Cable bacteria promote DNRA through iron sulphide dissolution

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Abstract

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

Introduction

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

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

Nitrogen (N) is an important nutrient in aquatic environments, and understanding nitrate reduction pathways is an important part of managing ever-increasing global loads (Conley et al. 2009). Denitrification is an important N-removing process, whereby nitrate is reduced to N_2 gas. In competition to denitrification is dissimilatory nitrate reduction to ammonium (DNRA), which retains N in the system. Thus, the balance of denitrification and DNRA can be an important control on whether a system is net N removing or recycling (An and Gardner 2002; Dunn et al. 2013; Gardner et al. 2006; Giblin et al. 2013; Kessler et al. 2018; Roberts et al. 2014). This is of great significance in estuaries in particular, as denitrification-dominated estuaries may remediate high nitrate concentrations, while DNRA-dominated estuaries are likely to pass large bioavailable nitrogen loads to coastal waters and embayments. Nitrification, the oxidation of ammonium to nitrate, can enhance N removal if coupled with denitrification. Nitrification is usually considered an aerobic process, but anoxic nitrification processes are known involving Mn and possibly Fe oxides (Hulth et al. 1999; Mortimer et al. 2004).

It is not yet known how cable bacteria control and influence the N cycle. Marzocchi et al. (2014) showed that nitrate can be used as alternative cathodic electron acceptor in the absence of oxygen. Whether the cable bacteria perform denitrification or DNRA is yet unclear, but recent work suggests that cable bacteria can reduce both nitrate and nitrite, but not N_2O (Risgaard ‐ Petersen et al. 2014). However, cable bacteria have not yet been observed *in situ* in a high-nitrate, anoxic environment, and it is not known whether cathodic nitrate-reduction occurs in a sub-oxic zone even in the presence of oxygen. If so, denitrification or DNRA directly as the cable bacteria's cathodic half-reaction may be important processes (Fig 1a). We hypothesize that these direct reactions by cable bacteria may contribute significantly to sediment nitrogen cycling.

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

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

In this study, we investigated the three hypothesized cable-bacteria-mediated nitrogen cycling reactions described above, as depicted in Fig 1. First, we measured rates of denitrification and DNRA and compared their relative contribution to nitrate reduction in sediments with and without cable bacteria to address whether cable bacteria could lead to a stimulation of DNRA relative to denitrification. Second, we repeated this experiment in sediment with active cable bacteria and inactivated cable bacteria to address if such a stimulation could be attributed to the ability of cable bacteria to perform DNRA or alternatively to promote DNRA by increasing Fe^{2+} availability. Third, we tested whether cable bacteria can promote anodic nitrification in oxygen-free environments, by two methods. One method involved addition of $^{15}NH_4^+$ to the deep, anoxic part of the sediment. If cable bacteria promoted anoxic, anodic nitrification, this would produce $^{15}N\text{-}NO_3^-$, which would subsequently be rapidly reduced to $^{15}N\text{-}N_2$. The other method involved adding different concentrations of $^{15}NO_3^-$ to the water overlying the sediment, resulting in varying penetration of $^{15}NO_3^-$ into the sediment. Therefore if anodic nitrification occurred, higher $^{15}NO_3^-$ concentrations would result in greater overlap of the zones of anoxic nitrification and $^{15}NO_3^-$ denitrification, and the measured rate of denitrification of ambient $^{14}NO_3^-$ would increase with $^{15}NO_3^-$ concentration.

