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The impact of suspended oyster farming on nitrogen cycling and nitrous oxide production in a sub-tropical Australian estuary

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ABSTRACT

In this study we quantified nitrate (NO_3^-) reduction (denitrification, anammox and DNRA) and N_2O production in sediments and epibiont communities associated with Sydney Rock Oyster (*Saccostrea glomerata*) farming. In sediments beneath an active suspended oyster farm, DNRA accounted for 98% of NO_3^- reduction with rates of up to 169 ± 45 μmol N m^{-2} h^{-1}. Much of this DNRA was fuelled by NO_3^- derived from nitrification. Reference sediments had significantly lower DNRA rates of 83.8 ± 28.2 μmol N m^{-2} h^{-1}, however this constituted 96% of the sites total NO_3^- reduction. Fatty acid analysis showed that sediment organic matter was more labile in the oyster impacted sediments, facilitating subtle shifts in sediment oxygen demand which increased the Fe^{2+} availability with respect to the reference sediments. The difference in DNRA rate between the sites was attributed to autotrophic oxidation of soluble Fe^{2+} in sediments underlying the oyster cultures. DNRA was absent in the oyster shell epibiont communities and rates of anammox and denitrification were lower than in the sediments. Production of NH_4^+ from the oysters and their associated epibionts was larger than DNRA and reached a rate of 206.2 μmol N m^{-2} h^{-1}. Nitrous oxide production rates were generally low compared to other aquaculture systems and the net flux of N_2O for the combined oyster cultivation system (i.e. sediments plus epibionts) was negative, i.e. there was N_2O consumption in the sediments beneath the oysters. Overall, subtropical suspended oyster farming systems favour inorganic N retention over N loss.

1. Introduction

Mollusc aquaculture is a significant contributor to the world's total aquaculture value and volume (11.5% and 22.8% respectively) (FAO, 2014) and bivalve aquaculture accounts for some 70% of global mariculture. In Australia, farming of the Sydney Rock Oyster (*Saccostrea glomerata*) generated ~3000 tonnes of product worth $33 M in 2012 (DPI, 2014). While bivalve aquaculture is clearly a major contributor to global fisheries supply, there is uncertainty over the impact that it has on estuarine nitrogen cycling.

On the one hand, some studies conclude that filter feeding bivalves can contribute to the net removal of nitrogen (N) from coastal environments through the assimilation of suspended nutrients and enhanced denitrification in underlying sediments (Edebo et al., 2000; Carlsson et al., 2012; Smyth et al., 2013). Indeed suspended bivalve cultivation has for many decades been regarded as a mechanism for the 'bio-remediation' of eutrophic estuaries (Petersen et al., 2014; Nielsen et al., 2016). Conversely however, there is also strong evidence to suggest that bivalve cultivation performs an ecosystem disservice by 1) promoting sulphidic conditions and nutrient export from the underlying sediments through the local deposition of faeces and pseudofaeces, and 2) enhancing nutrient recycling in the water column due to the constant excretion of inorganic nutrients by the cultivated organisms (Christensen et al., 2003; Nizzoli et al., 2006, 2011; Higgins et al., 2013; Murphy et al., 2016).

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Whilst these conflicting conclusions may be the result of local factors such as the type, extent and intensity of bivalve cultivation and/or the hydrodynamics, eutrophication status etc. of the study site, part of the uncertainty arises as few studies have comprehensively assessed the overall impacts of bivalve aquaculture on nutrient dynamics. Many studies have focussed exclusively on the exchange of dissolved and particulate nutrients between the water column and cultivated organisms or the impacts of biodeposition on the nutrient fluxes from the underlying sediments (Christensen et al., 2000; Higgins et al., 2013; Lavoie et al., 2016). Furthermore, there is ambiguity surrounding the magnitude and direction of N losses in bivalve impacted systems due to the presence and measurement of different nitrate reduction pathways including denitrification, anammox and DNRA. While the first two are mechanisms of permanent N loss, DNRA acts to retain N within the environment (Tiedje et al., 1982). Hence the balance between DNRA and N_2 production is crucial for understanding the net role of suspended aquaculture operations on local nutrient budgets. To date only a handful of studies have measured the individual sediment nitrate reduction pathways within aquaculture production systems (Gilbert et al., 1997; Christensen et al., 2000; Nizzoli et al., 2006; Castine et al., 2012; Murphy et al., 2016); no aquaculture related studies have measured all three nitrate reduction rates simultaneously and there are no published rates of NO_3^- reduction beneath suspended Sydney Rock Oyster farms.

DNRA is proposed to be favoured over denitrification as the labile organic carbon to NO_3^- ratio (OC: NO_3^-) increases, and also in the presence of sulphide and/or Fe^{2+} (Tiedje et al., 1982; Straub et al., 1996; An and Gardner, 2002; Giblin et al., 2013; Hardison et al., 2015); typical of the conditions usually found in organic rich sediments. Consequently DNRA would be expected to be stimulated in sediments receiving aquaculture biodeposits. In the few aquaculture related studies where sediment DNRA has been measured, its rate was between 4 and 25 fold higher than N_2 production (Gilbert et al., 1997; Christensen et al., 2000; Nizzoli et al., 2006; Murphy et al., 2016). The paucity of information regarding the contribution of DNRA to nitrate reduction in sub-tropical suspended bivalve dominated estuaries means that a reliable assessment of their ecological impact cannot be made. This is an important knowledge gap given that oyster farming occurs in 17% of the estuaries along the New South Wales coast (DPI, 2014).

Another reason for the uncertainty in our understanding of bivalve aquaculture on local estuarine N budgets is the fact that very few studies have included estimates of the contribution of biofilms associated with bivalve aquaculture on N cycling (Holmer et al., 2015; Welsh et al., 2015). Bivalve shells are colonised by microbial biofilms and/or a thick layer of epiphytes that may contribute significantly to N cycling, however much of the emphasis of past work has been on the sediments (Welsh and Castadelli, 2004; Heisterkamp et al., 2013; Caffrey et al., 2016). Of the two studies that have looked at the contribution of bivalve biofilms to biogeochemical cycling, both found that the biofilms made a similar, and at times greater, contribution to inorganic N fluxes, oxygen demand and denitrification as did the sediments (Holmer et al., 2015; Welsh et al., 2015).

