Environmental Chemistry and Microbiology Unit (ECMU)
Research Institute for the Environment and Livelihoods Darwin, NT 0909
Australia.

Developing quantitative PCR assays to target microbial nitrogen cycle genes: A new tool to monitor ecosystem function in sediment.

CHARLES DARWIN UNIVERSITY

Prepared for:
The INPEX-operated Ichthys LNG Project
Aquatic Health Unit, Northern Territory Department of Environment and Natural Resources and Power and Water Corporation

By
Dr Mirjam Kaestli, Prof Karen Gibb and Julia Fortune
March 2018

Document history

Version	Date	Author/Prepared/Edited by:	Issued to/reviewed by:
1	29 June 17	Mirjam Kaestli, Julia FortuneJulia Fortune	Karen Gibb
2	30 June 17	Mirjam Kaestli, Julia Fortune, Karen Gibb	Simon Townsend
3	4 Sept 17	Karen Gibb, Mirjam Kaestli	Lynda Radke, Simon Townsend
4	1 Nov 17	Mirjam Kaestli, Julia Fortune, Karen Gibb	Lynda Radke, Simon Townsend
5	20 Nov 17	Mirjam Kaestli, Julia Fortune, Karen Gibb	INPEX
6	12 Mar 18	Mirjam Kaestli, Julia Fortune, Karen Gibb	Final version

Funding acknowledgements

This research was funded by the INPEX-operated Ichthys LNG Project and was supported by the Power and Water Corporation. The project was administered by the NT Government. This sediment quality program represents the first systematic sediment monitoring program for Darwin Harbour.

Executive summary

The aim of this project was to develop and validate quantitative polymerase chain reaction (qPCR) assays targeting microbial nitrogen genes as a tool for monitoring ecosystem function of sediment.

The activity described in this report was developed from initial research co-funded by Power and Water Corporation (PWC), Northern Territory Government (NTG) and INPEX. The initial study showed that some bacterial nitrogen cycling genes (functional markers) detected in sediment and water of Darwin Harbour were different between human impacted and reference sites. These included *nosZ* genes associated with denitrification (the removal of nitrogen or nitrogen compounds that results in the escape of gaseous nitrogen), archaeal *amoA* genes associated with ammonia oxidation (the biological oxidation of ammonia or ammonium to nitrite), and nitrite oxidation genes.

An unknown was whether these high throughput and relatively inexpensive tests would be sufficiently sensitive and robust to use in sediment across the harbour and whether they were able to distinguish between impact and reference sites. Another unknown was how they compared to conventional N flux assays. The sediment quality subprogram under the Integrated Marine Monitoring and Research Program (IMMRP) in partnership with NT Government and INPEX provided an opportunity to address these gaps.

The N gene tests were based on bulk-sediment samples which potentially reflected many years of sedimentation and were more site-specific (impacted vs reference) than nutrient flux, while the latter showed high variability dependent on abiotic conditions encountered at the time of sampling and reflecting the heterogeneous nature of sediment with anoxic and oxic micro-niches in close proximity.

The microbial N-cycle gene qPCR results 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.

The N gene qPCR results also indicate that nitrogen cycling monitoring should target sediment and not water – this supports the accepted view that sediment plays a key role in nitrogen cycling in estuaries.

Denitrification efficiency (DE%) as measured by conventional nutrient flux is known to be depressed at impacted sites. We found that these sites also had less *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.

The archaeal ammonia oxidizing (AOA) qPCR test showed significantly less signal in highly impacted samples and ammonia oxidizing archaea have been shown to be adapted to nutrient-limiting conditions.

There was a strong association between nitrifying bacteria (the otu606 qPCR test for nitrite oxidizing bacteria) and reference samples with a clear distinction in qPCR results between impacted and reference sites. These nitrifying bacteria have been found to decrease under anoxic conditions in marine sediment.

More research is needed to fine-tune these assays and design additional assays from a pool of N-cycle genes which showed a significantly different abundance between impact and reference sediment in the initial experiment. More temporal and spatial data is needed from Darwin Harbour sediment to conduct an in-depth biological validation on the usefulness of these assays, and to refine the sampling depths to make them more consistent with those contributing to benthic fluxes.

We found that the composite of qPCR assays further improved the explanatory power of the assays for the level of impact or site and the combination of assays could provide a valuable cost-effective tool to map zones of impact in Darwin Harbour at higher spatial resolution than could be practically achieved using benthic flux measurements.

Contents

Acronyms

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
ARC-LP	Australian Research Council Linkage Project
bp	DNA nucleotide base pair
Ct	Cycle threshold
dbRDA	distance-based redundancy analysis
DE	Denitrification efficiency (nitrate to nitrogen gas)
DNA	Deoxyribo-nucleic acid
DNRA	Dissimilatory Nitrate Reduction to Ammonia
FDR	False Discovery Rate method to adjust P values accounting for multiple testing
LSWTP	Leanyer Sanderson Wastewater Treatment Pond
N_2	Nitrogen gas
NH_3	Ammonium
NO_2^-	Nitrite
NO_3^-	Nitrate
nosZ gene	Gene encoding nitrous oxide reductase (N_2O to N_2) in denitrification
OTU	Operational taxonomic units
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

List of figures

List of tables

Objectives

The aim of this project was to develop and validate quantitative PCR assays targeting microbial nitrogen genes as a tool to monitor ecosystem function.

1. Develop quantitative PCR (qPCR) assays targeting microbial genes encoding enzymes which drive the N-cycle. These qPCR assays measure the abundance of these N-cycle genes in sediment. A focus will be on those N-cycle genes whose abundance significantly differs between effluent impacted and reference sites in Darwin Harbour. A technical validation will be conducted to assess the suitability of the qPCR assays to correctly measure the abundance of the targeted N-cycle gene targets.

2. Collect sediment at impacted and reference sites and measure nutrients, N-genes using qPCR and N-species flux using chamber studies.

3. In a biological validation, compare the two sets of results (N-flux and qPCR on N-gene abundance) to determine whether the qPCR assays are an appropriate surrogate for N-flux and if it is a useful tool to measure ecosystem function in sediment.

1. Background

1.1 The sediment quality subprogram

Projects under the sediment subprogram theme of the Integrated Marine Monitoring and Research Program (IMMRP) have focussed on understanding sediment-metal dynamics in mangrove sediments. The subprogram also includes an assessment of ecosystem function using microbial nitrogen genes (N-genes). This report details the key outcomes of the development and validation of quantitative PCR assays that target microbial N genes. And discusses how this work can be advanced and considered in the design of a long-term monitoring program.

The specific aim of this project was to develop and validate quantitative PCR (qPCR) tests for microbial nitrogen genes as a tool to monitor ecosystem function. Research (2013-16) co-funded by PWC, NTG and INPEX showed that some bacterial nitrogen cycling genes detected in sediment and water were different between impacted and reference sites. This result provided field evidence that this approach had merit and lead to further work to identify which genes in particular should be developed further for routine assessment (Kaestli et al., 2017; manuscript in preparation). An unknown was whether these high throughput and relatively inexpensive tests would be sufficiently sensitive and robust to use in sediment across the harbour and how the results compared to the more conventional but much more time-consuming N flux assays.