Materials and methods

Sites and sediment collection

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

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

Signatures for cable bacteria activity

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

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

Nitrate reduction experiments

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

Collected sediment was sieved (0.5 mm), homogenised, and packed into short polymethylmethacyrlate (PMMA) core liners (L = 120 mm, ID = 42 mm). Cores were incubated in 10 L of oxygenated site water for approximately 3 weeks (with a maximum of 24 cores per bath). Three weeks was chosen as a time where we expect significant cable bacteria activity based on typical dynamics observed in Yarra River and other sediments (Burdorf et al. 2017). Table 1 shows the details of the nitrate reduction experiments performed. To avoid the development of e-SOx, the sediment was cut at 2 mm depth every 1-2 days; the frequency of cutting varied over the various experiments and is detailed in Table 1. Cutting is achieved by inserting a fine wire into the sediment at ~ 2 mm depth (just below the depth of oxygen penetration) using two pins, and pulling the wire through the sediment, effectively slicing a surface layer without removing it from the core. This action inhibits the respiration and carbon uptake of the cable bacteria (Pfeffer et al. 2012; Vasquez-Cardenas et al. 2015) and consequently their growth. This treatment is referred to as "no-cables". Cores with inactivated cable bacteria were prepared by cutting only once immediately (< 1 hour) before the experimental incubation. Therefore, this "inactivated-cables" treatment maintains the same biogeochemical conditions as an uncut core, but excludes the direct influence of the cable bacteria metabolism and e-SOx (Risgaard-Petersen et al. 2015). As shown previously, the Fe^{2+} pool is diminished by diffusion after cable bacteria are inactivated; as diffusion is slow over cm distances, the Fe^{2+} pool in anoxic layers of sediments with inactivated cable bacteria does not change significantly within one hour of inactivation and persists even 33 hours after inactivation (Risgaard-Petersen et al. 2012). To control for any small amount of oxygen entrained by inserting the cutting wire into the sediment, the wire was inserted into each "active-cables" and "inactivated-cables" sediment at every cutting time, but removed without drawing it through the sediment and inactivating the cable bacteria.

Cores were transferred to separate, individually stirred PVC tube (L = 240 mm, ID = 50 mm) filled with oxic site water. The overlying water in the PVC tubes were amended to a final concentration of 30 µM $^{15}NO_3$, and were sealed with a rubber stopper. After several hours (see Table 1), the stopper was gently removed. Samples of the overlying water were collected for ^{15}N-N_2 (12 mL in a glass vial (Labco Exetainer)) and $^{15}NH_4^+$ (6 mL in a polypropylene (PP) centrifuge tube, Falcon), both preserved with 100 µL 50% $ZnCl_2$. The surface 2 cm of sediment was then extruded, transferred to a beaker containing 2% $ZnCl_2$ and quickly and gently homogenised, then transferred to 12 mL glass vials for $^{15}N_2$ analysis. A 6 mL subsample of this slurry was also collected in a PP centrifuge tube for $^{15}NH_4^+$ analysis. For details of this method, see Kessler et al. (2018).

^{15}N-N_2 was measured by adding a 4 mL He headspace to the 12 ml glass vials, and analysed using a Sercon isotope ratio mass spectrometer (IRMS). $^{15}NH_4^+$ was extracted with 1:1 2 M KCl, shaken for 1 hours at 120 rpm. The supernatant after centrifuging was transferred to a glass vial, purged with He and the NH_4^+ converted to N_2 with alkaline hyperbromite (Risgaard-Petersen et al. 1995) and measured by IRMS. Denitrification and DNRA were measured as the rate of production of ^{15}N-N_2.

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

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

Nitrification experiments

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

$^{15}NH_4^+$ experiments

To test if cable bacteria can promote nitrification in anoxic sediments, ^{15}N experiments were performed with sediments from Yarra River, Australia and Vilhelmsborg Sø, Denmark.

Cores with treatments “active-cables”, “inactivated-cables” and “no-cables” were prepared, incubated and cut as described for the nitrate reduction experiments, except that the PMMA core liners were replaced with polypropylene tubes (L = 70 mm, ID = 20 m) created by cutting 60 mL syringes (Thermo). 0.1 mL of anoxic, 100 mM $^{15}NH_4Cl$ was injected via a hypodermic needle through a port 1.5 cm below the sediment surface into the centre of the tube. Tubes were then transferred to separate, individually stirred 50 mm ID PVC tube filled with oxic site water to prevent cross-contamination. After ≤ 60 min (see Table 1), tubes were removed from the bath and quickly extruded. Two, 1 cm slices were transferred to separate beakers containing 2% $ZnCl_2$ and quickly and gently homogenised, then transferred to 12 mL glass vials for later analysis of ^{15}N-N_2.

