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1 **Isolation and physiological characterization of psychrophilic denitrifying bacteria**
2 **from permanently cold Arctic fjord sediments (Svalbard, Norway)**
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27 Key Words: Psychrophile / denitrification /temperature

28 Running Title: Denitrifying Bacteria in Arctic Sediments

29 **ABSTRACT**

30 A large proportion of reactive nitrogen loss from polar sediments is known to be mediated by
31 denitrification. However, the microorganisms mediating denitrification in polar
32 environments remain poorly characterized. A combined approach of MPN enumeration,
33 cultivation, and physiological characterization was used to describe psychrophilic
34 denitrifiying bacterial communities in sediments of three Arctic fjords in Svalbard (Norway).
35 The physiological response of representative isolates to temperature was examined by
36 quantifying growth rates, nitrate depletion, and membrane lipid composition across a
37 temperature gradient. A most probable number (MPN) assay showed the presence of 10^3 -
38 10^6 cells of psychrophilic denitrifying bacteria g^{-1} of sediment. Seventeen denitrifying
39 strains displaying wide phylogenetic affiliations within the Proteobacteria were isolated using
40 a systematic enrichment approach with organic acids as an electron donor and nitrate as an
41 electron acceptor. Phylogenetic characterization of 16S rRNA gene sequences indicated that
42 the isolates belonged to five genera, including *Shewanella, Pseudomonas, Psychromonas*
43 (Gammaproteobacteria), *Arcobacter* (Epsilonproteobacteria), and *Herminiimonas*
44 (Betaproteobacteria). All the isolates were determined to be facultative anaerobes and
45 complete denitrifiers, showing stoichiometric conversion of nitrate to gaseous end products.
46 The growth response from 0 to 40°C indicated that all genera, except *Shewanella* were
47 psychrophiles (optimal growth <15 °C). Adaptation to low temperature was confirmed as
48 membrane fatty acid profiles showed a shift from primarily C16:0 saturated fatty acids to
49 C16:1 monounsaturated fatty acids at lower temperatures. This study provides the first
50 targeted enrichment and characterization of psychrophilic denitrifying bacteria from polar

51 sediments, and two genera, *Arcobacter* and *Herminiimonas*, are isolated for the first time in
52 permanently cold marine sediments.

53 **INTRODUCTION**

54 Nitrogen is a major limiting nutrient of biological productivity in the coastal
55 ocean (Rabalais, 2002; Howarth and Marino, 2006). The response of the nitrogen cycle
56 to anthropogenic disturbances is strongly influenced by the phylogenetic structure and
57 associated function of microbial communities responsible for nitrogen loss in coastal
58 marine ecosystems. Two microbially catalyzed respiration processes, denitrification and
59 anammox, convert dissolved inorganic nitrogen (NO_3^-, NO_2^-, NH_4^+) to gaseous N_2 and
60 comprise the largest sink of reactive nitrogen from the coastal ocean on a global scale.
61 Up to 50% of marine N removal is estimated to occur by denitrification and anammox in
62 continental shelf sediments (Codispoti, 2007). The relative contribution of sedimentary
63 denitrification and anammox to N removal varies strongly with water column depth, but
64 denitrification is generally considered the dominant pathway for N removal in shallow (<
65 100m) shelf sediments (Dalsgaard et al., 2005).

66 The Arctic Ocean is the shallowest of the world's ocean basins and is comprised
67 of 50% continental shelf. Substantial denitrification and anammox rates have been
68 measured on Arctic shelves, indicating that the Arctic basin has a significant role in
69 global N removal (Devol et al., 1997; Rysgaard et al., 2004; Gihring et al., 2010).
70 Future reductions in Arctic sea-ice cover may lead to diminished fluxes of organic matter
71 to sediments, resulting in major shifts in the biogeochemical cycling of nitrogen
72 (Piepenburg, 2005; Arrigo et al., 2008). Thus, an understanding of the diversity and
73 physiology of denitrifying bacteria from polar sediments is integral to understanding
74 climate change related effects on nitrogen cycling in the Arctic.

75 Though sedimentary denitrification comprises an important N sink in marine
76 ecosystems on a global scale and the majority of the seafloor is cold (< 5 °C), few studies
77 have addressed the physiological adaptation of denitrifiers to cold temperatures. Arctic
78 shelf sediments are characterized by permanently cold conditions, but rates of microbial
79 metabolism (e.g., hydrolysis, oxygen respiration, and sulfate reduction) from Arctic
80 sediments largely overlap with those of temperate sediments (Arnosti et al., 1998;
81 Thamdrup and Fleischer, 1998; Kostka et al., 1999). This apparent lack of temperature
82 limitation has been ascribed to the fact that microbes in these sediments are psychrophilic
83 (see Morita, 1975). The permanently cold conditions in Arctic sediments may exert a
84 strong selection for psychrophilic bacteria, but isolation of aerobic bacteria from Arctic
85 sediments has yielded a mix of psychrophilic and psychrotolerant bacteria (Groudieva et
86 al., 2004; Helmke and Weyland, 2004; Srinivas et al., 2009). Denitrifying bacteria have
87 been isolated from cold (≤ 4 °C) marine waters from temperate environments under
88 anaerobic conditions with nitrate as an electron acceptor (Brettar et al., 2001), but to date,
89 no study has systematically investigated psychrophilic denitrifying bacteria in
90 permanently cold sediments.

91 Shallow sediments in the Arctic Ocean basin have been shown to be active sites
92 of denitrification, but the microbial communities mediating this process are understudied.
93 Cultivation-independent methods have been used to study the community structure of
94 denitrifying bacteria in coastal marine sediments from primarily temperate ecosystems
95 (Braker et al., 2001; Mills et al., 2008), but horizontal gene transfer events of
96 denitrification genes make it difficult to reconstruct phylogenies (Heylen et al., 2006).
97 Therefore, cultivation of representative denitrifying bacteria is a crucial component to

98 improving detection of environmentally relevant taxa by cultivation-independent
99 approaches. A better understanding of the physiology of psychrophilic denitrifying
100 bacteria is also a necessity to better predict the role of low temperature in controlling
101 denitrification activity in polar sediments. In the present study, a primarily cultivation-
102 based approach was used to investigate the phylogeny and physiology of psychrophilic
103 denitrifying bacteria from Arctic fjord sediments. The objectives of this study were to:
104 (i) isolate and phylogenetically characterize psychrophilic bacteria capable of
105 denitrification; (ii) examine the physiology of cold adaptation in psychrophilic
106 denitrifying isolates; and (iii) detect isolated taxa in sediment samples using molecular
107 community fingerprinting.
108

109 **MATERIALS AND METHODS**

110 **Sample sites and sampling procedures**

111 Sediment cores were collected in August 2008 from three fjord sites within the
112 Svalbard archipelago (Table 1). At the time of collection, sediment surface temperatures
113 ranged from 1.3 - 6.5°C. Sediments from Smeerenburgfjorden (SM) were black clayey
114 and rich with organic matter, while the sediments from Ymerbukta (YM) and
115 Kongsfjorden (KF) were black sandy and reddish-brown loamy, respectively. Sediment
116 cores were retrieved with a Haps corer, and subsamples from the upper 0-5 cm depth
117 interval were collected aseptically into sterile conical tubes. Samples for cultivation were
118 transported at *in situ* temperature and stored at 1.5 °C until processed. Samples for
119 molecular characterization were frozen immediately and stored at -80 °C until further
120 analysis.