Also lacking in most aquaculture N cycling related studies is an assessment of how production influences N_2O dynamics. If suspended bivalve aquaculture enhances NO_3^-reduction, particularly denitrification in the underlying sediments, then one could reasonably expect a concomitant increase in N_2O production. Increased organic matter mineralisation and NH_4^+ production may also promote nitrification and N_2O production via nitrifier denitrification, especially under low oxygen conditions (Goreau et al., 1980). Bivalves themselves release N_2O produced by denitrifying organisms within surface biofilms and their digestive systems (Stief et al., 2009; Heisterkamp et al., 2013). Gut denitrification in particular can be an important source of N_2O, as differential expression of the genes for denitrification during the transition from oxic to anoxic conditions that occurs following ingestion can result in incomplete denitrification and high N_2O yields (Stief et al., 2009; Heisterkamp et al., 2016). Given the variable oxygen supply to sediments, the availability of organic matter, and the capacity of the animals themselves to release N_2O, one would assume that sediments associated with bivalve aquaculture would be hotspots of N_2O generation. However in high C loaded systems such as mangrove lined estuaries, N_2O can also be consumed (Erler et al., 2015b). While there are some measurements of N_2O production from individual bivalves (Heisterkamp et al., 2010), to date there are scant measurements of N_2O production from bivalve aquaculture systems (Welsh et al., 2015), and indeed from aquaculture operations in general (Hu et al., 2012). As yet there are no N_2O production rates available for Sydney Rock Oysters or Rock Oyster farms in Australia.

The overall objective of this study was to make a comprehensive evaluation of the impacts of oyster farming on the nitrogen dynamics of a sub-tropical estuary. More specifically we aimed to 1) determine the contribution of DNRA to NO_3^- reduction in sediments impacted by suspended oyster cultivation, 2) determine the relative contributions of the oysters, their shell epibiont communities, and the underlying sediments to inorganic N cycling, and 3) determine whether suspended oyster cultivation is a source or sink for N_2O.

2. Methods

2.1. Study site

Wallis Lake is a sub-tropical estuary system located on the south east coast of Australia. Geographical and geological details of the Wallis Lake estuary are given in Eyre and Maher (2010) and Maher and Eyre (2010). Two sampling sites were selected within the major oyster growing area of the estuary, one directly within an active oyster lease (in its 6th year of operation) (32.1835°S, 152.4884°E), and a second 'reference' site without active oyster cultivation (32.1816°S, 152.4829°E) (see Fig. 1 and KML file associated with this submission). Due to the patchy distribution of oyster leases in the studied estuary, a true un-impacted control site could not be studied, as it would have to be so far removed from the oyster cultivation area that it would not be comparable in terms of background biogeochemistry or hydrodynamics. Therefore we chose a 'reference' site as the comparison rather than a control. Both sites were within the tidal reach of the estuary.

2.2. Core collection and maintenance

Five undisturbed sediment cores (~25 cm) were manually collected in plexiglass core tubes (50 cm length x 9 cm internal diameter), from both sites for flux and process rate measurements during low tide on the 3rd Nov 2015 (water depth ~ 50 cm). Spare cores were also collected to gauge O_2 consumption. An additional set of 5 cores (~15 cm) were also collected from each site with PVC core tubes (20 cm length x 10 cm diameter) for later deployment of diffusive gradient and diffusive equilibration in thin films samplers (DGT and DET respectively). At each site, sediment samples (x3) were collected for C:N, loss on ignition (LOI), total Fe analysis, and fatty acid composition (polyunsaturated fatty acids (PUFA) and saturated fatty acid (SAFA). This involved colleting 10 cm of surface sediment using cut-off 50 ml syringes. Site water (100 L) was collected from a nearby boat ramp during the incoming flood tide.

All cores were transported to the field laboratory within 1 h and

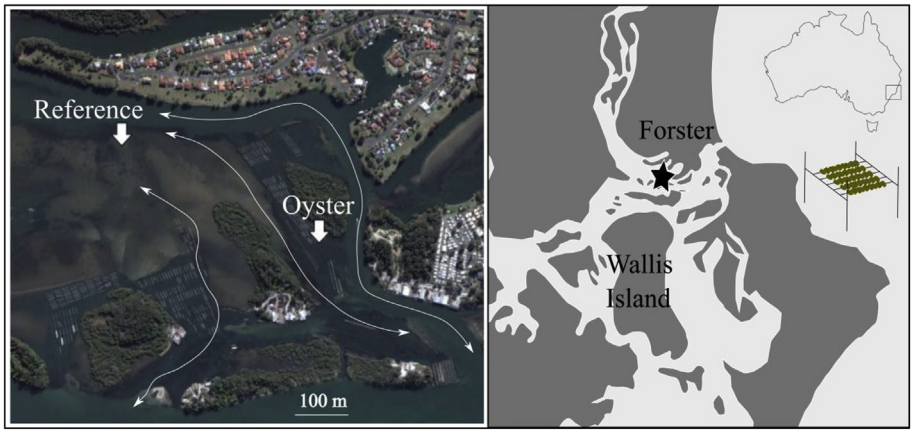

Fig. 1. Location of the Wallis Lake Estuary on the south eastern coast of Australia, near the city of Forster. The study location is shown as a star, and the satellite image (courtesy of Google Earth) shows the two sampling sites, the oyster site which sits amid an active oyster lease, and the reference site some 650 m away on an open sand/mud flat. A schematic of the stick cultivation used in this estuary is also provided. The tidal flow is indicated with white arrows. A more detailed map is provided as a KML file in the supplementary material.

submerged within incubation chambers containing the site water. Ambient temperature (20 °C) was maintained with a temperature control unit and O_2 concentrations were maintained at saturation with an aerator. Water within the cores was mixed using individual magnetic stirrers suspended ~10 cm above the sediment surface, which were driven by a central electric motor. The cores were left under ambient light conditions (i.e. in the dark) overnight to equilibrate, after which time about a third of the incubation water was replaced with new site water.

2.3. Sediment flux and process measurements

After the equilibration period the water within each core was mixed with the overlying water by inserting the aeration tube into each core for ~1 min and dark flux measurements were initiated by capping the long cores, measuring O_2 (Hach HQ40D), and taking samples for dissolved inorganic nitrogen (DIN which includes NH_4^+ and NO_3^-), and dissolved N_2O. The core lids contained a stoppered sampling port for insertion of the DO probe, and two valved openings for the extraction (and replacement from the incubation tank) of core water. Cores were gently lifted above the surface of the water before opening the DO sampling port. For core water sampling, the core was left submerged so that the core water could be replenished during liquid extraction via the second port. This procedure was repeated after 4 h, with this incubation period chosen based on oxygen consumption measured in the spare cores, so that the end time oxygen concentration remained above 80% of the initial saturation. The cores remained covered with a black plastic sheet during the dark incubation.

Following the flux incubations, the core lids were removed and the aeration tube was used to flush the cores. After ~2 h the isotope measurements were initiated by recapping the cores, collecting a water sample for determination of the ambient NO_3^- concentration (10 mL) and adding enough stock solution of ^{15}N-$NaNO_3^-$ (30 mmol L^{-1}, 98 At%, Aldrich) to achieve a final enrichment of ~30 μmol L^{-1} in the overlying water. After a 30 min pre-incubation period to allow diffusion of the added label to the sediment nitrate reduction zone (Nielsen, 1992), a water sample for NO_3^- was taken (10 mL) to enable calculation of the actual ^{15}N-NO_3^- addition by difference and the cores capped. After a 4 h dark incubation period, the cores were removed from the chamber, uncapped and a 25 mm internal diameter sub-core (55 cm) was used to collect a sub-sample of the sediment and water column. This sub-core was immediately emptied into a sample jar containing enough KCl to give a final concentration of 2M (calculated using the sub-core water volume, assuming a 30% porosity of the sediment). The jars were kept at 4 °C and intermittently shaken over a 24 h period after which a water samples were collected for the determination NH_4^+ concentration and ^{15}N-NH_4^+ isotopic analysis. The remaining core contents, after sub-core removal, were gently slurried and after a short settling period (~2 min) water samples were collected for future analysis of dissolved N_2O concentration and analysis of ^{15}N-N_2, and ^{15}N-N_2O.