1.2 What is a qPCR test?

A qPCR is used to detect a specific DNA target in a sample and also to determine the amount of the DNA compared to a standard (Figure 1).

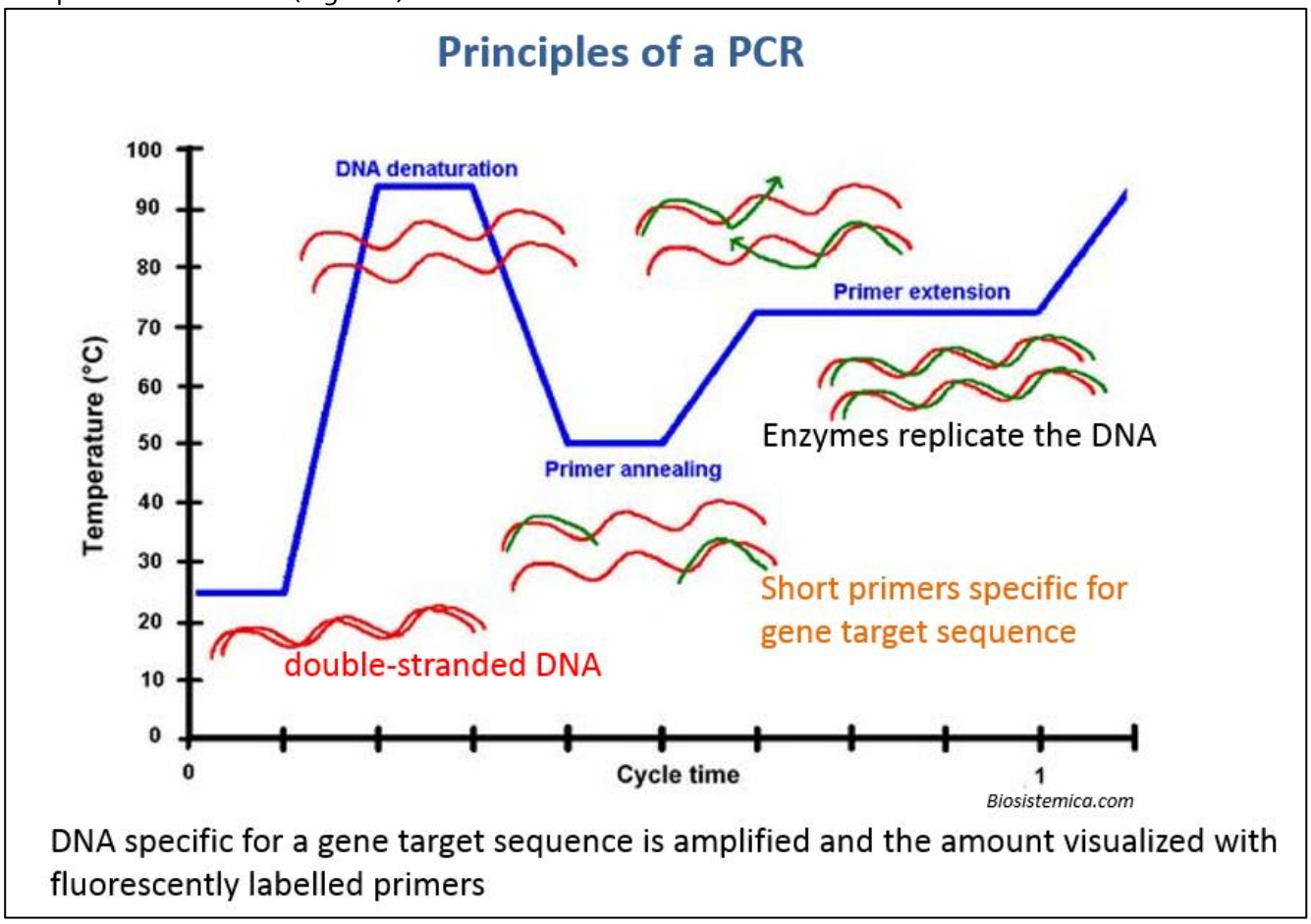


Figure 1: Principles of a PCR

1.3 DNA-based monitoring tools and microbes as bio-indicators

Ecosystem based approaches to marine monitoring are driving a need for efficient, low-cost bio-indicators of marine ecological quality. Microbes drive nutrient cycles but are not amenable to visual inspection and thus are largely excluded from detailed inventories. However new molecular based assessments of biodiversity and ecosystem function offer advantages over conventional methods and are becoming the norm for monitoring ecosystem health (Kopf et al., 2015, Karsenti et al., 2011; Bork et al., 2015; Gilbert et al., 2014; Duffy et al., 2013; Muller-Karger et al., 2104; Zampoukas et al., 2014.). These molecular methods promise to improve assessment by increasing the scope, depth and throughput of information and by reducing costs and reliance on specialised taxonomic expertise. The other attraction is that marine microbe communities can evolve rapidly in response to environmental shifts and can be used as indicators of change. In fact, marine microbes are considered sound 'early warning' indicators for the marine environment.

Inclusion of the assessment of biodiversity and function using sequencing approaches should add considerable value to a program, allowing a more holistic ecological assessment from a functional and taxonomic perspective. The up and down regulation of genes as a consequence of anthropogenic stress have been documented (Thureborn et al., 2013; Chen et al., 2013; Tacao et al., 2012).

In areas of more acute exposure (WWTP/point sources) the use of the DNA based surveillance will permit a comprehensive understanding of nitrogen cycling and functional response to measurable impacts such as eutrophication. The application could help 'map' out broader scale functional response such as across impact gradients to determine the extent of influence. One of the big sticking points is finding N-probes that are meaningful and relevant in the chosen context.

1.4 Why target the nitrogen cycle?

Darwin Harbour is a tropical estuarine ecosystem 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. Effluent contributes 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, contributes 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 (Burford et al., 2008; Butler et al., 2013; Aquatic Health Unit, 2015).

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 2). Nitrogen is essential in supporting primary productivity but too much nitrogen or poor removal can result in eutrophication compromising water quality. Denitrification efficiency (DE) is reduced in localised areas impacted by elevated nutrient loads (Smith et al., 2012). The ability to measure denitrification as a key process of nitrogen removal in Darwin Harbour would provide valuable insight into nitrogen processing and assimilatory capacity. 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. This research project will help to advance our understanding of nitrogen processing and cycling in intertidal sediments. Simple PCR assays that measure appropriately researched and validated probe targets will provide a

breakthrough in sediment function assessment. The validation of these assays in comparison to biogeochemical (nutrient) flux data will enable evaluation of this monitoring tool.