121 **Enrichment and isolation of denitrifying bacteria**

122 A bicarbonate buffered minimal saltwater medium (MSW) was prepared and
123 dispensed according to Widdel and Bak (1992), with the modifications of omitting
124 sulfate, resazurin, selenite, and tungstate. The medium contained the following
125 components per liter: NaCl (20 g), NH_4Cl (0.250 g), KH_2PO_4 (0.200 g), KCl (0.5 g),
126 $MgCl_2*6H_2O$ (3.0 g) and $CaCl_2*2H_2O$ (0.150 g) $NaHCO_3$ (2.5 g), trace element solution
127 (TES; 1 ml), vitamin B_{12} (1 ml), vitamin mix (1 ml) and thiamine (1 ml). The medium
128 was autoclaved and poured under strictly anoxic conditions with a N_2:CO_2 (80:20)
129 headspace, resulting in a final pH of 7.0. All enrichments and physiological screening of
130 the isolates was conducted in this medium with modifications to the electron donor and
131 NO_3^- concentration as indicated.

132 Enrichment experiments were conducted with 1 mM NO_3^- as the electron acceptor
133 and with either acetate (10mM), lactate (10mM), or a APB (acetate, propionate, butyrate,
134 10mM each) as the source of carbon and energy. Enrichments were inoculated with 10 %
135 (w/v) sediment from each sample site and incubated in the dark at 1.5 °C. Enrichments
136 were transferred to fresh medium every 10 days using a 10 % inoculum (v/v). After the
137 second transfer, the concentration of NO_3^- was raised from 1 mM to 5 mM in order to
138 prevent growth limitation by nitrate and cell lysis.

139 For isolation and purification, the MSW medium was supplemented with 10 mM
140 HEPES (Fisher Scientific) and 1.8 % molecular grade agar (Sigma-Aldrich) as a
141 buffering and solidifying agent, respectively. Streak plates were prepared and incubated
142 at 1.5 °C under aerobic conditions. Morphologically distinct colonies were picked using
143 sterile toothpicks and purified by multiple re-streakings onto fresh plates. The purity of

144 each culture was reconfirmed by PCR amplification and sequencing of the small-subunit
145 (SSU) rRNA gene. Culture stocks were preserved at -80 °C in 20 % glycerol.
146 Purified isolates were screened for nitrate depletion and gaseous nitrogen
147 production under denitrifying conditions in anaerobic MSW medium amended with ^{15}N-
148 enriched NO_3^- (98 atom %; Cambridge Isotope Laboratories, Inc., Andover, MA).
149 Cultures and uninoculated controls were prepared in 10-ml Hungate tubes. At the initial
150 time-point (immediately after inoculation) and after maximum cell density was achieved,
151 growth was terminated in duplicate cultures by the addition of 1 % (wt/vol) $HgCl_2$. Gas
152 samples for N_2O analysis were extracted from the headspace through the rubber septa cap
153 using a 100-µl gas-tight syringe and were immediately analyzed by gas chromatography
154 using a Shimadzu GC-8A gas chromatograph equipped with a Porapak-Q column and an
155 electron-capture detector. The production of N_2 was determined by the accumulation of
156 excess ^{15}N-N_2 using a membrane inlet mass spectrometer configured and calibrated
157 according to An *et al.* (2001). Nitrate depletion was confirmed using a colorimetric
158 method (Cataldo, 1975).
159 **Most probable number enumeration**
160 Psychrophilic denitrifying bacterial populations from Arctic fjords were
161 enumerated by the three-tube most-probable-number (MPN) assay using 10-fold serial
162 dilutions of fjord sediments in MSW growth medium. Tubes were incubated at ambient
163 sediment temperature (1°C) for two months. Lactate was chosen as the electron donor
164 for the MPN experiments, based on the vigorous growth and taxonomic coverage in
165 initial lactate-amended enrichments. Growth of denitrifying bacteria was monitored by
166 culture turbidity, depletion of added nitrate, and accumulation of N_2O in the vial

167 headspace as compared to nitrate-free controls. The MPN index was determined from
168 statistical tables published by the American Public Health Association (1969). Isolates
169 were obtained from the highest positive MPN dilutions and were subsequently identified
170 by SSU rRNA gene sequences. However, no physiological tests were performed on these
171 isolates.

172 **Total community profiling by TRFLP**

173 Genomic DNA from frozen sediment grabs was extracted in triplicate using a Mo-
174 Bio Power Soil™ DNA kit (Mobio Laboratories, Carlsbad, CA, USA) according to the
175 manufacturer's instructions. The 16S rRNA gene was amplified by PCR using the 27F
176 and 1492R general bacterial primers (Lane, 1991). The forward primer (27F) was
177 fluorescently labeled with 6-carboxy fluorescine (FAM) for Terminal Restriction
178 Fragment Length Polymorphism (TRFLP) profiling. PCR reactions were conducted
179 using EconoTaq Plus 2X master mix (Lucigen Corporation, Middleton, WI). A final
180 concentration of 0.33 μM and 0.25 μM was added for the forward and reverse primer,
181 respectively. PCR yields were column purified using the UltraClean™ PCR clean-up kit
182 (Mobio). A single enzyme digestion of PCR products was performed using the
183 restriction enzyme *Bsh* (Fermentas, Glen Burnie, MD). Digestion reaction products were
184 read by an ABI 310 genetic analyzer at the Florida State University sequencing facility
185 (Tallahassee, USA). Processing of TRFLP profiles was performed using Gene Mapper
186 software (Applied Biosystems, Foster City, CA). TRFLP profiles that had a total peak
187 area of less than 1000 were not included in the analysis.

188 **Phylogenetic analyses**

189 Genomic DNA of the recovered isolates was extracted using the Mo Bio
190 UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA)
191 according to the manufacturer's instructions. For 16S rRNA gene amplification, the 27F
192 and 1492R general bacterial primers (Lane, 1991) were used. PCR reactions were
193 conducted using EconoTaq Plus 2X master mix (Lucigen Corporation, Middleton, WI).
194 Primers were added to a final concentration of 0.5 μM, and the magnesium concentration
195 was adjusted to 4.0 mM with the addition of magnesium chloride. The resulting PCR
196 yield was column purified using the GenCatch (TM) PCR Cleanup Kit (Epoch Biolabs,
197 www.epochbiolabs.com). In some cases, long rRNA gene sequences were generated
198 using multiple sequencing reactions, and composite sequences were generated using the
199 software package Sequencher (Gene Codes, Ann Arbor, MI). Low quality data were
200 trimmed from the sequences prior to generating the composite sequences. Nearly full
201 length 16S rRNA gene sequences were submitted to Genbank under the accession
202 numbers [XXXX-YYYY].
203 Recovered sequences were aligned to known bacterial sequences using the
204 "greengenes" 16S rRNA gene database and alignment tool (DeSantis *et al.*, 2006).
205 Aligned sequences and close relatives were imported and alignments were manually
206 refined by visual inspection in the ARB software package (Ludwig *et al.*, 2004).
207 Sequences were exported from ARB using a bacterial 50 % conservation filter (excluding
208 positions at which less than 50 % of the sequences had the same base). These filtered
209 sequences were imported into the MEGA 4.0 software package (Tamura *et al.,* 2007),
210 and neighbor-joining phylogenetic trees were constructed using the maximum composite
211 likelihood substitution model with complete deletion of gapped positions (946

212 informational positions). The robustness of inferred tree topologies was evaluated by
213 1,000 bootstrap re-samplings of the data. Additionally, Bayesian analyses were
214 performed on the filtered sequence data (MrBayes ver. 3.1; Ronquist and Huelsenbeck,
215 2003) by running four simultaneous chains (3 heated, 1 cold) for four million
216 generations, sampling every 1000 generations. The selected model was the general time
217 reversible (GTR) using empirical base frequencies, and estimating the shape of the
218 gamma distribution and proportion of invariant sites from the data. A resulting 50 %
219 majority-rule consensus tree (after discarding the burn-in of 25 % of the generations) was
220 determined to calculate the posterior probabilities for each node. The split differential
221 between the two runs was below 0.01 after the completion of the run.