2.4. Sediment pore water solute concentrations

The DET gels for porewater NH_4^+ and Fe^{2+} determination, and the DGT gels for sulphide determination (hereafter referred to as S^{2-}), were prepared as described in Robertson et al. (2008) and Pagès et al. (2012). The DET NH_4^+ and DGT S^{2-} binding gels were assembled in commercially available probes (DGT Research LTD) with the diffusive gel layer of the DGT probe serving as a DET for Fe^{2+} (Pagès et al., 2012). A single DET/DGT- S^{2-} probe and a DET probe for NH_4^+ were inserted into the central portion of 5 replicate 10 cm diameter sediment cores from each site and the cores were incubated in the dark for 12 h, after which the probes were removed and processed as described in Pagès et al. (2012).

Briefly, the gels were immediately cut from the probes using a ceramic scalpel, the diffusive layer from the DGT probe (Fe^{2+} DET) was immediately transferred onto a transparent sheet on a flat-bed scanner, overlain with a ferrozine reagent staining gel (Robertson et al., 2008) and stained and scanned using the optimised technique of Bennett et al. (2012). The colorimetric S^{2-} DGT gels do not require staining and were scanned after pre-treatment with hydroxylamine to remove potential interference from iron oxides (Robertson et al., 2008). The scanned images were converted to greyscale intensity values and converted to Fe^{2+} and S^{2-} concentration distributions using an appropriate greyscale versus concentration calibration curve (Robertson et al., 2008; Bennett et al., 2012). To generate depth profiles for each individual gel, the Fe^{2+} and S^{2-} concentrations for each pixel were averaged across the 2 cm width of each gel for each 5 mm depth interval.

The NH_4^+ DET gels were removed from the probes in the same manner and immediately sliced into 1 cm depth increments which were transferred into 10 mL vials containing 1 mL of ultra-pure (Milli-Q) water.

2.5. Oyster flux and process measurements

Oysters for the incubations were obtained from the lease holder on the day of the core collection and placed into an aerated holding tank. For the flux incubations, 4 cores containing 4 whole oysters, and 4 cores containing 4 shucked oysters (i.e. animal removed so only the shell epibionts remained), were placed into the incubation tanks containing fresh site water. The cores were capped and fluxes were measured in the dark as per the sediment cores. However due to the high O_2 demand in the cores containing the whole oysters, the incubation time was reduced to 1.5 h (incubation time for the shell epibionts was still 4 h).

For the process measurements, the shells from 4 shucked oysters were added to each of 8 cores. After capping, a stock solution of ^{15}N-$NaNO_3^-$ (30 mmol L^{-1}, 98 At%, Aldrich) was added to 4 of the cores, and a stock solution of ^{15}N-NH_4Cl (30 mmol L^{-1}, 98 At%, Aldrich)) was added to the remaining cores to achieve final enrichment of ~30 μmol L^{-1} (note that the actual addition was later calculated by difference from water samples collected pre and post label addition). Water samples (60 mL) for determination of dissolved N_2O and DIN were collected at the start of the incubation. After a 4 h incubation water samples were collected for dissolved DIN, N_2O and for ^{15}N-N_2, ^{15}N-N_2O and ^{15}N-NH_4^+ analyses.

2.6. Sample processing, analysis and calculations

The C and N contents of KCl treated, freeze dried and ground sediments were measured using a Thermo Finnigan Flash EA 1112 interfaced with a Thermo Conflo III and a Thermo Delta V Plus IRMS. Sediment density was determined by weighing a known volume of sediment and porosity as loss of wet weight of the same sample following drying to constant dry weight at 70 °C. Subsamples of the dried sediment were used to determine organic matter (OM) content as loss on ignition, calculated as the weight differences between pre and post combusted sediments (500 °C, 4 h) and total Fe content was determined by ICPMS, after extraction of known weights of sediment with 1 M Nitric Acid. Sediment fatty acid composition was determined following the protocols of Moynihan et al. (2016).

All samples for DIN analysis and for ^{15}N-NH_4^+ isotopic analysis were collected via a syringe and filtered (0.45 μm) into 10 ml vials. Samples for ^{15}N-N_2 analysis, and for N_2O concentration analysis, were collected in 12 mL Exetainers™ to which 20 μL of a saturated solution of $HgCl_2$ was added to halt biological activity. All samples were capped without headspace and collected in triplicate. For ^{15}N-N_2 isotopic analysis and N_2O concentration analysis, a 2 mL He headspace was added to the Exetainers™ at atmospheric pressure and allowed to equilibrate on a shaker table for at least 12 h prior to analysis. Samples for ^{15}N-N_2O isotopic analysis were collected by siphoning core water into 300 mL glass serum bottles to overflowing. One mL of a saturated solution of $HgCl_2$ was added to halt biological activity and the bottles were capped without a headspace with screw cap septa. Prior to analysis, a 25 mL He headspace was added at atmospheric pressure and allowed to equilibrate for at least 12 h.

Concentrations of NH_4^+ and NO_x^- were measured colourimetrically on a Lachat QuickChem 8000 four channel Flow Injection Analyzer. This included the water containing the NH_4^+ DET gel strips. For the NH_4^+ DET gel probes, the original porewater concentration was determined by dividing the total quantity of NH_4^+ measured in the vial by the liquid volume in the 1 cm slice of gel.

Headspace gas samples from the Exetainers™ were analysed for the ^{15}N isotopic composition of N_2 on a Thermo Trace GC Ultra with a 25 m × 0.32 mm PoraPLOT Q column interfaced to a Thermo Delta V Plus IRMS. The ^{15}N isotopic composition of N_2O was analysed by a Thermo Fisher GasBench II interfaced to a Thermo Delta V Plus IRMS following He sparging and cryogenic trapping. The ^{15}N-NH_4^+ samples were oxidised to NO_2^- by hypobromite and then reduced to N_2O by sodium azide and acetic acid according to Zhang et al. (2007). The ^{15}N isotope ratio of the N_2O was analysed by IRMS as above. Prior to analysis, the samples were checked for presence of NO_2^-, this was not detected in the samples and no sample pretreatment was required prior to $^{15}NH_4^+$ analysis. Dissolved N_2O concentration was determined by the headspace equilibrium technique using a Thermo Finnigan Trace GC fitted with a poraPLOT-Q column (32 m long x 0.32 mm internal diameter) and an Electron Capture Detector, as previously described in Welsh et al. (2015).