1.5 Microbial nitrogen cycle genes and N transformations

Benthic flux studies are commonly used to measure productivity and N-cycling processes but these studies are time consuming, labour intensive and need expert knowledge (see next section). Currently these approaches do not permit routine landscape scale assessment of nitrogen removal. We need new tools to measure biogeochemical processes like DE in sediments so we can monitor nitrogen removal in Darwin Harbour, particularly while it is still relatively undisturbed. Benthic flux studies rely on the fact that microbes transform nitrogen (Figure 2). An alternative approach is to directly measure the microbe functional genes. These microbes include both taxonomic kingdoms of prokaryotes, bacteria as well as archaea. In addition, microbes are good biological indicators to measure water or sediment health, as they rapidly respond to change, and are ubiquitous and abundant.

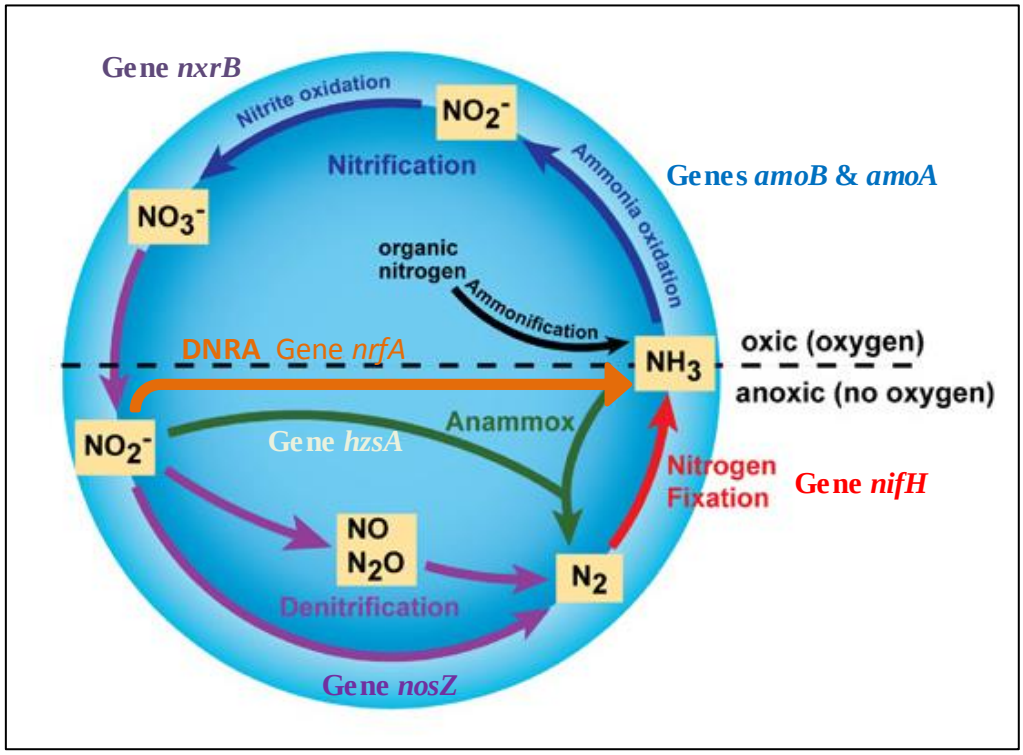


Figure 2: The Nitrogen (N)-Cycle showing microbial genes that drive steps in the transformation of N.

Recent innovations in ecosystem function research include functional gene analysis using DNA extracted directly from water or sediment. While these functional gene tests are technically straightforward to develop, there are few reports in the literature that show these DNA functional gene tests perform well in the field, and can be correlated to more familiar measure of ecosystem function such as nutrient flux assays. Nitrogen-cycle processes of interest in this study include nitrification and denitrification.

Nitrification is the oxidation of ammonia to nitrates. It is 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. 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 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.

Denitrification is an anaerobic process transforming nitrates back to dinitrogen gas (N_2). It is an essential step in wastewater treatment plants to remove excess nitrogen and several microbial genes are involved in the process including the *nosZ* gene which encodes the nitrous oxide reductase enzyme.

$$\underset{\text{Nitrate}}{NO_3^-} \rightarrow \underset{\text{Nitrite}}{NO_2^-} \rightarrow \underset{\text{Nitric Oxide}}{NO} \rightarrow \underset{\text{Nitrous Oxide}}{N_2O} \rightarrow \underset{\text{Nitrogen gas}}{N_2}$$

Denitrification is dependent on the availability of organic carbon and is tightly coupled to nitrification providing nitrates. 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.

1.6 Prior research - N-cycle microarray

The work reported here follows on from an ARC-L project "Microbiology of a tropical creek impacted by treated sewage effluent: Novel impact assessment methods using N-cycle functional markers and changes in community composition" (ARC L-P; Partners: PWC, INPEX-operated Ichthys LNG Project, NT Government Dept of Business, Department of Environment and Natural Resources). In the ARC L-P, 494 water and sediment samples were collected from Darwin Harbour in 2013 and 2014 and the bacterial community composition was compared between samples impacted by treated sewage effluent, urban runoff and reference samples with less anthropogenic impact. This work has recently been published in the peer-reviewed international journal Frontiers in Microbiology (Kaestli et al., 2017).

As part of this ARC L-P, the diversity of microbial N-cycle genes was measured in these samples using a novel N-cycle microarray developed by the research group of Lev Bodrossy at CSIRO Hobart. See Figure 3 for background information about the microarray technology.

