Recovery of denitrification and dissimilatory nitrate reduction to ammonium following reoxygenation of sediments from a periodically hypoxic temperate lagoon

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Abstract

Eutrophication and stratification events have increased the incidences of hypoxia and anoxia in coastal environments. The depletion of oxygen in these environments can lead to a shift in biogeochemical processes such as denitrification and dissimilatory nitrate reduction to ammonium (DNRA). The balance between denitrification and DNRA is critical because denitrification leads to a loss of bioavailable nitrogen and DNRA maintains it within the system. This study examined the effects of reoxygenation on denitrification and DNRA in sediments collected from a periodically hypoxic system (Gippsland Lakes, Australia) and subjected to experimental hypoxia using a modified 15N-isotope pairing technique. For freshly collected sediments, the ratio of denitrification : DNRA was generally < 1 indicating a dominance of DNRA. After 3–4 weeks of oxygenation, denitrification increased relative to DNRA resulting in a denitrification : DNRA ratio > 1. Profiles of 15N-N2 and 15NH4+ accumulation in these experiments showed denitrification and DNRA generally only took place in the surface ~ 2 cm of sediment. Prolonged experiments with reoxygenated sediments showed a decrease in the denitrification : DNRA to < 1 after 56 d coincident with deep accumulation of 15NH4+ within the sediment (2–5 cm) suggesting faunal irrigation enhanced DNRA deep in the sediment. An experiment with bromide tracer confirmed the occurrence of DNRA deep within the sediment at 2–6 cm was coincident with tracer penetration, and there was a significant positive relationship between bioirrigation and DNRA. We suggest DNRA deep within the sediment was enhanced by increased availability of organic carbon, reduced solutes, and possibly exposure of reworked sediment to NO3-.

The pressure of increased nutrient inputs from catchments coupled with stratification has increased the incidences of hypoxia (dissolved O2 < 80 μM) and anoxia (dissolved O2 = 0 μM) in coastal environments. Eutrophication-induced hypoxia is now observed on a global scale, including the Baltic Sea, Chesapeake Bay, and Neuse River Estuary (Diaz and Rosenberg 2008). Hypoxia has a profound effect on biogeochemical cycling processes (Middelburg and Levin 2009). Nitrogen is a key driver of eutrophication and associated hypoxia, and as such there is a great deal of interest in how nitrogen is cycled under these conditions. A key step in the nitrogen cycle is NO3 reduction to either N2 or NH4+, the former leading to removal of bioavailable nitrogen (denitrification) while the latter results in nitrogen recycling (dissimilatory nitrate reduction to ammonium [DNRA]). Several studies have focused on the factors (e.g., O2, temperature, S2-, iron, carbon) that control the ratio between the two competing NO3 reduction pathways. In general, it has been observed that Fe2+, free S2-, and high organic matter availability relative to NO3 enhance DNRA over denitrification (Bonaglia et al. 2013; Roberts et al. 2014; Kessler et al. 2018a; Caffrey et al. 2019). As a consequence, sediments previously exposed to hypoxic and anoxic conditions show high rates of DNRA relative to denitrification following reoxygenation (Roberts et al. 2012; Hylén et al. 2021).

Fewer studies have examined the influence of prolonged hypoxia on benthic macrofauna and their combined role in determining the ratio between denitrification and DNRA under these conditions (Bonaglia et al. 2013, 2014b). Sulfide can be toxic to benthic macrofauna, and environments that...
experience periodic hypoxia or anoxia generally support more tolerant species of benthic macrofauna and bacteria that opportunistically flourish in an O2-stressed environment (Diaz and Rosenberg 2008). Several adaptations such as O2 uptake regulation, lowered metabolic demands, and utilizing anaerobic metabolism allow preconditioned infauna to survive in a near anoxic environment (Childress and Seibel 1998). Sturdivant et al. (2012) observed that *Paraprionospio pinicola* were highly active in near-anoxic sediments of Chesapeake Bay, while there was a significant reduction in burrow length and depth. *Capitella capitata* is also known to survive for extended periods during hypoxia and up to 30 days before mortality under anoxia (Diaz and Rosenberg 1995).

In the absence of benthic macrofauna, dissolved O2 will penetrate only millimeters into cohesive sediments; however, burrowing fauna can lead to O2 penetration greater than 10 cm depth altering the vertical distribution of solutes in the sediment (Aller and Aller 1988; Wenzhöfer and Glud 2004). Benthic fauna also have pronounced effects on the nitrogen cycle increasing NO3 reduction rates into the sediment and also increasing the area of the oxic–anoxic interface which can stimulate both nitrification and denitrification (Pelegri and Blackburn 1995; Rysgaard et al. 1995; Nielsen et al. 2004). Over the short term, this has the potential to expose deeper anoxic sediments to NO3 and thus enhance DNRA; however, over the longer term, this could lead to more oxidized sediments and reduced rates of DNRA.