The measurement of denitrification and DNRA in estuarine and coastal sediments are largely based on the original isotope pairing technique (IPT) (Nielsen, 1992; Risgaard-Petersen and Rysgaard, 1995). The calculation techniques have evolved to account for the occurrence of anammox which violates the central assumptions of the IPT (Risgaard-Petersen et al., 2003; Trimmer et al., 2006). However in sediments where anammox and DNRA rates are high, there is also the potential for $^{15}NH_4^+$ supplied by DNRA to interfere with the IPT calculation (i.e. by allowing anammox to produce $^{30}N_2$ from the combination of $^{15}NH_4^+$ and $^{15}NO_3^-$). Recent updates to the IPT calculation procedure account for the occurrence of $^{30}N_2$ produced via anammox in slurry assays (Song et al., 2013) and also in intact sediment cores (Song et al., 2016). The calculation of N_2O production via denitrification is also possible with an additional calculation technique proposed by Salk et al. (2017). The calculation procedure in the sediment incubations follows that of (Salk et al., 2017) and is hereafter referred to as the IPT-DNRA. For the shell epibiont incubations, DNRA was not detected therefore the original IPT method of Rysgaard et al. (1993) was used for the NO_3^- and the NH_4^+ amendments. For the NH_4^+ amendments, the production of N_2 is actually coupled nitrification/denitrification, and the production of N_2O is assumed to come from the denitrification part of the process.

For the oyster incubations, we calculated areal rates by normalising the incubation fluxes per oyster and then multiplying by the stocking density of oysters (50 per m^2) at the time of the study.

Student's T tests were used to detect differences in rates or parameters between sites. For the DGT and DET data, we averaged the depth data to define a single concentration and used the Student's T test to compare the site means. The reported errors are standard deviations and the significance level for the Student's T test is 5%.

3. Results

3.1. Sediment parameters

Sediments at the oyster and reference sites had very similar density (1.84 ± 0.06 and 1.86 ± 0.04 g ml^{-1}, respectively) and porosity (0.47 ± 0.02 and 0.45 ± 0.03, respectively), suggesting they had similar granulometry and therefore that the reference site was a suitable comparison station. Additionally, loss on ignition data suggested that both sediments had similar organic matter contents of 1.6 and 1.3% for oyster impacted and reference sediments respectively. The molar C:N ratio of sediment material beneath the suspended oyster racks (8.3 ± 2.2) was lower but not significantly different to the reference site sediments (13.5 ± 3.0). The oyster impacted sediments however contained significantly more polyunsaturated fatty acids (PUFA) and had a lower SAFA (saturated fatty acid) to PUFA ratio than the reference site (Table 1). The presence of diatoms was significantly greater in the oyster sediments as indicated by higher contributions of their markers

Table 1
Relative contribution (% of total fatty acids) of polyunsaturated fatty acids (PUFA and 20:5ω3), and ratio of saturated fatty acids SAFA/PUFA and 16:1ω7/16:0 in oyster impacted and reference sediments. Errors are standard deviations.

	PUFA	SAFA/PUFA	20:5ω3	16:1ω7/16:0
Oyster	11.8 ± 1.7	3.9	4.3 ± 0.7	0.6
Reference	6.6 ± 3.3	9.5	2.3 ± 1.1	0.2

(20:5ω3, 16:1ω7/16:0) (Perez et al., 2013) compared to the reference site (Table 1). The oyster impacted sediments had significantly lower total iron content relative to the reference sediments (0.30 ± 0.05% and 0.48 ± 0.06% respectively).

The average porewater concentrations of Fe^{2+} and NH_4^+ were significantly higher in the oyster sediments relative to the reference sediments (32.1 ± 18.8 and 8.2 ± 6.2 μmol L^{-1} Fe^{2+} and 41.3 ± 15.7 and 75.8 ± 36.7 μmol L^{-1} for NH_4^+ respectively) (Fig. 2). For the oyster sediments Fe^{2+} was present from the surface of the sediment to the bottom of the gel probe sampler (100 mm depth). Porewater dissolved S^{2-} was absent throughout the depth profile in the reference sediments, but present in the oyster sediments as hotspots below 60 mm (Fig. 2).

3.2. Fluxes of DIN, O_2 and N_2O

Oyster impacted sediments had significantly higher rates of NO_3^- uptake and NH_4^+ production ($p < 0.05$) relative to the reference sediments. While O_2 uptake was higher for the oyster sediments than the reference sediments, the difference was not significant ($p > 0.05$) (Fig. 3). Nitrous oxide fluxes were also significantly different between the two sites ($p < 0.05$), with the reference sediment being a net source and the Oyster impacted sediment a net sink for N_2O (Fig. 3).

Nitrate uptake rates were similar for the whole animal and the shell epibionts, and based on a stocking density of 50 individuals per m^2, both rates were lower on an areal basis than the sediment uptake rates. In contrast, areal NH_4^+ production rates were significantly greater ($p < 0.05$) for the whole oysters relative to the sediments. Whole oysters had higher NH_4^+ production rates than the shell epibionts. For the oyster incubations (both whole and shell epibionts), O_2 consumption rates were similar to those of the sediment, although the O_2 consumption rate of whole oysters was significantly greater ($p < 0.05$) than that of the shells and their epibionts alone (Fig. 3).

More N_2O was produced when the animal was present relative to the empty shells, but the highly variable production by the whole oysters meant that this difference was not significant. The reference sediment production of N_2O was greater than the oyster shell fluxes, whereas the oyster farmed sediment was a net sink for N_2O.

3.3. Process rates

Rates of DNRA in the sediments were an order of magnitude higher than the rates of denitrification and anammox at both sampling sites, and the rate of DNRA in the oyster impacted sediments was double that of the reference sediments (Table 2). Rates of anammox were greater than rates of denitrification in both sediment types, and for the oyster shell epibionts, but the rates were variable and the differences were non-significant. Significantly lower rates of denitrification and anammox were measured in the oyster shell epibiont incubations relative to the sediment incubations when standardised on an areal basis. DNRA was not detected in the shell epibiont incubations.

The highest rates of N_2O production, based on the IPT calculations, were in the oyster impacted sediments (Table 2). The epibiont rates of N_2O production via denitrification and coupled nitrification/denitrification were similar, and the sum of both rates (Table 2) was similar to the directly measured net flux of N_2O (Fig. 3). Hence N_2O generated by the shell epibiont community was not consumed after it was produced.