The method varied slightly for the experiments performed with Vilhelmsborg Sø sediments as follows. Sediment was sieved (0.5 mm), poured into a glass aquaria, and incubated with aerated tap water. The overlying water was replaced weekly to avoid accumulation of metabolic products and to replenish nutrients. On the day of sampling, half of the cores were cut at a depth of 3 mm to inhibit cable bacteria activity, and this treatment is referred to as “inactivated-cables” as above. Sediment cores were extracted from the tank and were immediately incubated for 15 min in a water bath with acetylene (10% partial pressure) to inhibit nitrification activity (Berg et al. 1982) in the surface millimetre of sediment, thereby minimizing eventual diffusion of nitrate from the surface sediment layer to the lower layer where the anodic reaction occurred. Cores were subsequently extracted from the bath and 0.1 mL of a 100 mM solution of anoxic $^{15}NH_4Cl$ was injected at 1.5 cm depth as above. At each time-point (see Table 1), three cores were processed as follows: each cores was sliced at 3 and 21 mm depth. Sediment from zero to 3 mm depth (surface) and from 3 to 21 mm depth (bottom) was transferred into falcon tubes containing a solution of Allylthiourea (100 µM) to stop nitrification activity (Ginestet et al. 1998 and references therein) and gently stirred to minimize gas exchange with the atmosphere. 3 mm was chosen for the first slice to reflect the expected depth of the anodic cable activity in the cores based on microprofiler measurements (Fig S1) and previous experience with these sediments. These measurements were not possible with the Yarra River sediments. A total depth of 21 mm was chosen to closely match the 20 mm total depth analysed for the Yarra River cores. The solution was then left for a short period (< 2 min) to allow the coarser sediment particles to settle out, before an aliquot of the supernatant was transferred into 6 mL glass vials and fixed with 100 µL ZnCl 50% (w:w) for later ^{15}N-N_2 analysis.

$^{15}NO_3^-$ experiment

An alternative experiment to the $^{15}NH_4^+$ experiments was used to measure anoxic nitrification. In this experiment different concentrations of $^{15}NO_3^-$ were added to the water overlying the sediment. A full explanation of the rationale for this experiment is included in the discussion section.

Cores were prepared, incubated and cut as described for the nitrate reduction experiments using Yarra River sediment. Sediment was transferred to individual 50 mm ID PVC tube amended with different concentrations of $Na^{15}NO_3$ (see Table 1). PVC tubes were sealed with a rubber stopper and stirred for 3 hours, after which the surface 3 cm of sediment was extruded into a beaker containing 30 mL 2% $ZnCl_2$ and quickly and gently homogenised, then transferred to 12 mL glass vials. Two experiments were performed to span a large range of nitrate concentrations (see Table 1). D_{14} is defined as the rate of denitrification of ambient $^{14}NO_3^-$, and was calculated as $D_{14} = D_{15} \times p_{29}/(2 \times p_{30})$ following Nielsen (1992), where $D_{15} = p_{29} + 2 \times p_{30}$ is the rate of accumulation of $^{15}N\text{-}N_2$ and p_{29} and p_{30} are the rates of accumulation of $^{29}N_2$ ($^{14}N^{15}N$) and $^{30}N_2$ ($^{15}N^{15}N$) respectively.

Results

Nitrate reduction experiments

In the first nitrate reduction experiment (Fig 2a), the no-cables treatment showed appreciably lower rates of DNRA (2.3 µmol m^{-2} h^{-1}) than the active-cables treatment (12.8 µmol m^{-2} h^{-1}, $p < 0.005$). No difference was observed in denitrification rate ($p = 0.1$), resulting in a much greater contribution of DNRA in the active-cables treatment (%DNRA = 45 %) compared with the without-cables (%DNRA = 10 %). The second nitrate reduction experiment (Fig 2b) showed similar results, with similar rates of denitrification between treatments ($p = 0.2$) and slightly higher DNRA rates in the active-cables and inactivated-cables treatments leading to a significantly higher %DNRA in these treatments (5.0 % and 4.4 %) compared with the no-cables sediment (%DNRA = 2.3 %, $p = 0.03$). The active-cables and inactivated-cables treatments showed no significant differences in denitrification rate ($p = 0.9$), DNRA rate ($p = 0.8$) or %DNRA ($p = 0.6$). Notably, while the DNRA rates were similar in the two experiments, denitrification rates were approximately an order of magnitude higher in the second experiment (Fig 2b), resulting the much smaller values of %DNRA. The highest denitrification rate observed (300 µmol m^{-2} h^{-1}) would represent a decrease of < 20% in the added $^{15}NO_3^-$ concentration over the experimental incubation.

