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Measuring ecosystem function in Darwin Harbour sediment using microbial nitrogen cycling genes

Prepared for the Integrated Marine Monitoring & Research Program, Department of Environment & Natural Resources, NT Government

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Executive Summary

Darwin Harbour is considered to be slightly to moderately disturbed and nitrogen-limited with the extensive area of fringing mangroves found to be the main primary producer. However, it is subject to increasing human pressure. It is macrotidal and pollutants assumed to disperse rapidly, but some areas are poorly flushed and pollutants can be trapped inshore for long periods. Monitoring nutrient and contaminant loads in the sediment over time and recognizing potential changes to geochemical processes are vital to guarantee the ongoing ecosystem health of the harbour.

The N-cycle is a key process in nature converting nitrogen gas to nutrients and back to gas. Nitrogen is essential for supporting primary productivity but too much nitrogen or poor removal can result in eutrophication compromising water quality and giving rise to algal blooms. The ability to measure denitrification as a key process of nitrogen removal in Darwin Harbour provides valuable insight into nitrogen processing and assimilatory capacity. However, this is traditionally done through laborious core incubation experiments which are not suitable for routine monitoring.

In 2017, as part of the SP2 sediment subprogram theme of the Integrated Marine Monitoring and Research Program (IMMRP) in partnership with the NT Government and the INPEX-operated Ichthys LNG Project, we reported on the assessment of ecosystem function using microbial nitrogen genes. The aim of this earlier work was to advance our understanding of nitrogen processes and cycling in intertidal sediments in Darwin Harbour and to assess the usefulness of quantitative PCR (qPCR) microbial N gene assays as a routine monitoring tool. In that 2017 report the assays differentiated between sediments of different trophic status reflecting long-term average nutrient loads at a site, however the assays and nutrient flux data were not correlated. The 2017 study was based on bulk sediment reflecting many years of sedimentation. Consequently, the aim of this 2019-20 project was to extend the biological validation of these qPCR assays using newly sampled sediment from mainly East and West Arm in Darwin Harbour. This time, surface as well as bulk sediment were collected at each site and compared to flux data of core incubations.

In this second study the surface sediment contained significantly more denitrification gene *nosZ* compared to the bulk sediment and there were significant correlations between its relative abundance in the surface sediment and nutrient fluxes. These included a positive association with N_2 emission and a negative association with NO_x flux; both indicating active denitrification processes in the sediment-water interface. There were also significantly more *nosZ* genes at the mouth of the creeks compared to the upper estuaries. This likely reflects carbon loading with upper estuaries being more densely vegetated by mangroves with higher carbon to nitrogen ratios also likely favouring DNRA processes over denitrification. In contrast, the mouth of estuaries has a higher availability of oxic and anoxic sediment pockets in close proximity for coupled nitrification-denitrification processes. We also found a unimodal relationship between *nosZ* genes and Si as well as PO_4 fluxes with highest relative *nosZ* abundance at intermediate fluxes.

The strongest trend however was a negative linear association between the ammonia oxidizing gene *AOA1b* and silicate flux, an indicator of diatom activity. Diatoms play an important role in primary production in sediment and their activity reflects input of labile diatomaceous organic matter into the sediments producing anoxic conditions detrimental for nitrification.

In summary, the N-cycling indicator assays show promise as a fast monitoring tool to differentiate between sediment with functional nitrification-denitrification processes and sediment with low nitrification or denitrification rates such as due to extremes of carbon loading.

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Acronyms

AHU	Aquatic Health Unit, Water Resources Division, NT Government
AOA	Ammonia oxidizing Archaea
Anammox	Anaerobic ammonium oxidation
amoA gene	Gene encoding ammonia mono-oxygenase (NH_3 to NO_2^-) in nitrification
AOB	Ammonia oxidizing Bacteria
BMA	Benthic microalgae
bp	DNA nucleotide base pair
Ct	Cycle threshold in qPCRs
dbRDA	distance-based redundancy analysis
DE	Denitrification efficiency (nitrate to nitrogen gas)
DIC	Dissolved inorganic carbon (carbonates and CO_2)
DNA	Deoxyribo-nucleic acid
DNRA	Dissimilatory Nitrate Reduction to Ammonia
FDR	False Discovery Rate method to adjust P values accounting for multiple testing
N_2	Nitrogen gas
NH_4	Ammonium
NO_x	Nitrite and Nitrate
nosZ gene	Gene encoding nitrous oxide reductase (N_2O to N^2) in denitrification
OTU	Operational taxonomic unit reflected by specific 16s rRNA gene sequence
PCO	Principal Coordinate Ordination
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
rRNA	ribosomal ribonucleic acid
SYBR green	Fluorescent dye which stains double-stranded DNA

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Study Aim and Research Questions

The aim of this project was an extended biological validation of N-cycling indicator qPCR assays using newly sampled sediment from East and West Arm in Darwin Harbour. This was to assess the suitability of these assays as fast monitoring tools for sediment ecosystem function indicating the trophic status of the sediment and as potential replacement for laborious core incubation experiments.

The following research questions were addressed:

1. Do the N-cycling indicator assays reflect the trophic status of the sediment?
    a. Do they show a spatial pattern across sediment in Darwin Harbour reflecting the level of nutrient exposure at these sites?
    b. Is there a difference in N-cycling indicators between surface and deeper sediment cores reflecting more recent and long-term average nutrient exposure at a site?
2. Could N-cycling indicator assays be used instead of laborious core incubation experiments?
    a. Is there an association between N-cycling indicators and nutrient flux data?

1 Background

1.1 Darwin Harbour

Darwin Harbour is an estuarine ecosystem in the wet-dry tropics of northern Australia. It is subject to increasing human pressure (Aquatic Health Unit, 2016). Since it is macrotidal, pollutants are commonly assumed to disperse rapidly. However, some areas are poorly flushed and pollutants can be trapped inshore for long periods (Williams et al., 2006). Treated sewage effluent discharged from four wastewater treatment outfalls, are the dominant anthropogenic point-source of nutrients to the harbour. A study in 2009 showed that effluent contributed 71% of total phosphorus and 31% of total nitrogen of the annual catchment load entering the harbour (Skinner et al., 2009). In comparison, diffuse urban runoff based on 2004 land-use categorisation, contributed 16% of total phosphorus and 21% of total nitrogen (Skinner et al., 2009). A hydrodynamic model for Darwin Harbour raised concerns about the increasing significance of nutrient and pollutant inputs from diffuse urban sources in particular during the wet season (Drewry et al., 2009). Darwin Harbour is however considered to be relatively undisturbed in comparison to many Asian and Australian harbours notwithstanding development within its catchment and coastal environs (Burford et al., 2008;Butler et al., 2013;Aquatic Health Unit, 2015). The harbour system remains nitrogen-limited with the extensive area of fringing mangroves found to be the main primary producer.

1.2 The Nitrogen Cycle

A healthy harbour has many components, one of which is nitrogen (N) cycling. The N-cycle is a key process in nature converting nitrogen gas to nutrients in soil and water and back to gas released into the atmosphere (Figure 1). Nitrogen is essential in supporting primary productivity but too much nitrogen or poor removal can result in eutrophication compromising water quality. Maintaining the oligotrophic condition of the Harbour is important to ecosystem function whereas eutrophic conditions may give rise to poor water quality and algal blooms.

Denitrification or the reduction of nitrates to nitrogen gas is the main process in removing nitrogen. Several microbial genes are involved in the process including the *nosZ* gene which encodes the nitrous oxide reductase enzyme (Figure 1). Denitrification efficiency (DE) is reduced in localised areas impacted by elevated nutrient loads (Smith et al., 2012). Denitrification occurs under sub-oxic conditions and is dependent on the availability of reactive organic carbon. It is tightly coupled to nitrification which provides the substrate for denitrification i.e. nitrates.

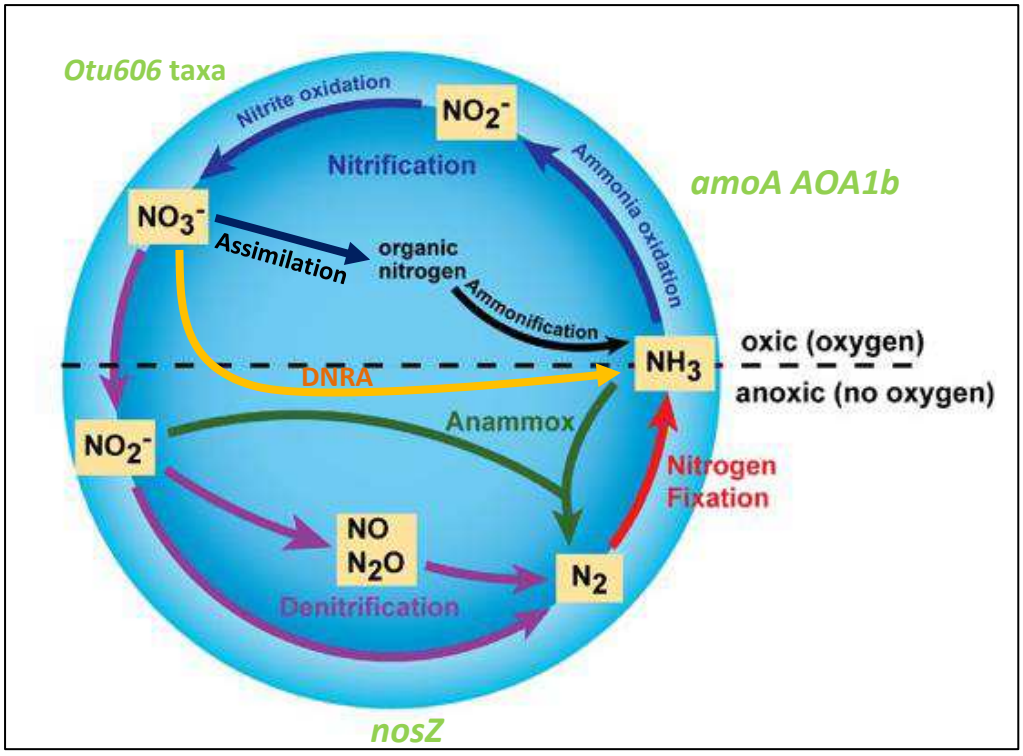


Figure 1: The nitrogen cycle
Three N-cycling indicator targets are shown in light green – graph edited from (Bernhard A, 2010)

Nitrification is the oxidation of ammonia to nitrates. It is performed under oxic conditions and a two-step process in which:

1. Ammonium (NH_4^+) or ammonia (NH_3) is oxidised to nitrite (NO_2^-); and
2. Nitrite is oxidised to nitrate (NO_3^-).

Step 1 is driven by AOA (ammonia oxidizing archaea) or AOB (ammonia oxidizing bacteria) encoding the archaeal or bacterial *amoA* gene (Figure 1). Ammonia oxidation is often the rate-limiting step in nitrogen-removal in wastewater treatment plants as it is easily inhibited such as through low oxygen content, too high substrate i.e. ammonia concentration, sulphides, organic compounds or heavy metals (Wittebolle et al., 2008). Step 2, the nitrite oxidation is driven by the enzyme nitrite oxidoreductase which is encoded by the *nxrB* gene in microbes.

Other processes such as dissimilatory nitrate reduction to ammonia (DNRA), anammox (anaerobic ammonia oxidation) or nitrate assimilation by benthic algae are known competitors for denitrification (Figure 1).

1.3 Sediment and Nutrient Fluxes

Sediments are critical biogeochemical components of aquatic ecosystems and often important sinks of nutrients and contaminants (SPA1 Project Sediment Fluxes)(Eyre et al., 2011). The efficiency of removal of excessive nitrogen is tightly linked to carbon loading which impacts on the sediment structure (Eyre and Ferguson, 2009). Carbon loadings that are too high can cause sub-oxic or anoxic

zones to develop in sediment, which leads to reduced nitrification and low nitrate levels. In comparison, low carbon loadings can lead to the dominance of sediment by oxic zones and thus a lack of denitrification which is dependent on anoxic conditions. The presence of sulphides also inhibits nitrification and denitrification, while in the presence of high carbon loading i.e. a high C:N ratio, other processes might compete for nitrates such as DNRA or assimilation by benthic algae (Dong et al., 2011).

The flux of nutrients into or out of benthic sediment is often measured through core incubation experiments, especially if the use of otherwise preferred *in situ* benthic chambers or landers is impeded by environmental conditions such as strong macrotidal currents which are also encountered in Darwin Harbour (Stockdale et al., 2009).

Some examples of nutrient fluxes include:

- **Nitrogen gas**: Positive N_2 fluxes are considered to be indicative of net denitrification, while negative N_2 fluxes indicate net nitrogen fixation rates. Benthic N_2 fluxes are coupled to nitrification during organic matter degradation near the sediment-water interface.
- **Ammonia**: Benthic ammonia efflux (i.e. ammonia emission into the water column) is often attributed to DNRA processes linked to anoxic diagenesis, increased organic matter deposition and sulphate reduction to sulphides (Kristensen, 2000;Giblin et al., 2013). Ammonia efflux can also indicate ammonification or nitrogen fixation. NH_3 fluxes near zero suggest low available ammonia in the water column and balance in ammonification and assimilation, and/or ammonification due to oxic breakdown of organic matter coupled with complete nitrification.
- **Dissolved inorganic carbon:** DIC mainly consists of dissolved carbon dioxide (CO_2) and carbonates. DIC fluxes are indicative of carbon decomposition rates, which are a proxy for carbon loading to the seafloor (Henrichs, 1992;Eyre and Ferguson, 2009). DIC uptake indicates higher primary production in the sediment. DIC emissions indicate net respiration rates in the sediment (such as at night in the absence of photosynthesis). Influx of particulate organic matter such as through land runoff can also lead to more heterotrophic activity and respiration, and DIC emission.
- **Silicate:** Benthic efflux of Si is primarily related to diatoms, which have high sinking rates due to their siliceous frustules (Haese et al., 2007). A positive Si flux indicates chemical dissolution of diatom frustules into the water column during the breakdown of organic matter. This can be enhanced by bio-irrigation i.e. the flushing by benthic organisms of their burrows with overlying water (Berelson et al., 2013). Si flux may also be a measure of benthic microalgae (BMA) (Srithongouthai et al., 2003;Serpetti et al., 2016). A negative flux or uptake of Si into the sediment indicates shell formation i.e. a growing microalgal population or phytoplankton sedimentation. Both, diatoms and BMA play an important role in primary production in sediment and compete for nitrogen-containing nutrients.

1.4 Our prior work on N-cycling indicator assays

The ability to measure denitrification as a key process of nitrogen removal in Darwin Harbour provides valuable insight into nitrogen processing and assimilatory capacity. However, this is traditionally done through laborious core incubation experiments which are not suitable for routine monitoring. Ecosystem based approaches to marine monitoring are driving a need for efficient, low-cost bio-indicators of marine ecosystem health. Microbes drive nutrient cycles but are not amenable

to visual inspection and thus are largely excluded from detailed inventories. As part of the SP2 sediment subprogram theme of the Integrated Marine Monitoring and Research Program (IMMRP) in partnership with the NT Government and the INPEX-operated Ichthys LNG Project, we published a report in 2017 on the assessment of ecosystem function using microbial nitrogen genes. We developed and validated quantitative PCR (qPCR) assays that target microbial N genes. The purpose was to advance our understanding of nitrogen processing and cycling in intertidal sediments in Darwin Harbour and to assess their usefulness as a routine monitoring tool.

Three qPCR assays were subjected to technical and biological validation. Biological validation involved comparing the abundance of target N-cycling genes and taxa in oligo-, meso- and (hyper) eutrophic sediments from Shoal Bay and East Arm (Darwin Harbour) in the dry and wet season in 2015 -2016.