For the sediments, the rate of N_2O production measured by the IPT was higher in the oyster impacted sediments relative to the reference sediments (Table 2). This is contrary to the directly measured net N_2O fluxes which were negative for the oyster

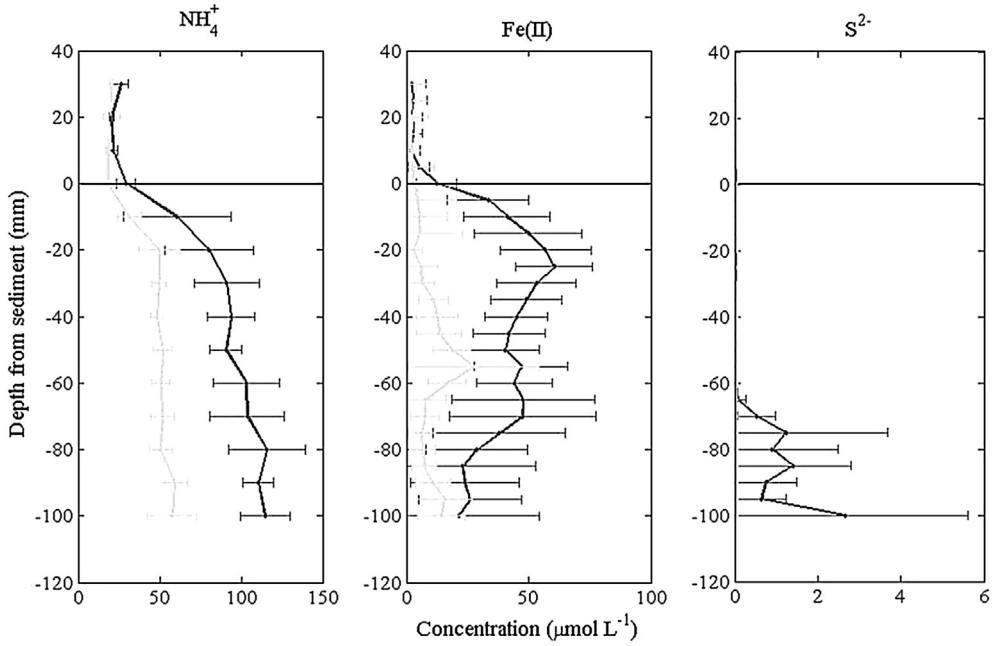

Fig. 2. Porewater concentration profiles (μmol l^{-1}) of NH_4^+, Fe^{2+} and S^{2-} in oyster impacted (dark grey) and reference (light grey) sediments. The solid horizontal line indicates the approximate location of the sediment surface. Data points are mean values from the individual profiles (n = 5) and error bars indicate the standard deviation of the mean.

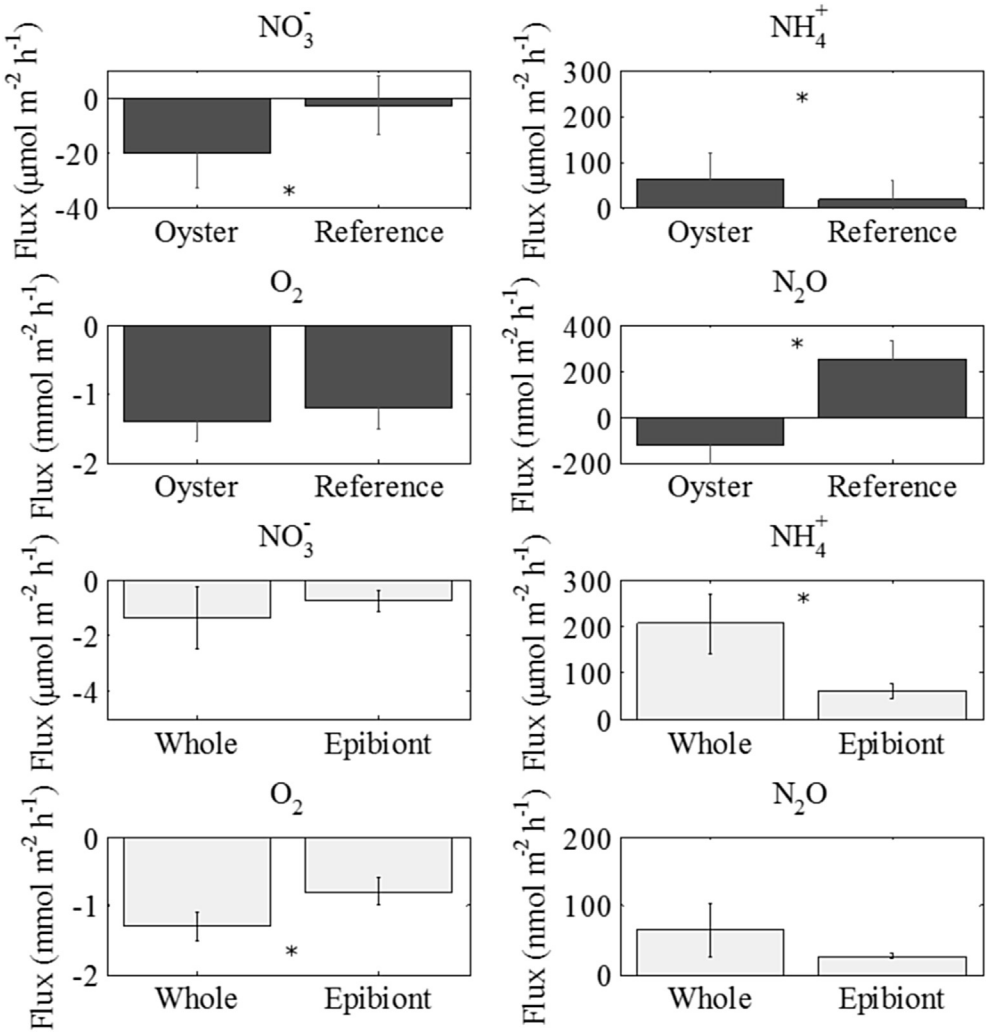

Fig. 3. Net fluxes of inorganic N (in µmol N m^{-2} h^{-1}), dissolved O_2 (in mmol O_2 m^{-2} h^{-1}), and dissolved N_2O (nmol N m^{-2} h^{-1}) for oyster impacted and reference sediments (upper panel), and for whole oysters and shucked oysters (shell + epibionts). The whole and shucked oyster rates are based on a stocking density of 50 individuals per m^2. Error bars indicate the standard deviation of the mean (n = 5) and an asterisk indicates a significant difference ($p < 0.05$) between the treatments for that parameter.

Table 2
Rates of nitrate reduction (µmol N m^{-2} h^{-1}) in Oyster impacted and reference sediments, and for oyster shell epibionts (nmol N m^{-2} h^{-1}) (oyster shell rate conversion to m^2 is based on a stocking density of 50 individuals m^{-2}). Gross rates of N_2O production based on the IPT are in units of nmol N m^{-2} h^{-1} for the sediments and for the shell epibionts. Shaded cells have units of nmol N m^{-2} h^{-1} while all other cells have units of µmol N m^{-2} h^{-1}. For the shell epibionts, both N_2O production via denitrification (DN) and coupled nitrification/denitrification (NTDNT) are shown. Errors indicate the standard deviation of the mean (n = 5).