222 **Fatty acid methyl ester analysis**

223 The response of membrane-derived fatty acid composition to shifts in temperature
224 was determined for a representative isolate of each genus under aerobic conditions at 1.5,
225 5 and 15 °C using the MSW medium supplemented with low levels of peptone (0.1 %),
226 yeast extract (0.1 %) and beef extract (0.05 %) as a carbon source. Freeze-dried cells (60
227 to 90 mg) were extracted using a modified Bligh and Dyer procedure (methanol-
228 chloroform-water,10:5:4). The solid cellular residue was recovered by centrifugation and
229 the solvent phase partitioned by addition of chloroform and water to a final ratio of
230 10:10:9. The lower chloroform layer containing the total lipid extract (TLE) was
231 removed and dried under N_2. Fatty acid methyl esters (FAME) were prepared by
232 treatment of the TLE by transesterification with freshly prepared 0.1 N methanolic NaOH
233 for 60 min at 37 °C (White, 1979). FAME were identified by GC-MS as described by

234 (Jahnke, 2004). The double-bond positions of FAME were determined by preparing
235 dimethyl disulfide adducts by heating at 35 °C for 35 min (Yamamoto, 1991).

236 **Nitrate Utilization and Optimum Growth Temperatures**

237 The growth rate and nitrate utilization potential were determined in batch culture
238 for representative isolates of each identified genus. A 5% (vol/vol) inoculum) from mid-
239 log phase cultures was added to MSW media amended with 10 mM lactate and 5 mM
240 NO_3^- for all isolates. Triplicate cultures were incubated at 5°C in 160 mL serum bottles,
241 and nitrate-free controls were used to test for fermentative growth. Growth was
242 monitored as optical density at 600 nm using a Shimadzu UV-Vis spectrophotometer.
243 Nitrate + nitrite and nitrite were determined by chemiluminescence detection after
244 reduction with vanadium (Braman and Hendrix, 1989) or iodide (Garside, 1982).

245 Optimum growth temperatures were determined for representative isolates in a
246 temperature gradient block incubator. Isolates were grown under denitrifying conditions
247 in MSW with 10mM lactate and 5mM NO_3^- at 7-10 temperatures between 0 °C and 30
248 °C. Optical density at 600 nm was monitored twice daily in a Spectronic 21
249 spectrophotometer by placing an entire Balch tube into the instrument. Specific growth
250 rates (μ) were calculated as the slope of the linear portion of the plot of the natural log
251 (ln) of O.D. versus time.

252

253 **RESULTS**

254 **Characterization of *in situ* communities**

255 Cultivatable denitrifying microorganisms were enumerated using an MPN serial
256 dilution assay at each site. MPN counts were 2.4×10^3 cells/g sediment, 6.1×10^5

257 cells/g sediment, and 3.0 x 10^6 cells/g sediment at sites SM, KF, and YM, respectively
258 (Table 1). Growth by denitrification in the MPN tubes was inferred from higher turbidity
259 as compared to control tubes (lactate only), as well as depletion of nitrate and
260 accumulation of N_2O. Isolation of bacteria from the highest positive dilutions, followed
261 by 16S rRNA gene sequencing and BLAST alignmnent, indicated that the cultivatable
262 denitrifier with the highest relative abundance at KF and YM was closely related to
263 *Psychromonas* sp., while *Shewanella* sp. and *Psychomonas* sp. were highly enriched at
264 SM.

265 DNA fingerprinting by TRFLP targeted to16S rRNA gene sequences indicated
266 that the SM and KF sediments had a highly similar community composition (Figure 1).
267 For both sites, the most dominant peaks were seen at fragment sizes of 56, 103, 107, 210,
268 242, and 389 base pairs (bp). The 16S rRNA profile from the YM site showed a distinct
269 community composition compared to the other two sites, and the most dominant peaks
270 were at fragment sizes of 56, 109, 242, and 391 bp. An *in silico* digest of 16S rRNA
271 gene sequences from the isolates obtained in this study (see following section) showed
272 that peaks from all three sites at 210 and 389 bp matched the predicted fragment sizes
273 from *Shewanella* and *Pseudomonas*. A peak at 395 bp corresponding to *Arcobacter* was
274 observed at sites SM and YM (Figure 1).

275 **Isolation and phylogenetic characterization**

276 A systematic enrichment strategy was used to isolate denitrifying bacteria from
277 one intertidal and two permanently cold sediments. The most rapid growth was observed
278 in the serum vials amended with sediments from SM, followed by YM and KF. Visual
279 observation of the plates indicated an abundance of slow growing, small colonies and

280 fewer, fast growing, slightly pigmented colonies. More than 200 colonies were obtained
281 from each enrichment on solid media plates, and based on colony morphology and
282 growth pattern, a total of 17 colonies were selected for further screening.
283 Phylogenetic analysis of near full-length 16S rRNA gene sequences showed
284 that the marine denitrifying isolates belonged to five genera within the Gamma-, Beta-
285 and Epsilonproteobacteria (Figure 2). Isolates were classified within the genera
286 *Arcobacter* (6 isolates), *Herminiimonas* (1 isolate), *Pseudomonas* (3 isolates),
287 *Psychromonas* (3 isolates), *and Shewanella* (4 isolates) based on 16S rRNA gene
288 sequence similarity (Table 2). *Arcobacter* isolates showed high sequence identity (>97%
289 BLAST similarity) to either *Arcobacter* sp. KT0913 (Heylen, 2006) or *Arcobacter*
290 *venerupis* F67-11(Levican et al., 2012).
291 **Fatty acid methyl ester profiles**
292 Representative isolates were grown in MSW medium at 1.5°C, 5°C, and 15°C
293 under aerobic conditions to examine the acclimation of membrane fatty acid composition
294 to low temperature (Table 3). At all growth temperatures, the primary FAMEs detected
295 in all isolates were C16:0, 16:1ω7c and 18:1 ω7c. These three fatty acids comprised
296 greater than 95% of the total extracted fatty acids in strains Y2B (*Psychromonas*), SL-1
297 (*Pseudomonas*) and SPB (*Herminiimonas*). In addition to 16:1ω7c and 18:1 ω7c, strains
298 YAS-1 and SAS1-1 (*Arcobacter*) also contained significant amounts of C14:0 (4 – 5%),
299 14:1ω7c (5 – 8%), and 16:1ω7t (5 – 10%). *Shewanella* strain YLB-1 had the most
300 diverse fatty acid profile and was the only strain that contained branched fatty acids (20 –
301 28 %), as well as eicoaspentaenoic acid (20:5ω3).

302 With decreasing growth temperatures, all strains except *Shewanella* YLB-1
303 exhibited a decrease in the relative abundance of the most abundant saturated fatty acid,
304 C16:0. *Arcobacter* and *Herminiimonas* strains also exhibited a lower concentration of
305 18:1 ω7c with lowered temperature. Concomitant with the relative decrease in saturated
306 and long chain fatty acids at low temperature, increases in monounsaturated acids were
307 observed that showed variation with respect to strain. *Psychromonas* Y2B and
308 *Herminiimonas* SP-B exhibited an increase primarily in 16:1ω7c, while *Arcobacter*
309 strains increased 14:1ω7c and 16:1ω7c. *Pseudomonas* SL-1 increased 16:1 ω7c and 18:1
310 ω7c in response to lowered temperature. Consistent with its unique fatty acid profile,
311 *Shewanella* YLB-1 exhibited unique shifts in fatty acids with lowered growth
312 temperature, including increases in C16:0 and 17:1ω8c and decreases in branched (i13:0
313 – i15:0) fatty acids and 14:1ω7c.

314 **Denitrification activity and optimal growth temperature**

315 Denitrification capacity was confirmed in all 16 isolates by higher biomass
316 accumulation in nitrate-amended media as compared to nitrate-free controls, as well as
317 near-stoichiometric conversion of nitrate to gaseous end products (N_2 and N_2O). Strains
318 were all facultative anaerobes, and produced either N_2O (*Shewanella* and *Psychromonas*)
319 or N_2 (*Arcobacter, Pseudomonas, Herminiimonas*) as the primary end-product of
320 denitrification.