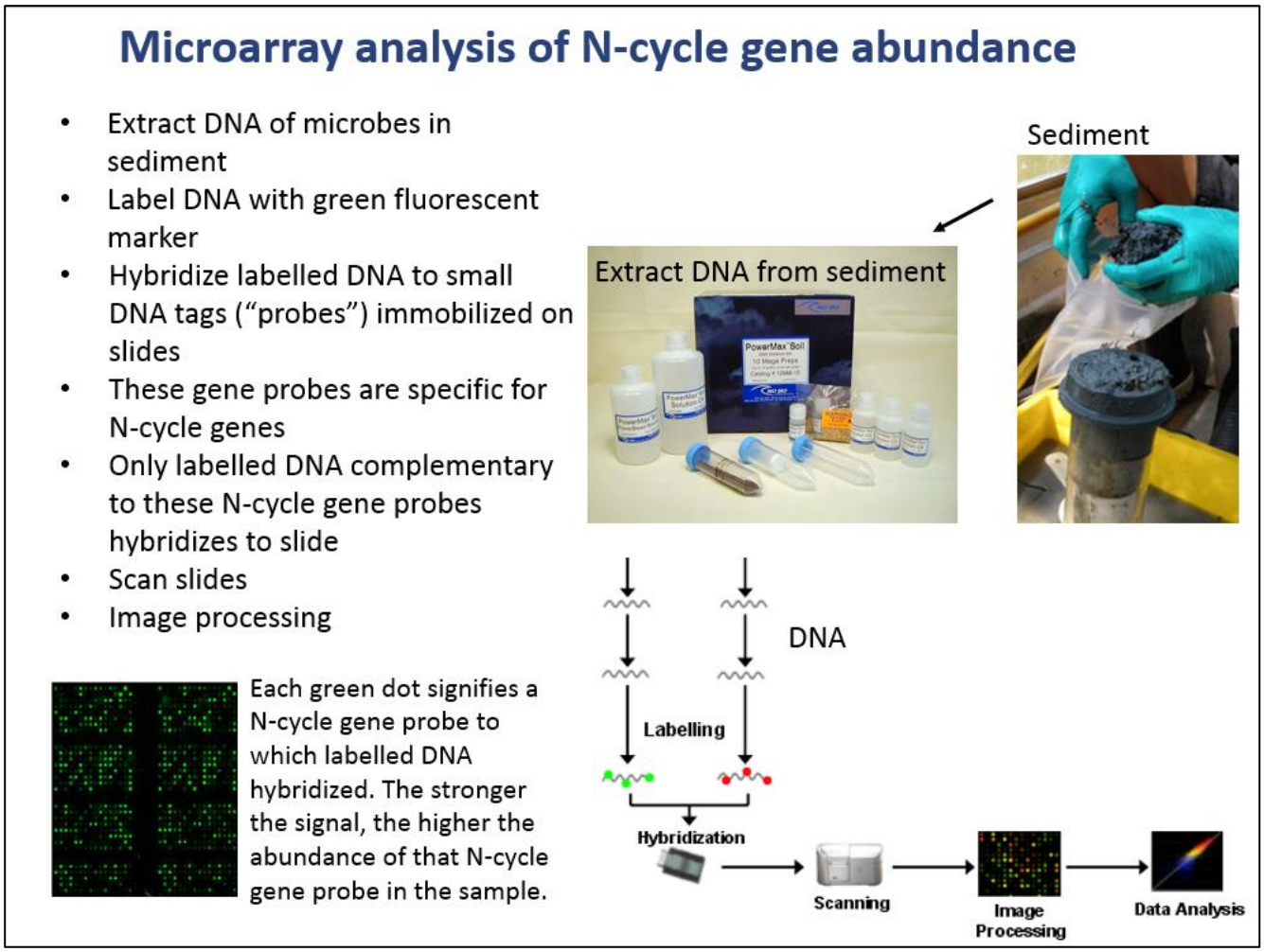


Figure 3: What is a microarray

The output of a microarray is often displayed in form of a heatmap (Figure 4), which shows which gene probes were found in a sample. The signal strength of the probe provides a guide to the relative amount of that DNA tag in a sample. Figure 4 shows the output of the ARC-LP microarray experiments as a heatmap. N-cycle gene signals (or probes) were detected in 174 sediment samples. Blue-coloured probes indicate no signals of the corresponding N-cycle probes while red and pink-coloured probes indicate a high abundance of these N-cycle genes in the corresponding sediment samples. These data allowed us to choose the N-gene probes that showed differences between impacted and reference sites and which formed the basis of the N-gene tests described in this report. As shown in Figure 4, there is also potential to choose probes for several steps in the N-cycle: nitrification ie ammonia oxidation (bacterial or archaeal *amoA*) and nitrite oxidation (*nxrB*), anammox (anaerobic ammonia oxidation) (*hzsA*), nitrogen fixation (*nifH*), denitrification (*nosZ*) and dissimilatory nitrate reduction to ammonia (*nrfA*).

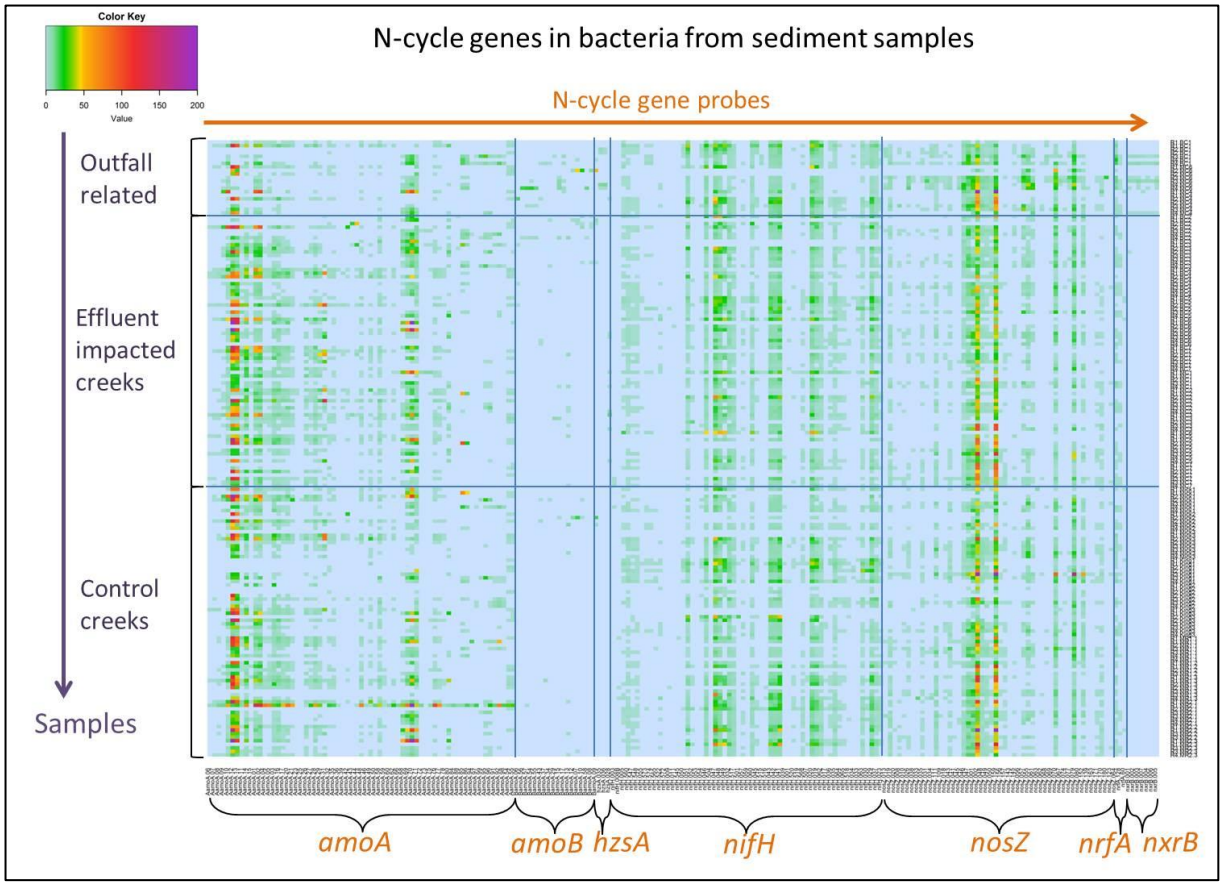


Figure 4: Heatmap of N-cycle gene abundance in sediment of Darwin Harbour

1.7 Benthic nutrient flux data

A benthic nutrient flux is the amount of a nutrient released or taken up by a unit area of sediment over a specific time interval. Benthic chambers, which sit on the seafloor, are the preferred method to measure benthic fluxes (Santschi et al., 1990; Nicholson and Longmore, 1999). However, these systems are expensive and difficult to deploy in extreme macro-tidal systems such as Darwin Harbour. Instead, *ex situ* core incubation experiments proffer a cost effective and widely used approach to measuring benthic fluxes, and have the added benefit of allowing for replication. While core incubations add layers of complexity and handling time, it is invaluable to have conventional flux measurements against which to compare the more novel N gene approach. The fluxes presented in this report proffer a first order estimation of N and metabolic processes across starkly different tidal creek systems of varying nutrient status.