	Denitrification	Anammox	DNRA	DNRA:NO3- reduction	N2O
Oyster impacted sediments	1.2 ± 0.4	1.6 ± 0.4	169 ± 45	0.98	70 ± 16
Reference sediments	1.3 ± 0.5	2.4 ± 0.9	83.8 ± 28.2	0.96	30 ± 6
Oyster shell epibionts	71 ± 22	121 ± 92	NA		19 ± 6 (DN) 13 ± 6 (NTDNT)

impacted sediment. This can only mean that there was an uptake of N_2O following production in the oyster impacted sediments. For the reference sediments, the net flux was higher than the rate associated with denitrification, indicating that other N_2O production mechanisms may have been present.

3.4. N budgets

An N mass balance budget was made for the oyster impacted and reference sites by splicing together the production rates calculated via the IPT-DNRA and the net fluxes for each sediment, and the process and flux rates measured in whole and "shucked" (epibionts) oysters after scaling for the cultivation density of 50 ind. m^{-2} (Fig. 4). Rates of nitrate reduction (i.e. denitrification, anammox and DNRA) are taken directly from the IPT-DNRA calculations. Nitrification must supply NO_3^- as NO_3^- uptake from the water column was insufficient to sustain the measured NO_3^- reduction rates by epibionts or in the sediments. Nitrification rates can therefore be calculated as the sum of the determined net NO_3^- fluxes and the summed rates of NO_3^- reduction from the IPT-DNRA. Nitrification coupled to DNRA is the deficit between the rate of DNRA and the NO_3^- available in the water column, in other words, the NO_3^- for

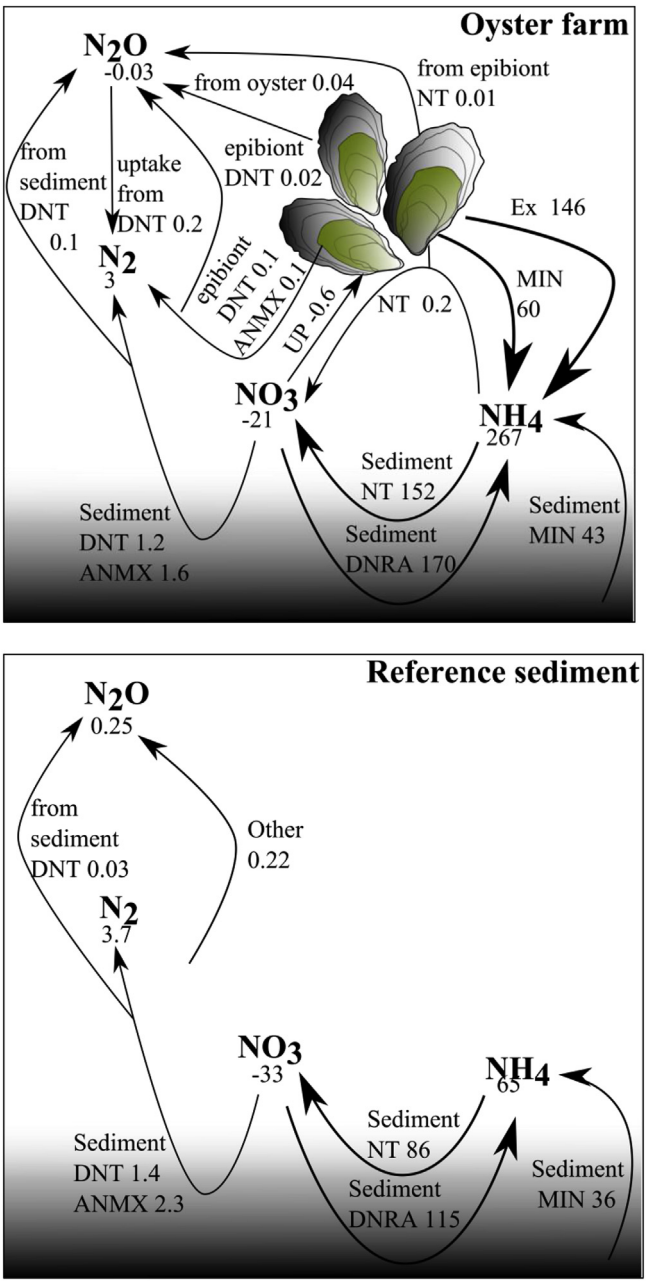

Fig. 4. Nitrogen mass balances for the oyster farm system and reference sites showing the rate of N transfer (μmol m^{-2} h^{-1}) through the different pathways. The major N pools are nitrate (NO_3), ammonium (NH_4), dinitrogen (N_2), and nitrous oxide (N_2O) and the values beneath these pools represents the net flux in μmol m^{-2} h^{-1} (positive and negative fluxes correspond to net release and uptake respectively). The pathways are dissimilatory nitrate reduction to ammonium (DNRA), denitrification (DNT), anammox (ANMX), nitrification (NT), mineralisation (MIN), uptake into the epibionts (UP) and oyster excretion (Ex).

DNRA that must have been supplied via nitrification.

The NH_4^+ pool is used for nitrification and is replenished via DNRA and ammonification. The rate of ammonification (ororganic N mineralisation) in the sediments was therefore calculated as the sum of the net flux of NH_4^+ and nitrification, minus the rate of DNRA.

For the oyster shell epibionts, rates of denitrification and anammox were calculated via the original IPT, as DNRA was not detected in the $^{15}NO_3^-$ incubations. Ammonification attributable to the shell epibionts was calculated as the net production of NH_4^+ plus the net NO_3^- flux, as NO_3^- production depends on nitrification of NH_4^+ and therefore nitrate reduction in excess of nitrate uptake must be based on NO_3^- produced by oxidation of NH_4^+ within the epibiont layer. The oyster NH_4^+ excretion rate was calculated as the difference in the NH_4^+ flux between the whole oyster incubations and the shucked (oyster shell plus epibionts) incubations.

The rate of nitrification could not be directly calculated because there was significant NO_3^- assimilation into the epibiont biomass (i.e. denitrification and anammox were less than 10% of NO_3^- uptake). Therefore an unknown amount of NO_3^- produced via nitrification could have been assimilated directly into the epibionts biomass. The calculated nitrification rate for the epibiont community therefore is the minimum rate. As such, the NO_3^- uptake rate into the epibionts is also a minimum estimate.

Overall, the gross NH_4^+ production rate was 267 μmol N m^{-2} h^{-1}. The largest source of NH_4^+ was sediment DNRA followed by oyster excretion. In the reference sediments the largest supply of NH_4^+ was also DNRA, however the gross NH_4^+ production rate was only a quarter of that (65 μmol N m^{-2} h^{-1}) at the oyster site.

4. Discussion

4.1. Nitrate reduction in oyster impacted and reference sediments

Nitrate reduction includes denitrification, anammox and DNRA. The first two processes act to remove inorganic N from the environment as N_2, whereas DNRA recycles N back into ecosystem (Tiedje et al., 1982; Christensen et al., 2000; An and Gardner, 2002). In this study we found that DNRA rates were significantly higher in the oyster sediments than the reference sediments. While the measured DNRA rates for both sediment types were relatively high compared to reported rates for non-aquaculture systems (see Table in Song et al. (2013)), they were within the range typically measured in aquaculture impacted sediments (Christensen et al., 2000, 2003; Nizzoli et al., 2006). However of more importance is the fact that the measured contribution of DNRA to total NO_3^- reduction in this study is the highest ever reported in the literature (98% contribution of DNRA to total NO_3^- reduction). The implication is that, at least for the sediments, denitrification is an ineffective means of N removal. Below we address the large contribution of DNRA to NO_3^- reduction in the context of our own study and with reference to other sediment systems.