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

Nitrification experiments

Fig 4a shows production of $^{15}N\text{-}N_2$ in the $^{15}NH_4^+$ nitrification experiments. In all three experiments, a small amount of $^{15}N\text{-}N_2$ (≤ 1 µmol m^{-2} h^{-1}) was measured in the deep layer of the active-cables cores, but was not significantly different compared to the controls ($p > 0.05$). Rates measured in the surface layer of sediment were approximately 5-10 times higher than rates in the bottom layer in the Yarra River experiments. In the Vilhelmsborg Sø sediment cores with added acetylene, the surface $^{15}N\text{-}N_2$ production was also negligible indicating the almost complete inhibition of nitrification activity by the acetylene.

For the $^{15}NO_3^-$ nitrification experiment in Yarra River sediments, no significant difference was seen in D_{14} (the rate of denitrification of ambient $^{14}NO_3^-$) with the presence of cable bacteria (Fig 4b, $p = 0.95$ and 0.1 after log-transformation). The solid lines in Fig 4b show regressions for the “no-cables” treatment, and the dashed lines show the expected value of the “active-cables” treatment if an anoxic rate of 5 µmol m^{-2} h^{-1} anoxic nitrification were occurring (representing 0.1 % of the calculated anodic electron transfer), with overlap of the denitrification and anoxic nitrification zone modelled as a square root function with no overlap at zero and complete reduction of the produced $^{14}NO_3^-$ reduced to $^{29}N_2$ at 5000 µmol L^{-1}. Note that the data presented in Figure 4b come from two separate experiments covering the ranges of 0 – 400 µmol L^{-1} and 500 – 5000 µmol L^{-1} nitrate (see Table 1), resulting in the discontinuity at 400 µmol L^{-1}.

Discussion

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

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

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

There are two possible explanations for the influence of Fe^{2+} on nitrate reduction pathway. First, several studies have suggested that Fe^{2+} can be a direct electron donor for DNRA bacteria (Coby et al. 2011; Kessler et al. 2018; Roberts et al. 2014; Robertson et al. 2016; Robertson and Thamdrup 2017; Weber et al. 2006), as depicted in Fig 1b. Mostly, it is suggested that Fe^{2+} reacts with nitrite (NO_2^-) rather than nitrate, and that the earlier step of nitrate reduction to nitrite is performed by other members of the denitrifying community (Robertson et al. 2016). This first step is usually slow, and nitrite does not usually accumulate in these sediments, instead being rapidly reduced to N_2 by denitrification and/or NH_4^+ by DNRA, depending on which community dominates (Roberts et al. 2014). The first nitrate reduction experiment supports this hypothesis, though the effect is smaller in the second experiment (Fig 2b). Secondly, it is known that Fe^{2+} can inhibit denitrification by disrupting intracellular electron transport (Carlson et al. 2012), which would lead to a similar increase in %DNRA. Both the active-cables and inhibited-cables treatments appear to have slightly reduced denitrification in both experiments (Fig 2a and b), although because neither denitrification nor DNRA rates are generally correlated with Fe^{2+}, this study cannot conclusively differentiate these two effects. It is likely that both effects are relevant, depending on other conditions, and that other factors influence both denitrification and DNRA. For example, microbes utilising the well-established sulphide-driven DNRA pathway (An and Gardner 2002; Brunet and Garcia-Gil 1996) may scavenge sulphide released by FeS dissolution in the anoxic zone. As the microbial communities responsible for Fe^{2+}- and sulphide-driven DNRA are not well established, it is difficult to separate these effects. Similarly, sulphide has known toxic effects on denitrification (Sørensen et al. 1980). Despite these additional influences, which may account for the weak correlation in Fig 3b, it would appear that the role of cable bacteria in the nitrogen cycle is to enhance the relative importance of DNRA by other members of the sediment microbial community through increasing Fe^{2+} availability.