Indeed, fauna have been shown to have a considerable influence over the relative rates of denitrification and DNRA. In sediments of Lake Ohio, USA, Nogaro and Burgin (2014) showed DNRA increased at high tubificid density and high NO3 concentration, but the relative importance of denitrification to DNRA shifted toward denitrification in bioturbated sediments. Pelegri and Blackburn (1996) attributed 20–40% of the measured NH4 efflux to DNRA in bioturbated sediments with low NO3 reduction rates owing to NO3 limitation. Few studies have specifically examined the relative importance of denitrification and DNRA in bioturbated sediments in saline environments, where SO4 reduction and the accumulation of S2− is more prominent (Bonaglia et al. 2013, 2014a). In reoxygenated Baltic Sea sediments, DNRA increased threefold in bioturbated sediments when compared to the non-bioturbated control while denitrification decreased by 50% (Bonaglia et al. 2013). The large increase in DNRA was attributed to higher SO4 reduction rates in the presence of the bioturbator *Marenzelleria* sp., which led to an increase in dissolved S2− at the sediment water interface inhibiting denitrification. Similarly, Bonaglia et al. (2014a) found increased rates of DNRA in the presence of the bivalve *Macoma balthica* in the Stockholm Archipelago of the Baltic Sea. In contrast, Dunn et al. (2009) found no significant enhancement of denitrification or DNRA with increased amphipod density in Morven Bay, Australia, because the increased abundance did not have a significant influence on the sediment characteristics.

The recovery trajectory of the nitrogen cycle in sediments affected by hypoxia is of considerable interest because it can exert a feedback on primary production and ecosystem recovery from eutrophication (Duarte et al. 2009). Using depth resolved profiles of denitrification and DNRA, we investigated how the rates and spatial distribution of these processes changed following reoxygenation of sediments affected by hypoxia over timescales of days to months. In addition, we quantified denitrification and DNRA in relation to bioirrigation to test the hypothesis that DNRA is enhanced through the irrigation of nitrate into deeper sediment layers.

**Materials and methods**

**Study site**

The Gippsland Lakes are a large coastal lagoon system in south-east Australia (Fig. 1). Three large lakes make up the lagoon system, Lake Wellington, Lake Victoria, and Lake King. Lake King North, the study site for this research, has a basin (7–10 m depth), which becomes stratified after periods of high river flow, leading to periodic bottom water hypoxia (Webster 2001; Scicluna et al. 2015). Increases in nutrient loads from the catchment have led to eutrophic conditions in the lakes and sediment release of phosphorus during summer can lead to blooms of toxic nitrogen-fixing cyanobacteria (Cook et al. 2010). The median NO3 concentration in the bottom water at this site between 1986 and 2012 is 0.8 μM, with a 90th percentile of 11 μM observed by Victorian EPA monthly monitoring. We have previously observed sediments at this study site to recolonize with *Capitellid* worms upon sediment reaeration (Scicluna et al. 2015).

**Sediment collection**

Sediment cores were collected from Lake King North (37°52′31.188′′S, 147°46′25.32′′E) in the periodically hypoxic region of the lakes at a depth of ~ 7 m using a core head with a check valve (Aquatic Research Instruments) connected to a 30 cm (h) × 6.6 cm (d) polyethylene core. The substratum was soft mud and approximately half of the core was filled with sediment. Care was taken not to disturb the surface layer of the sediment. The cores were stoppered with a rubber bung at the top and bottom and immediately transported to the laboratory for further treatment. In addition, ~ 40 L of site bottom water was collected for core incubations. Water quality parameters including dissolved O2 saturation (%), temperature (°C), salinity, pH, and turbidity were measured in the bottom waters using a calibrated Hydrolab DS5.

**Experimental approach**

We undertook three experiments to investigate how denitrification and DNRA rates change following reoxygenation over different timescales and the role of bioirrigation in this process. In Experiment 1, we retrieved cores from the Gippsland Lakes on five dates, fully reoxygenated them, and
quantified depth resolved denitrification and DNRA after 1 d, 1 week, and 3–4 weeks of oxygenation. In Experiment 2, we undertook longer term preincubations of up to 90 d on retrieved cores to allow the development of faunal activity as indicated by burrow development. We then quantified the depth resolved rates of denitrification and DNRA after 16, 39, 56, and 90 d. In Experiment 3, we undertook an oxic pre-incubation followed by anoxic incubation of 2 weeks followed by anoxic incubation of 6 weeks to allow the development of faunal burrows in the cores. We then quantified depth resolved rates of denitrification and DNRA, nutrient, sulfide, and bromide tracer profiles which we used to quantify bio-irrigation. Details of these experiments are as follows.