In this study we observed higher rates of DNRA in the oyster impacted sediments relative to the reference sediments. Firstly, it is important to note that the reference sediments are not a true control as they are also influenced, albeit to a lesser degree, by the nearby oyster cultivation. Tidal regimes in the area would potentially carry suspended OM such as oyster faeces or pseudofaeces to the reference site where it may settle and contribute to benthic metabolism and N cycling. As such it is not surprising that the LOI was similar between the two sediments, and that the reference sediments behaved similarly to the oyster sediments in terms of the contribution of DNRA to nitrate reduction. However the presence of the oysters did significantly increase the rate of DNRA relative to the reference site.

DNRA is thought to be favoured when the ratio of OC to NO_3^- availability is high (i.e. electron acceptor limitation) (Hardison et al., 2015). Indeed high electron donor availability (e.g. high C:N conditions) is the main driver of high DNRA rates in sediments (Kraft et al., 2014; Van Den Berg et al., 2015; Yoon et al., 2015). This is because more energy is generated per NO_3^- consumed via nitrate ammonification relative to denitrification (Tiedje et al., 1982). Hence DNRA bacteria reach their maximum growth rate at lower NO_3^- concentrations than denitrifying organisms (i.e. higher NO_3^- affinity). In addition, more electrons are transferred via DNRA than

denitrification (8 versus 5), hence there is greater capacity to oxidise OM via DNRA when NO_3^- is limiting (Tiedje et al., 1982).

In this study the OM content of the two sediment types were similar and both were NO_3^- limited. However the OM within the oyster sediments did appear to be more accessible to the microbial community (i.e. its lability was higher). This was confirmed by the fatty acid data which showed that the oyster sediments contained more "fresh" OM most likely derived from diatoms. Higher OM availability is often associated with high sediment O_2 demand which can favour DNRA (Gardner and McCarthy, 2009). So it is possible that the greater accessibility to OM could have favoured DNRA in sediments beneath the oyster cultures relative to the reference site. If this were the case then we would expect to see significantly higher O_2 demand in the oyster impacted sediments. Based on the plot of O_2 demand vs DNRA presented by Hardison et al. (2015), DNRA should not have made such a significant contribution to NO_3^- reduction in either the oyster or reference sediment. Furthermore the two sediment types should have been similar in terms of their DNRA activity. Hence it is unlikely that differences in OM lability alone between the sites were large enough to drive the significant differences observed in DNRA activities between the oyster and reference sediments.

While we did not observe significant differences in O_2 consumption between the two sediments, the more labile nature of OM in the oyster impacted sediments seems to have facilitated subtle shifts in redox potential that were detected in the DGT/DET probes. The more reduced nature of the oyster impacted sediments was observable as an increase in the availability of soluble Fe^{2+} and the presence of low concentrations of S^{2-} in the deeper strata. Total Fe analysis of the sediments showed that there was less solid phase Fe in the oyster sediments relative to the reference site, most likely due to the diffusion of soluble Fe^{2+} to bottom waters. At the reference site Fe was most likely locked up as insoluble Fe^{3+} hydroxides in the more oxidised sediments.

From a thermodynamic perspective, the oxidation of Fe^{2+} by NO_3^-, to produce Fe^{3+} and NH_4^+, is energetically comparable to denitrification (Straub et al., 1996). A number of previous studies have now shown that DNRA is stimulated by the addition of Fe^{2+} to sediment slurries (Roberts et al., 2014; Robertson et al., 2016; Robertson and Thamdrup, 2017). However the process is far more difficult to prove using intact sediment cores where a number of electron donors (e.g. Fe^{2+}, OM and sulphide) are available for oxidation (Behrendt et al., 2013). The Wallis Lake sediments incubated in this study are relatively unique compared to other sediments in which DNRA has been measured, in that they contain very little OM or sulphide. This leaves Fe^{2+} as the most likely driver of NO_3^- reduction. As such, the high rates of DNRA measured in this study, using IPT protocols capable of separating DNRA from N_2 production in intact cores, appear to be dependent on porewater Fe^{2+}. We further propose that the difference in DNRA rates between the two sites was driven by a subtle increase in OM lability which promoted the availability of reduced Fe^{2+} in the oyster impacted sediments. The implication of this finding is that the presence of oysters facilitates N retention within sediments by reducing sediment redox potential and encouraging DNRA via Fe^{2+} oxidation.

With respect to other estuarine systems, we found that rates of N_2 production via denitrification and/or anammox were dwarfed by DNRA in both sediments (DNRA was >95% of NO_3^- reduction in both sediments). DNRA rates in the oyster impacted and reference sediments were similar to other aquaculture systems, and hence the large contribution of DNRA to NO_3^- reduction was not the result of an increase in the absolute rate of DNRA, but rather from low rates of N_2 production.

Measurements of N_2 production within the Wallace Lake system made using N_2/Ar ratios (Eyre et al., 2013, 2016) range from 6 to 116 μmol N m^{-2} h^{-1} with the lower values corresponding to sediments characteristic of our sampling sites (i.e. mid estuary channels dominated by fluvial muds and sands). Given that our measurements of N_2 production are similar to previous measurements (Eyre et al., 2013, 2016) it seems as though it was the very low rates of denitrification and anammox in the Wallace Lake systems that lead to the high DNRA:N_2 ratio. Unlike other aquaculture related studies that often report a decrease in denitrification as a result of increased O_2 demand and sulphide generation (Christensen et al., 2000, 2003; Nizzoli et al., 2006), the Wallace Lake sediments were not highly organic or sulphidic in nature. Rather we suspect that the prevalence of DNRA over N_2 production is related to NO_3^- limitation and the subtle shift in redox conditions within the sediments. More broadly, the current study provides yet another example of the dominance of DNRA in Australian estuarine systems.

The prevalence of DNRA over denitrification and anammox in coastal systems was highlighted by Giblin et al. (2013) who found that DNRA accounted for over half of the overall nitrate reduction in at least a third of coastal sediment studies. For Australian systems, all studies that have measured DNRA and N_2 production have found that DNRA is a major, if not dominant, contributor to NO_3^- reduction (Dunn et al., 2012, 2013; Roberts et al., 2014; Robertson et al., 2016). Numerous other studies show that DNRA dominates NO_3^- reduction in tropical systems and that this is not necessarily related to increased OM availability (Dong et al., 2011; Dunn et al., 2012, 2013; Erler et al., 2013; Molnar et al., 2013). Other studies of DNRA in estuarine systems suggest that sulphide availability may limit denitrification and promote DNRA (An and Gardner, 2002; Bernard et al., 2015). This has also been suggested for a number of aquaculture impacted sediments (Christensen et al., 2003; Nizzoli et al., 2006). However Dong et al. (2011) report that sulphide did not facilitate DNRA and in fact promoted denitrifying activity in one of three tropical estuarine systems. Our data also indicates that sulphide did not inhibit denitrification, nor did it promote DNRA, as it was absent in the porewater at the reference site and only occurred at low concentration at depths greater than 60 mm at the oyster impacted site.

