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1 **Cable bacteria promote DNRA through iron sulphide dissolution**

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26 Key words: cable bacteria; nitrogen; denitrification; DNRA

27

28

29 **Abstract**

30 Cable bacteria represent a newly discovered group of filamentous microorganisms, which are  
31 capable of spatially separating the oxidative and reductive half-reactions of their sulphide-oxidising  
32 metabolisms over centimetre distances. We investigated three ways that cable bacteria might  
33 interact with the nitrogen (N) cycle: (1) by reducing nitrate through denitrification or dissimilatory  
34 nitrate reduction to ammonium (DNRA) within their cathodic cells; (2) by nitrifying ammonium  
35 within their anodic cells; and (3) by indirectly affecting denitrification and/or DNRA by changing the  
36  $\text{Fe}^{2+}$  concentration in the surrounding sediment. We performed  $^{15}\text{N}$  labelling laboratory experiments  
37 to measure these three processes using cable bacteria containing sediments from the Yarra River,  
38 Australia, and from Vilhelmsborg Sø, Denmark. Our results revealed that in the targeted systems  
39 cable bacteria themselves did not perform significant rates of denitrification, DNRA or nitrification.  
40 However, cable bacteria exhibited an important indirect effect, whereby they increased the  $\text{Fe}^{2+}$  pool  
41 through iron sulphide dissolution. This elevated availability of  $\text{Fe}^{2+}$  significantly increased DNRA and  
42 in some cases decreased denitrification. Thus, cable bacteria presence may affect the relative  
43 importance of DNRA in sediments and thus the extent by which bioavailable nitrogen is lost from the  
44 system.

45

## 46 Introduction

47 The recent discovery of electric currents linking spatial separated biogeochemical processes (Nielsen  
48 et al. 2010) and cable bacteria (Pfeffer et al. 2012) has set a new paradigm for sedimentary  
49 biogeochemistry (Nielsen and Risgaard-Petersen 2015; Nielsen et al. 2010). Briefly, cable bacteria  
50 are members of the family *Desulfobulbaceae*, which is composed of a range of sulphur oxidising and  
51 reducing bacteria. The predominant metabolism of cable bacteria is chemotrophic sulphide  
52 oxidation, but these organisms separate the oxidation and reduction half-reactions by conducting  
53 electrons along their long, filamentous bodies (up to 3 cm) (Meysman 2017), as represented in Fig 1.

54 The many biogeochemical implications of long distance electron transport are yet to be fully  
55 understood, but in addition to directly influencing the cycling of sulphur (S), cable bacteria also  
56 indirectly affect the cycling of other elements. The high production of protons in the deep, anodic  
57 part of the sediment results in a pH minimum, as low as < 6.5 (Malkin et al. 2014; Nielsen et al. 2010;  
58 Risgaard-Petersen et al. 2012). Such acidification of the pore water stimulates the dissolution of iron  
59 sulphide (FeS) and carbonate minerals in the sediment (Risgaard-Petersen et al. 2012), which  
60 strongly alters the availability of Fe, Ca, Mn, and P at depth (Rao et al. 2016; Sulu-Gambari et al.  
61 2016b; Van De Velde et al. 2016). These dissolved constituents are then free to diffuse to the  
62 surface, where oxygen availability and high pH (as induced by the cathodic reaction) favour the  
63 precipitation of Fe- and Mn-oxides, Fe-phosphates, and Ca(Mg)-carbonates (Seitaj et al. 2015; Sulu-  
64 Gambari et al. 2016a; Sulu-Gambari et al. 2016b). As a result of long distance electron transfer, cable  
65 bacteria generate electric fields, which can be measured as an increase in electric potential with  
66 depth (Damgaard et al. 2014). This electrogenic sulphur oxidation (e-SOx) is potentially widespread,  
67 with cable bacteria discovered in marine systems across the globe (Burdorf et al. 2017; Burdorf et al.  
68 2016; Malkin et al. 2014), and more recently in aquifers (Muller et al. 2016) and freshwater  
69 sediments (Risgaard-Petersen et al. 2015).

70 Nitrogen (N) is an important nutrient in aquatic environments, and understanding nitrate reduction  
71 pathways is an important part of managing ever-increasing global loads (Conley et al. 2009).  
72 Denitrification is an important N-removing process, whereby nitrate is reduced to N<sub>2</sub> gas. In  
73 competition to denitrification is dissimilatory nitrate reduction to ammonium (DNRA), which retains  
74 N in the system. Thus, the balance of denitrification and DNRA can be an important control on  
75 whether a system is net N removing or recycling (An and Gardner 2002; Dunn et al. 2013; Gardner et  
76 al. 2006; Giblin et al. 2013; Kessler et al. 2018; Roberts et al. 2014). This is of great significance in  
77 estuaries in particular, as denitrification-dominated estuaries may remediate high nitrate  
78 concentrations, while DNRA-dominated estuaries are likely to pass large bioavailable nitrogen loads  
79 to coastal waters and embayments. Nitrification, the oxidation of ammonium to nitrate, can  
80 enhance N removal if coupled with denitrification. Nitrification is usually considered an aerobic  
81 process, but anoxic nitrification processes are known involving Mn and possibly Fe oxides (Hulth et  
82 al. 1999; Mortimer et al. 2004).

83 It is not yet known how cable bacteria control and influence the N cycle. Marzocchi et al. (2014)  
84 showed that nitrate can be used as alternative cathodic electron acceptor in the absence of oxygen.  
85 Whether the cable bacteria perform denitrification or DNRA is yet unclear, but recent work suggests  
86 that cable bacteria can reduce both nitrate and nitrite, but not N<sub>2</sub>O (Risgaard - Petersen et al. 2014).  
87 However, cable bacteria have not yet been observed *in situ* in a high-nitrate, anoxic environment,  
88 and it is not known whether cathodic nitrate-reduction occurs in a sub-oxic zone even in the  
89 presence of oxygen. If so, denitrification or DNRA directly as the cable bacteria's cathodic half-

90 reaction may be important processes (Fig 1a). We hypothesize that these direct reactions by cable  
91 bacteria may contribute significantly to sediment nitrogen cycling.