Experiment 1: Short-term effects of reoxygenation on denitrification and DNRA

Sediment cores were collected on 15 December 2014, 3 February 2015, 5 October 2015, 10 February 2016, and 31 March 2016 to capture a range of in situ O2 conditions in the bottom waters and macrofaunal activity (i.e., assuming less activity in hypoxic sediments). On December 2014 and February 2016, the bottom water was hypoxic (< 80 μM), while the bottom water O2 concentration was 118–173 μM on other dates. Twelve sediment cores were collected on each date; four cores for each treatment. When the cores were brought to the laboratory (within 4 h of collection) they were aerated gently, avoiding disturbance of the sediment surface. Sediment cores were set up in a temperature-controlled water bath at in situ temperature (+ 1.0°C, Table 1). The cores were submerged in aerated site water (100% air saturation); and, a magnetic stirrer bar was suspended ~ 2 cm above the sediment surface to ensure the water column remained thoroughly mixed (but no sediment resuspension) throughout the incubation. The stirring speed was maintained at ~ 40 rpm to minimize any disturbance to the sediment surface.

Three treatments were tested in this experiment: Treatment I with 1 d of oxygenation, Treatment II with 1 week of oxygenation, and Treatment III with 1 month of oxygenation after collection. Denitrification and DNRA were determined using the isotope pairing technique (Nielsen 1992), but the treatment of the core at the end of the incubation was modified to quantify the depth distribution of denitrification and DNRA. On the day of the incubation, the four cores of each treatment (I, II, or III) were capped and the initial dissolved O2 concentration measured. A 12 mL filtered (0.45-μm filter Sartorius) sample for the initial NO3 concentration was collected before the addition of 1 mL 0.05 M 15N-NO3 (> 98%, Cambridge Isotope Laboratories) to the overlying water column to give a final 15N-NO3 concentration of ~ 100 μM. The tracer was mixed thoroughly throughout the water column without disturbing the sediment surface and a 12 mL sample for NO3 was filtered (0.45-μm PES (polystyrene sulfone) filter Sartorius) to determine the actual concentration of added tracer. The sample water removed from the core was replaced with site water, and the sediment core was then sealed.

Cores were preincubated for ~ 1 h, then sacrificed in a time series of up to 12 h (~ 1- to 3-h intervals) depending on the rate of O2 consumption throughout the experiment so as to maintain oxygen concentration within 20% of its initial concentration (Dalsgaard et al. 2000). At the end of the incubation, the O2 concentration was measured along with the sediment height in the core. The overlying water and sediment were then sampled to measure denitrification (15N-N2)
and DNRA ($^{15}$NH$_4^+$) as follows. A 1 mL aliquot of ZnCl$_2$ (50% w:v) was added to the overlying water column to terminate microbial activity. A water column sample for N$_2$ was collected and capped immediately in a 12 mL Exetainer (Labco) and preserved with 250 μL ZnCl$_2$ (50% w:v). Another 20 mL unfiltered sample for $^{15}$N-NH$_4^+$ was collected and frozen until analysis. To determine the profile of $^{15}$N produced in the sediment, the core was sliced at intervals of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 cm (Kessler et al. 2018a). Each 0.5- and 1.0-cm slice was rapidly placed into 40 and 80 mL ZnCl$_2$ (2% w:v), respectively, and slurried gently until the sample was homogeneous. We estimate < 10% of the labeled N$_2$ gas is lost during processing for this method (Kessler et al. 2013, 2018a). A 12 mL sample for N$_2$ was immediately collected in a 12 mL Exetainer (Labco) and stored inverted until analysis. A 6 mL sample for $^{15}$NH$_4^+$ analysis was also collected from the slurry mixture in a 12-mL centrifuge tube (Falcon). Irrigation is an additional process that will lead to loss of both $^{15}$NH$_4^+$ and $^{15}$N-N$_2$ from the sediment; however, integrated rates account for this because the accumulation of $^{15}$NH$_4^+$ and $^{15}$N-N$_2$ in the water column were also measured. The depth profiles will underestimate relative accumulation rates where rapid irrigation occurs, such as when we observed production of $^{15}$NH$_4^+$ 2–8 cm within the sediment. We note that > 50% of the tracer could always be accounted for within the sediment, so volumetric rates could be underestimated by as much as a factor of 2. Despite this, our data give a good indication of the depth distribution of process rates within the sediment, which was the key purpose.

**Experiment 2: Long-term effects of reoxygenation on denitrification and DNRA**

Sediment cores were collected on 13 June 2016 and incubated in oxygenated site bottom water at ambient temperature (~18.5°C) for up to 90 d before denitrification and DNRA was measured. Cores were separated into four time periods of oxygenation after collection, approximately 2, 5, 8, and 13 weeks, with four cores per treatment.

The same procedure described for Experiment 1 was followed to determine denitrification and DNRA with the following amendments. (1) Cores were incubated with $^{15}$N-NO$_3^-$ for up to 10 h depending on the O$_2$ consumption rate before all being sacrificed. These cores were not measured in a time series as this complicated the interpretation of bioirrigation rates which are highly heterogeneous. (2) The sediment slice was halved; one half was treated in ZnCl$_2$ as described in Experiment 1 and the other half placed in a 50-mL centrifuge tube (Falcon) and the headspace purged with Argon. The untreated slice was centrifuged at 4000 rpm for 10 min. The pore water was collected for the analysis of nutrients and frozen until analysis.