321 Based on the phylogenetic analysis, six strains were selected (SL-1, Y2B, YAS-1,
322 SAS-1, YLB, SP-B), for further physiological characterization. The isolates were grown
323 at 5 °C in MSW media with 5mM NO_3^- and 10mM lactate, and the complete depletion of
324 nitrate concomitant with exponential growth was observed (Figure 3). Isolates from the

325 the *Gammaproteobacteria* had the highest specific growth rates (Figure 3), with
326 *Shewanella* sp. YLB-1 growing fastest(μ, 0.54 d^{-1}), followed by *Pseudomonas* sp. SL-1
327 (μ, 0.28 d^{-1}) and *Psychromonas* sp. Y2B (μ, 0.23 d^{-1}). Growth rates for the
328 *Herminiimonas* sp. SPB isolate (0.20 d^{-1}) and both *Arcobacter* isolates (0.14 – 0.17 d^{-1})
329 were lower than the Gammaproteobacteria isolates. Nitrate utilization, estimated by
330 linear regression of nitrate depletion during exponential growth phase, was highest in
331 *Arcobacter* sp. SAS-1, *Shewanella* sp. YLB, and *Arcobacter* sp. YAS-1. There was no
332 strong correlation between growth rate and nitrate utilization rate.
333 All strains had optimal growth temperatures of 15 °C or less, except *Shewanella*
334 YLB-1, which had an optimal growth temperature of 18 °C (Figure 4). All strains
335 maintained substantial growth rates near 0 °C that were between 25 – 50% of the optimal
336 growth rate. Growth was not observed in any of the strains above 30 °C, and two strains,
337 *Psychromonas* Y2B and *Herminiimonas* SP-B, did not grow above 25 °C.
338

339 **DISCUSSION**

340 Denitrification is well recognized as a dominant pathway for the removal of
341 reactive nitrogen in marine sediments, including polar sediments. However, no prior
342 cultivation based studies have targeted denitrifying bacteria in permanently cold marine
343 sediments. Previous enrichment studies from Arctic sediments have often been
344 conducted under aerobic conditions, using complex cultivation media, short incubation
345 times, and incubation temperatures above *in situ* values (Srinivas et al., 2009; Kim et al.,
346 2010a; Yu et al., 2010). In this study, denitrifying bacteria were anaerobically enriched
347 in a minimal medium with defined electron donors. Enrichments were carefully

348 maintained at *in situ* temperatures and incubation times were lengthened (> 30 days) to
349 mimic *in situ* conditions. This approach allowed for the isolation of taxa whose role in
350 denitrification may have previously been overlooked.
351
352 **Characterization of *in situ* denitrifying communities**
353 Most probable number (MPN) enumeration indicated the presence of $2 \times 10^3 - 3$
354 $\times 10^6$ cells of denitrifying bacteria g^{-1} of sediment. Quantification of total bacterial
355 abundance by direct counts in Svalbard surface sediments has shown the presence of $2 \times$
356 $10^8 - 3 \times 10^9$ cells cm^{-3} of sediment, and site SM has been determined to have 2.1 – 4.7
357 $\times 10^9$ cells cm^{-3} (Sahm and Berninger, 1998; Ravenschlag, 2001). From these results, the
358 relative abundance of denitrifying bacteria can be estimated to contribute between less
359 than 0.01 % to 1.5 % of the total community. The relative abundance of denitrifying
360 bacteria was similar (0.17 %) for temperate estuarine sediments using a MPN-based
361 approach, but the same study found up to two orders of magnitude more denitrifying
362 bacteria using qPCR-based functional gene analysis (Michotey et al., 2000).
363 It is unclear why differences in denitrifying MPN cell numbers between sites did
364 not correspond with reported denitrification rates. While site SM exhibited high rates of
365 denitrification, it also had a lower number of denitrifying bacteria than site KF. The
366 choice of lactate as an electron donor for the MPN experiment may have biased the
367 growth in SM sediments, and also, the use of only an organic electron donor may have
368 limited the growth of autotrophic denitrifying bacteria. Site YM had the highest number
369 of denitrifying cells (3.0×10^6), which may have been influenced by the input of

370 macroalgal detritus in the intertidal zone. The C:N ratio of 19.9 ratio at site YM falls
371 near the median value reported for macroalgae (Atkinson and Smith, 1983)
372 Based on an *in silico* digest of 16S rRNA gene sequences from our isolates, three
373 isolates (*Shewanella, Pseudomonas, Arcobacter*) were putatively detected in the TRFLP
374 profiles from the fjord sediments. All of the genera isolated in this study except
375 *Herminiimonas* have been previously detected in polar marine sediments in 16S rRNA
376 gene clone libraries. Bowman et al. (2003) found 5 -10 % Shewanella and 2 -5 %
377 *Psychromonas* in clone libraries from the top 1cm of Antarctic coastal sediments.
378 Members of *Shewanella* and *Pseudomonas* have also been detected in clone libraries
379 from surficial sediments (0-5 cm) in the Beaufort Sea (Li, 2009). In Svalbard sediments,
380 *Pseudomonas* has been detected at Hornsund (Ravenschlag et al., 1999) and *Shewanella,*
381 *Psychromonas,* and *Arcobacter* have been detected near site KF in Kongsfjorden (Tian et
382 al., 2009). These studies provide further evidence for the widespread presence of the
383 genera isolated in this study in the surficial layers of permanently cold sediments.
384 However, further cultivation-independent studies are needed to confirm that the isolates
385 from this study are the primary taxa that perform denitrification *in situ*.
386 **Distribution of psychrophily and denitrification within the genera isolated**
387 Members of the genus *Shewanella* have been isolated and described from a wide
388 range of oceanic regions, including psychrophilic strains from deep-sea and polar
389 sediments (Kato and Nogi, 2001). *Shewanella* species are capable of respiring a diverse
390 set of electron acceptors, including metals (Fe, Mn), sulfur compounds, and nitrate (Hau
391 and Gralnick, 2007). Complete denitrification has been confirmed for a few *Shewanella*
392 isolates from the marine environment (Brettar et al., 2002; Zhao et al., 2006), but the

393 presence of the marker gene for dissimilatory reduction of nitrate to ammonium (nrfA) in
394 the genomes of *Shewanella* species indicates that this nitrate respiration pathway may be
395 more common within the genus than denitrification (Simpson et al., 2010). For example,
396 the described organism that shows the highest SSU rRNA gene sequence similarity to
397 *Shewanella* YLB-1, *Shewanella frigidimarina*, is unable to reduce nitrite, lacks the key
398 denitrification genes (nirS/K and nosZ), and possesses the nrfA gene (Kato and Nogi,
399 2001; Markowitz et al., 2012). The isolation of N_2 gas producing *Shewanella* strains in
400 this study further strengthens the evidence for the contribution of *Shewanella* to
401 sedimentary denitrification in permanently cold sediments.

402 Nearly all described species of the genus *Psychromonas* are psychrophilic, as the
403 name implies. This genus (along with *Shewanella*) is found in the order
404 *Alteromonadales*, and is readily isolated under aerobic conditions from sea-ice, marine
405 water columns, and sediments (Groudieva et al., 2003; Auman et al., 2006; Nogi, 2007).
406 While nitrate reduction to nitrite is common within the genus, the only evidence for
407 complete denitrification is nitrite reduction by *Psychromonas hadalis* (Nogi, 2007) and
408 the presence of nitrous oxide reductase genes in *Psychromonas ingrahamii* (Markowitz et
409 al., 2012). The confirmation of gaseous nitrogen production in the isolate *Psychromonas*
410 Y2B from this study provides more conclusive evidence for denitrification within the
411 genus *Psychromonas*.