92 Furthermore, little is known about indirect effects of cable bacteria on N cycling. A recent study  
93 showed that increased  $\text{Fe}^{2+}$  concentration promotes DNRA over denitrification in estuarine  
94 sediments (Roberts et al. 2014) and freshwater lake sediments (Robertson et al. 2016; Robertson  
95 and Thamdrup 2017). As cable bacteria can increase pore water  $\text{Fe}^{2+}$  through acidity generation and  
96 dissolution of  $\text{FeS}$ , we hypothesize that cable bacteria can promote DNRA by other members of the  
97 microbial community (Fig 1b).

98 In marine microbial fuel cells, a current is generated between a buried anode and cathode in the  
99 overlying water, allowing oxidising microbes such as *Desulfobulbus* to oxidise sulphide in the  
100 absence of an oxidant (Lowy et al. 2006). The possible occurrence of biologically-mediated anoxic,  
101 anodic nitrification is currently debated (He et al. 2009; Qu et al. 2014; Vilajeliu-Pons et al. 2018; Xu  
102 et al. 2015). As cable bacteria function analogously to a microbial fuel cell (Tender et al. 2002), we  
103 hypothesize that cable bacteria can promote anoxic nitrification (Fig 1c) either directly as part of  
104 their metabolism, or via symbiotic microbes using the anode provided by the cable bacteria.

105 In this study, we investigated the three hypothesized cable-bacteria-mediated nitrogen cycling  
106 reactions described above, as depicted in Fig 1. First, we measured rates of denitrification and DNRA  
107 and compared their relative contribution to nitrate reduction in sediments with and without cable  
108 bacteria to address whether cable bacteria could lead to a stimulation of DNRA relative to  
109 denitrification. Second, we repeated this experiment in sediment with active cable bacteria and  
110 inactivated cable bacteria to address if such a stimulation could be attributed to the ability of cable  
111 bacteria to perform DNRA or alternatively to promote DNRA by increasing  $\text{Fe}^{2+}$  availability. Third, we  
112 tested whether cable bacteria can promote anoxic nitrification in oxygen-free environments, by two  
113 methods. One method involved addition of  $^{15}\text{NH}_4^+$  to the deep, anoxic part of the sediment. If cable  
114 bacteria promoted anoxic, anodic nitrification, this would produce  $^{15}\text{N-NO}_3^-$ , which would  
115 subsequently be rapidly reduced to  $^{15}\text{N-N}_2$ . The other method involved adding different  
116 concentrations of  $^{15}\text{NO}_3^-$  to the water overlying the sediment, resulting in varying penetration of  
117  $^{15}\text{NO}_3^-$  into the sediment. Therefore if anoxic nitrification occurred, higher  $^{15}\text{NO}_3^-$  concentrations  
118 would result in greater overlap of the zones of anoxic nitrification and  $^{15}\text{NO}_3^-$  denitrification, and the  
119 measured rate of denitrification of ambient  $^{14}\text{NO}_3^-$  would increase with  $^{15}\text{NO}_3^-$  concentration.

120

121 **Materials and methods**

122 **Sites and sediment collection**

123 Sediment and water were collected from near to Scotch College (55°32'63.48"E 58°10'85.4"N) in the  
124 Yarra River Estuary, Melbourne, Australia. This site is usually located in the salt wedge of the estuary,  
125 and is characterised by periodical hypoxia in the bottom waters during low rainfall, combined with  
126 aphotic sediments due to the high turbidity of the overlying fresh water layer (Roberts et al. 2012).  
127 The site has been used previously for studies of the fate of nitrate during nitrate reduction (Roberts  
128 et al. 2012; Roberts et al. 2014) and investigation of the sediment has shown an *in situ* population of  
129 cable bacteria (Burdorf et al. 2017).

130 In addition to the experiments with sediment from the Yarra River, the anoxic nitrification  
131 experiment was supplemented with a similar experiment using riparian sediment from Vilhelmsborg  
132 Sø (56°04'00.9"N 10°11'01.7"E), an artificial freshwater lake near Aarhus, Denmark.

133

134 **Signatures for cable bacteria activity**

135 Cable bacteria development was monitored in the Yarra River experiments by high-resolution pH  
136 profiles. A 50 µm tip pH sensor (Unisense) was mounted on a motor-driven micromanipulator and  
137 profiles recorded at 50 µm steps near to the surface, and 200 µm steps below 2 mm. A reference  
138 electrode (REF201 Red Rod electrode; Radiometer Analytical, Denmark) was kept in the overlying  
139 water. Both electrodes were connected to a high-resistance ( $> 10^{13} \Omega$ ) multimeter (Unisense).

140

141 Cable bacteria development was monitored in the Vilhelmsborg Sø experiments by high-resolution  
142 Electric Potential (EP) depth profiles, measured with house-built microsensors (Damgaard et al.  
143 2014). The sensors were mounted on a motor-driven micromanipulator and profiles were recorded  
144 at 400 µm steps. A reference electrode (as above) was used. The EP sensor and the reference  
145 electrode were connected to a custom-made voltmeter with high internal resistance  $> 10^{14} \Omega$   
146 (Aarhus University, Denmark) connected to a 16-bit analog-to-digital converter (AD216, Unisense,  
147 Denmark). The EP profiles also served to identify the depth and intensity of anodic activity (Risgaard-  
148 Petersen et al. 2014).

149

150 **Nitrate reduction experiments**

151 To study the direct and indirect effects of cable bacteria on nitrate reduction,  $^{15}\text{N}$  experiments with  
152 cores having active cable bacteria ("active-cables") or inactive cable bacteria ("inactivated-cables")  
153 and cores without any cable bacteria ("no-cables") were performed.