**Experiment 3: Effect of bioirrigation on denitrification and DNRA**

Fourteen sediment cores were collected on 15 February 2017 and incubated in site bottom water at ambient temperature (~20°C) for 12 weeks to allow for burrow networks to develop in the cores before denitrification and DNRA were measured. A variable O$_2$ regime was simulated during this time; the cores were aerated for the 1st 4 weeks, then allowed to go anoxic for 2 weeks, and then they were reoxygenated for a further 6 weeks to simulate the variable O$_2$ regime observed in the Gippsland Lakes and select for anoxia tolerant species. The overlying water was replaced every 2 weeks with site water, and site salinity was maintained during this time.

On the day of incubation, the cores were placed in a temperature-controlled water bath at 18.5°C and a magnetic stirrer was suspended ~ 2 cm above the sediment surface to ensure the water column was thoroughly mixed. Cores were oxygenated individually to maintain ~ 100% saturation in the water column. The same procedure described for Experiment 2 was followed to determine bioirrigation (Br$^-$ tracer), denitrification and DNRA with the following amendments. (1) The sediment slice was halved; one half was treated in ZnCl$_2$ as described in Experiment 1 and the other half placed in a 50-mL centrifuge tube (Falcon) and the headspace purged with argon then centrifuged at 4000 rpm for 10 min. The pore water was collected for the analysis of $^{35}$S$^{2-}$, Br$^-$, $^{15}$N$_4$, NO$_3^-$, and $^{15}$NH$_4^+$. Immediately after centrifuging, the pore water was filtered (0.45-μm PES filter Sartorius) and a 1 mL subsample was preserved with 50 μL 0.06 M zinc acetate. The remaining sample was frozen until analysis. In-house tests...

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**Table 1.** Bottom water conditions in Lake King North on all sampling dates (mean ± SD). Cores in February 2016 were incubated at 19°C based on seasonal temperature for bottom water. The dates in 2014–2016 refer to samples collected for Experiment 1.

| Date       | O$_2$ Sat (%) | O$_2$ μM | Temp (°C) | pH    | Sal   |
|------------|---------------|----------|-----------|-------|-------|
| 15 Dec 2014| 2.7 ± 3.5     | 6 ± 8    | 19.5 ± 0.0| 7.33  | 29.9  |
| 03 Feb 2015| 55.4 ± 5.5    | 149 ± 15 | 13.1 ± 0.0| 6.74  | 31.3  |
| 05 Oct 2015| 65.3 ± 4.2    | 173 ± 11 | 13.8 ± 0.0| 7.68  | 31.6  |
| 10 Feb 2016| ~ 20*         | ~ 48     | nd        | nd    | nd    |
| 31 Mar 2016| 49.1 ± 6.1    | 118 ± 15 | 19.3 ± 0.1| 7.53  | 31.0  |

nd, no data.

*Estimated from O$_2$ measurement in the laboratory.
comparing this approach with extractions in an anoxic glove bag have shown the same concentrations for dissolved iron, indicating little oxidation of even the most rapidly oxidized species. (2) The cores were separated into two distinct groups: cores where bioirrigation was observed (bioirrigation velocity > 0.00 L m⁻² d⁻¹, n = 9) and cores where bioirrigation was not observed (bioirrigation velocity = 0.00 L m⁻² d⁻¹, n = 5) to explore drivers of denitrification and DNRA.

To determine bioirrigation rate, a modified version of the method described in Martin and Banta (1992) was used. A 12 mL filtered (0.45-μm PES filter Sartorius) water sample was collected from the overlying water column to determine the background concentration of Br⁻. Then 2.5 mL of 2 M KBr was added, at the same time as the NO₃⁻ tracer, to the overlying water column to give a final concentration of ~ 10 mM Br⁻ in the overlying water. The KBr was mixed thoroughly through the water column without disturbing the sediment surface before a 12 mL filtered (0.45-μm PES filter Sartorius) water sample was collected to determine the final concentration of added Br⁻. Br⁻ in the sediment pore water was collected from the same half slice used for nutrients. The cores were oxygenated individually before denitrification and DNRA were measured in the core.

Sample analysis

Nutrients (NH₄⁺ and NO₃⁻) were analyzed via flow injection analysis (Lachat Quickchem 8000 FIA), following the procedures in the Standard Methods for Water and Wastewater (APHA 1992) including standard and quality assurance checks. Sulfide was determined via the methylene blue method (Cline 1969). Br⁻ samples were diluted and analyzed on an ion chromatography (Dionex ICS 1100).

N₂ gas samples were analyzed on a Sercon 20–22 continuous flow isotope ratio mass spectrometer coupled to a gas chromatograph (GC-IRMS) after the introduction of a 4 mL He headspace. Sediment samples for ¹⁵N-NH₄⁺ were extracted with 1 : 1 2 M KCl and shaken at 120 rpm for 1 h and then centrifuged at 2000 rpm for 10 min. A volume of 7.5 mL of supernatant, or water sample was transferred into a 12 mL Exetainer (Labco) and sealed with a rubber septum. The headspace was purged with He to remove background N₂ and 200 μL of alkaline hypobromite was added to convert the NH₄⁺ to N₂ gas (Risgaard Petersen and Rysgaard 1995). The samples were shaken for 16 h overnight before analysis via GC-IRMS.