1.8 Conceptual Overview of Study Approach

Figure 5 shows a conceptual overview of the study approach. The steps in the process are summarised below.

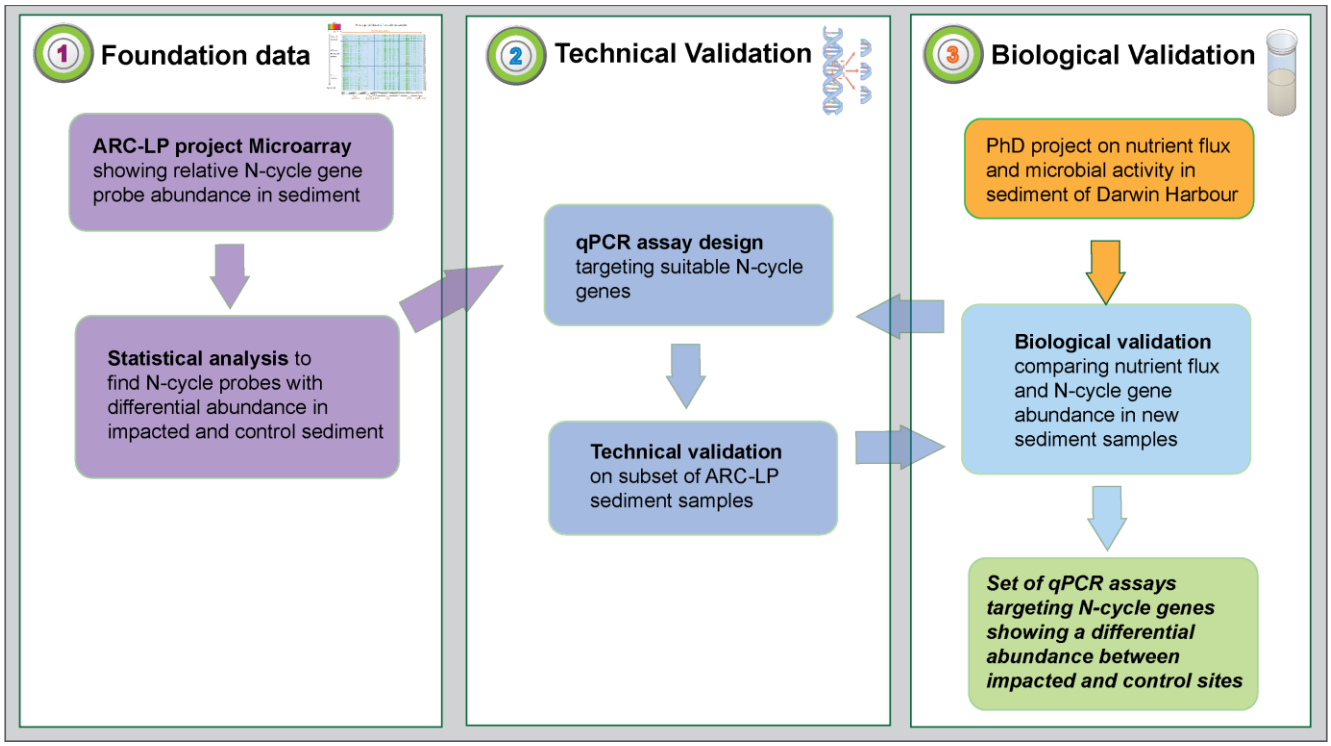


Figure 5: Flow chart of study approach

N-cycle gene microarray data obtained in the ARC L-P study provided the framework for this study. The data provided a first insight into the temporal and spatial distribution of N-cycle gene probes i.e. N-cycle microbial DNA tags in sediment of Darwin Harbour.

Statistical analyses including an indicator value analysis and a binomial model were used to find those N-cycle gene probes which showed the best differential abundance between sites impacted by treated effluent compared to sites from reference creeks with less human impact.

qPCR assays were designed using bioinformatics online tools focusing on the subset of N-cycle gene probes that showed the best differential abundance between impacted sites and those with less human impact.

Assays were first validated using a subset of ARC-LP sediment samples. These were the same samples that were used in the microarray experiment. The aim of this **technical validation** was to verify whether the assays replicated the differential abundances seen in the corresponding N-cycle gene probes of the microarray experiment. For this reason, samples were grouped according to impact level, as they were for the statistical analysis, and not according to creek.

For the **biological validation**, the microbial N-cycle gene abundance of sediment was compared to the results of nutrient flux experiments conducted on the same samples. These samples were grouped according to creek (except not for Buffalo Creek which showed a strong gradient from high human impact upstream to less impact at the mouth) to test the resolution of the assays on new sediment samples from across Darwin Harbour.

This process of designing qPCR assays based on a pool of suitable N-cycle gene probes followed by a technical and biological validation is repeated until a **set of qPCR assays** is ready and suitable to monitor impact in sediment.

2. Methods

2.1 Field Methods

2.1.1. Field methods for the technical validation

All field methods for the collection of sediment used for the microarray experiment in the ARC-LP and technical validation are described in Kaestli et al. (2017). In summary, sediment was collected from 30 sites in Shoal Bay and East Arm. For Shoal Bay, seven sites were chosen along the impacted Buffalo Creek while three sites each were located in two reference creeks, Micket and King Creeks. The distance between the mouth to the effluent outfall or most upstream site for the reference creeks was similar i.e. 4.6 – 4.8 km for all three creeks. Similarly for East Arm, there were seven sites along the impacted creek (Myrmidon Creek) as well as three sites each from two reference creeks (Short and Reference Creeks). A further site was chosen in this tributary 400 m downstream from the outfall and 200 m upstream from the Myrmidon main channel. Sediment was collected in duplicates with the help of a corer targeting the top 10 cm. All samples were kept on ice and processed within six hours of collection by aliquoting 7 g from the centre of each core into a tube followed by freezing until DNA extraction.

2.1.2. Study sites for the biological validation

Sites were two tributaries of Shoal Bay to the east of Darwin Harbour and two tidal creeks of the Elizabeth River estuary (Figures 6-7). Sites were chosen to represent a range of trophic conditions from oligotrophic to hypereutrophic to acquire an understanding of the likely extremes in nutrient loading and benthic fluxes. The tidal creek systems are typical of many tributaries of Darwin Harbour with margins fringed by dense mangrove forest. Mangrove habitats in the region are ecologically significant and provide a variety of ecosystem services, including acting as a sink and source particularly for nutrients and metals. Mangroves and intertidal zones cover a large proportion of Darwin Harbour and are known to contribute significantly to benthic and pelagic productivity (Burford et al., 2008; Alongi, 2002; Smith et al., 2012).