154 Collected sediment was sieved (0.5 mm), homogenised, and packed into short  
155 polymethylmethacrylate (PMMA) core liners (L = 120 mm, ID = 42 mm). Cores were incubated in 10 L  
156 of oxygenated site water for approximately 3 weeks (with a maximum of 24 cores per bath). Three  
157 weeks was chosen as a time where we expect significant cable bacteria activity based on typical  
158 dynamics observed in Yarra River and other sediments (Burdorf et al. 2017). Table 1 shows the  
159 details of the nitrate reduction experiments performed. To avoid the development of e-SO<sub>x</sub>, the  
160 sediment was cut at 2 mm depth every 1-2 days; the frequency of cutting varied over the various  
161 experiments and is detailed in Table 1. Cutting is achieved by inserting a fine wire into the sediment  
162 at ~ 2 mm depth (just below the depth of oxygen penetration) using two pins, and pulling the wire

163 through the sediment, effectively slicing a surface layer without removing it from the core. This  
164 action inhibits the respiration and carbon uptake of the cable bacteria (Pfeffer et al. 2012; Vasquez-  
165 Cardenas et al. 2015) and consequently their growth. This treatment is referred to as “no-cables”.  
166 Cores with inactivated cable bacteria were prepared by cutting only once immediately ( $< 1$  hour)  
167 before the experimental incubation. Therefore, this “inactivated-cables” treatment maintains the  
168 same biogeochemical conditions as an uncut core, but excludes the direct influence of the cable  
169 bacteria metabolism and e-SOx (Risgaard-Petersen et al. 2015). As shown previously, the  $\text{Fe}^{2+}$  pool is  
170 diminished by diffusion after cable bacteria are inactivated; as diffusion is slow over cm distances,  
171 the  $\text{Fe}^{2+}$  pool in anoxic layers of sediments with inactivated cable bacteria does not change  
172 significantly within one hour of inactivation and persists even 33 hours after inactivation (Risgaard-  
173 Petersen et al. 2012). To control for any small amount of oxygen entrained by inserting the cutting  
174 wire into the sediment, the wire was inserted into each “active-cables” and “inactivated-cables”  
175 sediment at every cutting time, but removed without drawing it through the sediment and  
176 inactivating the cable bacteria.

177 Cores were transferred to separate, individually stirred PVC tube (L = 240 mm, ID = 50 mm) filled  
178 with oxic site water. The overlying water in the PVC tubes were amended to a final concentration of  
179  $30 \mu\text{M } ^{15}\text{NO}_3$ , and were sealed with a rubber stopper. After several hours (see Table 1), the stopper  
180 was gently removed. Samples of the overlying water were collected for  $^{15}\text{N-N}_2$  (12 mL in a glass vial  
181 (Labco Exetainer)) and  $^{15}\text{NH}_4^+$  (6 mL in a polypropylene (PP) centrifuge tube, Falcon), both preserved  
182 with  $100 \mu\text{L } 50\% \text{ZnCl}_2$ . The surface 2 cm of sediment was then extruded, transferred to a beaker  
183 containing  $2\% \text{ZnCl}_2$  and quickly and gently homogenised, then transferred to 12 mL glass vials for  
184  $^{15}\text{N}_2$  analysis. A 6 mL subsample of this slurry was also collected in a PP centrifuge tube for  $^{15}\text{NH}_4^+$   
185 analysis. For details of this method, see Kessler et al. (2018).

186  $^{15}\text{N-N}_2$  was measured by adding a 4 mL He headspace to the 12 ml glass vials, and analysed using a  
187 Sercon isotope ratio mass spectrometer (IRMS).  $^{15}\text{NH}_4^+$  was extracted with 1:1 2 M KCl, shaken for 1  
188 hours at 120 rpm. The supernatant after centrifuging was transferred to a glass vial, purged with He  
189 and the  $\text{NH}_4^+$  converted to  $\text{N}_2$  with alkaline hyperbromite (Risgaard-Petersen et al. 1995) and  
190 measured by IRMS. Denitrification and DNRA were measured as the rate of production of  $^{15}\text{N-N}_2$ .

191 This nitrate experiment was performed twice, with slight modification. In the first experiment, the  
192 whole extruded 2 cm was slurried as above. In the second experiment, the extruded sediment was  
193 halved vertically. One half was slurried as above, while the second half was transferred to a 50 mL PP  
194 centrifuge tube which was then flushed for  $> 1$  min with Ar to prevent oxidation. These samples  
195 were centrifuged and 1 mL of the filtered ( $0.22 \mu\text{m}$ ) supernatant added to 0.5 mL 0.01 M ferrozine  
196 and stored in the dark. These samples were analysed for total dissolved iron concentration  
197 spectrophotometrically following Stookey (1970), by measuring the intensity of the purple ferrozine  
198 complex in an ammonium acetate buffer after addition of hydroxylamine hydrochlorite, with all  
199 reagents made as described in (Viollier et al. 2000). Fe(III) is negligible in the filtered pore water  
200 (Roberts et al. 2014), and so the total dissolved iron concentration was treated as being  $\text{Fe}^{2+}$ .

201 ANOVA was used to compare treatments in each experiment using the software R (v 3.2.0) following  
202 Crawley (2012). As denitrification and DNRA rates depend on a number of factors (e.g. carbon,  
203 temperature) which may vary between experiments, we compare the contribution of DNRA to total  
204 nitrate reduction, defined as  $\% \text{DNRA} = 100 \times \text{DNRA} / (\text{denitrification} + \text{DNRA})$ . This approach is  
205 similar to previous work on the relative importance of these processes (Kessler et al. 2018; Roberts  
206 et al. 2014).

207

208 **Nitrification experiments**

209 Two types of experiment were performed to measure anoxic nitrification, with each experiment  
210 replicated. Table 1 summarises the details and procedures of the experiments performed.

211 *<sup>15</sup>NH<sub>4</sub><sup>+</sup> experiments*

212 To test if cable bacteria can promote nitrification in anoxic sediments, <sup>15</sup>N experiments were  
213 performed with sediments from Yarra River, Australia and Vilhelmsborg Sø, Denmark.

214 Cores with treatments “active-cables”, “inactivated-cables” and “no-cables” were prepared,  
215 incubated and cut as described for the nitrate reduction experiments, except that the PMMA core  
216 liners were replaced with polypropylene tubes (L = 70 mm, ID = 20 m) created by cutting 60 mL  
217 syringes (Thermo). 0.1 mL of anoxic, 100 mM <sup>15</sup>NH<sub>4</sub>Cl was injected via a hypodermic needle through  
218 a port 1.5 cm below the sediment surface into the centre of the tube. Tubes were then transferred  
219 to separate, individually stirred 50 mm ID PVC tube filled with oxic site water to prevent cross-  
220 contamination. After ≤ 60 min (see Table 1), tubes were removed from the bath and quickly  
221 extruded. Two, 1 cm slices were transferred to separate beakers containing 2% ZnCl<sub>2</sub> and quickly and  
222 gently homogenised, then transferred to 12 mL glass vials for later analysis of <sup>15</sup>N-N<sub>2</sub>.