Faunal identification

The destructive nature of the profiling method did not allow for faunal counts per core, however, to characterize the species present specimens for faunal identification were collected from additional cores collected during Experiments 2 and 3. The cores were treated in the same manner as the experimental oxygen regime and were sieved using a 1-mm sieve followed by a 300-μm sieve. Specimens were identified using Wilson et al. (2003) or they were sent to Robin Wilson at Melbourne Museum for identification.

Calculations

Denitrification and DNRA were calculated from the accumulation of ¹⁵N-N₂ and ¹⁵N-NH₄⁺ over time (Dalsgaard et al. 2000). In the calculation of denitrification and DNRA rate, the sediment volume was corrected to account for the ZnCl₂ added to the sediment slice. Given the large variance in bioirrigation rate, denitrification and DNRA were determined for individual cores by calculating the excess of ¹⁵N-N₂ and ¹⁵N-NH₄⁺ accumulated over the incubation within each core. The mean and standard error are reported for the four cores on each date, with the exception of the Br⁻ experiments. The rates calculated for individual slices are “potential rates” and are reflective of the ¹⁵N-N₂ and ¹⁵N-NH₄⁺ production.

To determine total denitrification and DNRA rates in the core, excess ¹⁵N was integrated across both the sediment slices and the overlying water column. Rates of ¹⁵N denitrification and DNRA (D14 and D15) were calculated based on the accumulation of ²⁹N₂ and ³⁰N₂ according to Nielsen (1992) and Risgaard Petersen and Rysgaard (1995). For profiles, we present D15 rates and integrated whole core rates are presented as D14. The D15 rates should be seen as potential rates as they reflect the higher concentration of ¹⁵NO₃⁻ added above background, D14 rates were not able to be calculated for individual slices as the ratio of ¹⁴NO₃⁻ to ¹⁵NO₃⁻ was not determined in each slice. Regression analysis was run in Sigmaplot 14.0 and t-test was performed in Microsoft Excel.

Quantification of bioirrigation

Bioirrigation rate was modeled using a one-dimensional (1D) nonlocal exchange function proposed first by Emerson et al. (1984). The modeling procedure largely followed the method of Heilskov et al. (2006), irrigation velocity modeled as a lognormal distribution in the sediment.

\[
\alpha = \alpha_{\text{max}} - \frac{\exp \left( -\frac{\ln(\omega/\theta)}{2\sigma} \right)}{(x-\theta)\sigma \sqrt{2\pi}}.
\]

(1)

Bioirrigation parameters \(\alpha_{\text{max}}, \theta, \sigma, \) and \(m\) were adjusted so that predicted Br⁻ profiles matched the observed using a least-squares criterion (e.g., see Supporting Information Fig. S1) (Heilskov et al. 2006). Some profiles had zones of more intense bioirrigation, and for these zones two lognormal profiles were added and fit, such that

\[
\alpha = \alpha_1 + \alpha_2.
\]

(2)

All fit parameters for all 14 simulations are included in Supporting Information Table S2 and are shown graphically in Supporting Information Figs. S1, S2.
The 1D transport equation for Br\(^{-}\) concentration \(c\) was solved using R (v 2.15.2) with the freely available diagenetic model code ReacTran (Soetaert and Meysman 2012).

\[
\frac{\partial c}{\partial t} = \frac{\partial}{\partial x} \left( \frac{\partial D_x}{\partial x} \right) - \frac{\partial}{\partial x} \left( \frac{c}{\phi_a} (c - c_{\text{OW}}) \right).
\]

(3)

The meaning for all parameters and the values chosen are provided in Supporting Information Table S1.

Finally, the irrigation velocity \(v\) is calculated as

\[
v = \int_0^1 \frac{dx}{\alpha}.
\]

(4)

**Results**

**Water column conditions**

Water column conditions at Lake King North, Gippsland Lakes, are recorded in Table 1. On all sampling occasions, O\(_2\) was undersaturated in the bottom waters; hypoxia (< 80 \(\mu\)M O\(_2\)) was observed in December 2014 and February 2016 (Table 1). On all other sampling dates, O\(_2\) concentration ranged between 118 and 173 \(\mu\)M. Bottom waters were saline on all sampling occasions. February 2015 represents a period of recovery after a long period of hypoxia (see December 2014; 2.7% O\(_2\)).

**Faunal identification**

In sediments collected for Experiments 2 and 3, the dominant species present was *Orthopriopospiro cirriforma*, other species included *Hesionidae* sp., *Captellid* sp., and *Priapulus tuberculatospinusus*. While Captellids have been previously reported as abundant in the sediments of Lake King North in the Gippsland Lakes, only one specimen was recorded in the cores collected. Because all cores were not sieved due to the experimental method, it is possible that other species that have been previously identified in Gippsland Lakes were present in the cores and not detected.