412 *Pseudomonas* is readily isolated from marine sediments, and the genus contains
413 many denitrifying representatives (Zumft, 1997). One marine strain, *Pseudomonas*
414 *stutzeri* ZoBell, has been used as a model organism for the study of denitrification

415 (Lalucat et al., 2006). *Pseudomonas* is a ubiquitous denitrifying genus, and its
416 occurrence in permanently cold marine sediments is not unexpected.

417 Isolates from the genus *Arcobacter* have been obtained from a variety of marine
418 environments, including hydrothermal vents, tissue from mussels, and the water column
419 off the coast of Europe and Africa (Eilers et al., 2000; Huber et al., 2003; Levican et al.,
420 2012). Some strains of *Arcobacter* are able to oxidize sulfide to produce filamentous
421 sulfur (Wirsen, 2002), which may be coupled to denitrification under anaerobic
422 conditions (Lavik et al., 2009). Substantial rates of sulfate reduction have been measured
423 in surface sediments at sites SM and YM (Arnosti and Jorgensen, 2006; Sawicka et al.,
424 2010), which may supply sulfide for autotrophic denitrification by *Arcobacter* species.
425 Reduction of nitrate to nitrate is ubiquitous within the genus *Arcobacter*, and complete
426 denitrification has been confirmed for *Arcobacter* isolates from activated sewage sludge
427 (Heylen, 2006). However, no denitrifying strains from the marine environment have
428 been described. The *Arcobacter* isolates from this study are the first confirmed
429 denitrifying isolates from the marine environment, as well as the first reported
430 psychrophilic strains.

431 The psychrophilic nature of *Herminiimonas* is not surprising given that isolates
432 have been obtained from an Antarctic glacier (Garcia-Echauri et al., 2011), a deep
433 (3042m) Greenland glacial ice core (Loveland-Curtze et al., 2009), and Greenland sea ice
434 brine (Møller et al., 2011). At least two other isolates of *Herminiimonas* have been
435 shown to reduce nitrate, and the *Herminiimonas arsenicoxydans* genome contains the
436 nirK gene (Lang et al., 2007; Muller, 2006). The isolate *Herminiimonas* SP-B from this
437 study is the first confirmed denitrifying *Herminiiomonas* isolate from the marine

438 sediments, which broadens the potential functional role of *Herminiimonas* in marine
439 sediments.

440 **Adaptation of denitrifying bacteria to low temperatures**

441 The optimal growth temperature, Topt, has been established as the primary
442 parameter to distinguish psychrophilic from psychrotolerant and mesophilic bacteria
443 (Morita, 1975). The relatively high growth rates of psychrophilic bacteria at low
444 temperature reflect the adaptations necessary to maintain cellular metabolism at low
445 temperatures. These adaptations include expression of enzymes that are efficient at low
446 temperatures, production of cryoprotectant molecules, and the ability to maintain
447 membrane fluidity by altering lipid composition (D'Amico et al., 2006). In the present
448 study, low temperature adaption was confirmed in psychrophilic denitrifying bacteria by
449 growth, nitrate depletion, and by a comparison of membrane lipid composition at low
450 temperature. The optimal growth temperatures and high rates of growth at 0°C (25-50%
451 of Topt) of the current isolates reflect the highly psychrophilic nature of our isolates. For
452 all isolates except *Shewanella* YLB-1, we observed optimum temperatures for growth
453 (Topt) that were amongst the lowest reported for the genera (Table 4). Furthermore, we
454 isolated the first confirmed psychrophilic *Arcobacter* strains, and we present the lowest
455 Topt for *Herminiimonas*, a taxon that is often isolated from permanently cold habitats.

456 A comparison of the three most abundant fatty acids (C16:0, C16:1, C18:1) from
457 our isolates to literature values show the highest values of C16:1 unsaturated fatty acids
458 in our isolates grown at 5 °C (Table 4). Very few psychrophilic isolates have been grown
459 at 5 °C or less for FAME analysis, which precludes a fair comparison between our strains
460 and previously isolated psychrophiles. However, a decrease in C16:0 and an increase in

461 16:1ω7c with decreasing growth temperature was the main adaptation consistent amongst
462 all isolates except *Shewanella* YLB-1 (Table 4). These results are consistent with
463 previous research that demonstrated the importance of monounsaturated fatty acids for
464 low temperature growth of *Photobacterium profundum* (Allen et al., 1999). The genus
465 *Shewanella*, in contrast, uses a strategy that involves regulating branched fatty acids and
466 eicosapentaenoic acid in addition to monounsaturated fatty acids (Wang et al., 2009).
467

468 **Conclusion**

469 A total of 17 strains of psychrophilic denitrifying bacteria were isolated from
470 Arctic fjord sediments with varying depth and organic carbon content. This study reports
471 the first systematic enrichment of psychrophilic bacteria under denitrifying conditions in
472 permanently cold marine sediments. The taxa isolated in this study are routinely detected
473 by cultivation-independent techniques in surficial sediments, but only *Pseudomonas*
474 species have been previously recognized in marine sediments for their ability to denitrify.
475 The genera *Arcobacter* and *Herminiimonas* have not been previously isolated from
476 permanently cold marine sediments, and there are no reports of psychrophilic marine
477 *Arcobacter* strains. Growth experiments revealed optimal temperatures for growth of the
478 current isolates that were amongst the lowest reported for all genera, with the exception
479 of *Shewanella*. Concordantly, monounsaturated fatty acids, necessary for low
480 temperature growth, were higher than previously reported concentrations. These results
481 confirm the strongly psychrophilic nature of the present isolates and corroborate the
482 hypothesis that denitrification activity in permanently cold sediments is maintained at
483 relatively high levels due to the activity of psychrophilic bacteria.

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Table 1. Sample site descriptions and most probable number (MPN) estimates of denitrifying bacteria

Sample Site (Abbr.)	Latitude	Longitude	Depth	Sediment temperature	Sediment C:N	Denitrification Rate (µmol N m^{-2} d^{-1})	MPN (cells g^{-1})
Kongsfjörden (KF)	78°59.43' N	12°17.87' E	51m	1.3°C	11*	34 (± 12)*	6.1 X 10^5
Smeerenbergfjörden (SM)	79°42.01' N	11°05.20' E	211m	1.6°C	7.2*	289 (± 5)*	2.4 X 10^3
Ymerbukta (YM)	78°16.84' N	14°02.97' E	intertidal	6.5°C	19.9	N.D.	3.0 X 10^6

*data from Gihring et al. (2010). Denitrification rates were measured by Isotope Pairing Technique (Nielsen, 1992)
N.D., not determined

763 **Table 2.** Phenotypic and genotypic characterization of denitrifying isolates. (N.D., not determined; APB, combination of acetate, propionate and
764 butyrate)

Genus (phylum) and isolate	Sample site	Electron donor	Primary denitrification endproduct	Closest Isolate by BLAST (Accession Number)	BLAST % Similarity
Herminiimonas (*Betaproteobacteria*)					
SP-B	SM	APB	N_2	*Herminiimonas fonticola* CCQ (EU636040)	98%
Arcobacter (*Epsilonproteobacteria*)					
KLS-1	KF	Lactate	N_2	*Arcobacter* sp. KT0913 (AF235110)	97%
SAS-1	SM	Acetate	N_2	*Arcobacter* sp. KT0913 (AF235110)	98%
SL-3	SM	Lactate	N_2	*Arcobacter* sp. KT0913 (AF235110)	97%
Y2S	YM	APB	N_2	*Arcobacter venerupis*F67-11 (HE565359)	97%
YAPB-1	YM	APB	N_2	*Arcobacter venerupis*F67-11 (HE565359)	97%
YAS-1	YM	Acetate	N_2	*Arcobacter venerupis*F67-11 (HE565359)	97%
Pseudomonas (*Gammaproteobacteria*)					
SL-1	SM	Lactate	N_2	*Pseudomonas frederiksbergensis*(HQ242750)	97%
SLB-2	SM	Lactate	N_2	*Pseudomonas* sp. ice-oil-499 (DQ521397)	99%
UL-1	SM	Lactate	N_2	*Pseudomonas brenneri*(FM877472)	99%
Psychromonas (*Gammaproteobacteria*)					
SL-2	SM	Lactate	N_2O	*Psychromonas ingrahamii*(CP000510)	97%
YAB-1	YM	Acetate	N.D.	*Psychromonas* sp. IC004 (U85849)	98%
Y2B	YM	APB	N_2O	*Psychromonas* sp. IC004 (U85849)	96%
Shewanella (*Gammaproteobacteria*)					
KLB-1	KF	Lactate	NA	*Shewanella vesiculosa*(NR_042710)	99%

