescens* strain GP59 that were regulated in the

621   biofilm environment. In absence of strain NL23 in the Ref300C-23C and the 900N-30C biofilm

622   cultures, the *nirK* gene of strain GP59 was upregulated by 5 to 10 times compared to the

623   planktonic pure cultures that was also cultivated under denitrifying conditions. On the contrary,

624   the relative transcript levels of this gene did not change between the 0% NaCl biofilm cultures

625   and the planktonic pure cultures, while the relative transcript levels of the NL23 *nirK* were 49

626   times higher in the 0% NaCl biofilm cultures. These results suggest coordination in the

627   expression of *nirK* between these two strains in the 0%NaCl biofilm cultures.

628   The gene clusters encoding the three other denitrification reductases (*nap, nor, nos*) in

629   strain NL23 showed higher relative transcript levels in the 0% NaCl biofilm cultures. *napGH*

630   was however down regulated in these biofilm cultures. *napGH* is located in a separate

631   chromosomic region than the *napABCDEF* operon. NapGH and NapC have redundant function

632   of transferring electrons to NapB across the membrane. It was proposed that NapC transfers

633   electrons from the menaquinol, whereas NapGH do it from ubiquinol (Simon 2011). The

634   physiological consequence of *napGH* transcript decrease in the biofilm is unknown.

635   Observations on *napEDABC* found in the denitrifier *Shewanella denitrificans* OS217, and

636   *napDAGHB* in the respiratory NO_3^- ammonifier *Shewanella oneidensis* MR-1 suggest that

637   NapGH is more involved in the ammonification system (Simpson et al. 2010). Despite the

638   denitrifying conditions applied in both types of cultures, the biofilm environment has induced

639   strong up regulation of denitrification genes in *H. nitrativorans* strain NL23. This may be in

640   response to the rapid processing of NO_3^- by *M. nitratireducenticrescens* strain JAM1/GP59

641   (Mauffrey et al. 2015) that could generate rapidly high level of NO_2^-, which is toxic for strain

642   NL23.

643

644 **Conclusion**

645   The OB taken from the Biodome denitrification system underwent substantial changes in

646 its bacterial community when subjected to environmental changes. Cultivating the OB in the

647 homemade ASW medium with different formulations (varying NaCl, NO_3^- and methanol

648 concentrations, and temperature) or in the commercial IO medium showed much higher

649 proportions of *Methylophaga* spp. in these biofilm cultures compared to the OB. These results

650 concur with the growth of *M. nitratireducenticrescens* strain GP59 in these cultures. The

651 population of *Hyphomicrobium* spp showed a more complex trend. It was not detected in the

652 biofilm cultures cultivated in ASW at 2.75% NaCl, but persisted in the biofilm cultures

653 cultivated in ASW at low NaCl concentration, and also cultivated in the IO medium. Other

654 denitrifiers affiliated to *Marinobacter* spp. and *Paracoccus* spp. were isolated from the biofilm

655 cultures. Moreover, metatranscriptomic analysis revealed that denitrifying bacteria affiliated to

656 *Stappia* spp. were metabolically active in the biofilm cultures. The biofilm environment has

657 favored the upregulation of the denitrification pathway in *M. nitratireducenticrescens* strain

658 GP59 and *H. nitrativorans* strain NL23 compared to planktonic pure cultures, despite the facts

659 that these two types of cultures were grown under denitrifying conditions. All these results

660 demonstrated the dynamics and the plasticity of the denitrifying biofilm to sustain environmental

661 changes and illustrate a comprehensive picture of the microbial community of the biofilm and its

662 adaptation to these changes. This could benefit in the development of optimal denitrifying

663 bioprocess under marine conditions. For instance, the fact that non-methylotrophic, denitrifying

664 bacteria are present in the biofilm could suggest adaptation of denitrification process to another

665 source of carbon such as ethanol or acetate.

666

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669

670 **References**

671

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853

854

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.