223 The method varied slightly for the experiments performed with Vilhelmsborg Sø sediments as  
224 follows. Sediment was sieved (0.5 mm), poured into a glass aquaria, and incubated with aerated tap  
225 water. The overlying water was replaced weekly to avoid accumulation of metabolic products and to  
226 replenish nutrients. On the day of sampling, half of the cores were cut at a depth of 3 mm to inhibit  
227 cable bacteria activity, and this treatment is referred to as “inactivated-cables” as above. Sediment  
228 cores were extracted from the tank and were immediately incubated for 15 min in a water bath with  
229 acetylene (10% partial pressure) to inhibit nitrification activity (Berg et al. 1982) in the surface  
230 millimetre of sediment, thereby minimizing eventual diffusion of nitrate from the surface sediment  
231 layer to the lower layer where the anodic reaction occurred. Cores were subsequently extracted  
232 from the bath and 0.1 mL of a 100 mM solution of anoxic <sup>15</sup>NH<sub>4</sub>Cl was injected at 1.5 cm depth as  
233 above. At each time-point (see Table 1), three cores were processed as follows: each cores was  
234 sliced at 3 and 21 mm depth. Sediment from zero to 3 mm depth (surface) and from 3 to 21 mm  
235 depth (bottom) was transferred into falcon tubes containing a solution of Allylthiourea (100 μM) to  
236 stop nitrification activity (Ginestet et al. 1998 and references therein) and gently stirred to minimize  
237 gas exchange with the atmosphere. 3 mm was chosen for the first slice to reflect the expected depth  
238 of the anodic cable activity in the cores based on microprofiler measurements (Fig S1) and previous  
239 experience with these sediments. These measurements were not possible with the Yarra River  
240 sediments. A total depth of 21 mm was chosen to closely match the 20 mm total depth analysed for  
241 the Yarra River cores. The solution was then left for a short period (< 2 min) to allow the coarser  
242 sediment particles to settle out, before an aliquot of the supernatant was transferred into 6 mL glass  
243 vials and fixed with 100 μL ZnCl 50% (w:w) for later <sup>15</sup>N-N<sub>2</sub> analysis.

244 *<sup>15</sup>NO<sub>3</sub><sup>-</sup> experiment*

245 An alternative experiment to the <sup>15</sup>NH<sub>4</sub><sup>+</sup> experiments was used to measure anoxic nitrification. In this  
246 experiment different concentrations of <sup>15</sup>NO<sub>3</sub><sup>-</sup> were added to the water overlying the sediment. A  
247 full explanation of the rationale for this experiment is included in the discussion section.

248 Cores were prepared, incubated and cut as described for the nitrate reduction experiments using  
249 Yarra River sediment. Sediment was transferred to individual 50 mm ID PVC tube amended with  
250 different concentrations of Na<sup>15</sup>NO<sub>3</sub> (see Table 1). PVC tubes were sealed with a rubber stopper and



251 stirred for 3 hours, after which the surface 3 cm of sediment was extruded into a beaker containing  
252 30 mL 2% ZnCl<sub>2</sub> and quickly and gently homogenised, then transferred to 12 mL glass vials. Two  
253 experiments were performed to span a large range of nitrate concentrations (see Table 1). D<sub>14</sub> is  
254 defined as the rate of denitrification of ambient <sup>14</sup>NO<sub>3</sub><sup>-</sup>, and was calculated as  $D_{14} = D_{15} \times p_{29} / (2 \times p_{30})$   
255 following Nielsen (1992), where  $D_{15} = p_{29} + 2 \times p_{30}$  is the rate of accumulation of <sup>15</sup>N-N<sub>2</sub> and p<sub>29</sub> and  
256 p<sub>30</sub> are the rates of accumulation of <sup>29</sup>N<sub>2</sub> (<sup>14</sup>N<sup>15</sup>N) and <sup>30</sup>N<sub>2</sub> (<sup>15</sup>N<sup>15</sup>N) respectively.

257

258 **Results**

259 **Nitrate reduction experiments**

260 In the first nitrate reduction experiment (Fig 2a), the no-cables treatment showed appreciably lower  
261 rates of DNRA ( $2.3 \mu\text{mol m}^{-2} \text{h}^{-1}$ ) than the active-cables treatment ( $12.8 \mu\text{mol m}^{-2} \text{h}^{-1}$ ,  $p < 0.005$ ). No  
262 difference was observed in denitrification rate ( $p = 0.1$ ), resulting in a much greater contribution of  
263 DNRA in the active-cables treatment (%DNRA = 45 %) compared with the without-cables (%DNRA =  
264 10 %). The second nitrate reduction experiment (Fig 2b) showed similar results, with similar rates of  
265 denitrification between treatments ( $p = 0.2$ ) and slightly higher DNRA rates in the active-cables and  
266 inactivated-cables treatments leading to a significantly higher %DNRA in these treatments (5.0 % and  
267 4.4 %) compared with the no-cables sediment (%DNRA = 2.3 %,  $p = 0.03$ ). The active-cables and  
268 inactivated-cables treatments showed no significant differences in denitrification rate ( $p = 0.9$ ),  
269 DNRA rate ( $p = 0.8$ ) or %DNRA ( $p = 0.6$ ). Notably, while the DNRA rates were similar in the two  
270 experiments, denitrification rates were approximately an order of magnitude higher in the second  
271 experiment (Fig 2b), resulting the much smaller values of %DNRA. The highest denitrification rate  
272 observed ( $300 \mu\text{mol m}^{-2} \text{h}^{-1}$ ) would represent a decrease of  $< 20\%$  in the added  $^{15}\text{NO}_3^-$  concentration  
273 over the experimental incubation.

274 Fig 3a shows that pore water average  $\text{Fe}^{2+}$  was significantly enhanced in the active-cables and  
275 inactivated-cables treatments compared with the no-cables control ( $p < 0.005$ ). The contribution of  
276 DNRA to nitrate reduction (%DNRA) was only weakly correlated with pore water  $\text{Fe}^{2+}$  (Fig 3b,  $p =$   
277  $0.2$ ), and similarly neither the rates of denitrification ( $p = 0.1$ , Fig S2a) nor DNRA alone correlated  
278 strongly with  $\text{Fe}^{2+}$  ( $p = 0.8$ , Fig S2b).