2. Anoxic nitrification at the cable bacteria anode

No evidence for anoxic nitrification was observed at either site (Fig 4). The measured nitrification rates in the anoxic bottom layer (where the $^{15}NH_4^+$ was added) are consistently slightly higher in the active-cables treatments, but this effect is never statistically significant. The $^{15}N\text{-}N_2$ measured at the surface sediment is presumably due to a small leak of $^{15}NH_4^+$ solution to the surface through pores and fractures in the sediment during the injection. Indeed the high variation in the mean rates in the surface layers (s.e. = 20 % - 150 %) are consistent with random fractures in the sediment. It is possible that if DNRA dominates (see earlier discussion), then $^{15}NO_3^-$ produced by anoxic nitrification would be reduced back to $^{15}NH_4^+$, which we would not detect using this method. As the %DNRA was never above 50 % in either nitrate reduction experiment, and was usually approximately 10 % (Fig 2), we would still expect denitrification to be measureable in this case. With the sediment from Vilhelmsborg Sø, the finding that anoxic nitrification is negligible is consistent with the experiment from the Yarra River.

For the $^{15}NO_3^-$ experiment in Yarra River sediments, varying concentrations of $^{15}NO_3^-$ were added to the oxic water overlying the sediment. This should have resulted in increasingly deeper penetration of NO_3^-, and therefore an increasingly deep zone of denitrification. If anoxic nitrification occurred, it would be expected that there is an additional source of $^{14}NO_3^-$ in the zone of denitrification, increasing as the depth of the denitrification zone increases. Therefore, there D_{14} should be enhanced in the presence of cable bacteria if anoxic nitrification is occurring. There is no evidence of deviation of the “active-cables” treatment toward the dashed line in Fig 4b. Thus, this experiment provides additional evidence that anoxic nitrification does not occur either as part of cable bacteria activity, or by nitrifiers in the presence of (anodic) cable bacteria. It is noteworthy that the higher concentration treatment shows an increase in D_{14} with $^{15}NO_3^-$ concentration. This indicates that one or more of the assumptions of the isotope pairing technique are not met in this experiment, most likely that the system has not reached a steady state (Nielsen 1992; Risgaard - Petersen et al. 2003). As the present experiment is in any case exploiting a weakness in the isotope pairing technique, this does not invalidate the above finding, but does mean that the rates of D_{14} found cannot be treated as representative.

3. Implications for cable bacteria-rich environments

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

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

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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 $^{15}NH_4^+$ nitrification experiment, which was performed with Vilhelmsborg Sø sediment as indicated.

Sample collection	Treatments	Details	Cut dates†	Experiment date
Nitrate reduction experiments				
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
$^{15}NH_4^+$ nitrification experiments				
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 Sø)	12 x active-cables 12 x inactivated-cables	30, 75, 100, 120 min	1 h before expt.	30/03/17
$^{15}NO_3^-$ nitrification experiments				
17/11/16	12 x active-cables 12 x inactivated-cables	10, 30, 100, 400 µM $^{15}NO_3^-$	1 h before expt.	07/12/16
30/01/17	12 x active-cables 12 x no-cables	500, 1000, 2500, 5000 µM $^{15}NO_3$	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_2 (red), H_2S (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_2 (solid), H_2S (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^{2+} and %DNRA. (a) average Fe^{2+} 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^{2+} 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 ^{15}N-N_2 production in the $^{15}NH_4^+$ 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 $^{15}N_2$ lost to the overlying water column is not considered. (b) D_{14} during the $^{15}NO_3^-$ nitrification experiment using Yarra River sediment. The dashed line represent the deviation expected if anoxic nitrification occurred at a rate of 5 umol m^{-2} h^{-1} (0.1 % of total cable bacteria anodic electron transfer) and all of this nitrification resulted in $^{29}N_2$ at 5000 µmol L^{-1} nitrate. Error bars represent standard error. N = 3.