These 3 assays were:

- a qPCR assay targeting the ***AOA1b amoA*** gene for **ammonia oxidation to nitrite**. We mainly found archaeal ammonia oxidizers (AOA) in the Darwin Harbour sediment, as opposed to bacterial ammonia oxidizers, and previous studies also reported the predominance of archaeal ammonia oxidizers over bacterial ammonia oxidizers in oligotrophic sediment (Martens-Habbena et al., 2009). The *AOA1b* amoA gene occurs in the genus *Nitrosopumilus*, a common marine Crenarchaeota which is well adapted to highly nutrient limiting conditions (Martens-Habbena et al., 2009). We found that this gene was more abundant in oligotrophic sediment. Potentially, it is also sensitive to hypersaline conditions and it showed a higher abundance at intermediate salinity levels (Bernhard et al., 2010).
- a qPCR assay targeting the *Nitrospirales* taxa ***otu606*** which are known to be involved in **nitrite oxidation to nitrate**. These nitrifying bacteria have been found to decrease under anoxic conditions in marine sediment (Devereux et al., 2015) and we found a strong association of this group with oligotrophic sediment. High nitrite flux can 'feed' high denitrification - conditions that tend to occur at oligotrophic sites and high *otu606* signals might be indicative of good ecosystem function.
- a qPCR assay targeting the **denitrification *nosZ* gene** responsible for the reduction of nitrates to nitrogen gas. Denitrification efficiency (DE%) as measured by conventional nutrient flux is known to be depressed at impacted sites. We found that these sites also had fewer *nosZ* genes. However, there was no correlation between the *nosZ* gene abundance and DE%. This is in line with previous work by others which showed that the abundance of the *nir* gene which is also involved in denitrification was not correlated with absolute rates of denitrification. Instead it was correlated with the long-term optimal denitrification rate at given environmental conditions.

Core incubation experiments showed a strong seasonal trend with dinitrogen and DIC fluxes considerably higher in the wet season compared to the dry season at most sites. This was especially the case at Shoal Bay sites, which receive large freshwater inputs during that time. The much higher wet season N_2 fluxes likely signify a change from predominantly oxic to sub-oxic organic matter degradation processes in response to the higher seafloor carbon loadings (measured as DIC) at this time. Indeed, harbour-wide primary production was estimated to be more than two times higher in the wet season compared to the dry season in a study by (Burford et al., 2008).

The N_2 and NO_x fluxes were highly variable including between replicates from the same site. The variability was most evident at hypereutrophic sites and was likely caused by greater heterogeneity in the array of oxic, sub-oxic and anoxic micro-niches in the sediment caused by higher (and perhaps patchier) carbon loadings. The high variability may also reflect, in part, the dependence of biogeochemical processes on conditions such as water temperature, nutrients, DO and salinity which rapidly change with tides and seasons.

The N-cycling indicators and flux data were not correlated. The N-gene tests were based on bulk-sediment samples which potentially reflected many years of sedimentation. Accordingly, the assays provided a clearer picture of impact and were particularly able to differentiate between long-term eutrophic and oligotrophic sites. This goes in line with previous research showing that the sediment microbiota depends on past and current conditions and only changes slowly.

N-cycle bacteria were more abundant in sediment than water, which confirms that sediment plays a key role in nitrogen cycling. For routine assessment, sediment should be monitored for N-cycling, not water.

The combination of qPCR assays further improved the explanatory power of the assays for location. A combination of tests has also been shown in medical diagnostics to improve sensitivity and specificity (Johnson et al., 2015). The composite of the qPCR test results or the microbial N-cycle fingerprint significantly differed between all Shoal Bay sites. This also reflects our previous finding that the sediment microbiota differed between Shoal Bay creeks but was more uniform in the oligotrophic East Arm (Kaestli et al., 2017).

1.5 What will this present project add?

The 2017 SP2 study was extended in 2019-20 using sediment samples from mainly oligotrophic and a few mesotrophic sites from East and West Arm of Darwin Harbour. These sediment samples were also used in core incubation experiments to measure benthic O_2, dissolved inorganic carbon (DIC), silicate and nutrient fluxes. Two sediment depths were chosen: 0-10 cm and 0-<2cm. The 0-10 cm depth samples enabled the results of this study to be compared to those of SP2. Samples from this depth range reflect the long-term trophic status of a site while the shallow < 2cm sample contains the microbial community mainly involved in nutrient turnover with a higher likelihood to be correlated to the flux data.

2 Methods

2.1 Sediment collection

Sediment was collected by the AHU team in August and September 2019 from 25 sites in Darwin Harbour, namely from West and East Arm as well as along Elizabeth River (Figure 2 and Table 1) (Radke et al., 2020). Cores were withdrawn from all sites where a total depth did not exceed 2 m. This depth allowed the pole corer to be used with relative ease and provided access to sites during most tidal regimes. Sediment was collected in duplicates and from 2 depths i.e. 0-10 cm and 0-<2 cm. In total this resulted in 100 sediment samples. Samples were stored at -20C until processing.

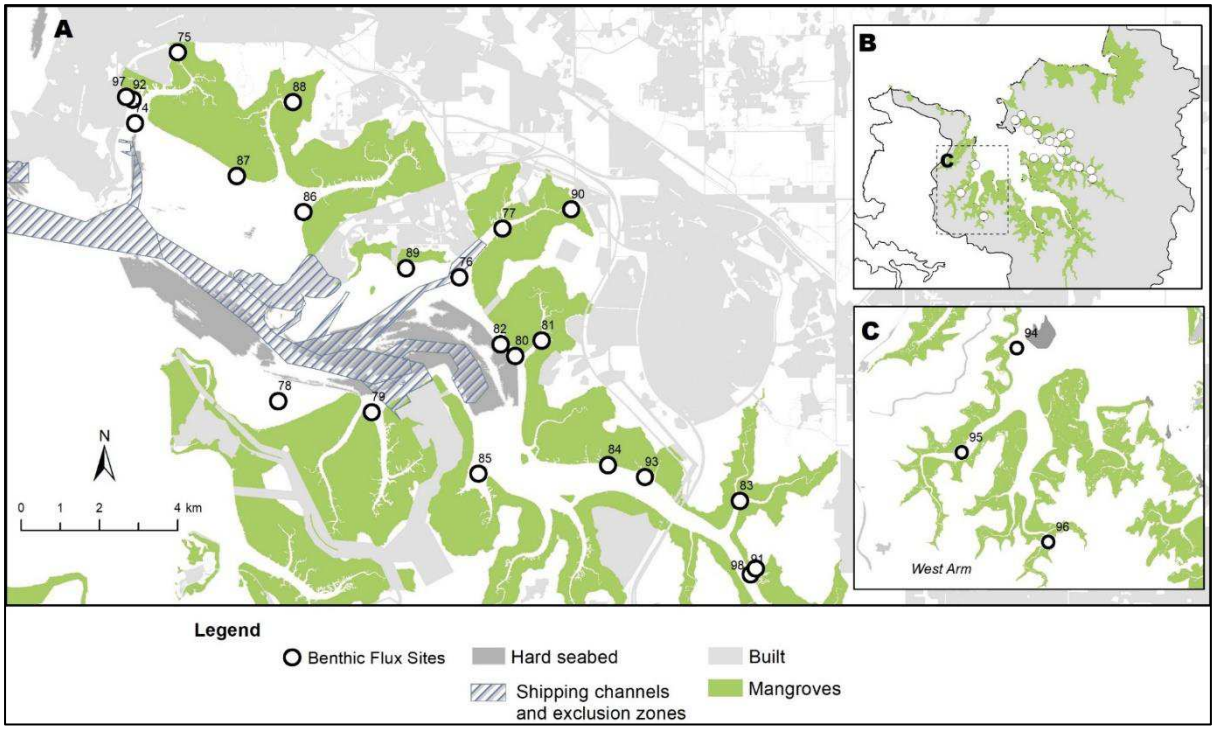


Figure 2: Sediment collection sites in Darwin Harbour

Figure provided by NTG AHU. Site numbers pertain to the wider sediment quality survey described in Radke et al., 2020.

ID	Location
74	Stormwater drain at Dinah
75	Upper Sadgroves Creek
76	Mouth of Hudson Creek
77	Upper Hudson Creek
78	East Arm
79	Lightening Creek
80	Myrmidon mouth
81	Myrmidon
82	Mudflat between Myrmidon and Hudson
83	Upper Mitchell/Brooking
84	Elizabeth River downstream boat ramp
85	Elizabeth River Bladen Point
86	Mudflat outside Blessers Creek
87	Mudflat in Charles Darwin National Park
88	Upper Reichardt Creek
89	Front of RSET in East Arm
90	Upper Hudson Creek
91	Elizabeth River RSET (seaward edge)
92	Seaward edge RSET Sadgroves Creek
93	Elizabeth River: downstream of bridge
94	West Arm
95	Upper West Arm
96	Uppermost West Arm
97	RSET Sadgroves (Landward)
98	RSET Virginia (Landward)

Table 1: Sample sites

2.2 Core incubation and flux experiments

Flux experiments were performed by the AHU team to measure benthic O_2, DIC, nutrient and silicate fluxes (all dark measurements) (Radke et al., 2020).

Negative fluxes denote sediment uptake and positive fluxes sediment efflux.

A net zero flux represents either a balance between analyte production and consumption in the sediments and/or rates that are below the detection limit.

2.3 DNA extraction from sediment

DNA was extracted from 5-7g of sediment per sample using the DNeasy PowerMax Soil DNA extraction kit (Qiagen Labs USA) following the manufacturer's instructions. DNA elutes in 5mL were precipitated and re-eluted in 100 µL of 10mM Tris buffer. All DNA samples were diluted to 10 ng/uL (measured by Nanodrop) prior qPCR assays.

2.4 qPCR assays and normalization

Seven qPCR assays were developed, and a technical and initial biological validation performed:

1. 16s EMP – targeting a 16s rRNA gene segment present in most Bacteria and Archaea
2. *nosZ* – targeting general denitrification with higher abundance at oligotrophic sites
3. AOA.1 – Archaeal ammonia oxidation with higher abundance at oligotrophic sites
4. *otu606* – nitrifying bacterial taxa with higher abundance at oligotrophic sites
5. *nosZ.55.2a – targeting a denitrification gene with higher abundance at eutrophic sites*
6. *nosZ.55.2bc – targeting a denitrification gene with higher abundance at eutrophic sites*
7. *nosZ.57 - targeting a denitrification gene with higher abundance at eutrophic sites*

The SYBR green based 16s EMP qPCR targets the 16s rRNA gene of both Archaea and Bacteria and is based on the Earth Microbiome Project using extensively validated primers (http://www.earthmicrobiome.org/) 16s_515FB_FOR (5'-GTGYCAGCMGCGCGGTAA-3') and 16s_806RB_REV (5'-GGACTACNVGGGTWTCTAAT-3') (amplicon length ~ 390 bp). Final primer concentrations were 0.2 µM and the QuantiTect SYBR Green PCR mix (Qiagen) was used in a reaction volume of 20 µL. qPCR conditions were 2 min at 50°C, 15 min at 95°C, 40 cycles of 15 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C and the fluorescence acquiring step for 15 sec at 80°C. A melt curve analysis from 95° to 60°C was conducted at the end to ensure the specificity of the detected amplicons.

A SYBR green based *nosZ* qPCR assay (Henry et al., 2006) was used to quantify the abundance of *nosZ* genes in the samples. Final primer concentrations were 1 µM (forw 5' WCSYTGTTCMTCGACAGCCAG, rev 5' ATGTCGATCARCTGVKCRTTYTC) with qPCR conditions: 15 min at 95°C, 6 cycles of 15 sec at 95°C, 30 sec touchdown starting at 67°C and a decrease of 1°C for every of 6 cycles, 30 sec at 72°C and 40 cycles of 15 sec at 95°C, 30 sec at 62C° and 30 sec at 72°C with fluorescence acquiring for 15 sec at 81°C followed by a melt-curve analysis as per above. The QuantiTect SYBR Green PCR mix (Qiagen) was used in a reaction volume of 20 µL.

Probe-based Taqman assays were used for the other assays (see Appendix 7.1 for primer and probe sequences). All probes had a FAM dye at the 5' end and a black-hole quencher (BHQ) at the 3' end (Biosearch Technologies, USA). The PerfeCTa qPCR ToughMix (MPBio) was used in reaction volumes of 20 µL with final concentration of 400 nM of primers and 250 nM of the probes. The following conditions were used: 10 min at 95°C and 50 cycles of 15 sec at 95°C and 60 sec at 60°C. The specificity of the PCR products was checked on a 2% agarose gel to verify the correct amplicon length.

The PCR efficiency and R^2 of the linear dynamic range were established for all assays using DNA serial dilutions over 6 logs in triplicates with DNA from a sediment sample collected from oligotrophic Kings Creek in July 2014 (MCC225 A). All qPCR assays were run on a Rotor-Gene Q 5-plex (Qiagen). A no-template control (NTC) was included in each run.

A technical validation (i.e. determine PCR efficiency and linear dynamic range) and biological validation on a small sample set (i.e. 2 sediment samples each from hypereutrophic, mesotrophic and oligotropic sites in Shoal Bay) were conducted on the seven qPCR assays. Four assays (16s EMP; *nosZ*; *AOA1b*; *otu606*) passed these tests and the sediment DNA samples (n=100) were screened across these. The PCR efficiencies of these tests were between 90 and 93% while the EMP assay had an efficiency of 79%.

All qPCR Ct values were normalized by the microbial DNA present in that sample. The 16s EMP assay was used to measure microbial DNA and the Pfaffl method for normalization which accounts for differences in PCR efficiencies between the target N-cycling and normalizing qPCR assays (Pfaffl, 2001). DNA from the above-mentioned sediment sample from oligotrophic Kings Creek (MCC225 A) was used as reference sample and positive control and included in each qPCR run.

Final qPCR results represent the relative N-cycling gene abundance normalized by the microbial load in that sample and relative to a reference sediment sample from an oligotrophic site in Darwin Harbour.

For instance, a qPCR result of 2 for the *nosZ* gene assay indicates that the sediment sample had twice the amount of *nosZ* genes compared to the reference sediment and normalized for the number of microbes in these samples. A result of 0.5 would indicate half the amount of *nosZ* genes.

2.5 Data Analysis

Data analysis was conducted with the software R (https://www.r-project.org/) and for multivariate analysis, the software Primer-E (Plymouth, UK).

qPCR assay results were checked for their approximate normal data distribution and scatter and box plots were plotted to compare N-cycling indicators vs. sediment depths and fluxes. Linear and unimodal fitted lines were added to the scatter plots – the latter by fitting a quadratic polynomial term. Linear Pearson and rank-based Spearman correlations were computed and mixed effect models using REML were used to explore whether there was an association between qPCR assay results (outcome) and predictors sediment depth, Darwin Harbour area and nutrient fluxes. Harbour sites were included as random intercept. Model averaging (R package MuMIn) was used to explore whether any of the qPCR assay results (surface sediment only) contributed to explaining the flux results (outcomes).

Multivariate analysis explored whether the composite of qPCR assay results (Euclidean distance of square root transformed qPCR values normalized to z values) differed between Darwin Harbour areas and distance linear models were used to explore how much the flux data explained the qPCR composite result.

N-cycling indicator abundance was also mapped using ArcMap 10.4.1 with shape files of Darwin Harbour provided by the NT Government.

3 Results

3.1 Technical and initial biological validation of 7 qPCR assays

The two tests for denitrification (*nosZ*.552a/bc and 57) failed the linear requirements for a semi-quantitative assay, were less suitable for sediment compared to water samples and were sensitive to varying salinity concentrations. As a result, these three tests were not considered further. The remaining four assays (16s EMP; *nosZ*; *AOA1b*; *otu606*) passed these initial tests and all sediment samples were screened by these assays.

3.2 Linear correlation between the N-cycling indicator tests

There was no correlation between *nosZ* and *AOA1b* nor *otu606* (Figure 3). In contrast, there was a significant positive correlation between *AOA1b* and *otu606* (R 0.41, P<0.001). An exception were samples from Hudson creek and Myrmidon mouth which did not follow this linear fit and showed a low *otu606* but high *AOA1b* abundance. Upper West Arm was unusual as both duplicate cores had high *otu606* but low *AOA1b* counts in the surface sediment and low *otu606* in the deeper 1-10cm core.

3.3 The N-cycling indicators vs sediment depth

There were on average 240 ng more microbial DNA per gram sediment in the 0-10 cm core compared to the 0-2 cm samples (P<0.001) (Figure 4).

There were significantly more *nosZ* genes in the surface 0-2 sediment compared to the deeper core (P<0.001) while there was no difference in the normalized relative abundance of *otu606* and *AOA1b* genes (P>0.2) (Figure 4).