279 **Nitrification experiments**

280 Fig 4a shows production of  $^{15}\text{N-N}_2$  in the  $^{15}\text{NH}_4^+$  nitrification experiments. In all three experiments, a  
281 small amount of  $^{15}\text{N-N}_2$  ( $\leq 1 \mu\text{mol m}^{-2} \text{h}^{-1}$ ) was measured in the deep layer of the active-cables cores,  
282 but was not significantly different compared to the controls ( $p > 0.05$ ). Rates measured in the  
283 surface layer of sediment were approximately 5-10 times higher than rates in the bottom layer in the  
284 Yarra River experiments. In the Vilhelmsborg S $\phi$  sediment cores with added acetylene, the surface  
285  $^{15}\text{N-N}_2$  production was also negligible indicating the almost complete inhibition of nitrification  
286 activity by the acetylene.

287 For the  $^{15}\text{NO}_3^-$  nitrification experiment in Yarra River sediments, no significant difference was seen in  
288  $D_{14}$  (the rate of denitrification of ambient  $^{14}\text{NO}_3^-$ ) with the presence of cable bacteria (Fig 4b,  $p = 0.95$   
289 and  $0.1$  after log-transformation). The solid lines in Fig 4b show regressions for the “no-cables”  
290 treatment, and the dashed lines show the expected value of the “active-cables” treatment if an  
291 anoxic rate of  $5 \mu\text{mol m}^{-2} \text{h}^{-1}$  anoxic nitrification were occurring (representing  $0.1\%$  of the calculated  
292 anodic electron transfer), with overlap of the denitrification and anoxic nitrification zone modelled  
293 as a square root function with no overlap at zero and complete reduction of the produced  $^{14}\text{NO}_3^-$   
294 reduced to  $^{29}\text{N}_2$  at  $5000 \mu\text{mol L}^{-1}$ . Note that the data presented in Figure 4b come from two separate  
295 experiments covering the ranges of  $0 - 400 \mu\text{mol L}^{-1}$  and  $500 - 5000 \mu\text{mol L}^{-1}$  nitrate (see Table 1),  
296 resulting in the discontinuity at  $400 \mu\text{mol L}^{-1}$ .

297

298 **Discussion**

299

300 **1. Nitrate reduction performed by or in the presence of cable bacteria**

301 The enhancement of DNRA in the active-cables treatment (Fig 2a) indicates that cable bacteria can  
302 influence nitrate reduction, but does not differentiate between two possible mechanisms: direct  
303 cathodic reduction by the cable bacteria, or an indirect influence on the N cycle due to their  
304 biogeochemical fingerprint (i.e. Fe-DNRA stimulation). The second nitrate reduction experiment  
305 tested these hypotheses by adding a third treatment (inactivated-cables, Fig 2b). In this experiment,  
306 there is no difference between denitrification, DNRA or %DNRA between the active-cables and  
307 inactivated-cables treatments. Therefore, despite the known ability of cable bacteria to use nitrate  
308 (or nitrite) as the cathodic electron acceptor (Marzocchi et al. 2014), it appears that the cable  
309 bacteria themselves do not contribute significantly to DNRA in the presence of oxygen. Higher DNRA  
310 rates and %DNRA in the inactivated-cables compared to the no-cables treatment indicates that  
311 sediments with a history of cable bacteria exhibit increased DNRA, even though direct DNRA by the  
312 cable bacteria was precluded. Therefore, we conclude that some by-product of cable bacteria's  
313 biogeochemical fingerprint leads to increased DNRA rates.

314 Recent studies have established a relationship between DNRA and Fe<sup>2+</sup> in Yarra River sediments  
315 (Kessler et al. 2018; Roberts et al. 2014; Robertson et al. 2016), and so we propose that the  
316 increased pore water Fe<sup>2+</sup> concentration as induced by the activity of cable bacteria (Sulu-Gambari et  
317 al. 2016a; Sulu-Gambari et al. 2016b) may be responsible for the enhanced DNRA observed in the  
318 active-cables treatment. In the second nitrate reduction experiment, Fe<sup>2+</sup> was significantly enhanced  
319 in the presence of cable bacteria (Fig 3a), presumably because of solubilisation of FeS by the acidity  
320 generated by the cable bacteria's anodic reaction and/or equilibrium dissolution due to depletion of  
321 pore water sulphide (Rao et al. 2016; Risgaard-Petersen et al. 2012; Sulu-Gambari et al. 2016a; Sulu-  
322 Gambari et al. 2016b) (see Fig 1c). This result is also consistent with the recent findings of Otte et al.  
323 (2018), who found significant correlations of both Fe<sup>2+</sup>-oxidising and Fe<sup>3+</sup>-reducing bacteria with  
324 cable bacteria in both marine and freshwater systems. Specifically, the Fe<sup>2+</sup>-oxidising genera  
325 *Pedomicrobium*, *Hoeflea*, *Chlorobium* and *Rhodopseudomonas* were identified as being correlated  
326 with cable bacteria. Notably, a member *Hoeflea* has been associated with nitrate-dependent iron  
327 oxidation (Sorokina et al. 2012), though there are many other possible candidates that may be  
328 present in our sediments. The contribution of DNRA to nitrate reduction was weakly correlated with  
329 Fe<sup>2+</sup>, with higher Fe<sup>2+</sup> increasing %DNRA. While the weakness of this correlation reflects the  
330 complexity of the relationship between Fe<sup>2+</sup> and nitrate reduction pathways, this general response is  
331 consistent with our previous observations (Kessler et al. 2018; Roberts et al. 2014; Robertson et al.  
332 2016) showing a link between Fe<sup>2+</sup> availability and DNRA.