4.2. Nitrogen and N_2O in the oyster shell epibiont community

Most studies looking at the impacts of bivalve aquaculture on N cycling have focussed on sediment biogeochemistry with less emphasis placed on the role(s) of shell biofilms and epibionts (Nizzoli et al., 2006, 2011; Holmer et al., 2015). Building on previous findings, we have found that the oysters and their associated epibiont communities were a more important source of NH_4^+ to the water column than the underlying sediment. Areal denitrification and anammox rates associated with the oysters however were much lower than those of the sediments and did not significantly contribute to overall N-loss. DNRA was not detected in any of the incubations with whole or shucked oysters.

The only other study that has measured N_2O production in a bivalve aquaculture environment found that the cultured organisms themselves were a large source of N_2O with rates of up to 3 μmol m^{-2} h^{-1} (Welsh et al., 2015). From a per animal perspective, the N_2O production by the Sydney Rock Oyster in this study (782 pmol ind^{-1} h^{-1}) is within the range of rates published for other bivalves (Stief et al., 2009; Heisterkamp et al., 2010, 2013; Svenningsen et al., 2012; Heisterkamp et al., 2013). However upscaling based on an average stocking density of 50 individuals m^{-2}, the combined areal rate of N_2O production by the animals and epibionts was 0.07 μmol m^{-2} h^{-1}, well below that found by Welsh et al. (2015).

In contrast to the study of (Welsh et al., 2015), who found that

N_2O as a proportion of N_2 production was <1% for clams, in the current study we calculated a value of 10%. This figure excludes N_2 production in the oysters digestive systems (which was not measured). This relatively high specific yield of N_2O suggests an inherent inefficiency of nitrification and denitrification within the shell epibiont community. Other research has shown a direct correlation between high N_2O:(N_2O + N_2) ratios and the presence of sulphides (Dalsgaard et al., 2013), but sulphide accumulation is unlikely to be a factor in the epibiont communities. More likely is that O_2 variability may have prevented denitrification from progressing to completion.

In this study, the measurement of gross (via the isotope pairing technique) and net (via direct concentration measurement) fluxes provides an insight into the dynamics of N_2O. For instance, for the oyster impacted sediments the net flux of N_2O was negative, while the isotopic measurements yielded a positive production. The isotopic estimates are based on the change in ^{15}N composition of the available N_2O and represent gross production via denitrification. The discrepancy between the net and gross production rates indicates that while N_2O is most certainly being produced in the sediments, consumption is also occurring at a higher rate, resulting in a net uptake of N_2O. In contrast, to the sediments, the gross and net production rates of N_2O were similar for the oyster shell epibiont incubations. This suggests that N_2O uptake after production was negligible. Overall, the oyster cultivation system was a small net sink for N_2O (Fig. 3). This was largely unexpected given that OM rich sediments and bivalves themselves are sources of N_2O (Welsh et al., 2015). The consumption of N_2O however is not an uncommon feature within aquatic sediments that are NO_3^- limited (Erler et al., 2015a; Maher et al., 2016). In the studied sediments, the small fluxes of N_2O are likely related to the dominance of DNRA over denitrification and the intense competition for NO_3^-.

For the reference sediments, the measured N_2O flux was higher than the production calculated via isotope pairing. The N_2O produced via the isotope pairing method is assumed to derive from denitrification and nitrifier denitrification only (Trimmer et al., 2006), therefore it is possible that the additional N_2O produced in the reference sediments was a by-product of NH_4^+ oxidation by nitrifiers.

4.3. Implications for estuarine N budgets

The major impacts of the oyster cultivation system on inorganic N processing are illustrated in Fig. 4. We must stress that the approach taken in this study relies on the separate incubation of oysters and sediments rather than a whole system approach. As such our study gives a snapshot of the impact of sediments and oysters on the dynamics of the inorganic and gaseous N pools. Some of the key observations that can be gleaned from this budget include; the fact that oysters and their associated epibiont community are the major contributor to inorganic N (in the form of NH_4^+) production; the system is NO_3^- limited and reliant on nitrification to supply the NO_3^- required for nitrate reduction (i.e. prevalence of coupled nitrification - DNRA/denitrification); DNRA dominates nitrate reduction with or without the presence of the oysters.

With respect to DNRA, it should be noted that in the reference sediments DNRA was the dominant pathway for NO_3^- reduction. We suspect that the composition of the OM in this region of the estuary is such that it promotes DNRA over denitrification and anammox. It is unclear whether other habitats in the estuary have similar contributions from DNRA to NO_3^- reduction. Given that other studies measured quite high rates of denitrification in other regions of the Wallace Lake estuary (Eyre et al., 2013, 2016), it is likely that the DNRA:N_2 ratio in other regions of the estuary may be lower than what we observed.

From an estuarine N budget perspective, it is clear from our study that oysters facilitate the retention of N within the system. So while their filter feeding activity can remove suspended N from the water column, the rates of NH_4^+ regeneration and the prevalence of DNRA tend to trap N and recycle NH_4^+. Unlike most other aquaculture related N cycle studies, the presence of oysters did not facilitate high sediment oxygen demand or sulphidic conditions. However the presence of the oysters caused subtle shifts in sediment redox characteristics which favoured DNRA. This raises an important point about the control mechanisms on DNRA.

In the last decade there has been a concerted effort to try and understand the controlling mechanisms between N_2 production processes and DNRA (Koop-Jakobsen and Giblin, 2010; Giblin et al., 2013; Hardison et al., 2015; Plummer et al., 2015). DNRA occurs as a fermentative heterotrophic process, where NO_3^- acts as an electron acceptor for the re-oxidation of NADH, as a true heterotrophic respiration or as an autotrophic process in which free sulphides (H_2S, HS^- and S_2^-) or Fe^{2+} are oxidised with NO_3^- serving as electron acceptor. Therefore the availability of C can be the principal determinant for the expression of DNRA in sediments as it provides an energy source and also leads to O_2 depletion, and H_2S and Fe^{2+} production via sulfate and Fe reduction, respectively. However in this study we have shown that sediments do not have to be particularly reduced or rich in organic material for DNRA to dominate. It appears as though the presence of reduced Fe has had a major influence on the rate of DNRA beneath the oyster cultures, and in the non-oyster impacted sediments the nature of the sediments (i.e. high C:NO_3^-) appears favourable for DNRA. What is clear though is that inorganic N cycling within the oyster culture system favours N retention rather than N loss.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ecss.2017.05.007.

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