SLB-1	SM	Lactate	N_2O	*Shewanella* sp. gap-d-13 (DQ530458)	97%
YLB-1	YM	Lactate	N_2O	*Shewanella frigidimarina*(AJ300833)	99%
UA-1	SM	Acetate	N.D.	NA	

Table 3. Temperature wise distributions (% distribution) of fatty acids in psychrophilic denitrifying bacteria isolated from Svalbard. Fatty acids that contributed less than 1% in all samples are not shown.

Isolate	YLB-1			Y2B			YAS-1			SAS-1			SL-1			SP-B		
	1.5°C	5°C	15°C	1.5°C	5°C	15°C	1.5°C	5°C	15°C	1.5°C	5°C	15°C	1.5°C	5°C	15°C	1.5°C	5°C	15°C
Saturated																		
C12:0	0.4	0.5	1.5				0.1	0.1	0.4	0.6		0.2						
C14:0	3.5	2.7	3.7	0.6	0.5	0.6	4	4.2	4.7	4.6	5.3	4.4	0.5	0.5	0.6	0.2	0.2	0.1
C15:0	5.9	4.6	4.6	0.1	0.3	0.1						0.1	0.2	0.4	0.1			
C16:0	**15.7**	**12.5**	**12.6**	**15.4**	**17.7**	**23**	**9.8**	**11.3**	**12.6**	**9.5**	**10.5**	**13.1**	**15.6**	**19.1**	**22.7**	**15.2**	**16.9**	**16.1**
C17:0	2.4	1.8	1.1		0.1									0.1				
Branched																		
i13:0	**5.9**	**6.1**	**9.9**															
i14:0	0.7	3.3	3.6															
i15:0	**9.2**	**12.7**	**12.5**															
Unsaturated																		
14:1ω7c	0.8	1.9	4.9	0.2	0.1	0.1	**7**	**5.5**	**5.1**	**8.1**	**6.6**	**4.2**	0.1	0.1	0.1			
15:1ω8c	1.6	1.1	1.3	0.1	0.1								0.1	0.1				
16:1ω9c	1.5	1.1	1														0.1	0.1
16:1ω7c	**25.8**	**27.6**	**25.6**	**67.2**	**62.7**	**58.9**	**56.9**	**57.1**	**48.6**	**52.2**	**58.9**	**50.6**	**65.7**	**61.4**	**60.7**	**72.3**	**66.8**	**60.5**
16:1ω7t	0.4						**7.3**	**4.5**	**9.6**	**8.1**	**nd**	**6.6**						
16:1ω5c		0.2	0.2				2.5	2.1	1.7	2.5	2.1	1.7					0.5	0.5
17:1ω8c	**9.9**	**8.1**	**5.6**	0.1	0.2	0.1						0.1	0.2	0.3	0.1	0.1		0.1
18:1ω7c	**4.4**	**5.3**	**4.4**	**15.8**	**16.6**	**15.9**	**11.7**	**14.5**	**16.1**	**14**	**15.4**	**18.1**	**17.3**	**16.8**	**14.4**	**10.3**	**11.8**	**17.6**
b19:1w6^	0.4	0.4	0.4				0.1	0.2	0.1	0.1	0.1	0.1				0.9	3	3.9
20:5	1.7	2	1.5															
∑X:1	49.7	51.5	47.1	83.5	80.3	75.7	85.7	84.1	81.5	85.1	83.5	81.8	83.6	79.7	76.2	84.2	79.5	79.4

Table 4. Comparison of representative isolates from the present study to described isolates. (Topt, optimal growth temperature; FAME Temp, growth temperature for fatty acid analysis). **[Note: References in table will be given a number abbreviation]**

Species/ Strain	Study	Environment	Topt	NO3 reduction	FAME Temp	C16:0	C16:1	C18:1
Psychromonas profunda	Xu	Deep Atlantic Sediments (2770m)	4	+	4	31	44	NR
Psychromonas Y2B*	**This study**	**Ymerbukta**	**5**	+	**5**	**18**	**63**	**17**
*Psychromonas ingrahamii***	Auman	Arctic Sea Ice (Point Barrow)	5	+	4	19	67	4
Psychromonas hadalis	Nogi	Japan Trench (7542m) sediments	6	+	NA	31	37	NR
Psychromonas kaikoae	Nogi	Japan Trench (7434m) sediments	10	+	10	15	52	2
Psychromonas boydii	Auman	Arctic Sea Ice (Point Barrow)	0-10[‡]	+	4	26	45	4
Psychromonas antarcticus	Mountfort	McMurdo Ice Shelf pond sediments	12	-	12	24	58	3
Psychromonas marina	Kawasaki	Okhotsk Sea WC	15	+	15	44	39	3
Psychromonas arctica	Groudieva	Svalbard Water Column	20	-	4	7-16	50	7-16
Psychromonas macrocephali	Miyazaki	Marine Sediments adajacent to whale carcass	20	+	20	27	51	4
Psychromonas ossibalaenae	Miyazaki	Marine Sediments adajacent to whale carcass	20	+	20	25	56	1
Psychromonas japonica	Miyazaki	Marine Sediments adajacent to whale carcass	21	+	21	22	53	3
Psychromonas agarivorans	Hosoya	Marine Sediments (Japan)	20-25	-	20	38	35	3
Psychromonas aquimarina	Miyazaki	Marine Sediments adajacent to whale carcass	20-25	+	20	29	49	2
*Shewanella halifaxensis**	Zhao	Emerald Basin (215m) sediments	10	+	10	20	19	7
Shewanella sediminis	Zhao	Emerald Basin (215m) sediments	10	+	10	17	33	7
Shewanella psychrophila	Xiao	West Pacific (1914m) sediment	10-15	+	10	13	38	9
Shewanella gelidimarina	Bowman	Antarctic Sea Ice	16	+	10	6-11	27-37	1-8
Shewanella YLB*	**This study**	**Ymerbukta**	**18**	+	**5**	**13**	**28**	**7**
Shewanella vesiculosa	Bozal	Shetland Island Antarctic marine sediments	15-20	+	20	10	25	0
Shewanella arctica	Kim	Tempelfjorden Svalbard sediment	20	+	20	17	NR	3
Shewanella frigidimarina	Bowman	Antarctic Sea Ice	21	+	10	5-17	38-55	3-7
*Shewanella denitrificans**	Brettar	Gotland Deep oxic-anoxic WC	20-25	+	28	13	31	3
*Shewanella baltica**	Brettar	Gotland Deep oxic-anoxic WC	20-25	+	28	4	24	2
Arcobacter YAS-1*	**This study**	**Ymerbukta**	**10**	+	**5**	**11**	**57**	**15**
Arcobacter SAS-1*	**This study**	**Smeerenbergfjorden**	**10**	+	**5**	**11**	**59**	**15**