333 There are two possible explanations for the influence of Fe<sup>2+</sup> on nitrate reduction pathway. First,  
334 several studies have suggested that Fe<sup>2+</sup> can be a direct electron donor for DNRA bacteria (Coby et  
335 al. 2011; Kessler et al. 2018; Roberts et al. 2014; Robertson et al. 2016; Robertson and Thamdrup  
336 2017; Weber et al. 2006), as depicted in Fig 1b. Mostly, it is suggested that Fe<sup>2+</sup> reacts with nitrite  
337 (NO<sub>2</sub><sup>-</sup>) rather than nitrate, and that the earlier step of nitrate reduction to nitrite is performed by  
338 other members of the denitrifying community (Robertson et al. 2016). This first step is usually slow,  
339 and nitrite does not usually accumulate in these sediments, instead being rapidly reduced to N<sub>2</sub> by  
340 denitrification and/or NH<sub>4</sub><sup>+</sup> by DNRA, depending on which community dominates (Roberts et al.  
341 2014). The first nitrate reduction experiment supports this hypothesis, though the effect is smaller in  
342 the second experiment (Fig 2b). Secondly, it is known that Fe<sup>2+</sup> can inhibit denitrification by  
343 disrupting intracellular electron transport (Carlson et al. 2012), which would lead to a similar

344 increase in %DNRA. Both the active-cables and inhibited-cables treatments appear to have slightly  
345 reduced denitrification in both experiments (Fig 2a and b), although because neither denitrification  
346 nor DNRA rates are generally correlated with  $\text{Fe}^{2+}$ , this study cannot conclusively differentiate these  
347 two effects. It is likely that both effects are relevant, depending on other conditions, and that other  
348 factors influence both denitrification and DNRA. For example, microbes utilising the well-established  
349 sulphide-driven DNRA pathway (An and Gardner 2002; Brunet and Garcia-Gil 1996) may scavenge  
350 sulphide released by FeS dissolution in the anoxic zone. As the microbial communities responsible  
351 for  $\text{Fe}^{2+}$ - and sulphide-driven DNRA are not well established, it is difficult to separate these effects.  
352 Similarly, sulphide has known toxic effects on denitrification (Sørensen et al. 1980). Despite these  
353 additional influences, which may account for the weak correlation in Fig 3b, it would appear that the  
354 role of cable bacteria in the nitrogen cycle is to enhance the relative importance of DNRA by other  
355 members of the sediment microbial community through increasing  $\text{Fe}^{2+}$  availability.

356

## 357 **2. Anoxic nitrification at the cable bacteria anode**

358 No evidence for anoxic nitrification was observed at either site (Fig 4). The measured nitrification  
359 rates in the anoxic bottom layer (where the  $^{15}\text{NH}_4^+$  was added) are consistently slightly higher in the  
360 active-cables treatments, but this effect is never statistically significant. The  $^{15}\text{N-N}_2$  measured at the  
361 surface sediment is presumably due to a small leak of  $^{15}\text{NH}_4^+$  solution to the surface through pores  
362 and fractures in the sediment during the injection. Indeed the high variation in the mean rates in the  
363 surface layers (s.e. = 20 % - 150 %) are consistent with random fractures in the sediment. It is  
364 possible that if DNRA dominates (see earlier discussion), then  $^{15}\text{NO}_3^-$  produced by anoxic nitrification  
365 would be reduced back to  $^{15}\text{NH}_4^+$ , which we would not detect using this method. As the %DNRA was  
366 never above 50 % in either nitrate reduction experiment, and was usually approximately 10 % (Fig 2),  
367 we would still expect denitrification to be measureable in this case. With the sediment from  
368 Vilhelmsborg Sø, the finding that anoxic nitrification is negligible is consistent with the experiment  
369 from the Yarra River.

370 For the  $^{15}\text{NO}_3^-$  experiment in Yarra River sediments, varying concentrations of  $^{15}\text{NO}_3^-$  were added to  
371 the oxic water overlying the sediment. This should have resulted in increasingly deeper penetration  
372 of  $\text{NO}_3^-$ , and therefore an increasingly deep zone of denitrification. If anoxic nitrification occurred, it  
373 would be expected that there is an additional source of  $^{14}\text{NO}_3^-$  in the zone of denitrification,  
374 increasing as the depth of the denitrification zone increases. Therefore, there  $D_{14}$  should be  
375 enhanced in the presence of cable bacteria if anoxic nitrification is occurring. There is no evidence of  
376 deviation of the “active-cables” treatment toward the dashed line in Fig 4b. Thus, this experiment  
377 provides additional evidence that anoxic nitrification does not occur either as part of cable bacteria  
378 activity, or by nitrifiers in the presence of (anodic) cable bacteria. It is noteworthy that the higher  
379 concentration treatment shows an increase in  $D_{14}$  with  $^{15}\text{NO}_3^-$  concentration. This indicates that one  
380 or more of the assumptions of the isotope pairing technique are not met in this experiment, most  
381 likely that the system has not reached a steady state (Nielsen 1992; Risgaard - Petersen et al. 2003).  
382 As the present experiment is in any case exploiting a weakness in the isotope pairing technique, this  
383 does not invalidate the above finding, but does mean that the rates of  $D_{14}$  found cannot be treated  
384 as representative.

385

## 386 **3. Implications for cable bacteria-rich environments**

387 Since the discovery of cable bacteria and their complex metabolism, many questions have arisen  
388 about their ability to affect other biogeochemical processes. There is strong evidence that cable  
389 bacteria can reduce nitrate (or nitrite) at their cathode (see Fig 1) (Marzocchi et al. 2014). That work  
390 was performed under laboratory conditions in high-nitrate ( $> 250 \mu\text{M}$ ), anoxic water. As yet, *in situ*  
391 observations of cable bacteria have not been reported in such an environment, but this remains a  
392 viable ecological niche for such activity. The present work shows that when the overlying water is  
393 oxygenated, cable bacteria do not contribute significantly to DNRA, as might be expected from  
394 traditional thermodynamic redox cascades (Froelich et al. 1979), or at least that cable bacteria DNRA  
395 occurs at low rates relative to total nitrate reduction.