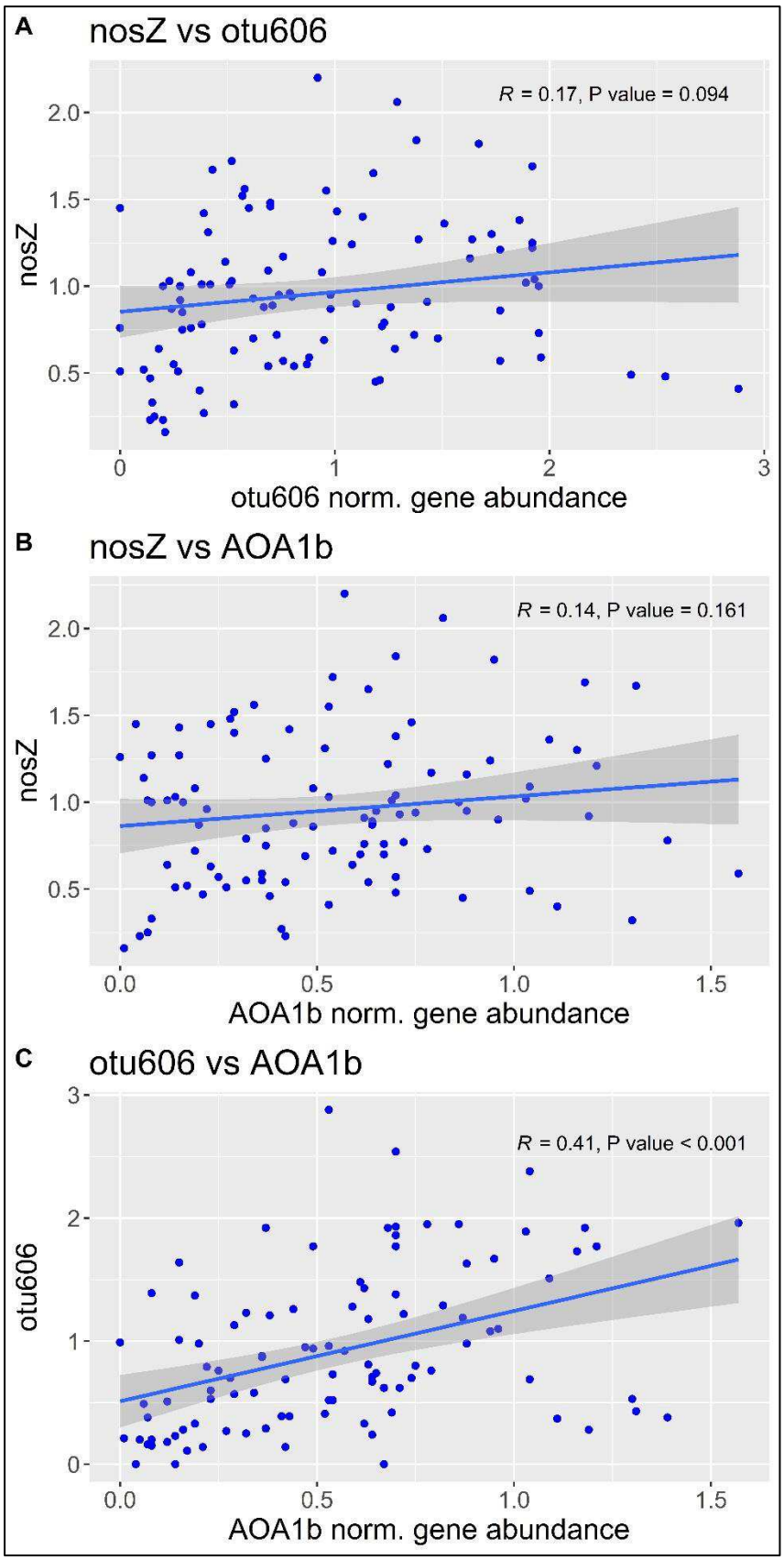


Figure 3: Correlation between N-cycling indicators.
R is the Pearson correlation index indicating the linear fit between the N-cycling gene abundances. The grey shade indicates the 95% confidence interval of the linear fit.

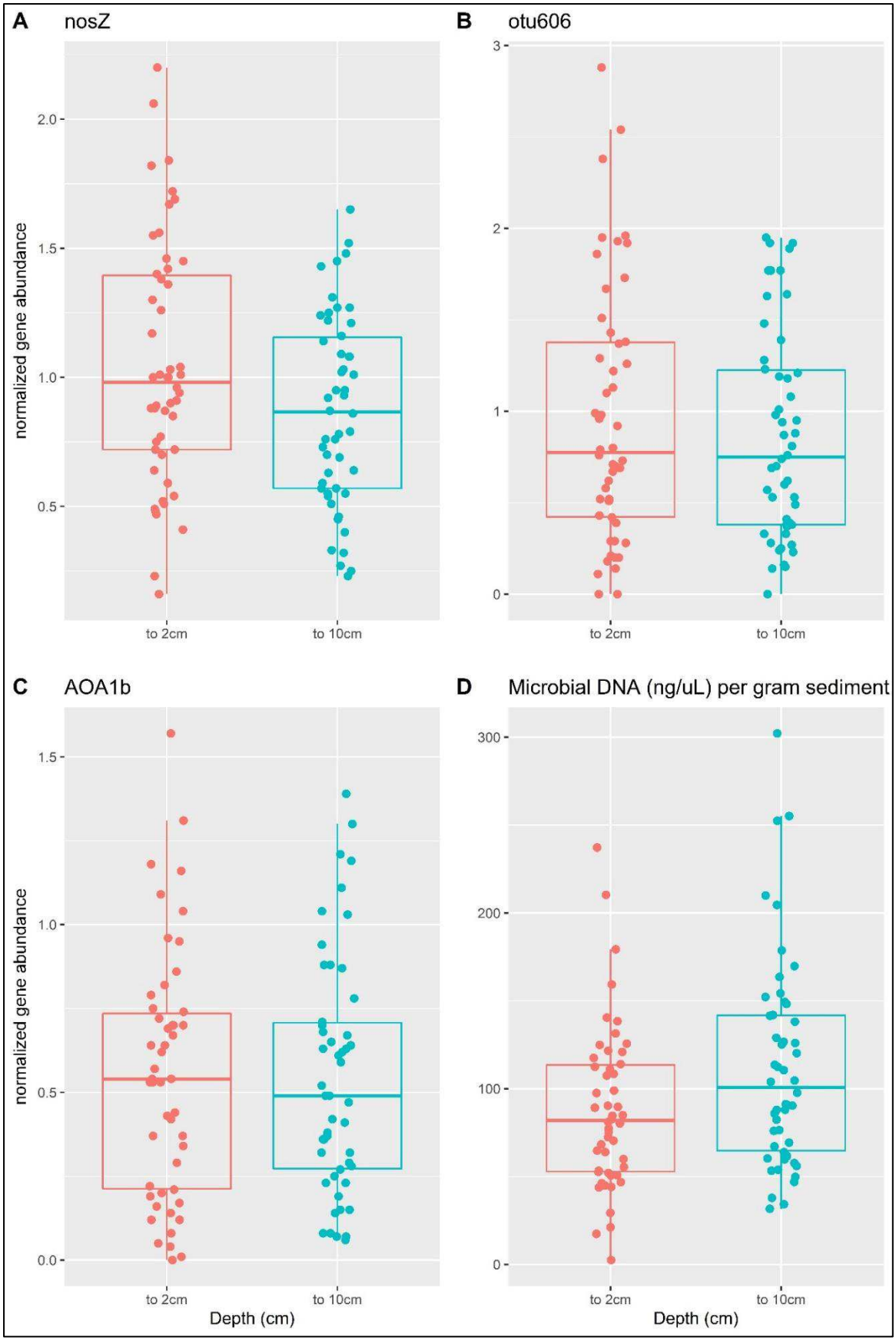


Figure 4 N-cycling genes and sediment depth

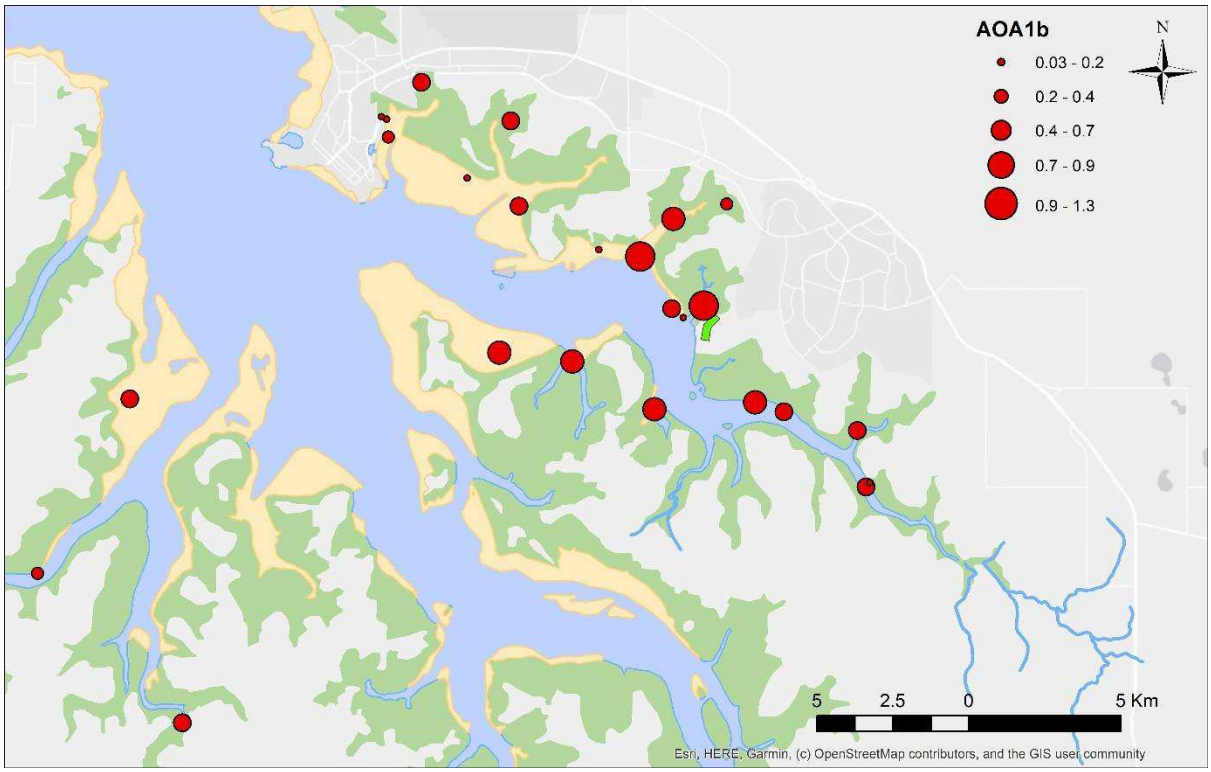
3.4 The N-cycling indicators across Darwin Harbour

The relative abundance of the N-cycling indicators in the top-2cm sediment varied between the Darwin Harbour sites (Figure 5 and Appendix 7.2).

For ammonia oxidizing *AOA1b*, the highest relative load was measured in Myrmidon and at the mouth of Hudson creek. The lowest levels were recorded at the urban Darwin sites, the mudflats of Charles Darwin National Park, the front of RSET in East Arm and at the mouth of Myrmidon.

For nitrite oxidizing *otu606*, levels were similar to *AOA1b* also recording higher levels in Myrmidon but also at uppermost of West Arm. Lowest levels were at Sadgroves near the city, the Charles Darwin mudflats, the mouth of Myrmidon and upper Elizabeth (RSET Virginia).

For the denitrification gene *nosZ*, there was a distinct trend of higher levels at the mouth of the creeks and mudflats and lower levels in the upper estuaries. An exception was the Charles Darwin mudflat which also had low *AOA1b* and *otu606* counts. The sites East Arm, Lightening Creek had the highest counts and in contrast to *AOA1b* and *otu606*, the stormwater drain at Dinah beach also showed higher *nosZ* levels.



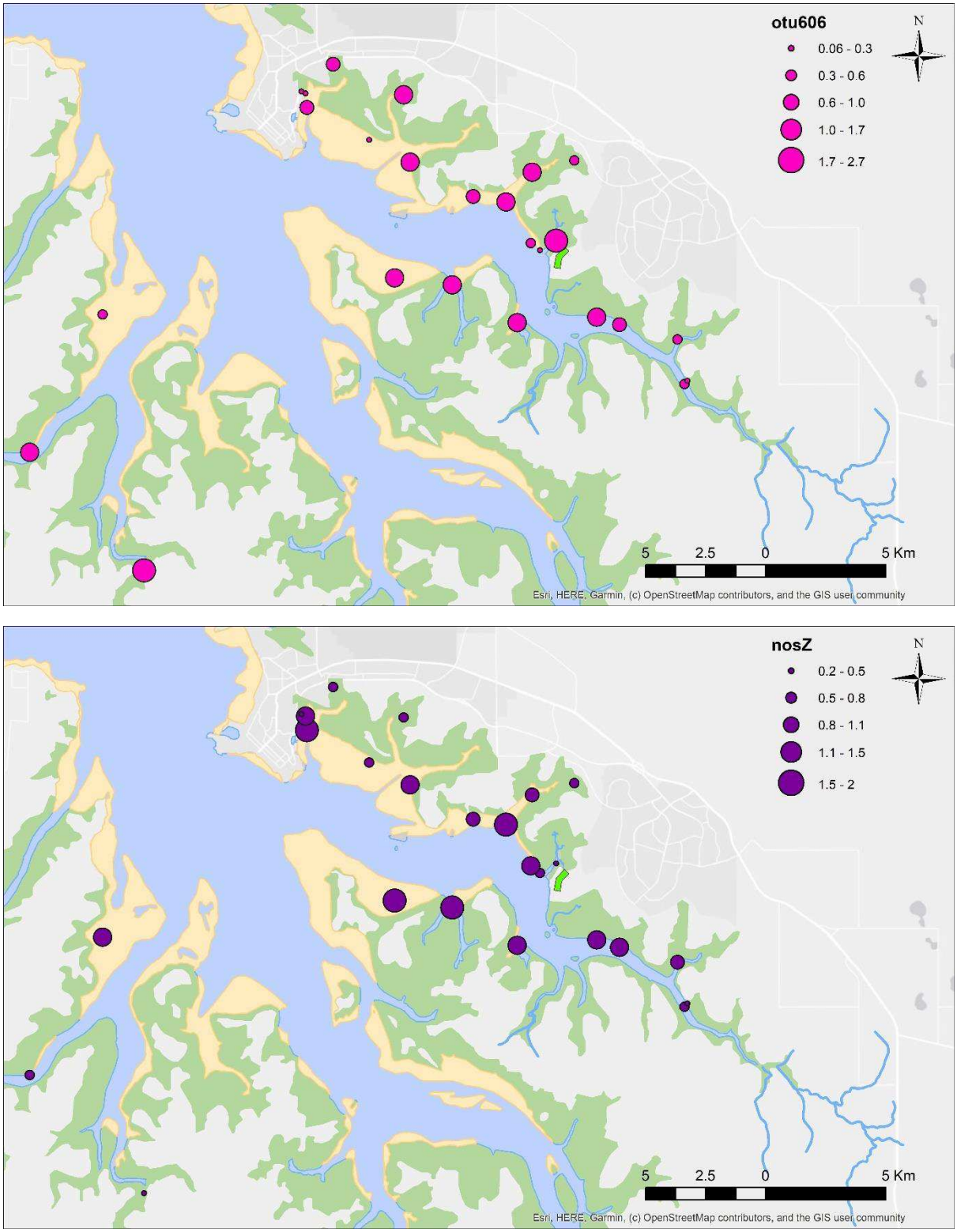


Figure 5 A-C: Relative abundance of N-cycling indicators at Darwin Harbour sites
The breaks in the indicator legends are based on natural breaks (Jenks method).

Of these assays, *nosZ* was the only gene that showed a consistent spatial pattern with more *nosZ* genes at the mouth of creeks and mudflats compared to upper creeks in a mixed multivariable model also accounting for sediment depth and sites (P<0.001)(Figure 6).

None of indicators showed a consistent trend for whole harbour areas (e.g. all urban sites vs Elizabeth river sites etc).