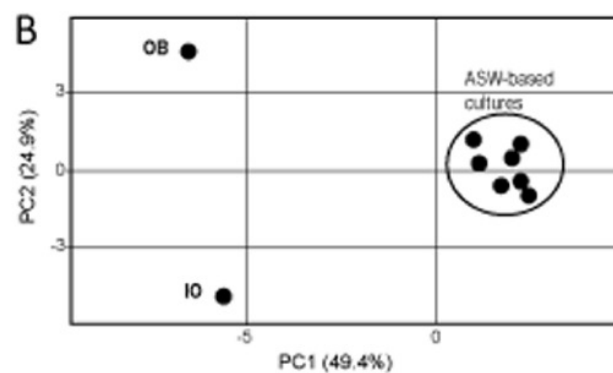
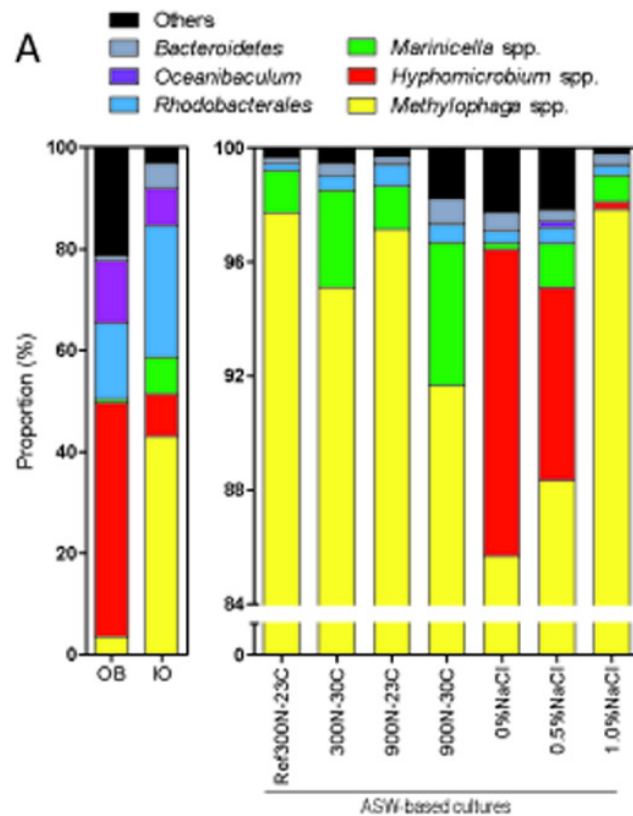



Figure 2

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.

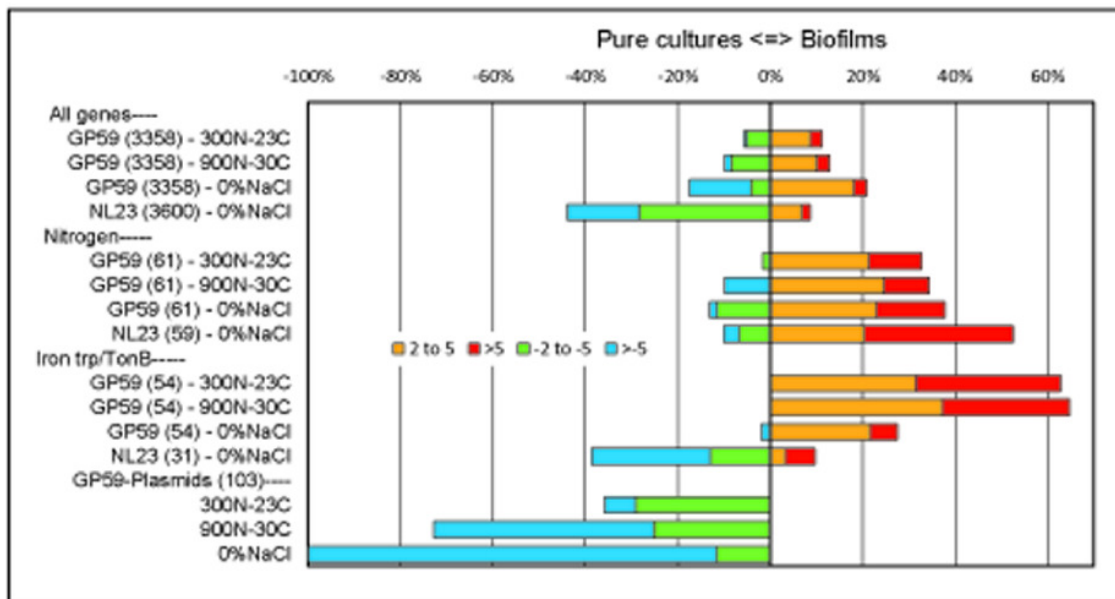



Figure 3

Relative gene expression profiles of selected metabolic pathways of *M. nitratireducenticrescens* 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.