Arcobacter halophilus	Donachie	Hypersaline Hawaiian lagoon water	18-22	+	20	26	26	26
Arcobacter venerupis	Levican	Mussels - Ebro delta Spain	18-37	+	NR	NR	NR	NR
Arcobacter marinus	Kim	Seaweeds,starfish,and water - East Sea	30-37	+	37	26	28	24
Arcobacter nitrofigilis	McClung	Spartina Marsh sediments -Sapelo Island, GA	10-35‡	+	37	32	31	13
*Arcobacter sp.**	Heylen	Activated Sewage sludge	-	+	-	-	-	-
Herminiimonas SPB*	**This study**	**Smeerenbergfjorden**	**10**	+	**5**	**17**	**67**	**12**
Herminiimonas fonticola	Fernandes	Spring water- Portugal	30	-	30	26	46	7
Herminiimonas glacei	Loveland-Curtze	Greenland Ice Core	30	-	28	31	12	6
*Herminiimonas arsenicoxydans***	Muller	Arsenic contaminated sludge	25	+	25	27	31	5
Herminiimonas saxobsidens	Lang	Lichen-rock interface	NA	+	28	33	19	9
Herminiimonas aquatilis	Kampfer	Drinking water (Uppsala, Sweden)	25	NA	25	12	48	9
Pseudomonas SL-1*	**This study**	**Smeerenbergfjorden**	**10**	+	**5**	**19**	**61**	**17**
Pseudomonas meridiana	Reddy	Cyanobacterial mats McMurdo Antarctica	22	+	25	34	29	22
Pseudomonas proteolytica	Reddy	Cyanobacterial mats McMurdo Antarctica	22	+	25	24	21	29
Pseudomonas antarctica	Reddy	Cyanobacterial mats McMurdo Antarctica	22	+	25	25	30	31
Pseudomonas alcaliphila	Yumoto	Seawater from the the coast of Japan	27	+	27	18	19	50
Pseudomonas marincola	Romanenko	Brittle Star, Fiji Sea (480m)	25-28	+	28	20	19	32
Pseudomonas pohagensis	Weon	Beach Sand, Korea	25-30	+	28	31	39	7
*Pseudomonas stutzeri**	Moss	Bile	NA	+	37	19	23	23
*Pseudomonas stutzeri**	Rossello-Mora	multiple strains (need access to paper)		+				

* Complete Denitrification confirmed by gas production

** Complete Denitrification inferred by genome analysis

‡ No Topt reported, growth range given instead

References: (McClung et al., 1983; Rosselló-Mora et al., 1994; Mountfort et al., 1998; Brettar et al., 2001; Yumoto et al., 2001; Brettar et al., 2002; Kawasaki et al., 2002; Nogi et al., 2002; Groudieva et al., 2003; Xu et al., 2003; Reddy et al., 2004; Donachie et al., 2005; Fernandes et al., 2005; Zhao et al., 2005; Auman et al., 2006; Kämpfer et al., 2006; Weon et al., 2006; Zhao et al., 2006; Lang et al., 2007; Nogi, 2007; Xiao et al., 2007; Miyazaki et al., 2008; Romanenko et al., 2008; Bozal et al., 2009; Hosoya et al., 2009; Loveland-Curtze et al., 2009; Auman et al., 2010; Kim et al., 2010b; Kim et al., 2012; Levican et al., 2012; Bowman, 1997; Heylen, 2006; Muller, 2006)

765 **Figure Legends**
766
767 **Figure 1.** TRFLP profiles of the 16S rRNA gene from surficial (0-5 cm) sediments. The
768 Shannon Index (H) is given for each site. Peaks that were tentatively matched to isolated
769 strains included *Shewanella* sp. (210), *Pseudomonas sp.* (389), and *Arcobacter* sp. (395).
770
771 **Figure 2.** 16S rRNA gene sequence based phylogenetic tree of isolated psychrophillic
772 denitrifiers from fjord of Svalbard showing relatedness of isolated strains with previously
773 characterized clones and isolated representative of database. Tree was generated by
774 neighbor-joining method and tested with bootstrap (1000). Nodes supported by bootstrap
775 values greater than70% are indicated by numeric values. The scale bar represents 0.02
776 substitutions per nucleotide position. **[add brackets for phyla and remove extra**
777 **isolates not in the paper, also reqsequencing will be done for UL-1, SL-2, SLB-1,**
778 **Y2B before new tree is made]**
779
780 **Figure 3.** Growth and nitrate utilization of the selected psychrophilic denitrifiers at 5°C
781 under denitrifying conditions (10mM lactate, 5mM NO_3^-). The average specific growth
782 rate (μ) and nitrate utilization rate (mM L^{-1} d^{-1}) are given to the right of the figure.
783
784 **Figure 4.** Temperature response of growth under denitrifying conditions (10 mM lactate,
785 5 mM NO_3^-). Error bars represent the standard deviation of triplicate measurements.

Figure 1.

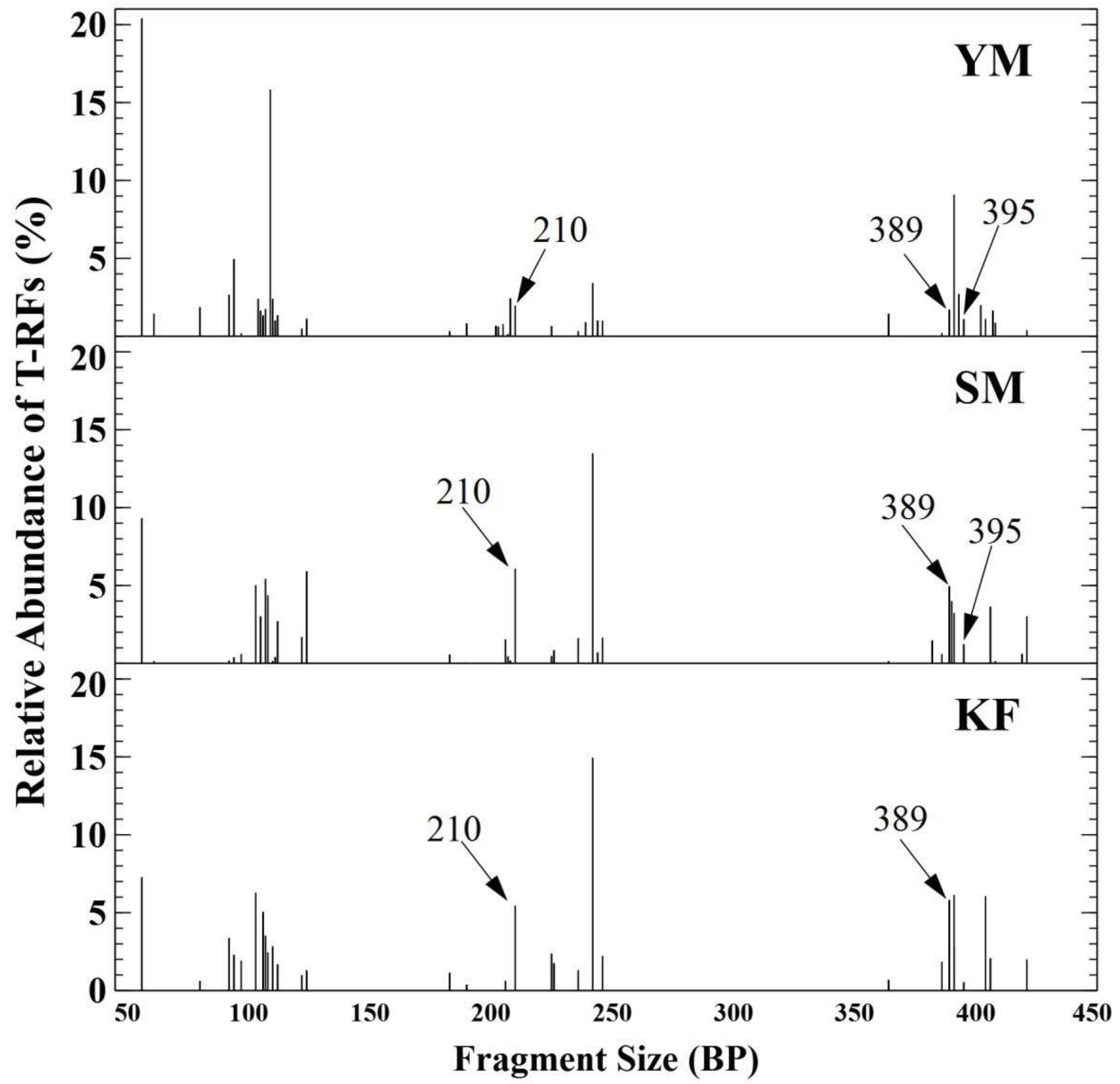

Figure 2.