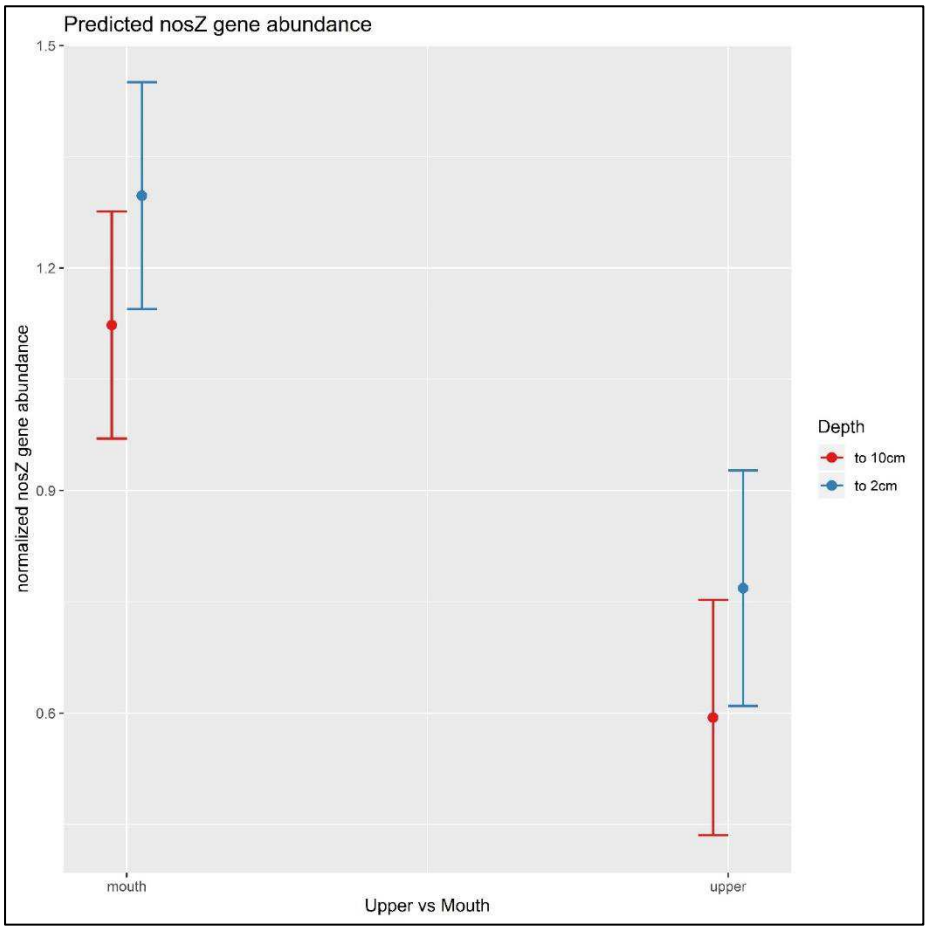


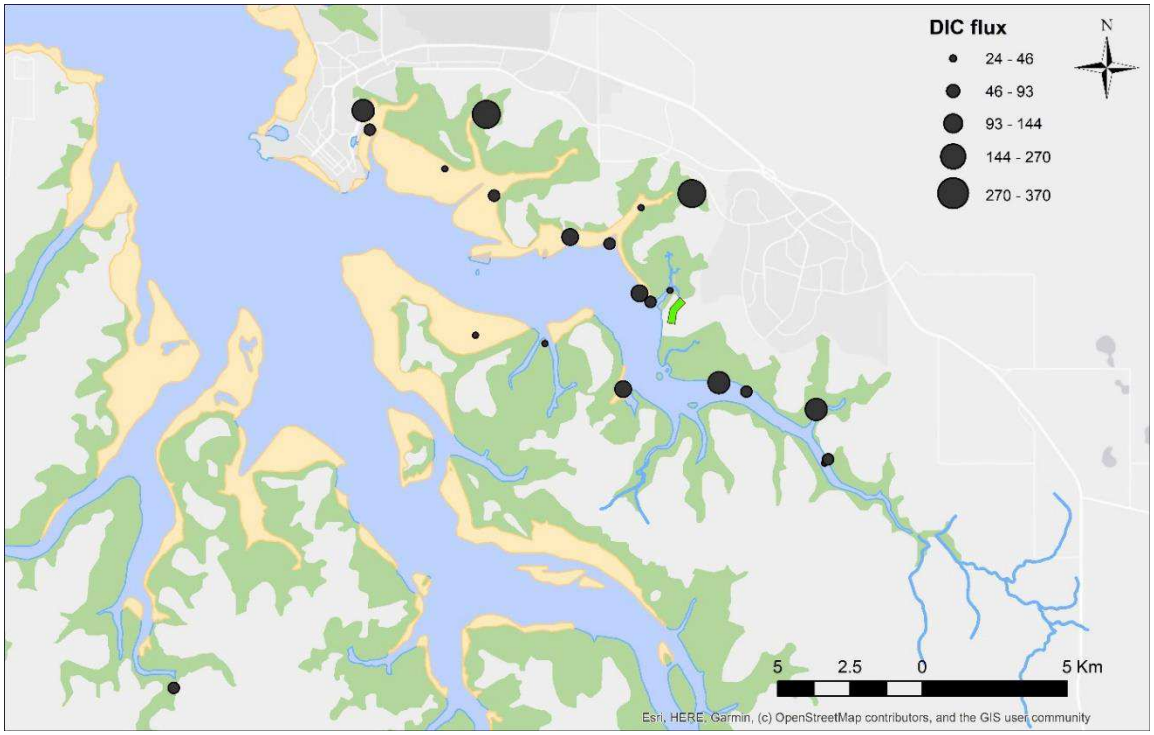
Figure 6: Predicted *nosZ* gene abundance at mouth vs upper estuaries

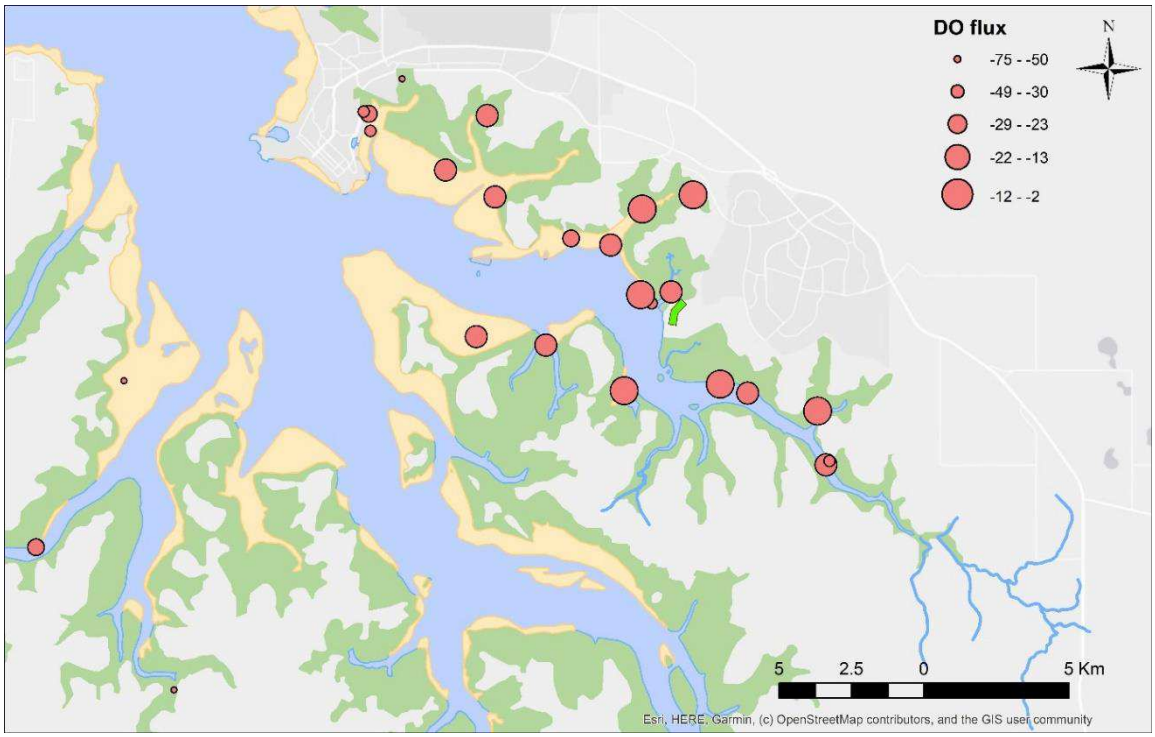
3.5 The nutrient fluxes in the sediment in Darwin Harbour

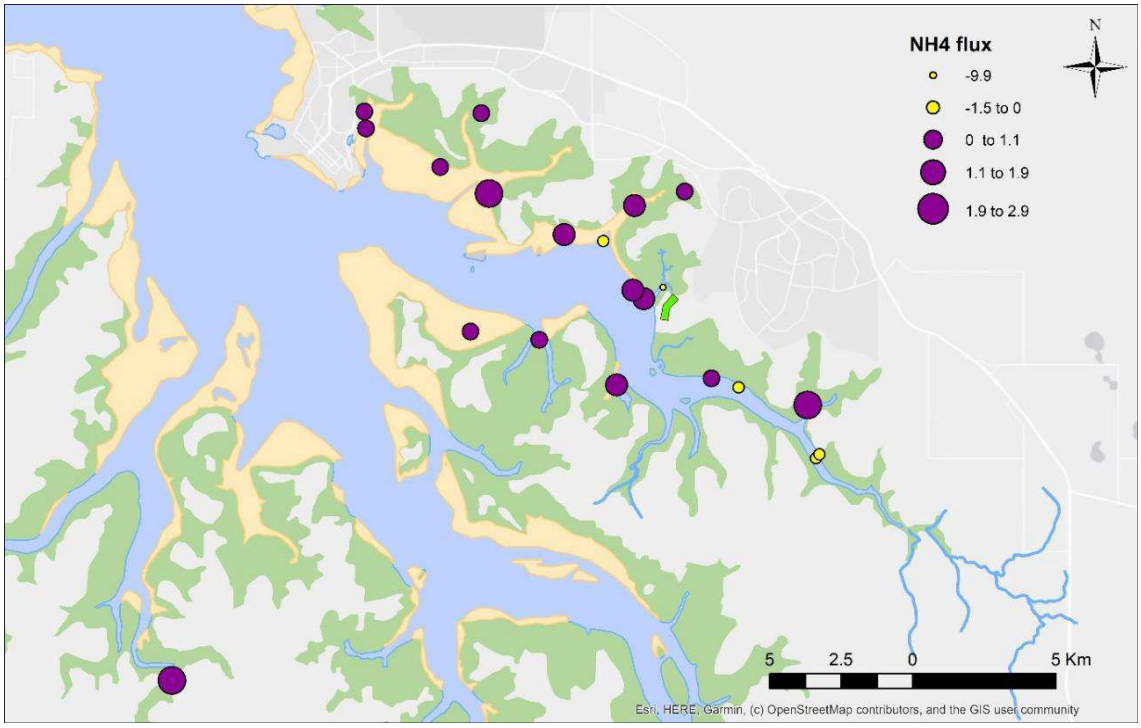
Figure 7 shows the flux levels of DIC, NH_4, NO_x, N_2, DO, PO_4 and silicate across Darwin Harbour.

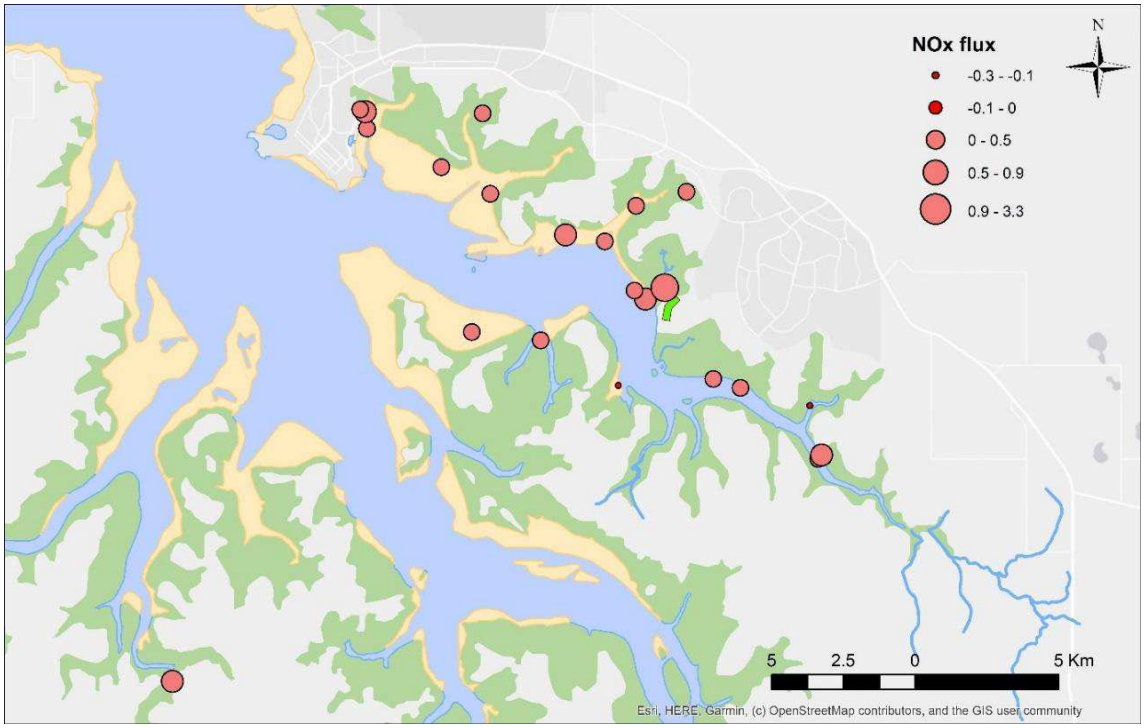
DIC showed an opposite trend to *nosZ* with the highest DIC emissions in the upper creeks and lower levels in the mudflats and mouths. The highest uptake of DO into the sediment was at the urban sites, at (uppermost) West Arm, Myrmidon mouth and the Elizabeth River RSET. Larger ammonia levels were emitted at upper Mitchell creek, uppermost West Arm and the mudflat outside Blesser creek. Ammonia uptake occurred at the mouth of Hudson creek, in Myrmidon creek at various sites along Elizabeth river. Myrmidon creek, the front of RSET in East Arm and the urban sites had larger NO_x emissions while there was a NO_x uptake at upper Mitchell creek and Elizabeth River Bladin

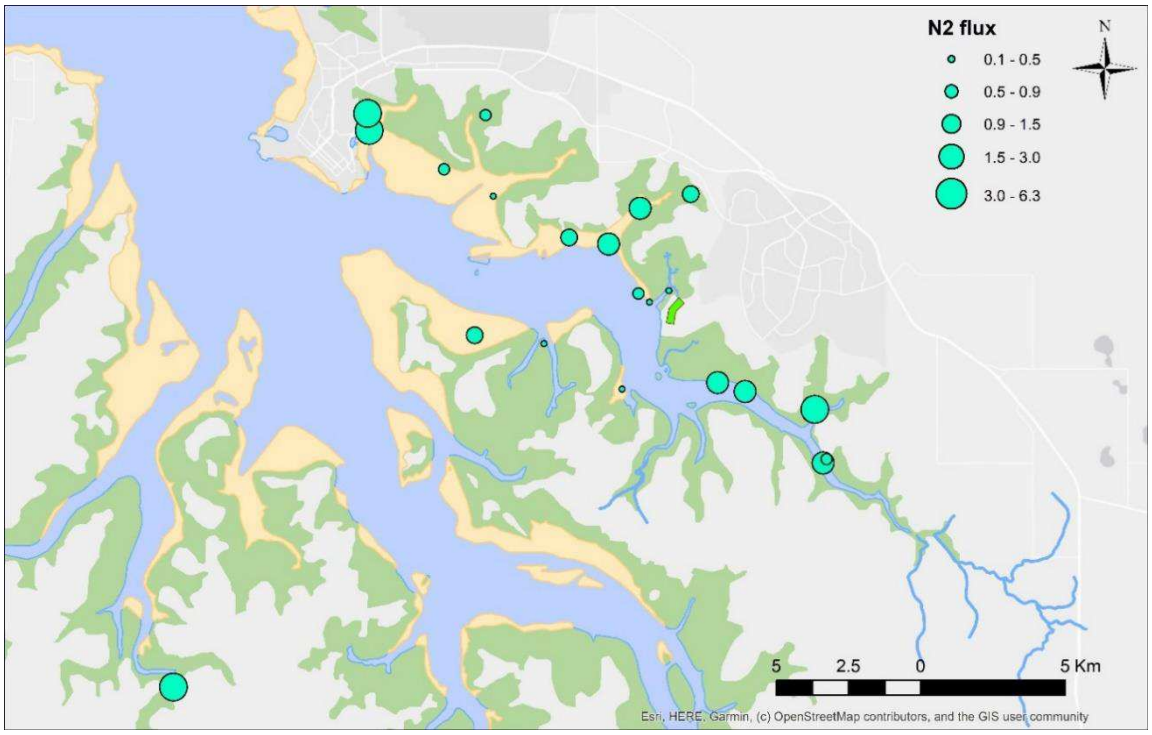
Point. The largest nitrogen gas emissions were at the urban sites and upper Mitchell creek while N2 fluxes were close to zero at Myrmidon creek, Bladin Point and the mudflat outside Blesser creek. Phosphate efflux was largest in Myrmidon and Hudson creek as well as at uppermost West Arm. The largest silicate efflux was in upper West arm and Hudson creek as well as at some urban and Elizabeth river sites while they were smallest at Myrmidon creek.