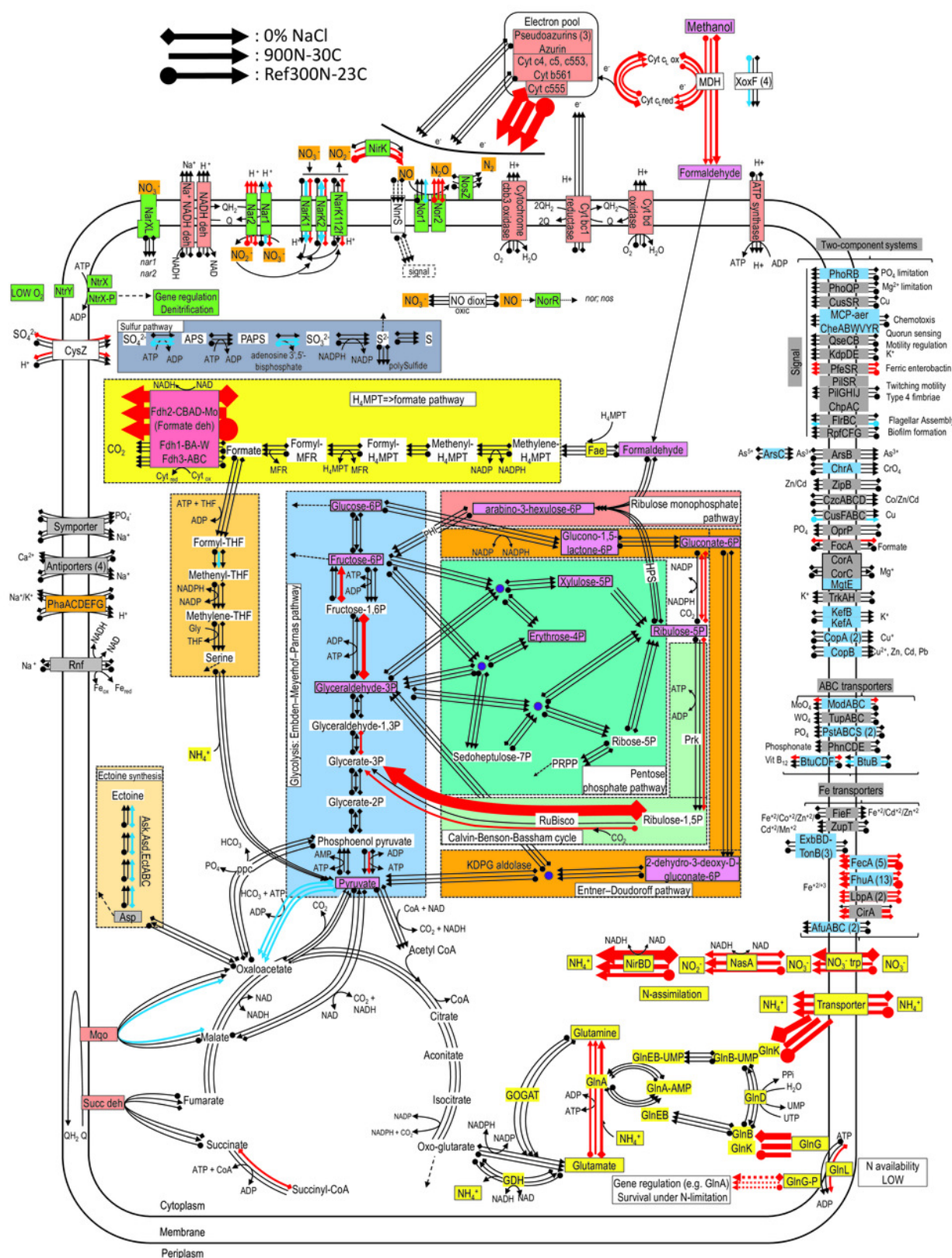



Figure 4

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

See Figure 3 legend.