**Experiment 1: Short-term effects of reoxygenation on denitrification and DNRA**

The highest rates of denitrification were most commonly observed at the sediment–water interface (Fig. 2). Except for December 2014, DNRA potential in the surficial sediments in all months was comparable to or less than denitrification potential (Fig. 2). In December 2014, after a prolonged period of near anoxia (Table 1), DNRA potential (Fig. 2b) was higher than denitrification potential (\(^{15}\)N-N\(_2\) potential; Fig. 2a) in the surficial sediments after 1 d of oxygenation before decreasing over the proceeding weeks. Peaks in DNRA potential below 1 cm depth in December 2014, February 2015, and October 2015 indicate that NO\(_3^-\) was being transported deep into the sediments.

With the exception of October 2015, DNRA made up 47–57% of total NO\(_3^-\) reduction after 1 d of oxygenation across the sampling dates; after 1 month of oxygenation, the contribution of DNRA to total NO\(_3^-\) reduction decreased to 25–46%. After 1 week of oxygenation, NO\(_3^-\) sourced from the water column for NO\(_3^-\) reduction increased from 5% to 14%, with NO\(_3^-\) supplied by nitrification ranging from 86% to 96% over the incubation period (Fig. 2).

The importance of DNRA in the Gippsland Lakes is confirmed through the denitrification : DNRA ratio where on several occasions the ratio was < 1.0, meaning DNRA was the dominant NO\(_3^-\) reduction pathway. However, after prolonged oxygenation (1 month), the denitrification : DNRA ratio was significantly higher (paired t-test, \(p < 0.05\)) than after 1 d on 4 out of 5 occasions (Fig. 2). The one exception (when there was little change) occurred, when in situ conditions were already oxic during October 2015.

**Experiment 2: Long-term effects of reoxygenation on denitrification and DNRA**

Overall NO\(_3^-\) reduction increased at 90 d. Denitrification was observed in the surface 2 cm of sediment only and rates in the top 0.5 cm increased markedly from 0.5 to 1.25–1.5 \(\mu\)mol L h\(^{-1}\) after 39 d. DNRA was highest in the surface 0.5 cm at 16 d (0.5 \(\mu\)mol L h\(^{-1}\)) and decreased over time, reaching a minimum at 90 d (~ 0.2 \(\mu\)mol L h\(^{-1}\)). DNRA increased at depth (2–4 cm) after prolonged oxygenation with the highest rates being observed at 56 and 90 d (Fig. 3). Integrated rates of denitrification increased from 7 \(\mu\)mol m\(^{-2}\) h\(^{-1}\) at 16 d to a maximum of 22 \(\mu\)mol m\(^{-2}\) h\(^{-1}\) at 39 d then decreased slightly. Integrated rates of DNRA remained relatively constant at ~ 12–15 \(\mu\)mol m\(^{-2}\) h\(^{-1}\) over the course of the experiment. The ratio of denitrification : DNRA was lowest at 16 d (0.6) and highest at 39 d (1.7) before decreasing again thereafter.

**Experiment 3. Effect of bioirrigation on denitrification and DNRA**

The cores with no bioirrigation (bioirrigation velocity = 0.00 L m\(^{-2}\) d\(^{-1}\)) had average denitrification and DNRA rates of 31.6 ± 1.9 and 3.8 ± 0.4 \(\mu\)mol m\(^{-2}\) h\(^{-1}\), respectively (Fig. 4a). The cores where bioirrigation was present (bioirrigation velocity > 0.00 L m\(^{-2}\) d\(^{-1}\)) had a similar denitrification rate of 27.0 ± 1.7 \(\mu\)mol m\(^{-2}\) h\(^{-1}\) but a doubled DNRA rate of 9.0 ± 2.0 \(\mu\)mol m\(^{-2}\) h\(^{-1}\) (Fig. 4b). There was a significant difference between means for DNRA (t-test, \(p < 0.01\)) and the denitrification : DNRA ratio (t-test, \(p < 0.05\)) in the irrigated vs. nonirrigated cores. No significant difference was recorded for denitrification rate between irrigated and nonirrigated cores (t-test, \(p > 0.05\)). There was a clear difference in the pattern of denitrification and DNRA with depth, with denitrification dominating at the sediment surface and DNRA dominating deeper in the sediment, particularly in the irrigated cores (Fig. 4e,f). Depth profiles of NO\(_3^-\) and NH\(_4^+\) were
similar in the two core groups, but small amounts of NO$_3^-$ were observed at 2 cm depth in the irrigated cores (Fig. 4g,h). The bromide profiles showed penetration to 4.5 cm depth in the nonirrigated cores, compared to 8 cm in the irrigated cores (Fig. 4i,j). The S$^{2-}$ profiles show S$^{2-}$ moving slightly closer to the surface (3.5 cm) in the nonirrigated cores compared to the irrigated cores (> 4 cm, Fig. 4h). Below 6 cm, S$^{2-}$ was highest in the irrigated cores.