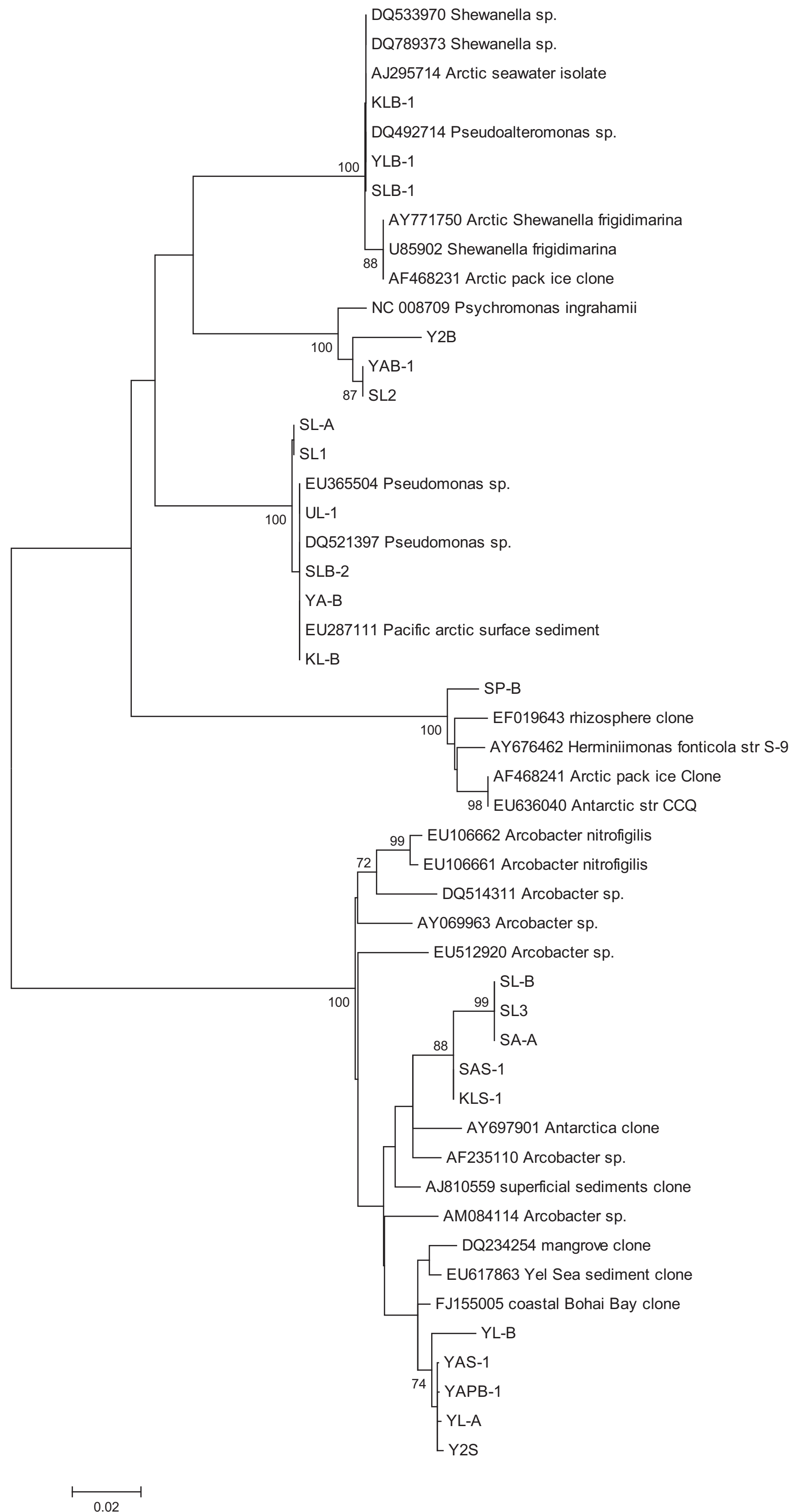

Figure 3.

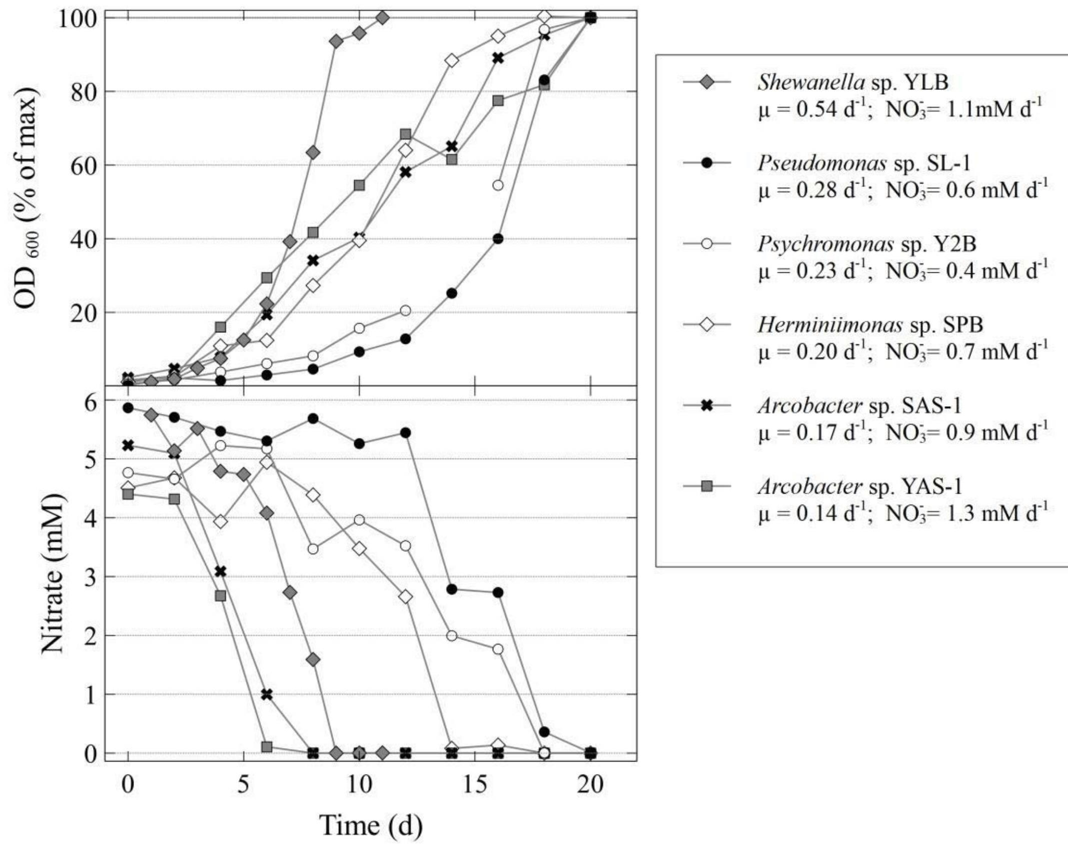

Figure 4.