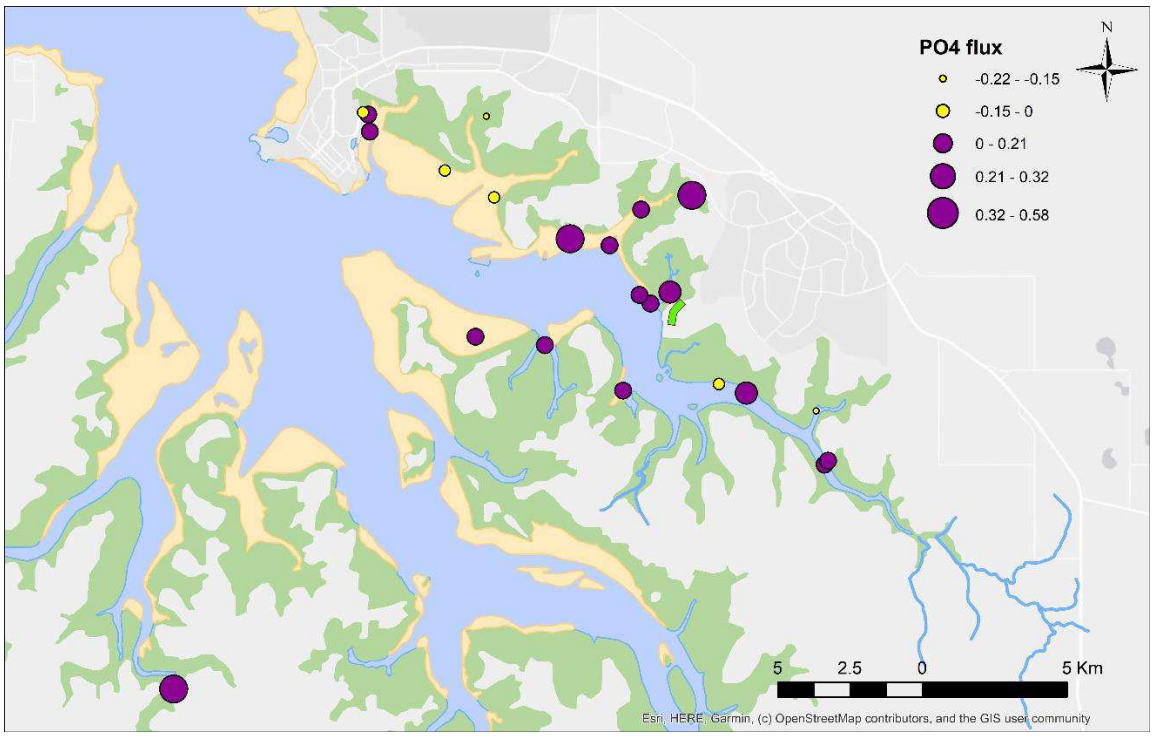





NH4 flux
-9.9
-1.5 to 0
0 to 1.1
1.1 to 1.9
1.9 to 2.9
N
5 2.5 0 5 Km
Esri, HERE, Garmin, (c) OpenStreetMap contributors, and the GIS user community


NOx flux
-0.3 - -0.1
-0.1 - 0
0 - 0.5
0.5 - 0.9
0.9 - 3.3
N
5 2.5 0 5 Km
Esri, HERE, Garmin, (c) OpenStreetMap contributors, and the GIS user community


N2 flux
0.1 - 0.5
0.5 - 0.9
0.9 - 1.5
1.5 - 3.0
3.0 - 6.3
N
5 2.5 0 5 Km
Esri, HERE, Garmin, (c) OpenStreetMap contributors, and the GIS user community


PO4 flux
-0.22 - -0.15
-0.15 - 0
0 - 0.21
0.21 - 0.32
0.32 - 0.58
N
5 2.5 0 5 Km
Esri, HERE, Garmin, (c) OpenStreetMap contributors, and the GIS user community

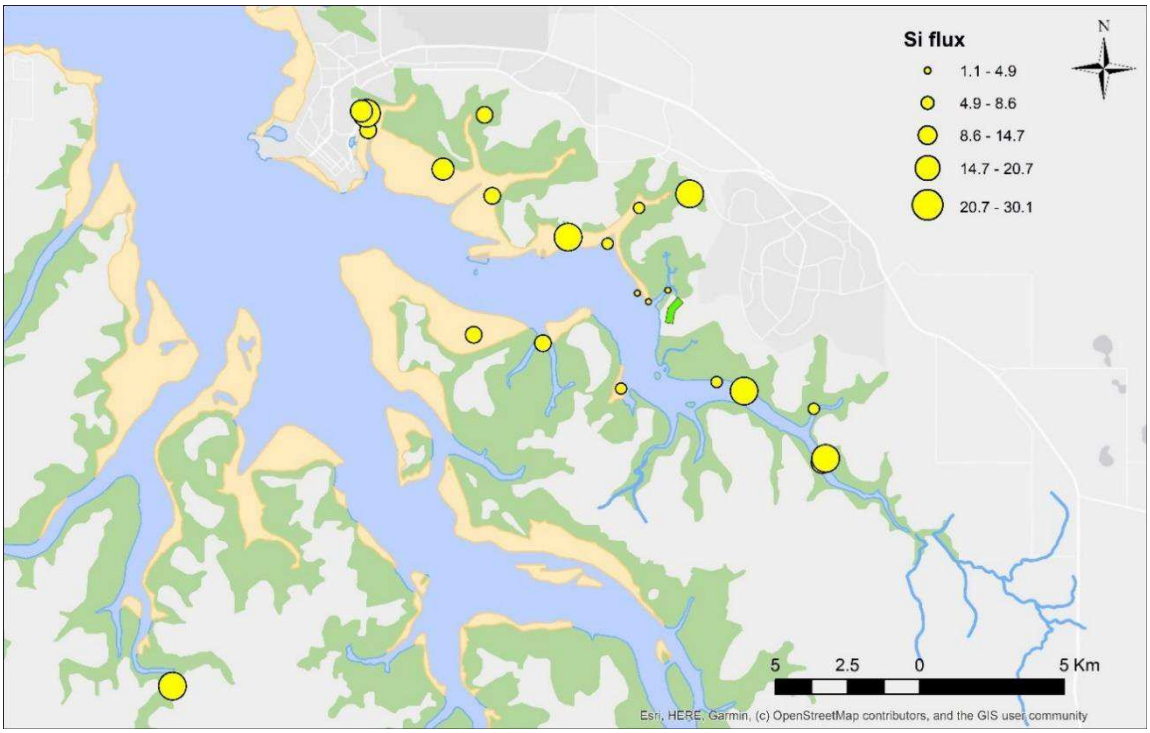


Figure 7: Flux levels in Darwin Harbour
Units are mmol/m^2/d. Natural breaks (Jenks method) except where they crossed zero.

3.6 The correlation between N-cycling indicators and nutrient fluxes

Table 2 and the scatter plots in Figure 8 and Appendix 7.3 show correlations between the N-cycling indicators and nutrient fluxes.

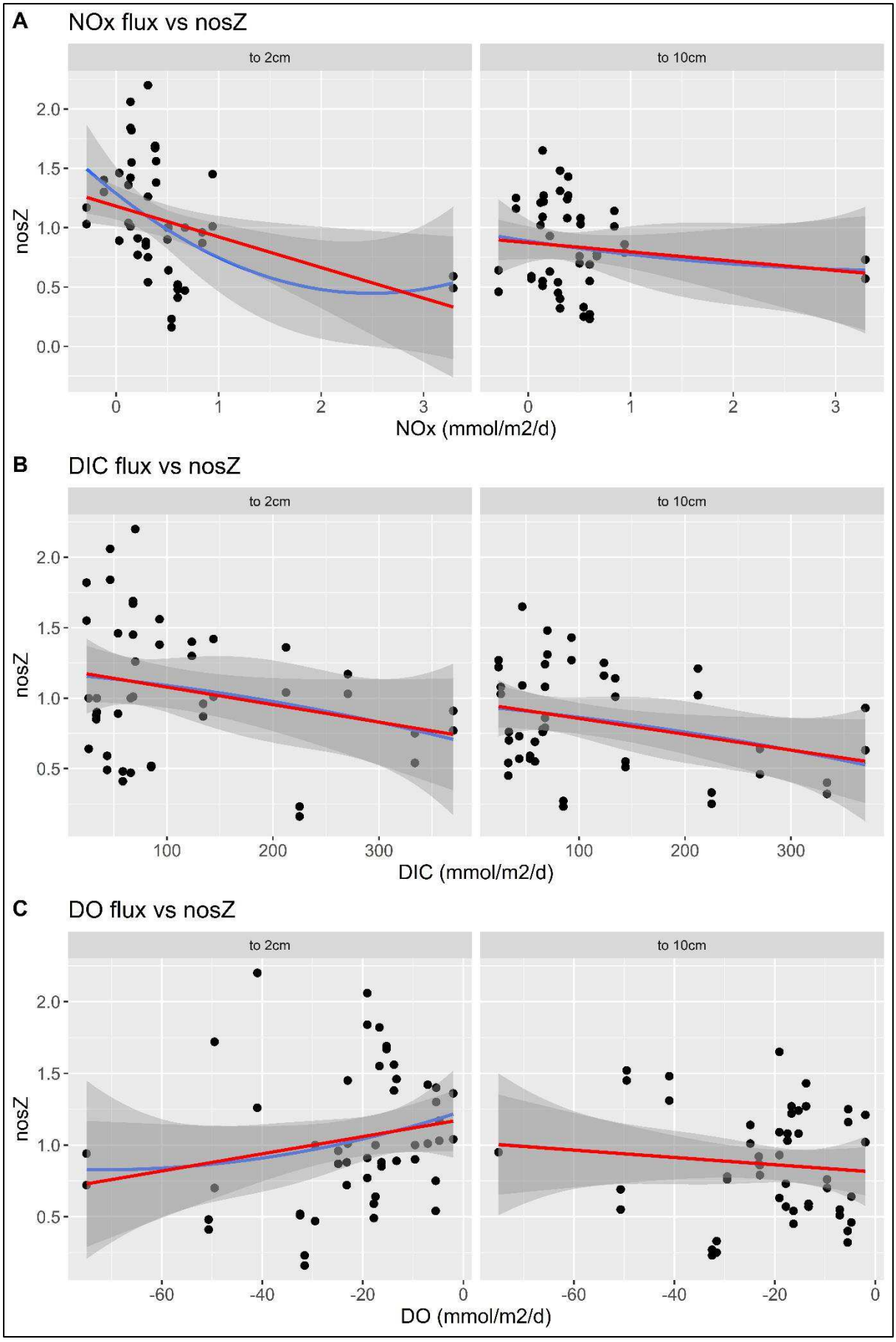
There was a strong negative correlation between NO_x flux and the *nosZ* gene abundance in the 0-2cm sediment layer (linear Pearson R -0.36, P=0.015 and rank-based Spearman Rho -0.51, P<0.001) and i.e. the higher the *nosZ* gene abundance, the smaller the NO_x efflux. Figure 8 indicates a unimodal-like relationship between the *nosZ* gene abundance and PO_4 and Si flux with highest *nosZ* gene abundance at intermediate fluxes.

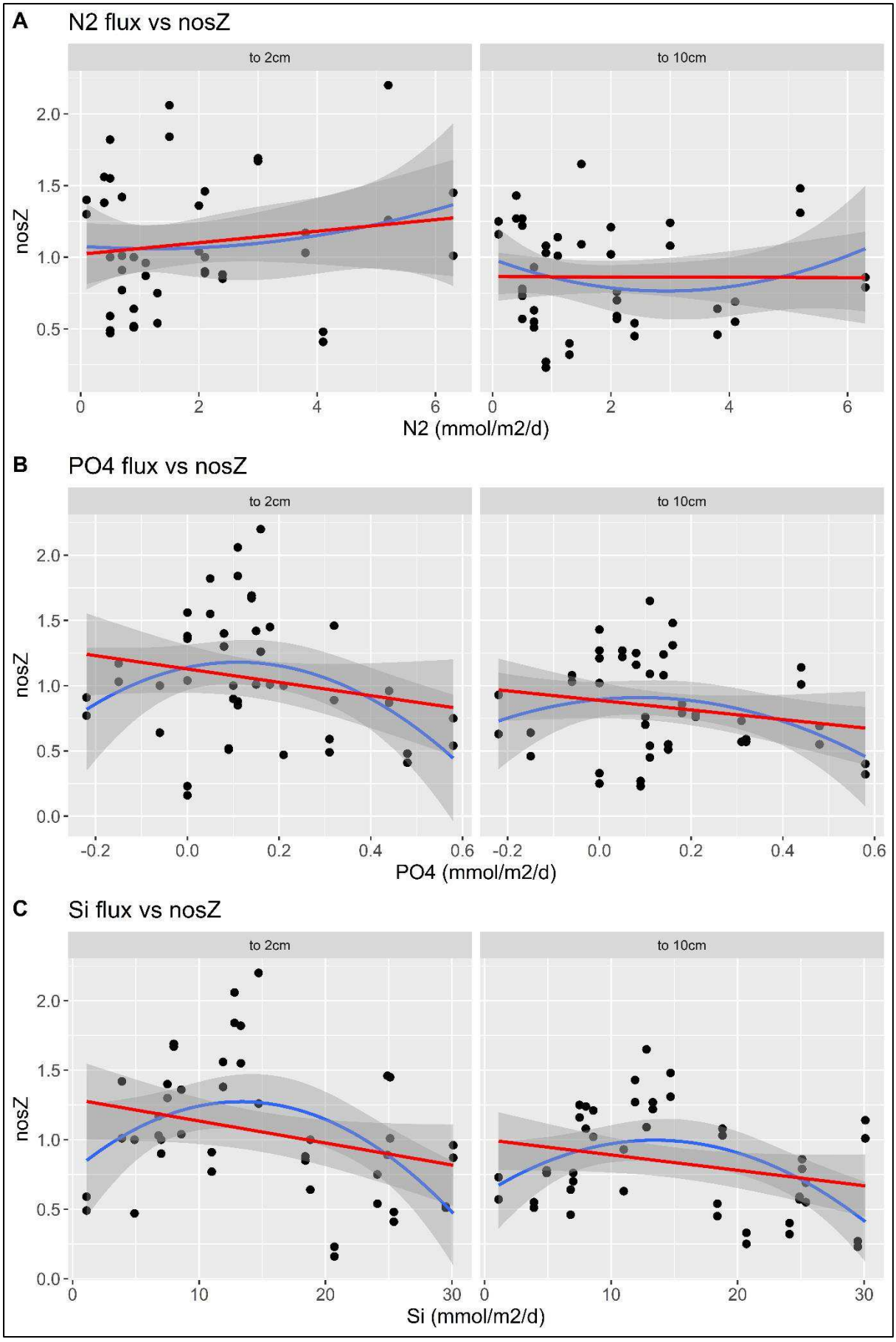
There were more *AOA1b* in those surface sediment samples with a higher DO efflux (Rho 0.37, P=0.008). The strongest linear negative correlation was found between surface *AOA1b* and silicate flux (Pearson R -0.56, P<0.001, Spearman Rho -0.51, P<0.001). The higher the silicate emission from the sediment, the less *AOA1b* in the sediment.

		Sediment Depth			
		to 2 cm		to 10 cm	
		rho	P value	rho	P value
nosZ	NH4	-0.05	0.739	0.04	0.789
	NOx	-0.51	<0.001***	-0.14	0.356
	N2	0.06	0.709	-0.14	0.361
	DIC	-0.11	0.464	-0.23	0.138
	DO	0.36	0.011*	-0.08	0.595
	PO4	-0.18	0.253	-0.17	0.277
	Si	-0.26	0.094	-0.17	0.281
AOA1b	NH4	-0.20	0.191	-0.24	0.117
	NOx	-0.37	0.014*	-0.17	0.262
	N2	-0.04	0.797	0.06	0.707
	DIC	-0.15	0.334	-0.05	0.735
	DO	0.37	0.008**	0.21	0.140
	PO4	-0.03	0.872	0.16	0.312
	Si	-0.51	<0.001***	-0.37	0.013*
otu606	NH4	0.02	0.883	-0.19	0.224
	NOx	-0.17	0.264	-0.23	0.128
	N2	-0.03	0.846	0.00	0.998
	DIC	-0.10	0.504	-0.17	0.272
	DO	0.13	0.376	0.35	0.012*
	PO4	0.06	0.697	-0.08	0.622
	Si	-0.26	0.085	-0.26	0.085

Table 2: Rank-based Spearman's rho correlation

Note that no adjustment for multiple testing was undertaken and P values should be interpreted with caution. * P<0.05; ** P<0.01; *** P<0.001 (yellow highlighted). Red or green colours indicate gradient from no colour to red for a negative correlation and no colour to green for a positive correlation. Note that while Spearman correlations are rank-based, they only detect monotonic correlations i.e. strictly in- or decreasing but no e.g. unimodal associations.