The following sites were chosen as they represent a range of trophic conditions in Darwin Harbour:

1. Buffalo Creek (Figure 6) is a hypereutrophic tidal creek that receives approximately 6,800 ML of treated wastewater per annum. A clear concentration gradient has been reported previously (Drewry et al., 2010; Smith et al., 2012) with a median NH_4^+ concentration of 857 µg/L and median NOx values of 31 µg/L (Fortune, 2015). Similarly, algal biomass (as Chlorophyll-a) is high in the creek with values that can exceed 150 µg/L in the upper to mid sections. The system is also subject to localised anoxia. This is particularly pronounced in the upper reaches during the slack of neap tides, and at the sediment-water interface.

2. Myrmidon creek (Figure 7) is consistent with a mesotrophic tidal creek where approximately 3,600 ML of treated wastewater enters this creek system in most years (Power Water Corp pers comm). Although nutrients and algal biomass are considered elevated at the discharge point of the Palmerston Treatment facility, the dense mangrove system which the discharge emanates through prior to reaching the tidal creek possibly provide some mediation. The discharge appears to be largely diluted by the physical forces of tide in this open creek whereby the detection of sewage is negligible at the creek's confluence of the Elizabeth River estuary (Drewry et al., 2010; DLRM, 2014).

Figure 6: Area 1. Buffalo Creek, Micket Creek and King Creek (control) sites.

Figure 7: Area 2. Myrmidon Creek, Reference Creek and Short Creek (control) sites.

3. Micket and King Creeks (Figure 6) represent typical oligotrophic tidal creeks of the Shoal Bay region. Shoal Bay is a broad shallow coastal embayment where tidal creeks from the Howard and Micket catchments flow into the bay. Micket and King Creeks are two of the three tidal creeks which flow into the embayment. Major flows from Howard River system via Hope inlet also flow into Shoal Bay delivering continued dry season freshwater flows from the Howard Springs aquifer.

4. Reference Creek and Short Creek (Figure 7) are typical oligotrophic tidal creeks of the Elizabeth Estuary in Darwin Harbour. These systems represent reference tidal creeks for Myrmidon Creek (Mesotrophic) and are currently not subject to point source inputs.

Naming conventions, site coordinates and impact category are shown in Table 1.

Table 1. Site location and sampling year.

Station	Dry 2015	Wet 2016	Short name	Lat	Long	Condition
SITE 1	28/7/15	31/3/16	Buff Ck 1 (BC1)	-12.35887	130.91054	Hypereutrophic
SITE 2	28/7/15	31/3/16	Buff Ck 2 (BC2)	-12.34738	130.90974	Eutrophic
SITE 3	9/7/15	17/2/16	Micket*	-12.36251	130.93801	Oligotrophic
SITE 4	9/7/15	17/2/16	King*	-12.37464	131.01808	Oligotrophic
SITE 5	23/7/15	17/3/16	Myrmidon	-12.50365	130.94893	Mesotrophic
SITE 6	23/7/15	17/3/16	Short Ck*	-12.5171	130.95342	Oligotrophic
SITE 7	23/7/15	17/3/16	Ref Ck*	-12.5507	130.94033	Oligotrophic

*Indicates control sites.

Samples were collected during the dry season of 2015 and wet season 2015-16. Each sampling period lasted 4-5 days around neap tides. Macrotidal conditions limited boat access and the sampling sites and times were chosen based on those areas that were accessible in practice.

2.1.3 Field sampling for the biological validation

Core incubation set-up and initial sampling commenced in May 2015 as part of a pilot study to assess the utility of the approach and streamline the methods in preparation for a comprehensive assessment at a harbour-wide scale. Following this small pilot project a number of sites were chosen for benthic flux measures and DNA was also extracted from these samples as part of the validation of qPCR assays. Sediment samples for DNA extraction for the biological validation were collected the same way as the samples for the technical validation i.e. by using a corer and targeting the top 10 cm of the sediment. 7 g of sediment were aliquoted within hours of collection and frozen until processed for DNA extraction using the PowerMax Soil DNA Isolation kit (MoBio, Carlsbad, CA, USA) following the manufacturer's instructions. DNA eluent in 5 mL were precipitated and eluted in 100 µL before further processing as described in (Kaestli et al., 2017). The water physico-chemistry of corresponding water samples is listed in the Appendix (A4).

2.2 Benthic Flux Determination for the Biological Validation

2.2.1 Benthic flux incubations

Three replicate cores (Figure 8a) were taken from each site. Cores (7 cm id, 35 cm long) were pressed into the sediment, capped and slowly withdrawn, a plastic plug was then placed in the bottom of the core, before they were returned to the surface. The cores were then returned to the laboratory within 2.5 hours, where they were then placed in a water bath and kept at in situ temperature (Figure 8b).

Water column samples in cores were processed using standard methods (Dalsgaard et al., 2000; Cook et al., 2004). Cores were left to equilibrate for several hours in a water bath while being aerated and stirred. Water temperature was maintained at ambient in-situ field temperatures. Most incubations were maintained at 25-26˚C in line with field measurements for dry season and up to 31.5˚C for wet season conditions. The overlying water was then replenished with harbour water, the cores were then capped and flux measurements commenced. Measurements were taken at ambient light levels in the laboratory or with supplementary lights (LED) elevating light to 150 µmol m^{-2} s^{-1} for some incubation treatments. Light did not reach the sediments for Buffalo Creek sites during sampling and therefore only dark incubations were performed for these sites. A similar approach was taken for dry season sampling at Micket and King Creeks. Samples were taken from the overlying water in the core over 4-5 hours, which allowed the dissolved oxygen (DO) to drop by about 20% from its original in situ value. As the sample was withdrawn, site water was replenished from a gravity fed reservoir. Samples were filtered through a GF/F (Sartorius) filter (0.45µm) with the exception of N_2 /Ar samples.