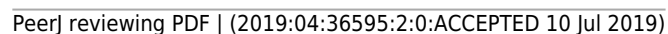
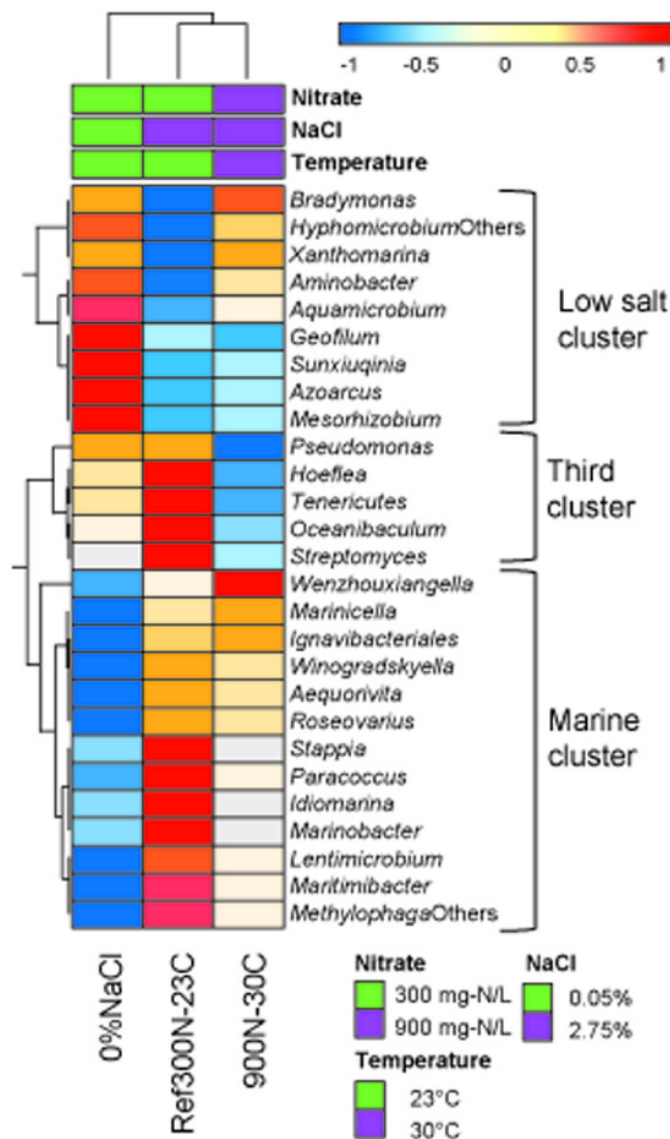



Figure 5

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) .

Biofilm culture conditions

1 **Table 1. Biofilm culture conditions**

Name	Medium	NO$_3^-$	Methanol	NaCl	Temp	Specific[a] denitrification rates
		mM (mg-NO$_3$-N/L)[c]	% (v/v)[c]	% (w/v)	°C	mM-NO$_x$ h^{-1} mg-protein^{-1}
Ref300N-23C[b]	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	3.0[d]	23	0.0611

16 The original biofilm was cultured in triplicates in these conditions at pH 8.0. The carriers were
17 transferred 5 times in fresh medium around each week before measuring the denitrifying
18 activities.
19 [a] From Payette *et al.* (2019).
20 [b] Reference biofilm cultures.
21 [c] The C/N ratio was 1.5 in all biofilm cultures
22 [d] The exact amount of NaCl added in the IO medium is not known. See Payette *et al.* (2019) for
23 the IO composition. For comparison, the amount of Na$^+$ and Cl$^-$ in the ASW medium is 3.2%.
24 In gray are changed parameters from the reference biofilm cultures.
25 IO: Instant Ocean medium.

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					0.02		
" "	*Hoeflea*	0.30	0.76	0.03	0.01			0.06	0.12	0.06
" "	*Nitratireductor*	1.40	0.03							
" "	*Hyphomicrobium**	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)*10^2	6.0(3.2)*10^1	1.3(0.5)*10^2	1.3(0.9)*10^2	2.8(1.0)*10^4	5.3(6.5)*10^4	1.1(0.9)*10^4
Alphaproteobacteria;Rhodobacterales;	*Litorisediminicola*	0.38	1.43							
" "	*Lutimaribacter*	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					0.01		
" "	*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	0.05	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**		0.09	0.11	0.04	0.13	0.52	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)*10^3	5.6(4.6)*10^4	2.3(0.6)*10^5	1.9(0.5)*10^5	2.1(0.4)*10^5	2.1(1.4)*10^5	4.7(2.0)*10^4	3.6(2.7)*10^4	1.5(0.2)*10^5
Others		9.86	4.27	0.25	0.37	0.53	2.00	2.28	0.69	0.25
Total of reads		348358	319265	9610	14048	8532	13915	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.