A
NOx flux vs nosZ
to 2cm
to 10cm
nosZ
NOx (mmol/m2/d)
B
DIC flux vs nosZ
to 2cm
to 10cm
nosZ
DIC (mmol/m2/d)
C
DO flux vs nosZ
to 2cm
to 10cm
nosZ
DO (mmol/m2/d)


A
N2 flux vs nosZ
to 2cm
to 10cm
nosZ
N2 (mmol/m2/d)
B
PO4 flux vs nosZ
to 2cm
to 10cm
nosZ
PO4 (mmol/m2/d)
C
Si flux vs nosZ
to 2cm
to 10cm
nosZ
Si (mmol/m2/d)

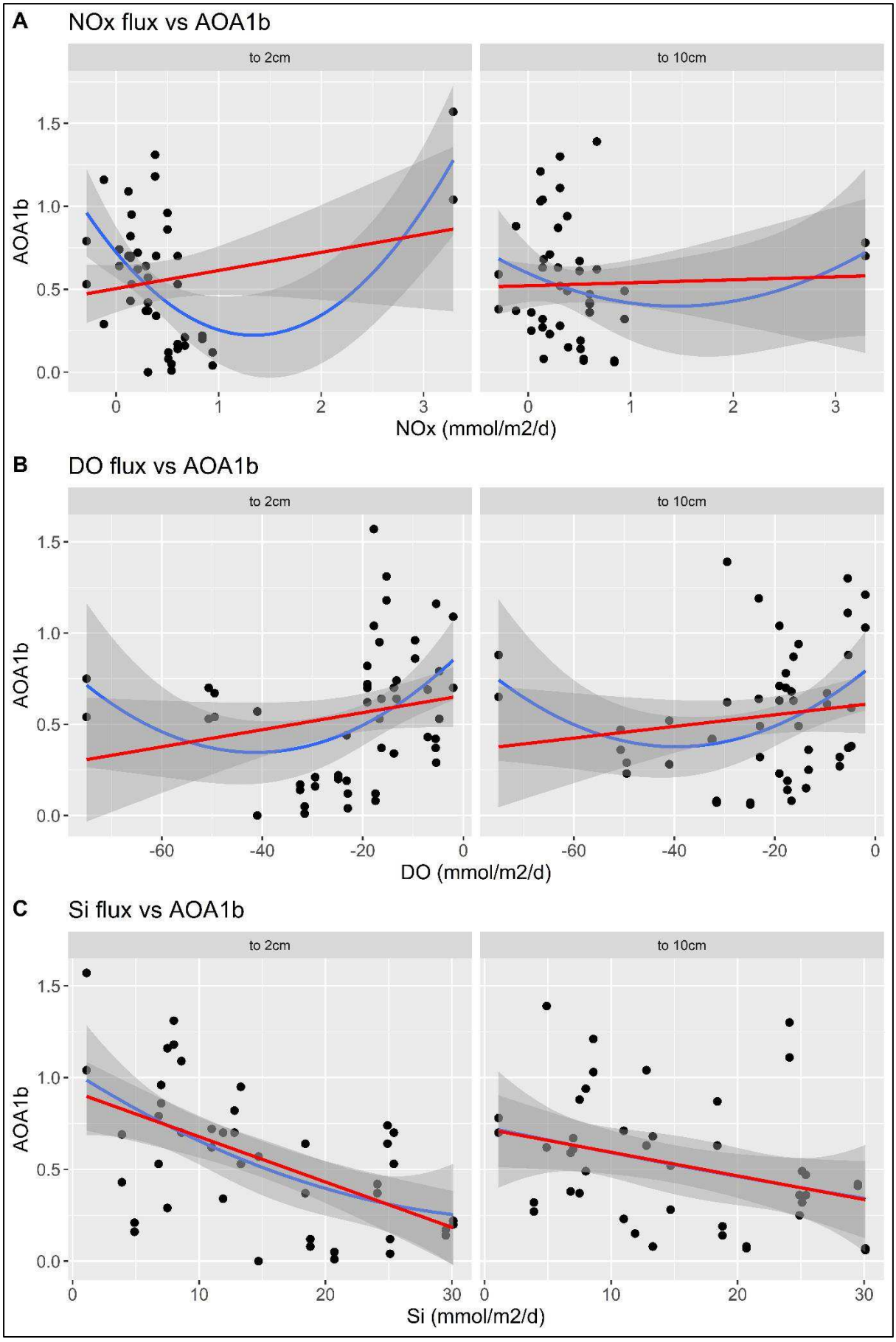

A
NOx flux vs AOA1b
to 2cm
to 10cm
AOA1b
NOx (mmol/m2/d)
B
DO flux vs AOA1b
to 2cm
to 10cm
AOA1b
DO (mmol/m2/d)
C
Si flux vs AOA1b
to 2cm
to 10cm
AOA1b
Si (mmol/m2/d)

Figure 8: Scatter plots of nutrient fluxes and N-cycling indicators.
Blue lines show unimodal fitted lines while red lines are linear fits. Shaded areas indicate 95% confidence intervals.

In order to explore whether the observed unimodal relationship between the relative abundance of *nosZ* and Si or PO_4 fluxes was also driven by differences in oxic status of the sediment, the size and colour of the dots marking the samples were adjusted to reflect the DO flux of these samples (Figure 9). These showed a weak trend of more anoxic sediments when silicate or phosphate flux were high and more oxic sediments with lower fluxes. However, there were several exceptions to this trend.

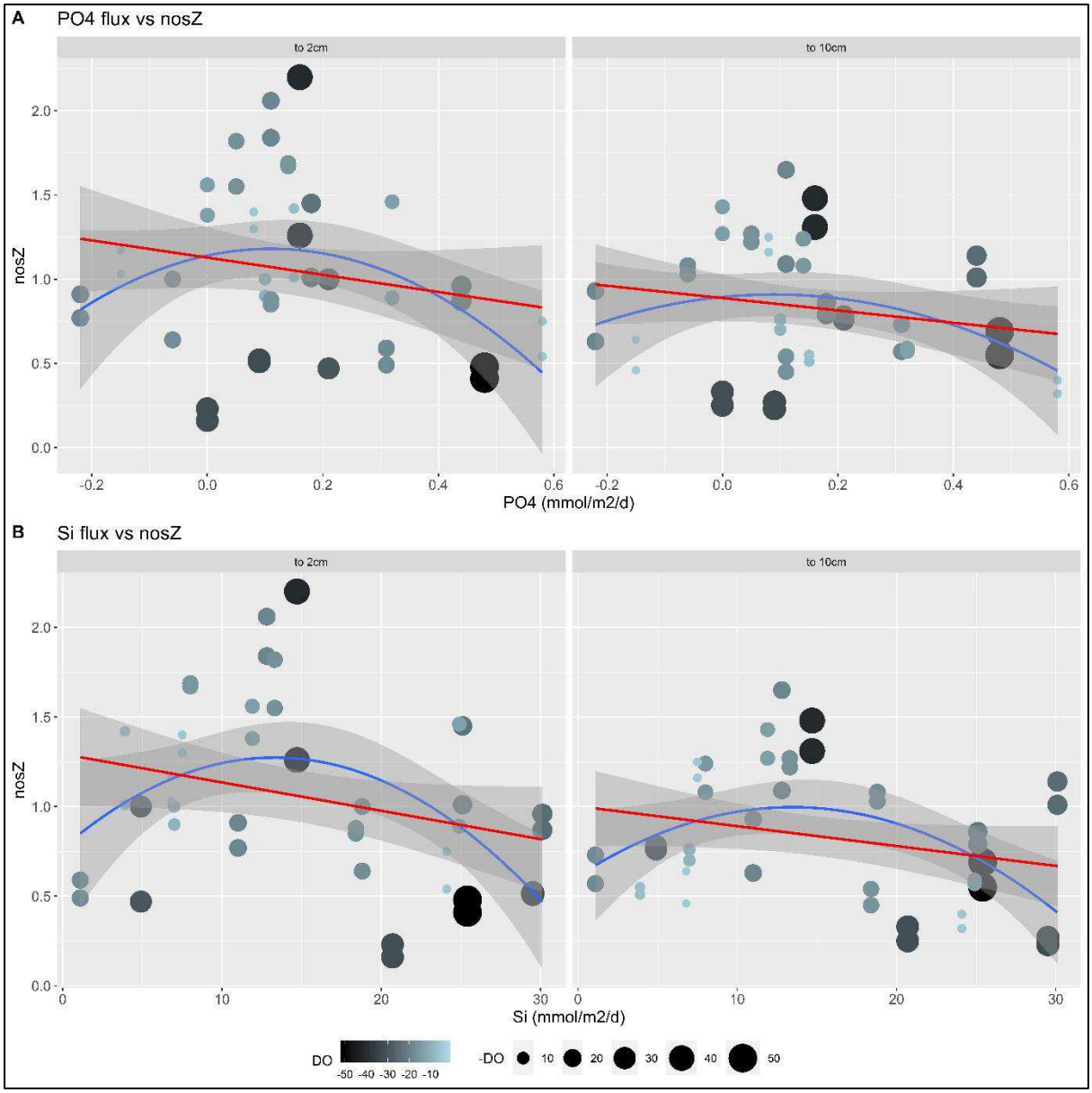


Figure 9: Unimodal relationship between *nosZ* and Si or PO_4
The blue lines mark the unimodal and the red line the linear fit. Shaded areas indicate 95% confidence intervals. The larger and darker the dot, the more anoxic the sediment based on negative DO flux.

3.6.1 Do nutrient fluxes predict N-cycling indicator abundances?

3.6.1.1 Do any fluxes explain the nosZ variance?

While there was no significant Pearson correlation between *nosZ* and N_2, there was a significant positive association in a multivariable mixed model accounting for sampling depth and spatial variance (upper creeks vs mouth as fixed factor and sites as random effect) (P=0.039) (Figure 10). Figure 10 also indicates a weak interaction (P=0.067) between sediment depth and N_2 flux i.e. the positive association between *nosZ* and N_2 was weaker at deeper sediment depth compared to the surface sediment.

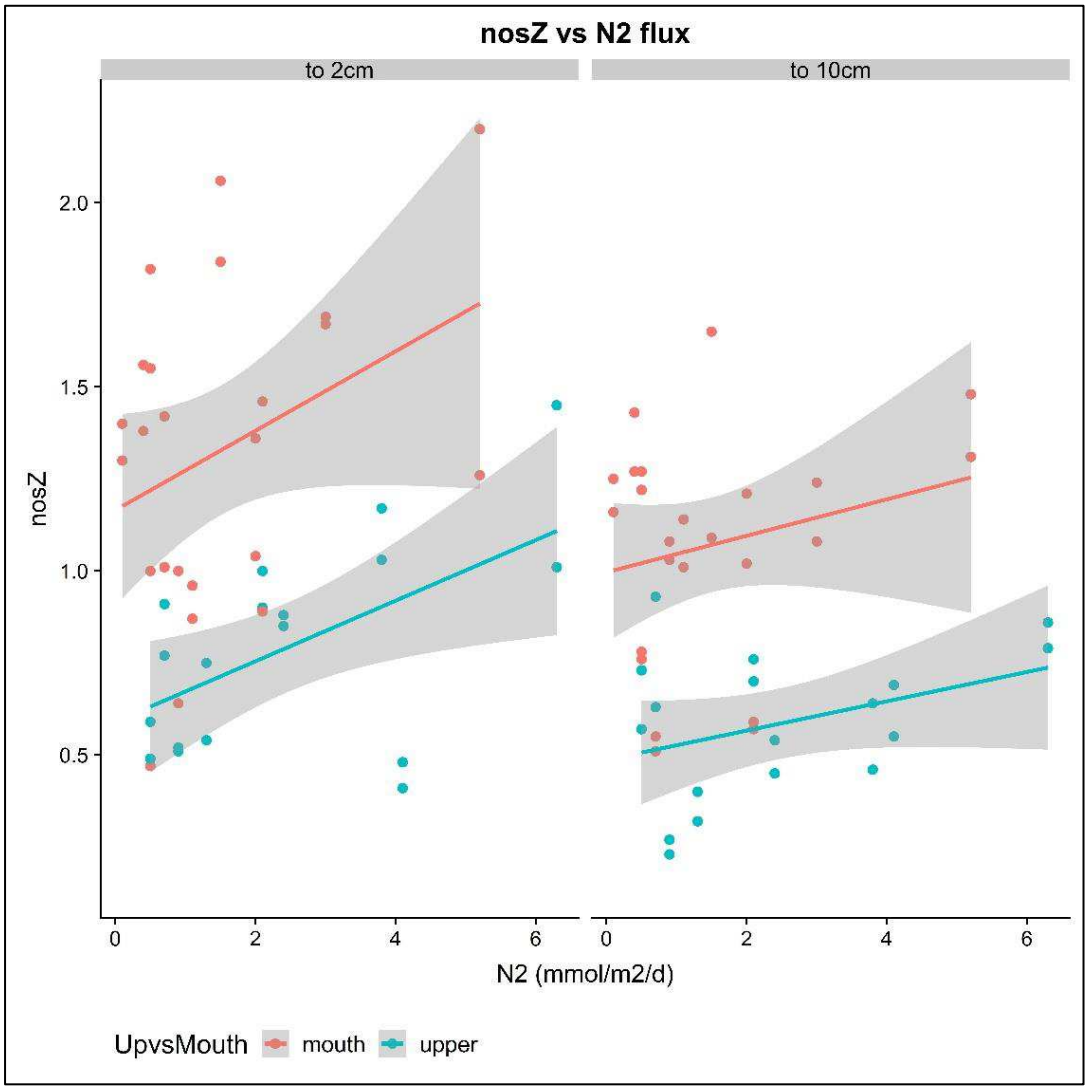


Figure 10: Scatter plots of *nosZ* and N_2 while accounting for spatial variance.
Gray shades indicate 95% confidence intervals.

There was a significant negative linear as well as rank-based correlation between *nosZ* and NOx at shallow sediment depth, this was further confirmed by a significant interaction term between depth and NO_x in a multivariable mixed model accounting for spatial variance (P=0.023) (Figure 11).