396

397 Cable bacteria appear to play a role in the N cycle through the dissolution of FeS by the acid-  
398 generating anodic half-reactions. This increased  $\text{Fe}^{2+}$  pool then serves as a driver for DNRA following  
399 recent observations  $\text{Fe}^{2+}$  directly and indirectly enhancing DNRA, including in the Yarra River (Kessler  
400 et al. 2018; Roberts et al. 2014; Robertson et al. 2016; Robertson and Thamdrup 2017). As the  
401 relative rates of DNRA and denitrification are of global interest as global N loads increase (Conley et  
402 al. 2009; Gruber and Galloway 2008; Steffen et al. 2015), understanding the conditions under which  
403 DNRA may be enhanced (or denitrification suppressed) is critical. If cable bacteria are significantly  
404 enhancing DNRA, then the stable, seasonally hypoxic systems most closely associated with cable  
405 bacteria (Burdorf et al. 2017; Burdorf et al. 2016; Malkin et al. 2014; Nielsen 2016; Seitaj et al. 2015)  
406 may become more N-recycling during the seasonal cable bacteria dominance. This is particularly  
407 interesting as the Fe-cycling associated with cable bacteria has been shown to buffer against euxinia  
408 (Seitaj et al. 2015). The proposed mechanism is that the  $\text{Fe}^{2+}$  solubilised at the anodic end of cable  
409 bacteria diffuses upwards, creating an iron oxide layer at the surface. This iron oxide layer provides a  
410 firewall against free sulphide diffusing out of the sediment once sulphide supply exceeds cable  
411 bacteria demand. This work suggests that the net value of cable bacteria as mediators of water  
412 quality may be limited, as the same  $\text{Fe}^{2+}$  release may inhibit N removal from estuarine and coastal  
413 waters by directly inhibiting denitrification and/or favouring its recycling through DNRA.

414

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564

565

## Tables

Table 1: Details of experiments performed. "Details" provides the incubation times and/or nitrate concentrations used in that experiment. All experiments were performed with Yarra River Sediments except the third  $^{15}\text{NH}_4^+$  nitrification experiment, which was performed with Vilhelmsborg S $\emptyset$  sediment as indicated.

Sample collection	Treatments	Details	Cut dates†	Experiment date
<b>Nitrate reduction experiments</b>				
30/01/17	12 x active-cables 12 x no-cables	2, 4, 6, 8 h	06/02, 13/02, 15/02, 17/02, 19/02, 1 h before expt.	23/02/16
16/02/18	8 x active-cables 8 x inactivated-cables 8 x no-cables	6 h	Daily 17-20/02, Twice daily 21-25/02, 1 h before expt.	26/02/18
<b><math>^{15}\text{NH}_4^+</math> nitrification experiments</b>				
17/11/16	9 x active-cables 12 x inactivated-cables	20, 40, 60 min	1 h before expt.	06/12/16
30/01/17	8 x active-cables 8 x no-cables	30, 60 min	06/02, 13/02, 15/02, 17/02, 19/02, 1 h before expt.	20/02/17
28/02/17 (Vilhelmsborg S $\emptyset$ )	12 x active-cables 12 x inactivated-cables	30, 75, 100, 120 min	1 h before expt.	30/03/17
<b><math>^{15}\text{NO}_3^-</math> nitrification experiments</b>				
17/11/16	12 x active-cables 12 x inactivated-cables	10, 30, 100, 400 $\mu\text{M } ^{15}\text{NO}_3^-$	1 h before expt.	07/12/16
30/01/17	12 x active-cables 12 x no-cables	500, 1000, 2500, 5000 $\mu\text{M } ^{15}\text{NO}_3^-$	06/02, 13/02, 15/02, 17/02, 19/02, 20/02, 21/02, 1 h before expt.	22/02/17

† Active-cables treatments were never cut. Inactivated-cables treatments were cut only once, on the day of the experiment. No-cables treatments were cut approximately every two days as described.

## Figure captions

Figure 1: Schematic of cable bacteria showing typical sediment depth-profiles of O<sub>2</sub> (red), H<sub>2</sub>S (green) and pH (black). Also shown in italics are the anodic and cathodic half-equations for cable bacteria metabolism and the proposed reactions involving the N cycle: denitrification and DNRA at the cathode (A), Fe-DNRA at the pH minimum (B) and anoxic nitrification (NIT) at the anode (C).

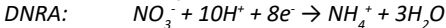
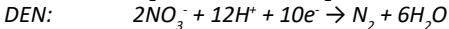
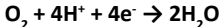
Figure 1 (print version): Schematic of cable bacteria showing typical profiles of O<sub>2</sub> (solid), H<sub>2</sub>S (long dash) and pH (short dash). Also shown in italics are the anodic and cathodic half-equations for cable bacteria metabolism and the proposed reactions involving the N cycle: denitrification and DNRA at the cathode (A), Fe-DNRA at the pH minimum (B) and anoxic nitrification (NIT) at the anode (C).

Figure 2: summary of nitrate reduction experiments results. Shown are rates of denitrification and DNRA and %DNRA for the active-cables (A), inactivated-cables (I) and no-cables (N) treatments. (a) shows the first experiment (23/02/2016, N=12) and has only treatments A and N. (b) shows the second experiment (26/02/2018, N=8), with all three treatments. Note the different axes to assist visualisation. Error bars represent standard error.

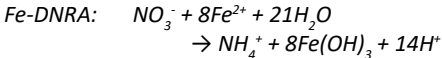
Figure 3: The link between cable bacteria, Fe<sup>2+</sup> and %DNRA. (a) average Fe<sup>2+</sup> concentration in the upper 20 mm of sediment is significantly lower in the no-cables treatment than the active-cables or inactivated-cables treatments ( $p = 0.01$ ). Data shown is from the same experiment as shown in Fig 2b. Error bars represent standard error. N = 6-8. (b) %DNRA is weakly correlated with Fe<sup>2+</sup> for the same data shown in panel a ( $p = 0.2$ ). Marker colour denotes the data as being part of the active-cables (A), inactivated-cables (I) and no-cables (N) treatments. N = 20.

Figure 4: Results of anoxic nitrification experiments in Yarra River (YR) and Vilhelmsborg SØ (VS) sediments. (a) Rate of <sup>15</sup>N-N<sub>2</sub> production in the <sup>15</sup>NH<sub>4</sub><sup>+</sup> nitrification experiment. Each experiment shows both an active-cables (A) and either an inactivated-cables (I) or no-cables (N) treatment. Surface and bottom refer to the surface and deep sediment layers. N = 3 for experiment 1 & 3 and N = 4 for experiment 2. "surface" is 0-10 mm depth for YR and 0-3 mm depth for VS. "bottom" is 10-20 mm depth for YR and 3-21 mm depth for VS. Note that rates are minimum rates, as <sup>15</sup>N<sub>2</sub> lost to the overlying water column is not considered. (b) D<sub>14</sub> during the <sup>15</sup>NO<sub>3</sub><sup>-</sup> nitrification experiment using Yarra River sediment. The dashed line represent the deviation expected if anoxic nitrification occurred at a rate of 5  $\mu\text{mol m}^{-2} \text{h}^{-1}$  (0.1 % of total cable bacteria anodic electron transfer) and all of this nitrification resulted in <sup>29</sup>N<sub>2</sub> at 5000  $\mu\text{mol L}^{-1}$  nitrate. Error bars represent standard error. N = 3.