There was a positive relationship between DNRA and bioirrigation velocity (DNRA = 1.32 [bioirrigation velocity] + 9.45; $r^2 = 0.53$; $n = 16$; $p < 0.01$; Fig. 5). No relationship was observed between denitrification and bioirrigation velocity.

### Discussion

The aim of this study was to better understand how rates of denitrification and DNRA recover following reoxygenation. Ideally, this study would have followed the recovery of a natural system to best understand how in situ dynamics proceed. Finding and sampling a system in this fashion is, however, logistically challenging in the context of coastal systems which often stratify and de-stratify stochastically based on prevailing weather conditions. Instead, we used an experimental approach which offered a high degree of control over the oxygenation regime and avoid confounding environmental factors. Based on previous experience, we also knew this...
approach would allow the establishment of faunal burrow networks. This approach, does, however, impose a high degree of artificiality on our measurements. Of key relevance here are carbon delivery rates. We did not add any carbon to our sediments as we have previously observed infaunal colonization of retrieved cores did not require this. The lack of carbon delivery is likely to mean that the rate of process recovery after hypoxia in our experiments is likely to differ from in situ. For Experiment 3, we also de-oxygenated the cores for a period to simulate anoxia in the Gippsland Lakes and select for species with a life cycle tolerant of anoxia. It has been previously observed that P. pinnata (a close relative of the O. cirriformia observed in our sediments) dominates burrowing activity in severely hypoxic sediments (Sturdivant et al. 2012) suggesting the fauna present in our experiments have environmental relevance. The relationship with bioirrigation observed in this experiment (discussed below) may only pertain to the species observed in Experiments 2 and 3.

We were able to identify the dominant faunal species present, however, we were not able to count fauna within each core as our core slicing and sampling approach precluded this. Instead, our approach allowed us to use the depth distribution of $^{15}$N-N$_2$ and $^{15}$NH$_4^+$ production within the cores as well as measured rates of bioirrigation to infer the role of faunal activity in controlling these processes. We therefore argue that although faunal density data were not measured, the nature of our measurements has allowed us to reasonably infer the role of fauna controlling the dynamics observed here. We also note that there was a broad agreement with our measured rates of DNRA and the proportion of DNRA relative to denitrification compared to a previous coastal in situ study (further discussed below) which gives us confidence that the rates presented in this study have meaning within a broader environmental context.

Our results suggest that sediment denitrification and DNRA rates are likely to undergo a multi-phase recovery process with processes initially occurring only at the surface sediment, and then at depth following faunal colonization. It is well known that the presence of Fe$^{2+}$, $S^{2-}$, and high organic carbon to NO$_3^-$ ratios enhance DNRA (Tiedje 1988; Brunet and Garcia-Gil 1996; Algar and Vallino 2014; Robertson et al. 2016; Wong et al. 2021). As such, conditions favorable to DNRA would be
expected to occur in systems with a hypoxic history. Immediately after reoxygenation, the surface sediments will become progressively reoxidized, partly through oxygen diffusion, but also through the activity of cable bacteria (Seitaj et al. 2015).

This will result in oxidation of sulfides, and the transient accumulation of Fe$^{2+}$ which can stimulate DNRA on the timescale of days to weeks (Kessler et al. 2018b). This is consistent with our observations in Experiment 1 that DNRA remained elevated relative to denitrification in the 1st day to 2 weeks after

![Figure 5](image-url)  
Fig. 5. Experiment 3: DNRA rate ($^{14}$N-NH$_4$$^+$; μmol m$^{-2}$ h$^{-1}$), denitrification rate ($^{14}$N-N$_2$; μmol m$^{-2}$ h$^{-1}$), and denitrification : DNRA ratio vs. bioirrigation velocity (L m$^{-2}$ d$^{-1}$). DNRA rate ($p < 0.01$) was significantly correlated with bioirrigation velocity ($r^2 = 0.46$) and the denitrification : DNRA ratio ($p = 0.02$) showed a significant relationship with bioirrigation velocity ($r^2 = 0.37$).
the reoxygenation of cores. After 3–4 weeks, there was a significant increase in rates of denitrification relative to DNRA consistent with the reoxidation of sulfides and Fe\(^{2+}\) on this timescale. Experiment 2 showed the same pattern in the sediment surface layers with a large increase in denitrification relative to DNRA being observed in the 0.5 cm layer after 39 d of reoxygenation.