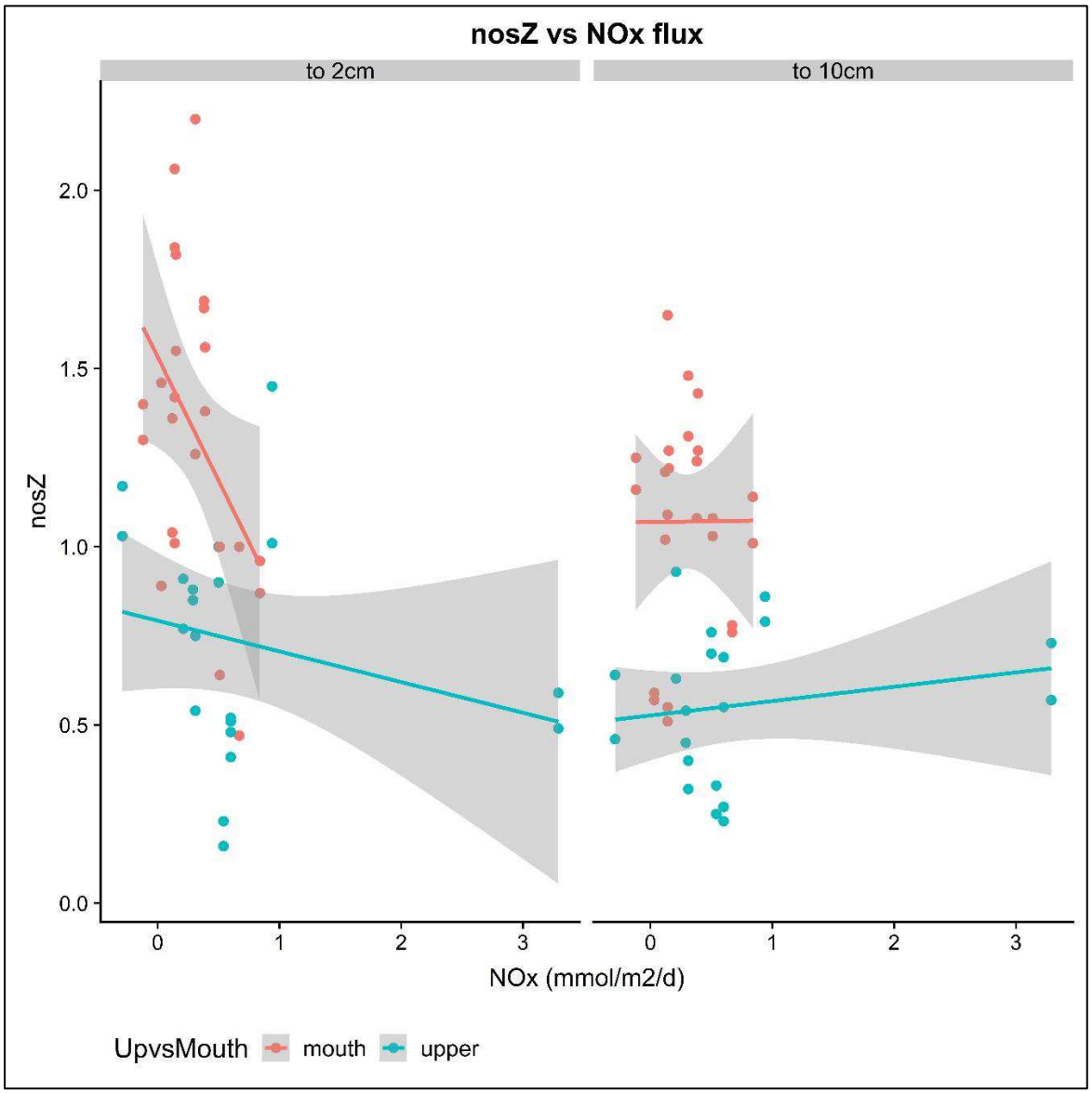


Figure 11: Scatter plots of *nosZ* and NO_x while accounting for spatial variance.
Grey shades indicate 95% confidence intervals.

3.6.1.2 *Do any fluxes explain the AOA1b variance?*

A mixed model with sites as random effect showed a strong negative association between *AOA1b* and Si flux confirming the negative correlation between these variables (P=0.004)(Figure 12).

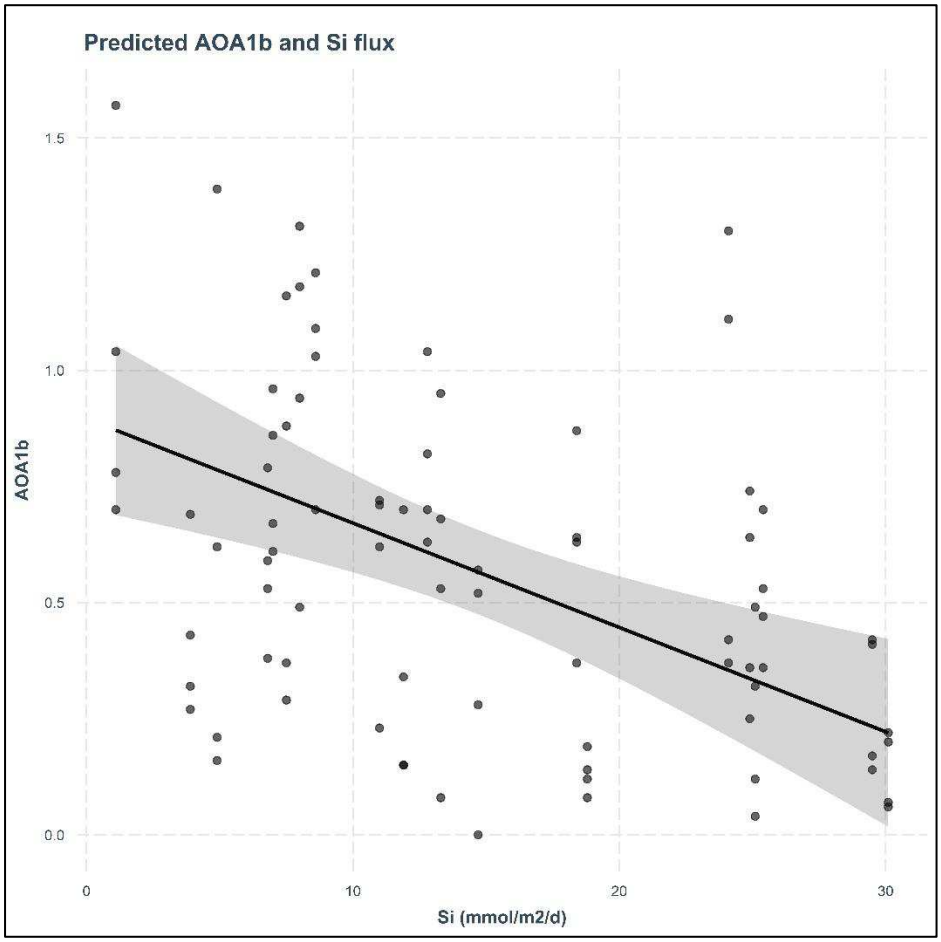


Figure 12: Predicted *AOA1b* vs Si flux

3.6.1.3 *Do any fluxes explain the otu606 variance?*

While the relationships between *otu606* and fluxes were similar to those of *AOA1b* and fluxes, they were generally less clear.

3.6.2 Do N-cycling indicators predict the nutrient fluxes?

None of the indicators at shallow nor deeper sediment depth were a significant predictor for nutrient flux while accounting for the variance across sites (data not shown).

3.7 The N-cycling indicator profile

The three N-cycling indicators were combined in a multivariate analysis to a Euclidean distance matrix. This matrix retains the information on how similar the combination of the 3 assays are between any two sediment samples. Figure 13 shows that the average N-cycling indicator profile of East Arm sediment samples (sites 78-79) clearly differed from all other samples. This was likely due to their consistently higher *nosZ*, *AOA1b* and *otu606* levels. Urban (sites 74-75, 92, 97) and neighbouring Charles Darwin National Park (sites 86-88) shared a more similar N-cycling indicator profile likely due to their lower *AOA1b* and medium to higher *nosZ* gene abundance. Sediment samples from Elizabeth River (sites 83-85, 91, 93, 98) showed the most consistent average profile i.e. smallest average cluster, which was indistinguishable from sediment samples from West Arm and Hudson Creek and also partially overlapped with samples from Myrmidon creek.

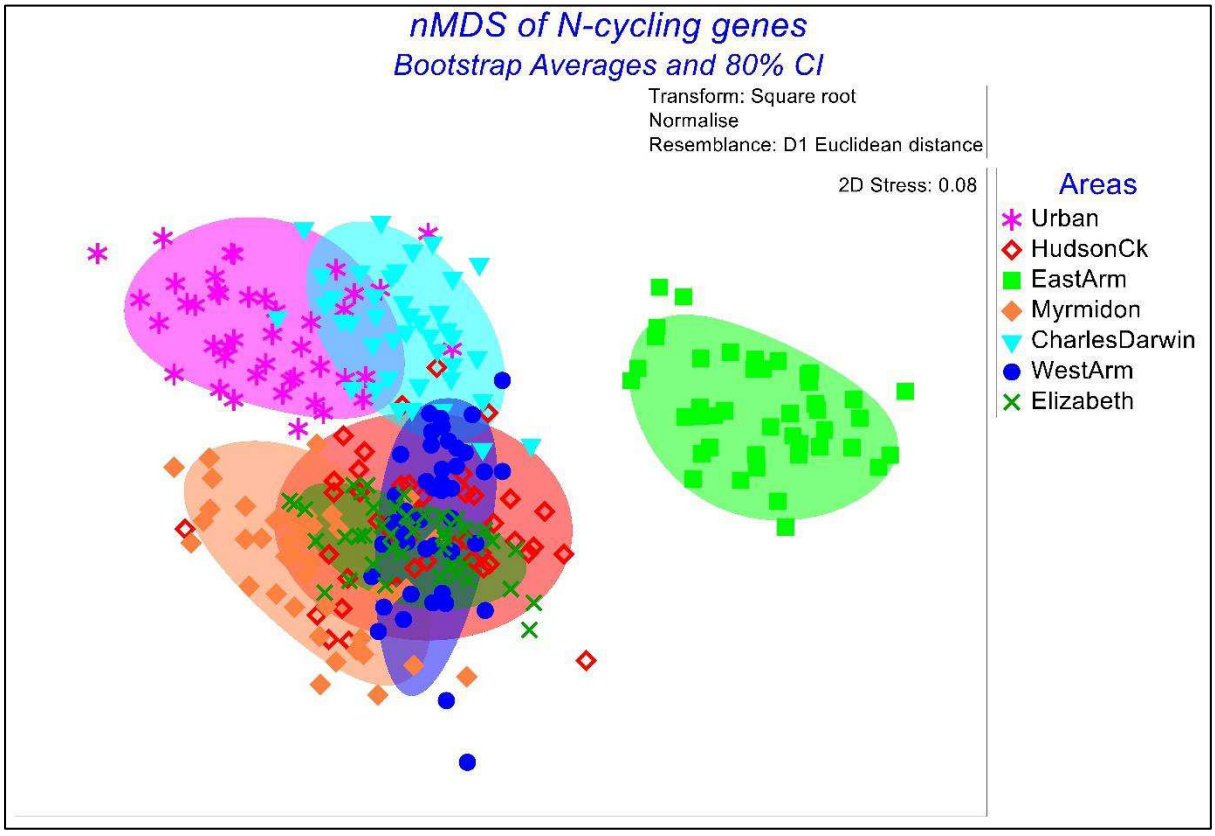


Figure 13: nMDS of the 3 N-cycling indicators across Darwin Harbour areas
The shades indicate the nMDS regions for which we are 80% confident that they contain the N-cycling indicator averages for the corresponding Harbour area. The dots mark 43 bootstrap averages per group. The closer the group shades, the more similar the N-cycling indicators are for these areas and the more apart the clusters, the more distinct the composite of the indicators in these estuaries.

The separation of sediment sa mples according to their N-cycling profile into East Arm vs Charles Darwin/Urban vs remaining samples was based on all sediment samples from both depths. This separation was less evident if only the shallow or only the deeper sediment profiles were compared.

3.7.1 The N-cycling indicator profile and nutrient fluxes

In a distance linear model exploring the association of flux data with the composite of N-cycling genes, silicate flux explained most and more than 10% of the variance in the N-cycling indicator profile of all sediment samples (P=0.003) (Table 3). Ammonia was the only other flux which explained more than 5% (P=0.007) while both, longitude and latitude also explained 5.5% each (P<0.01).

Flux data and latitude/longitude	Proportion	P value
Si	10.7%	0.003**
Lon	5.6%	0.003**
Lat	5.5%	0.009**
NH_4	5.1%	0.007**
DO	4.0%	0.026*
NO_X	3.4%	0.040*
PO_4	2.7%	0.088
DIC	1.7%	0.229
N_2	0.6%	0.711

Table 3: Variance explained of N-cycling indicator profile by fluxes
Marginal results of distance linear model.

The dbRDA in Figure 14 shows an association of the N-cycling indicator profile at some Hudson Creek sites with increased silicate sediment efflux. However, the first two dbRDA axes only explained 23.5% of the N-cycling profile i.e. the nutrient fluxes are not a good predictor for the N-cycling profile and combining the N-cycling profile with the flux data resulted in no clear clustering according to harbour area.

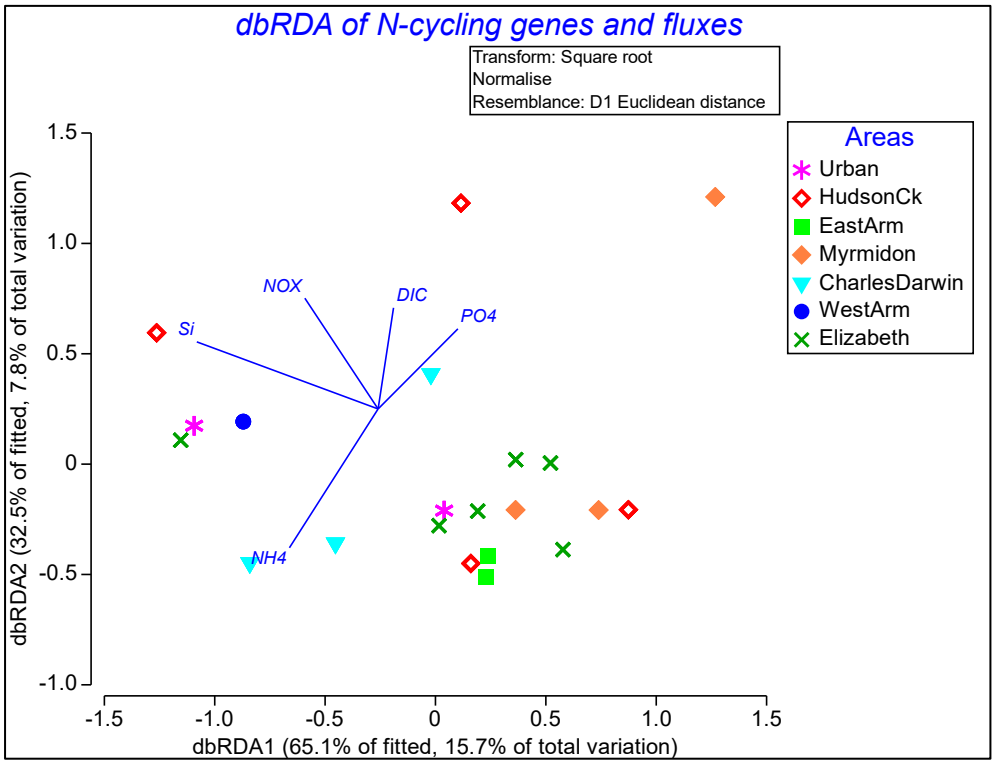


Figure 14: dbRDA of N-cycling indicator profile and nutrient fluxes

4 Discussion

4.1 The spatial variance of N-cycling indicators

One of the main objectives of this project was to assess whether the N-cycling indicator assays can reflect the trophic status of the sediment and thus, be used as a fast and relatively inexpensive tool to map nutrient load and ecosystem function across the harbour. While the first round of biological validation (Kaestli et al., 2018) had a wide range of sediments from oligo- to hypereutrophic sites from Shoal Bay to East Arm and from the dry and wet season, this round mainly contained meso- to oligotrophic sediment from a smaller area (i.e. East and West Arm in Darwin Harbour) and from the dry season only. Thus, we asked the question: can these assays differentiate between sediment from mainly oligotrophic sites?

We found that *nosZ* showed the clearest spatial pattern with consistent lower relative abundance in the upper estuaries compared to the mouths of the creeks. The upper estuaries also had higher DIC emissions and there was a negative correlation between *nosZ* abundance and DIC flux. DIC flux is a proxy for carbon loading, an important control on the efficiency with which microbes perform denitrification, i.e. carbon loading determines how efficient coastal sediments recycle nitrogen to N_2 gas (Eyre and Ferguson, 2009). As Eyre and Ferguson discuss, on either side of the denitrification efficiency optimum zone, there is a reduction in denitrification sites as the sediment loses its three-dimensional complexity. At low organic carbon loadings, a thick oxic zone with low macrofauna biomass exists, resulting in limited anoxic sites for denitrification, and at high carbon loadings, there is a thick anoxic zone and a resultant lack of oxygen for nitrification and associated nitrate production. Upper mangrove estuaries tend to be more densely vegetated with more detritus i.e. higher carbon loadings with higher C:N ratios and in the dry season less water flow would further contribute to anoxic conditions. Thus, low *nosZ* abundance might indicate a lack of available nitrates due to reduced oxic sediment pockets leading to low nitrification rates. We also found significantly higher DIC levels, more DO uptake and significantly more microbial DNA per gram sediment at these upper sites compared to the mouth of the creeks, all indicating more heterotrophic microbial activity and some of these microbes would also compete for the already sparsely available nitrates. High C:N ratios also favour DNRA over denitrification processes further contributing to lower relative *nosZ* gene abundances in upper estuaries (Giblin et al., 2013).