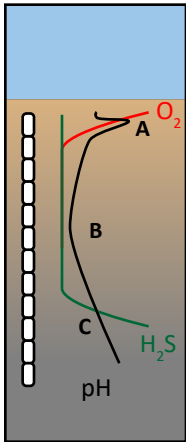
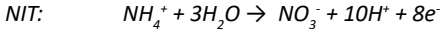
### A. Cathodic reaction of cable bacteria:

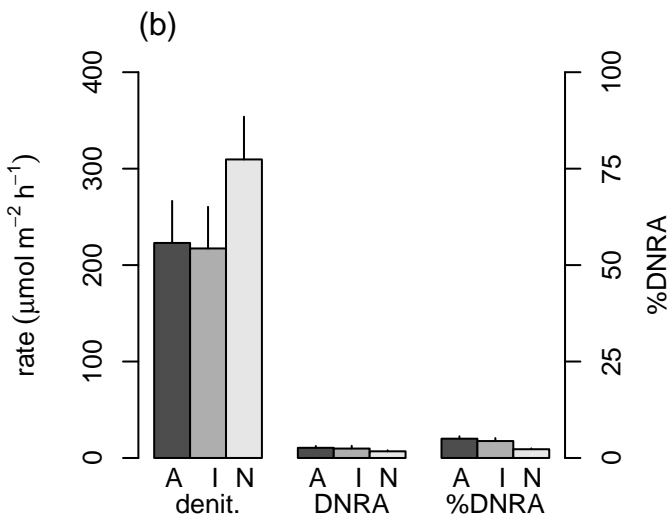
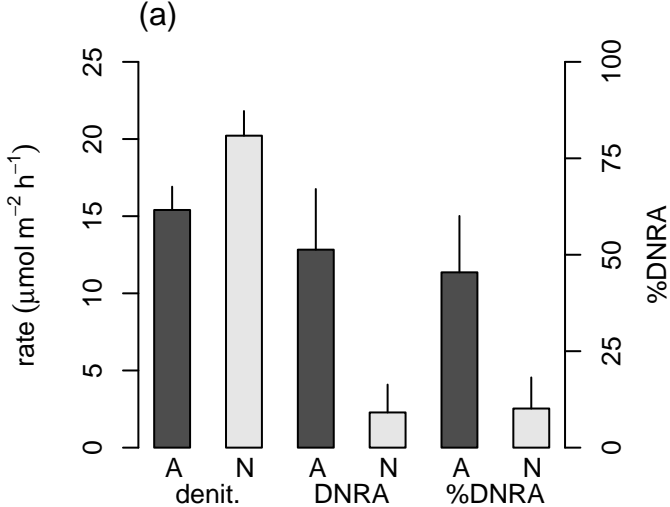


### B. pH minimum

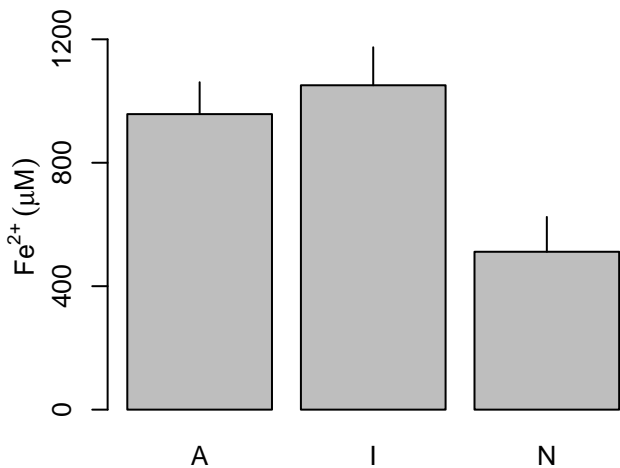


### C. Anodic reaction of cable bacteria:

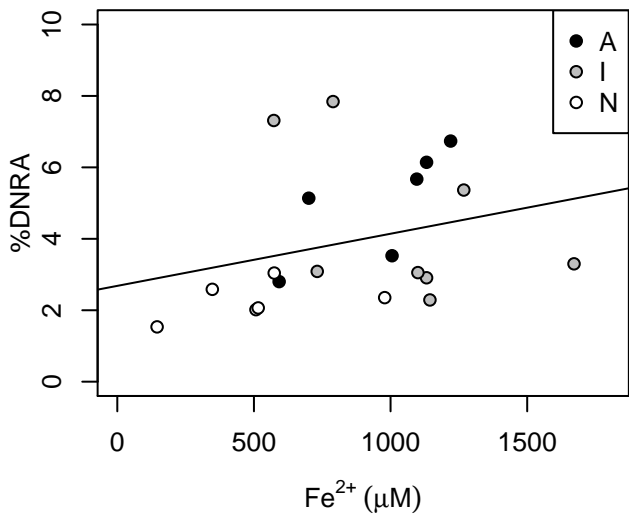


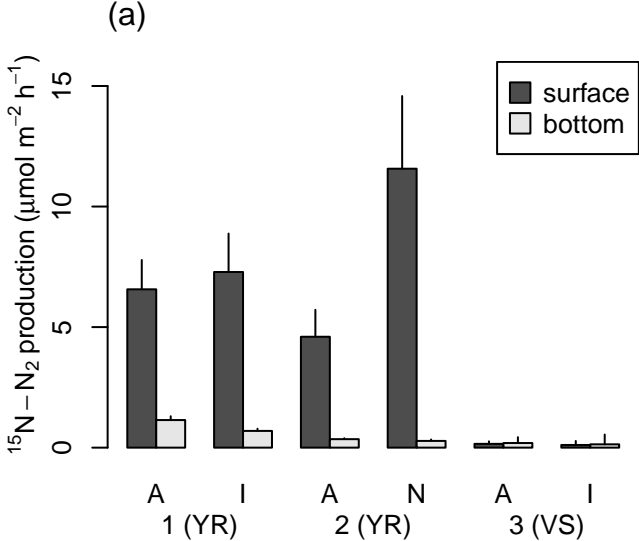


(a)



(b)





(b)