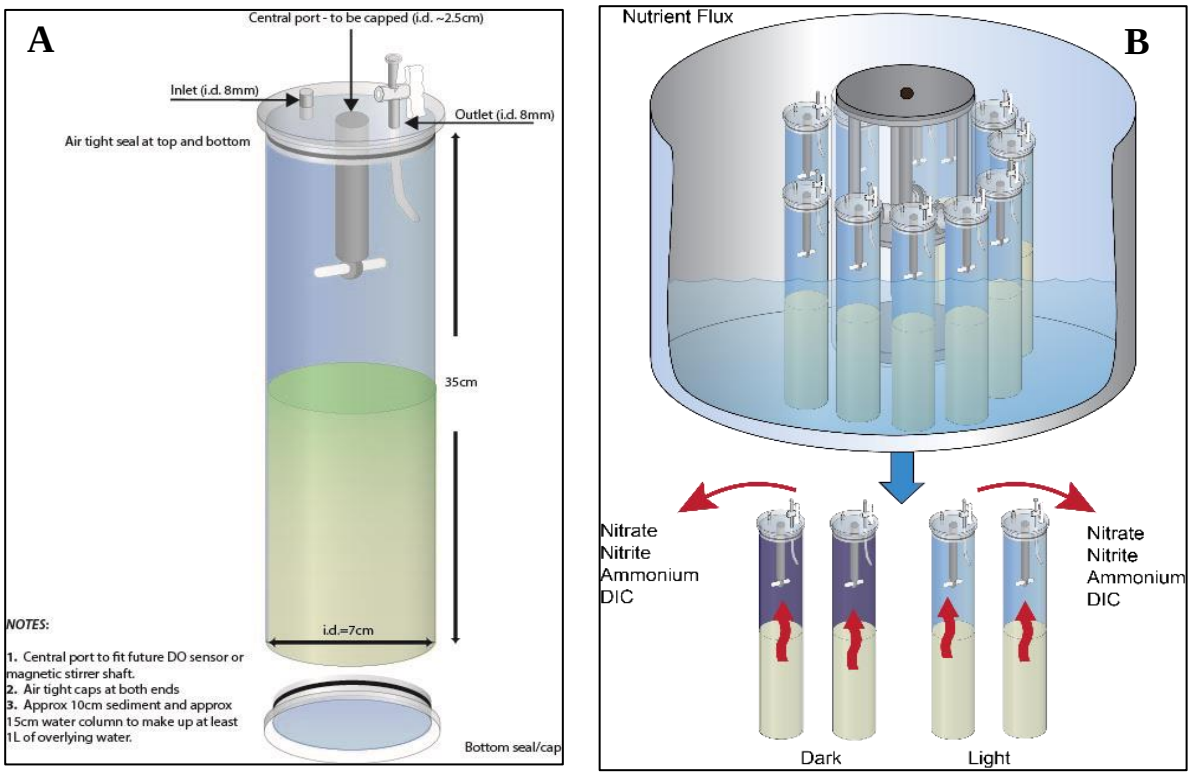


Figure 8: Benthic flux incubations. a) Core cylinder specifications and b) Flux measures from cores in laboratory chamber under light and dark treatments.

Filtered nutrient samples were frozen in 125mL polypropylene screw cap bottles. Dissolved oxygen was measured with a DO sensor inserted through a central port hole in the top of the core and an initial and final measure taken. Samples for N_2 /Ar analysis were taken in triplicate into 12-mL gas-tight glass exetainers, fixed with 10 µL saturated mercuric chloride, and stored submerged at *in situ* temperature until analysis. DIC samples were collected in 40ml glass vials and fixed with saturated mercuric chloride. Fluxes were calculated by monitoring the concentration change of individual analytes at the start and end of the incubation period (Dalsgaard et al., 2000; Potts et al. 2005). Concentrations were corrected for the addition of replacement water and any concentration change in a "blank" core containing only water.

2.2.2 Nutrient Analysis

Dissolved inorganic nutrients, NH_4^+, NO_2^- and NO_3^- were drawn from benthic chamber samples and analyzed by automated flow injection analysis using standard methods (Eurofin labs Brisbane). Ammonium was analysed by the automated phenate method and NOx (nitrate and nitrite) by automated cadmium reduction method. Dissolved inorganic nitrogen (DIN) was calculated from the sum of NH_4^+ and NOx for each sample. Dissolved inorganic carbon was analyzed on a Shimadzu carbon analyzer at the Australian Institute of Marine Science, Townsville. N_2 from the benthic chamber samples and O_2 from incubations were measured using a membrane inlet mass spectrometer (MIMS) at the University of Canberra using methods described by Kana et al. (1994). N_2 concentrations were determined from changes in the N_2:Ar ratios (± 0.05%).

2.3 Desktop Analysis to develop qPCR Assays

2.3.1 Statistical analysis of microarray experiment

Heat maps of the N-cycle gene array of the ARC-LP project (Figure 4) were analysed to identify N-cycle probes or genes in sediment which were associated with human impacts such as treated effluent or urban runoff. Box plots of the normalized abundance of the N-cycle probes were conducted in Primer-E (Plymouth, UK) comparing impacted vs reference creeks. A Canonical Analysis of Principal coordinates (CAP) ordination

constrained for creeks was used to assess the predictive ability of the N-cycle probes for the origin of the sample i.e. the creek sampled. Indicator value (IndVal) analyses were performed in R (package labdsv) to identify the N-cycle probes with the highest IndVal values, and therefore the strongest association with impacted samples – or vice versa with reference samples. Similarly, the association of N-cycle probes with impacted or reference samples were assessed using negative binomial models (DESeq2 in phyloseq in R). Multiple sequence alignments (http://www.ebi.ac.uk/Tools/msa/clustalo/) were generated to assess the relatedness of the N-cycle probe sequences (70 bp length) and a phylogenetic neighbour joining tree (FigTree) was constructed based on the global alignment to find clusters of probes associated with group of samples such as control or human impacted samples.

2.3.2 Bioinformatics to design qPCR assays

1. The N-cycle probes were 70 bp which are too short for qPCR assays. Consequently, the 70 bp probe sequences were pasted into ncbi Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find a 100% bacterial hit of the corresponding gene (e.g. *nosZ* for a *nosZ* probe) and the full gene sequence was extracted in Genbank (https://www.ncbi.nlm.nih.gov/genbank/). The sequence length was typically 600 to 700 bp.

2. Several sequences of the same gene (e.g. *nosZ* or 16s rRNA gene) from different bacterial taxa were extracted from Genbank and a multiple alignment was generated (http://www.ebi.ac.uk/Tools/msa/) for each gene to identify conserved and variable regions. In order to design assays specific for a N-cycle probe or OTU and avoid false positive results, it was important to identify variable sequence regions specific for the target sequence.

3. The target sequence was pasted into the Biosearch technologies online qPCR assay design software (https://www.biosearchtech.com/support/tools/design-software/realtimedesign-software). Suitable regions for primer and probe design were narrowed down to the above identified variable regions and for N-cycle probes, to regions covering the original probe where possible. Default primer and probe melt i.e. annealing temperature and GC content specifications recommended by the software for the Rotor Gene Q were followed. The software also checked for no or low primer secondary structures indicated by the Gibbs Free Energy delta G, self- or heterodimers and no nucleotide runs.

4. The primers and probe of the highest scored qPCR assays were checked for their specificity in Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

2.4 qPCR Assay Conditions and Data Normalisation

The 'delta delta Ct' method was the approach taken to quantify the N-gene test results (Livak and Schmittgen, 2001). This involves comparing the Ct values (cycle threshold = the number of cycles required for the fluorescent signal to cross the threshold i.e. exceed the background level) of the samples of interest with a control or calibrator. The Ct values of both the calibrator and the samples of interest were normalized to an appropriate endogenous housekeeping gene.