Spatial patterns for the other N-cycling indicators and sites were more diffuse. The urban sites, in particular RSET Sadgroves landward showed a very low relative abundance of all N-cycling indicators indicating low nitrification as well as low denitrification. This site also had the highest microbial DNA load per gram sediment, high DO uptake and high Si efflux indicating high microbial activity and likely high competition for nutrients but also high diatomaceous organic matter loadings and sediment anoxia inhibiting the coupled nitrification-denitrification process. An exception of the urban N-cycling profile was the stormwater drain at Dinah Beach, which had more nitrifying indicators. This site also had fewer microbes overall and high *nosZ* levels and high N_2 flux emissions indicating an active denitrifying microbial population matching N-cycling gene abundance and flux data.

The combination of N-cycling indicator assays improved the spatial resolution to a certain degree. The N-cycling profiles of the East Arm sites differed from all other sites and so did the urban and neighbouring Charles Darwin NP sites which shared a similar N-cycling profile. The East Arm

clustering was mainly driven by high *nosZ* gene levels while the urban and Charles Darwin NP sites shared the lowest *AOA1b* levels. None of the three assays showed the same level of area-specific clustering if analysed on their own. However, not even the combination of N-cycling assays succeeded in separating whole areas such as West Arm, Hudson, Elizabeth River and Myrmidon i.e. these showed all overall similar N-cycling profiles.

4.2 N-cycling indicators and sediment depth

In this study, we also differentiated between different sediment depths accounting for bulk sediment (0-10cm) reflecting many years of sedimentation, on-going degradation and long-term average nutrient levels compared to surface sediment (0-2cm) representing the active nutrient flux microbiota. Accordingly, our second main objective was to assess whether the N-cycling indicator assays differed between sediment depths. We found clear evidence for more *nosZ* genes in the shallow sediment compared to the bulk sediment while there was no difference in the *AOA1b* and *otu606* indicator levels. While denitrification deeper in the sediment contributes to on-going diagenesis of organic matter, our findings indicate that denitrification processes were more common in the sediment-water interface at the sites examined. Unless there is bioturbation such as through burrowing activity, a decrease in denitrification with increasing sediment depth could point to a decrease in available carbon and fewer oxic pockets for concomitant nitrification (Kristensen, 1988).

4.3 N-cycling indicators and nutrient flux

The third objective examined whether the N-cycling indicators measured in the surface sediment were associated with the nutrient flux data (as measured by core incubations). In contrast to the previous study on bulk sediment, we indeed found several significant associations such as a negative correlation between NO_x flux and *nosZ* gene abundance in the surface sediment. Thus, the more *nosZ* genes, the smaller the NO_x efflux suggesting that NO_x were indeed reduced to N_2 gas instead. There was also a significant positive association between *nosZ* and N_2 emission after accounting for sampling depth and spatial variance indicating net denitrification rates at sites with more *nosZ*. It was expected that both these relationships between nutrient fluxes and *nosZ* genes were considerably stronger or only evident in the surface sediment layer and less so in the bulk sediment.

The *nosZ* gene abundance showed a unimodal relationship with phosphate and silicate flux. For both, the relative *nosZ* abundance was highest at intermediate fluxes. Higher phosphate release could be due to sulphate reduction in anoxic sediments causing the release of phosphate bound to sulphides (L Radke, pers. communication). Similarly, high silicate fluxes indicate high diatomaceous organic matter breakdown and anoxic sediments with nitrification-denitrification coupling broken.

There was also a positive association between *AOA1b* genes and ammonia uptake. This association was mainly driven and evident in sediment from Myrmidon which had the largest ammonia uptake and largest *AOA1b* relative gene abundance suggesting active nitrification. Myrmidon also had similarly large *otu606* abundance and the largest NO_x emission.

The strongest association however was a negative correlation between *AOA1b* and silicate flux – the higher the silicate emission, the less *AOA1b* in the sediment. Silicate flux was also the only flux which explained more than 10% of the variance in the combined N-cycling indicator profile. Silicate flux reflects diatom activity (Haese et al., 2007). Diatoms are one of the main primary producers in benthic communities and thus, silicate flux also reflects input of labile diatomaceous organic matter into the sediments. When organic matter levels increase, degradation of organic carbon produces sub-oxic and ultimately anoxic conditions and inhibition of nitrification. To a lesser degree, the

silicate flux is also an indicator for benthic microalgal activity (BMA) which would compete with nitrifying bacteria for nitrogen containing nutrients.

4.4 How do these results compare to our previous findings?

The previous study on N-cycling indicators was conducted on a wide range of oligo- to hypereutrophic bulk sediments across the dry and wet season. The indicators differed between long-term eutrophic and oligotrophic sites with low N-cycling indicator levels in eutrophic sediment from Buffalo Creek. In this study we found consistent low N-cycling indicator levels at the urban RSET Sadgroves site which had an otherwise high microbial load, high levels of diatoms, high DIC efflux and high DO uptake reflecting anoxic conditions and conditions with DNRA favoured over denitrification.

In contrast, other mesotrophic sites such as the stormwater drain of Dinah beach with medium DIC efflux and microbial load still had low nitrification but high denitrification indicator levels (and concomitant high N_2 efflux) while Myrmidon creek with very low diatom levels and low DIC efflux, had low *nosZ* levels and matching low N_2 efflux but high nitrification indicator levels and concomitant high ammonia uptake and NO_x efflux. In the previous study, Myrmidon also showed near-zero N_2 flux in the dry season (in contrast to the wet season) matching findings of this study which was also conducted in the dry season.

The distinct difference in *nosZ* gene levels at the mouth vs upper estuaries is a new finding of this study with no such sites investigated in the previous report.

In the previous study, the composite of N-cycling indicators further improved the explanatory power of the assays for the level of human impact. In this report, we focused on sites in East and West Arm of the harbour and found that the assays had insufficient resolution to differentiate areas in this part of the harbour. This also matches our previous work which found that the microbiota differed less between mainly oligotrophic sites in East Arm compared to Shoal Bay (Kaestli et al., 2017).

In the previous study on bulk sediment, the N-cycling indicators were not a useful surrogate for N fluxes with no correlation between any of the indicators and fluxes. In this report, we differentiated between surface and bulk sediment and we indeed found several associations between N-cycling indicators in the surface sediment and benthic fluxes such as between *nosZ* and N_2 (positive association after accounting for spatial variance) and NO_x fluxes (negative association). There was also a negative correlation between nitrification indicator *AOA1b* and ammonium flux although this relationship was mainly driven by one extreme site in Myrmidon creek with large *AOA1b* levels at that site and the largest ammonia uptake. This site is close to a side creek which receives treated effluent from the nearby Palmerston waste water stabilisation ponds.

One of the main findings of this work i.e. a strong negative association between AO1b and silicate flux, is a new finding as silicate flux was not measured in the previous study.

5 Conclusions

This follow-up study added significant value to the previous report on the development and validation of microbial N-cycling indicator assays to evaluate sediment ecosystem function. While the assays in this project did not have sufficient resolution to differentiate most areas in East and West Arm based on their sediment nitrogen cycling gene profile, this was likely due to the similar and mainly oligotrophic nature of the sediment in this area. Nevertheless, we found several clear patterns in the relative abundance of the N-cycling indicators, in particular for the denitrification gene *nosZ.* These included a significant difference in abundance between bulk and surface sediment and between upper estuaries and the mouth of creeks. This likely reflects carbon loading and availability of oxic as well as anoxic sediment pockets for coupled nitrification-denitrification. The relative abundance of *nosZ* genes in the surface sediment was also associated with N_2 and NO_x fluxes and high levels of the nitrification target *AOA1b* matched high ammonia uptake.

The strongest pattern however relates to the negative correlation of nitrification genes and input of diatomaceous organic matter causing anoxic conditions.

In summary, the N-cycling indicator assays show promise as fast monitoring tools to differentiate between sediment with functional nitrification-denitrification processes and sediment with low nitrification or denitrification rates such as due to extremes of carbon loading.

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7 Appendix

7.1 Primer and probe sequences

AOA1b

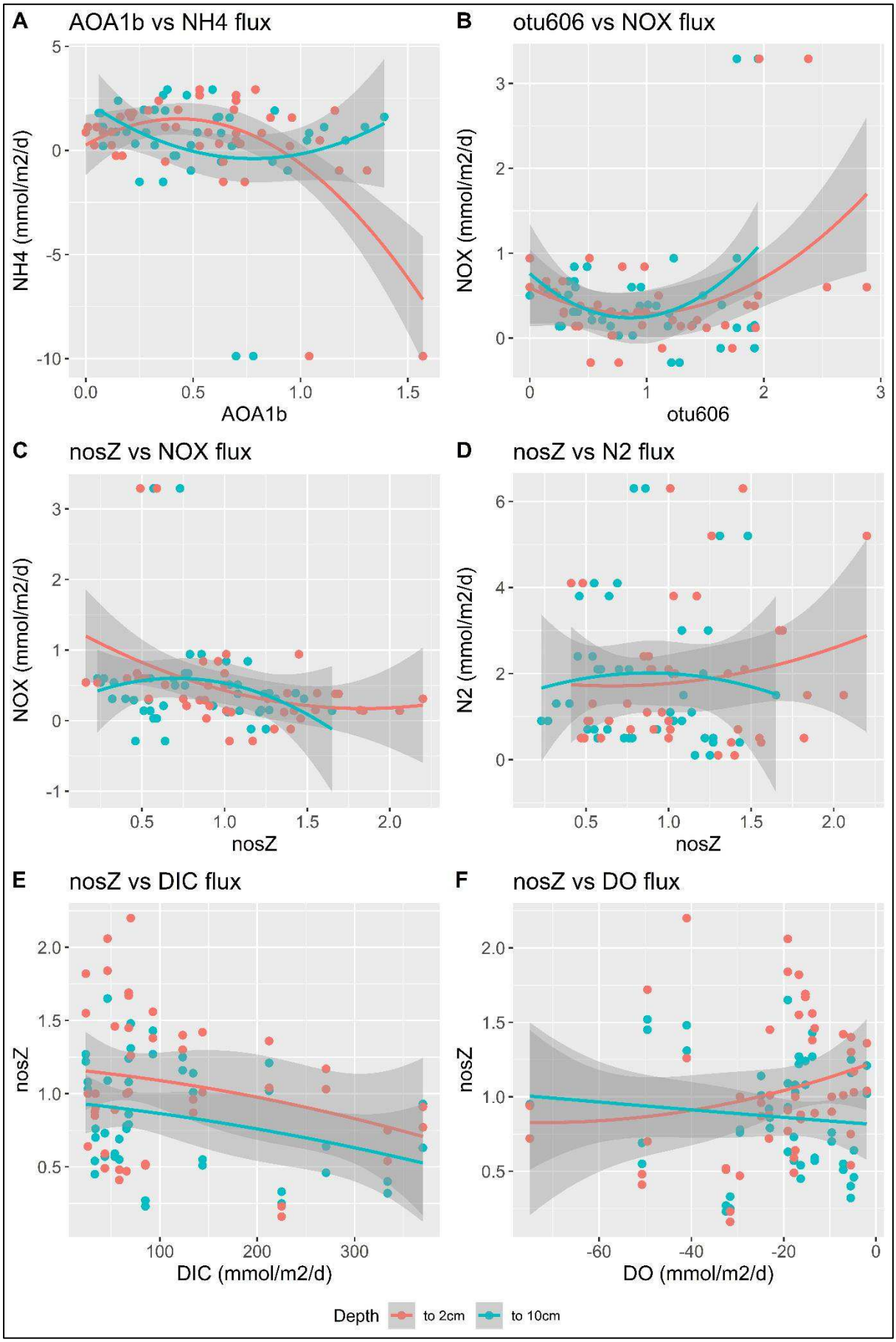
Name	Sequence
F_AOA.01b	CTGGGCTTGGACTTCGTACAC
P_AOA.01b	ATCGCAAACGTTGATGCTAATTGTGGG
R_AOA.01b	GCCTGGAACGCCTGTAAATG

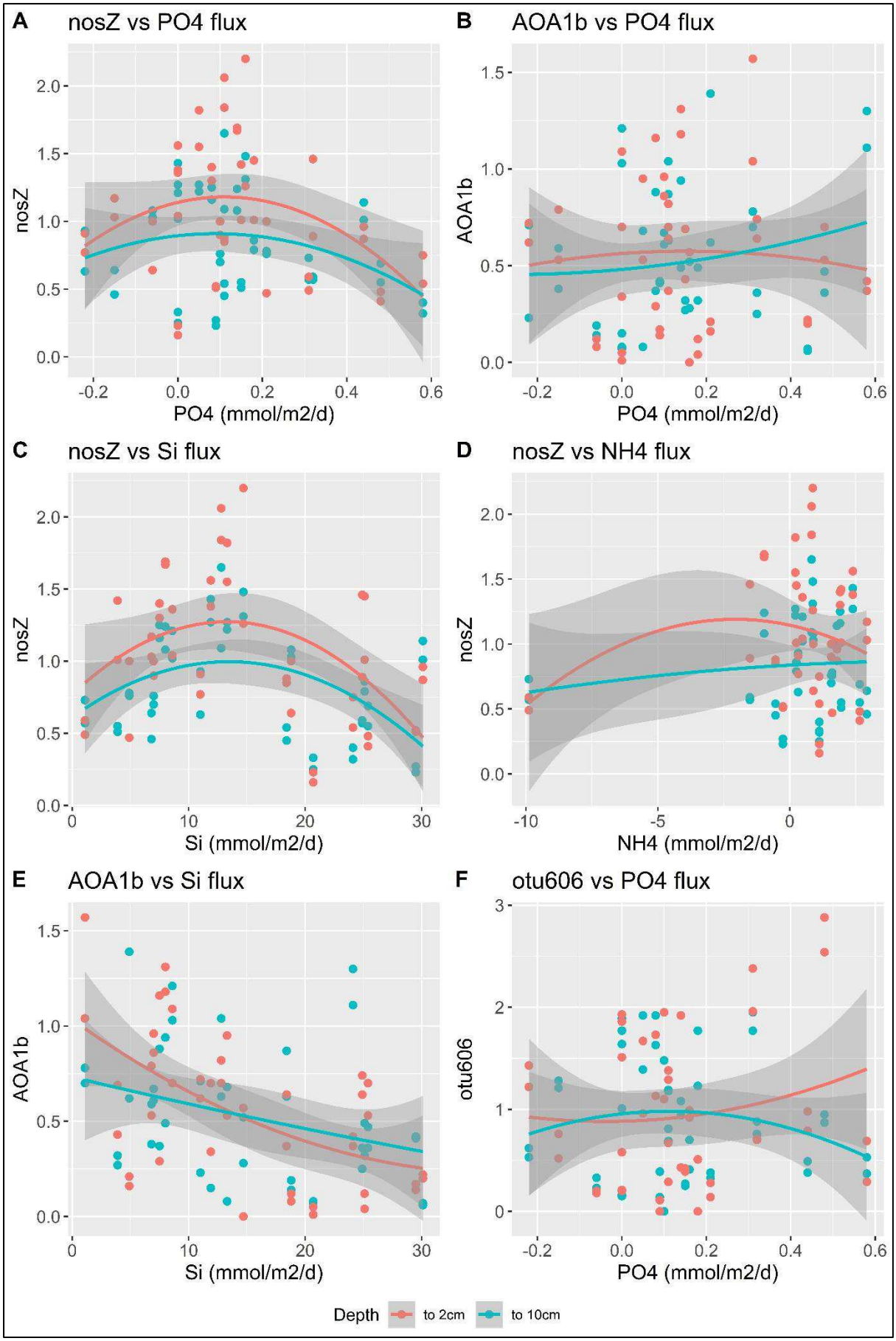
OTU606

Name	Sequence
F_*otu606*	GAAGGCCGGTGGTGAAGAC
P_*otu606*	CCTCTGGGCAATGACTGACGCTG
R_*otu606*	TCGCCACCCACACCTAGTAC

7.2 The N-cycling indicator abundance at all sites

7.3 Scatter plots of N-cycling indicators and nutrient fluxes




A
nosZ vs PO4 flux
nosZ
PO4 (mmol/m2/d)
B
AOA1b vs PO4 flux
AOA1b
PO4 (mmol/m2/d)
C
nosZ vs Si flux
nosZ
Si (mmol/m2/d)
D
nosZ vs NH4 flux
nosZ
NH4 (mmol/m2/d)
E
AOA1b vs Si flux
AOA1b
Si (mmol/m2/d)
F
otu606 vs PO4 flux
otu606
PO4 (mmol/m2/d)
Depth
to 2cm
to 10cm

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