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

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

	Sequence identity with *H. nitrativorans*	Number of OTUs	Number reads		Proportion of reads %	
			OB	IO	OB	IO
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
	Sequence identity with *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

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

Affiliation of the isolates isolated from the reference biofilm cultures (Ref300N-23C)

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, Flavobacteriales	
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

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

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

	Ratio TPM Biofilm cultures / TPM pure cultures				
	GP59			NL23	
Genes	Ref300N-23C	900N-30C	0%NaCl	0%NaCl	Genes
Denitrification					
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		
nosRZDFYL	ns	ns	ns	5.2	*nosRZDFYLX*
nnrS (3)	ns	ns	ns	ns	*nnrS* (2)
nsrR (2)	2.1	2.5	ns	ns	*nsrR*
DnrN	2.3	2.8	-3.0	ns	*nnrU*
NOdiox (2)	ns	ns	ns	4.0	*nnrR*
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	ns	*ntrYX*
glnA	2.0	3.1	5.1	19	*glnA*
				-2.1	*glnA* (3)
gltBD: GOGAT	ns	ns	ns	ns	*gltBD*
glnB	ns	ns	ns	14	*glnB*
GDH	ns	ns	ns	ns	GDH
glnD	ns	ns	ns	-2.9	*glnD*
glnE	ns	ns	ns	-3.2	*glnE*
glnK	18	24	25	22	*glnK*
NH₄ trp	11	11	11	20	NH₄-trp
glnLG	ns	2.1	5.9	ns	*glnLG*

37   Data values are the ratio of the Biofilm TPM divided by the planktonic pure-culture TPM. When the
38   ratios were <1, the negative inverse value (-1/ratio) was calculated. Compared to each other, the positive
39   ratio means higher relative transcript levels in the biofilm, and negative ratio higher relative transcript
40   level in the planktonic pure cultures. ns: no significant changes.
41   *glnA*: glutamine synthetase; *gltBD*: glutamate synthase; GDH: glutamate dehydrogenase; *glnB*: nitrogen
42   regulatory protein P-II 1; *glnK*: nitrogen regulatory protein P-II 2; *glnD*: uridylyltransferase; *glnLG*:
43   nitrogen regulation sensor histidine kinase and response regulator; trp: transporter; Diox: dioxygenase;
44   *nnrS*: involved in response to NO; *nsrR*: NO-sensitive transcriptional repressor; *DnrN*: NO-dependent
45   regulator; *nasAnasBD*: assmilatory nitrate and nitrite reductase; *ntrXY*: Nitrogen regulation proteins;
46   *narXL*: nitrate/nitrite sensor histidine kinase and response regulator; *narK*: nitrate/nitrite transporter;
47   *nosRE*: NO- reductase transcription regulator and activation protein.

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*				
Streptomyces	6103	729	2499	-
•*Bacteroidetes*				
Sunxiuqinia	212	504	2639	*norB*
Geofilum	77	109	11870	-
Aequorivita	18218	25569	1398	*nirK, norB;C;D;Q, nosZ;DFY*
Winogradskyella	8182	7917	169	*nirK, norC, nosZ;L;D*
Lentimicrobium	3032	3370	1671	-
Xanthomarina	190	4555	9598	*nirK, norB;D;C;Q, nosZ;L;D*
•*Ignavibacteriales*	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;J;I, nosZ;R;D, nirK*
Hyphomicrobium	0	1679	30057	*narH;I, norB;C;Q;D;E, nosZ;D;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;Q;E, nosZ;D;R*
Stappia	39926	30885	39745	*napABC, napADFE-nnrS, 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*
Methylophaga	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;I, nirK, nirS, norB;C;D;Q, nosZ;R,*
Wenzhouxiangella	132	2857	83	-
•Other bacteria	113200	243523	155554	*
•Archaea	113	297	303	
•Eukarya	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

51 sequences were annotated for function and affiliation. The TPM of the genes affiliated to specific bacteria
52 taxa were then summed. Denitrification genes identified by annotations from respective affiliated bacterial
53 taxa were sorted out. * Denitrification genes were found scattered in other bacterial taxa.