A. Cathodic reaction of cable bacteria:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

DEN: $2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O$

DNRA: $NO_3^- + 10H^+ + 8e^- \rightarrow NH_4^+ + 3H_2O$

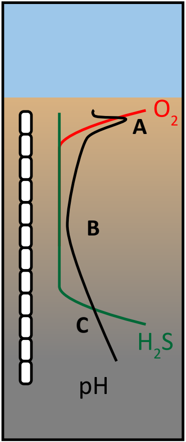
B. pH minimum

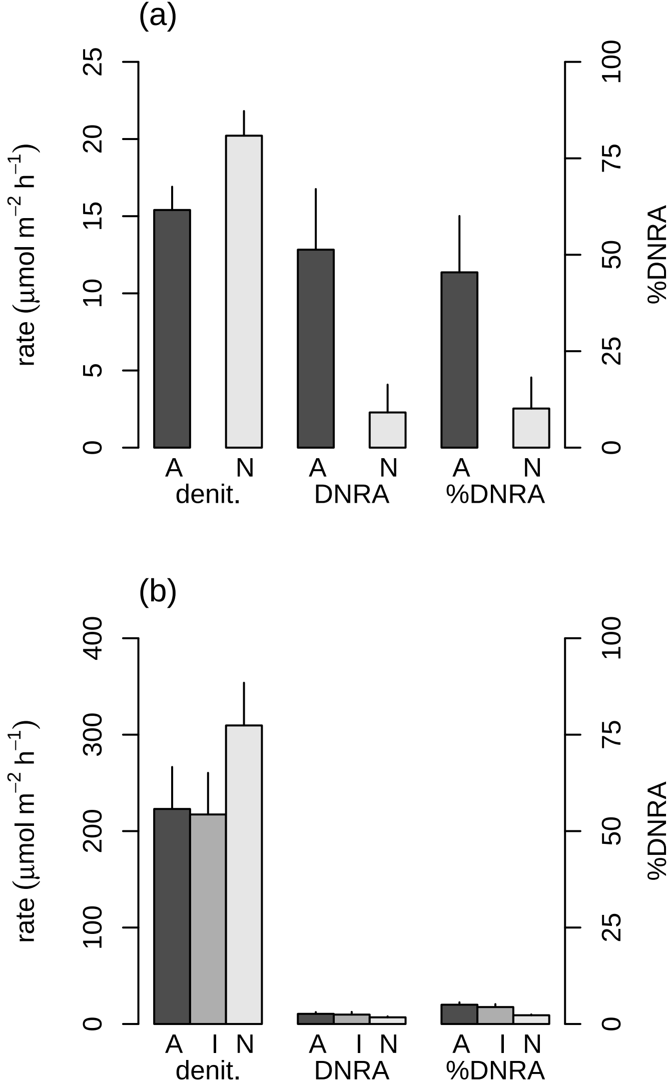
Fe-DNRA: $NO_3^- + 8Fe^{2+} + 21H_2O \rightarrow NH_4^+ + 8Fe(OH)_3 + 14H^+$

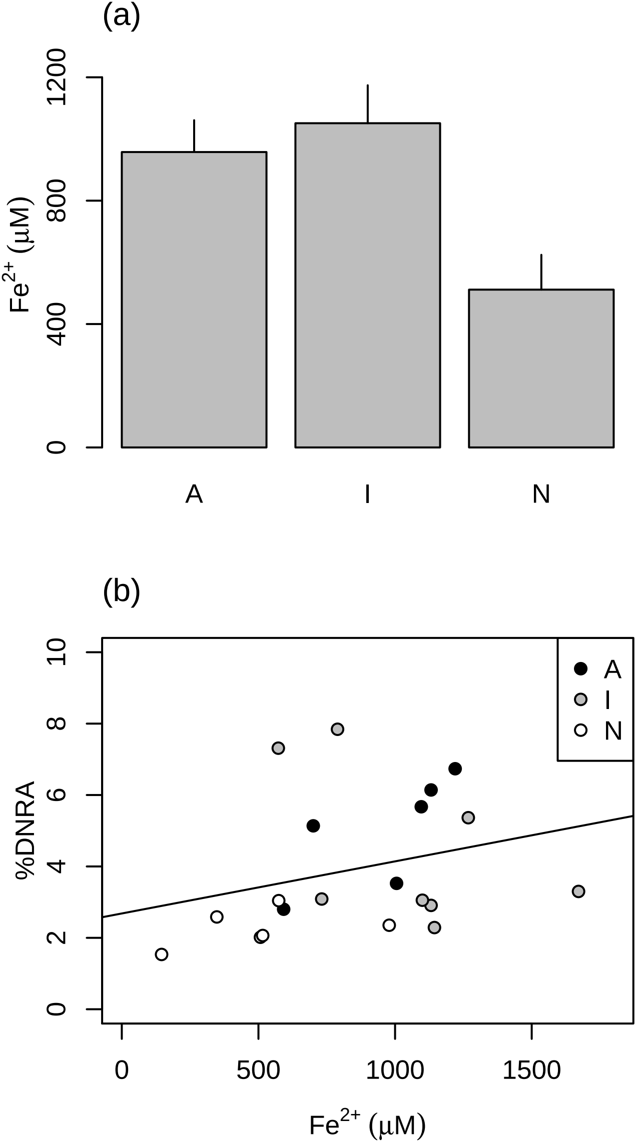
C. Anodic reaction of cable bacteria:

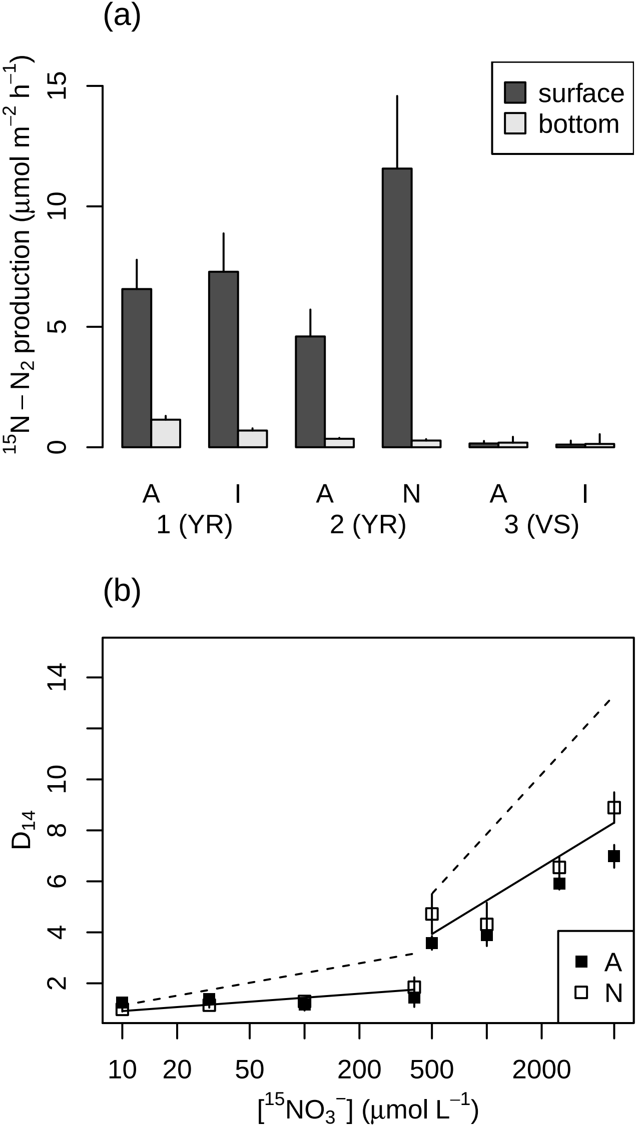
$$H_2S + 4H_2O \rightarrow SO_4^{2-} + 10H^+ + 10e^-$$

NIT: $NH_4^+ + 3H_2O \rightarrow NO_3^- + 10H^+ + 8e^-$




(a)
rate (μmol m−2 h−1)
%DNRA
0
5
10
15
20
25
0
25
50
75
100
A
N
A
N
A
N
denit.
DNRA
%DNRA
(b)
rate (μmol m−2 h−1)
%DNRA
0
100
200
300
400
0
25
50
75
100
A
I
N
A
I
N
A
I
N
denit.
DNRA
%DNRA


(a)
1200
800
400
0
Fe2+ (μM)
A
I
N
(b)
10
8
6
4
2
0
%DNRA
A
I
N
0
500
1000
1500
Fe2+ (μM)



(a)
$^{15}N-N_2$ production (μmol m^{-2} h^{-1})
0
5
10
15
surface
bottom
A
I
A
N
A
I
1 (YR)
2 (YR)
3 (VS)
(b)
D_{14}
2
4
6
8
10
14
A
N
10
20
50
200
500
2000
[$^{15}NO_3^-$] (μmol L^{-1})