Over timescales of weeks to months following hypoxia, there may be a recovery of faunal communities and we observed increased burrow development in the cores over this period. After 56 d, there was a marked increase in \(^{15}\text{NH}_4^+\) accumulation deep within the sediment which most likely reflected the irrigation of NO\(_3^-\) into the sediment by fauna. Experiment 3 also showed irrigated sediments had accumulation of \(^{15}\text{NH}_4^+\) at depths of 2–8 cm concurrent with bromide tracer penetration to this depth and there was a significant relationship between bioirrigation rate and DNRA (Fig. 5). Sediment irrigation has the potential to enhance rates of DNRA as it transports NO\(_3^-\) from the overlying water into the deeper reduced layers of sediment with higher potential for DNRA. However, the effect of this is likely to be time dependent as the sediments progressively become more oxidized following hypoxia. The lack of a relationship between bioirrigation and denitrification in this study was unexpected as denitrification has been shown to be enhanced by faunal irrigation in previous studies (Stief 2013). We believe the most likely explanation for this discrepancy is the anoxic history of these sediments compared to the sediments in previous studies. This leads to more reducing conditions, and hence DNRA is favored over denitrification with depth.

These results are consistent with previous work by Bonaglia et al. (2013), which showed that DNRA was increased in the presence of fauna in sediment affected by hypoxia. In that study it was suggested this was due to enhanced organic carbon inputs associated with fauna. In accordance with this hypothesis, we observed higher concentrations of S\(^2-\) at >6 cm depth in irrigated sediments (Fig. 3h) despite irrigation-driven O\(_2\) input. This strongly suggests increased organic matter inputs at depth supporting the hypothesis of Bonaglia et al. (2013). In addition to this mechanism, and associated cascading effects of increased reduced solutes, we hypothesize that the exposure of new sediment to NO\(_3^-\) by faunal activity also plays a role in the enhancement of DNRA. In anoxic sediment not exposed to NO\(_3^-\), fermention coupled to NO\(_3^-\), Fe(III), and SO\(_4^{2-}\) reduction will be the dominant metabolic pathway. The nrfA enzyme that mediates DNRA is constitutive and will be expressed in the absence of NO\(_3^-\) under these conditions (Jørgensen 1989). When this sediment is exposed to NO\(_3^-\), DNRA will therefore dominate, until a community which expresses the non-constitutive denitrification pathways establishes itself. Indeed, recent work has shown high DNRA potential in systems previously not exposed to NO\(_3^-\), and that there is a time lag of days for denitrification to become established after exposure to NO\(_3^-\) (Wong et al. 2021). This proposed mechanism suggests that the behavior of fauna may also play a role in controlling the ratio of denitrification : DNRA. Kristensen et al. (2012) identified reworking and ventilation as two distinct forms of behavior by sediment fauna. Reworking is the physical mixing of sediment, which is likely to continually expose formerly anoxic sediment to O\(_2\). Ventilation is the pumping of water through burrows, which is likely to lead to the introduction of oxygen deeper into the sediment leading to a more continuous exposure of the sediment in the burrow walls to both oxygen and NO\(_3^-\). We therefore speculate that these two distinct benthic faunal behaviors may lead to different nitrogen recycling pathways, with reworking fauna favoring DNRA in comparison to ventilating fauna. Further studies are encouraged to explore this hypothesis.

As previously noted, quantifying the dynamics of denitrification and DNRA in a natural system following hypoxia is complicated logistically and few studies have undertaken this. A study on the artificial reoxygenation of the By Fjord observed similar rates of DNRA and denitrification as well as denitrification : DNRA ratios to the present study. Of key importance, rates of DNRA increased from 200 to 500 μmol m\(^{-2}\)d\(^{-1}\), a year after reoxygenation had commenced and remained elevated co-incident with recolonization by fauna (Brabandere et al. 2015). Although there was a slight drop in the proportion of DNRA relative to denitrification, the ratio of denitrification : DNRA remained close to 1 for 2 years following reoxygenation. This highlights the long recovery times of denitrification and DNRA following hypoxia and the need to consider these processes with respect to faunal recolonization.

Our findings have some important implications for ecosystem resilience to eutrophication and hysteresis during recovery. In well-established faunal communities, where the sediments maintain an oxygenated state, denitrification will be favored and this will maintain the system in a relatively healthy state where nitrogen is removed through denitrification (Stief 2013). We speculate that once a system has become periodically anoxic, recovery of fauna resistant to hypoxia and anoxia will lead to enhanced rates of DNRA, maintaining nitrogen within the system, potentially leading to more algal growth and hypoxic/anoxic conditions. Indeed, this mechanism may be one feedback that prevents eutrophied ecosystems returning to their former state after nutrient inputs have been reduced (Duarte et al. 2009). It is only after prolonged oxic conditions that the sediment once again becomes more denitrifying, with high rates of DNRA relative to denitrification still observed 2 years after reoxygenation in By Fjord (Brabandere et al. 2015). The present study as well as recent literature suggests fauna can both buffer and amplify the effects of eutrophication on the nitrogen cycle in a similar manner to what has been previously shown for phosphorus (Norkko et al. 2012; Scicluna et al. 2015). The timescales of recovery of the nitrogen cycle following hypoxia and the potential interacting effects of faunal presence and species
composition should be further explored in systems experiencing and recovering from hypoxia.

Data availability statement
All data are available upon request from the authors.

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Conflict of interest

None declared.