Normalization was done by measuring bacterial load and this was achieved by amplifying the 16SrRNA housekeeping gene fragment from template DNA using 500 nM of primers (forward 331F 5'TCCTACGGGAGGCAGCAGT; reverse 797R 5'GGACTACCAGGGTATCTAATCCTGTT) and 50 ng/µL of non-acetylated bovine serum albumin with the following conditions: 2 min 50°C; 15 min 95°C and 40 cycles of 15 sec 94°C, 30 sec 62°C, 30 sec 72°C and a fluorescence acquiring step for 15 sec at 83°C. This step used SYBR green chemistry which requires primers but no internal probe (Nadkarni et al., 2002).

To normalise N-gene results against bacterial load, the Ct value for each 16s qPCR sample was subtracted from the Ct value for that sample's N-gene target.

This is represented as:

$$\Delta Ct\ (sample) = Ct\ (target, sample) - Ct(16s, sample)$$

The calibrator was a sediment sample from the highly impacted site BC2 in upper Buffalo creek collected in the dry season 2014 for the ARC-LP project. The calibrator target gene abundance was normalized against its bacterial load (same as above).

$$\Delta Ct\ (calibrator) = Ct\ (target, calibrator) - Ct(16s, calibrator)$$

The normalized calibrator gene abundance was then subtracted from the normalized sample target gene abundance.

$$\Delta\Delta Ct\ = \Delta Ct\ (sample) - \Delta Ct\ (calibrator)$$

The PCR efficiency of each assay was assessed to ensure that all efficiencies were within 10% of each other. The average PCR efficiency for all qPCR assays was 90% or 1.8 and the ratio of normalized target gene abundance of the sample compared to the calibrator was calculated as follows.

$$1.8^{-\Delta\Delta Ct}\ = abundance\ ratio$$

2.4.1 qPCR chemistries - SYBR green and Taqman

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). The following qPCR conditions were applied: 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.mThe QuantiTect SYBR Green PCR mix (Qiagen) was used in a reaction volume of 20 µL. A melt curve analysis from 95° to 60°C was conducted at the end of each run to ensure the specificity of the detected amplicons.

Twelve probe-based Taqman qPCR assays were designed (see desktop studies) to measure the abundance of N-cycle genes and/or bacterial taxa associated with ammonia oxidation and nitrite oxidation (ie nitrification). All probes had a FAM dye at the 5' end and a black-hole quencher (BHQ) at the 3' end (Biosearch Technologies, USA) (see Appendix A1 for primer and probe sequences).

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 linear dynamic range were established for all assays using DNA serial dilutions of effluent samples or Pseudomonas culture isolates. All qPCR assays were run on a Rotor-Gene Q 5-plex (Qiagen). A no-template control (NTC) was included in each run.

2.4.2 DNA standards from bacterial cultures

As part of the technical quality assurance, bacterial standards were prepared with known DNA concentrations. The standards were bacterial DNA extracted from in-house cultures of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Burkholderia thailandensis*, *Burkholderia vietnamiensis*, *Burkholderia diffusa*, *Burkholderia cepacia-complex*, *Burkholderia ubonensis*, *Achromobacter spp.*, *Cupriavidus spp.*, *Ralstonia spp.*, *Acidovorax spp.*, *Comamonas spp*. and *Bradyrhizobium spp.*

2.5 Technical & Biological Validation

2.5.1 Technical validation

Twenty sediment and 20 water samples from Darwin Harbour collected in the previous ARC-LP project were used as a technical validation set to assess the abundance of the N-cycle target genes between the treated effluent and urban runoff impacted and reference samples. These samples were aliquoted and diluted to 10 ng/µL for the technical validation steps. The aim of the technical validation was to assess the accuracy of the qPCR assays to reflect the microarray probe results. Therefore, the samples were grouped according to impact level as per the grouping of samples in the microarray experiment. Microbial data arising from the ARC-LP and published in Kaestli et al. (2017) were used to determine the impact level. For the reference or control samples, various sites along the salinity gradient of the reference creeks were chosen to account for salinity-driven effects as the more impacted sites were also further upstream with more freshwater inputs in particular during the wet season.

The 40 sediment and water samples from both, the dry and wet season were grouped into

- Treated effluent:
    - Leanyer Sanderson Wastewater Treatment pond (LSWTP)
    - LSWTP outfall or sediment from outfall
    - PWTP outfall or sediment from outfall
- High impact (hypereutrophic):
    - Buffalo Ck upstream– same site as BC1 of the biological validation (Table 1)
    - Myrmidon creek side branch which receives treated effluent of the PWTP
- Moderate impact (eutrophic):
    - Buffalo Ck downstream – same area as BC2 of the biological validation (Table 1)
- Low impact (mesotrophic):
    - Buffalo Ck – site close to boat ramp
    - Myrmidon Ck downstream – same area as Myrmidon site of the biological validation (Table 1)
- Reference (oligotrophic):
    - King Ck upstream – same area as King Ck site of the biological validation
    - Reference Ck up- and downstream – includes area of the biological validation site

Further, a standard dilution series of two effluent and two *Pseudomonas* culture DNA samples were prepared over 7 logs from 100 ng/µL to 10 fg/µL. These were used to assess the qPCR efficiency for the delta delta Ct method.

2.5.2 Biological validation

For the biological validation of the qPCR assays, 32 sediment samples from the Darwin Harbour flux study (Section 2.1.2) were subject to DNA extraction.

2.5.3 Statistical analysis of technical & biological validation

The qPCR results were compared between groups of samples in multiple regression analyses in Stata with the standard errors clustered for sites. The qPCR results were log transformed (plus 0.0001 to avoid log transformation of zeros) and explanatory variables were creeks (or level of impact for the technical validation), seasons and for the technical validation, type of sample (water vs sediment). Interaction terms between two variables were also tested with continuous variables centred at their mean. The residuals of the multiple regression models were tested for normality and random distribution and the explanatory variables were tested for multicollinearity. The qPCR results were also associated with the nutrient flux data in multiple regression analyses and the combination of qPCR assays was compared to flux data in Primer-E v7 (Plymouth, UK) using distance linear models based on the lowest Akaike information criterion (AIC). The qPCR assay

results were log transformed and a Chi2 distance matrix applied which was visualized with a principal coordinate ordination (PCO). PERMANOVA tests were used to compare the composite of qPCR results between groups of samples, namely a cross design with fixed factors sites or level of impact and season.

3. Results

3.1 Benthic Flux Measurements

A clear seasonal influence was apparent in most flux measurements, particularly the N2 flux (fN_2) and DIC flux (fDIC) which showed a distinct increase in flux rates in the wet season. The NOx flux (fNOx) rates were highly variable with no clear trend. This was likely a consequence of the highly temporal response to nitrate, which might change over a matter of minutes to hours depending on tidal conditions (see Appendix A5-8).

3.1.1 NH4 Flux

NH_4 fluxes (f NH_4) were most apparent in impacted sediment from Buffalo Creek with negative mean fluxes (i.e. ammonia assimilation) in the dry season (Figure 9). During the wet season, the highly impacted BC1 cores had highly variable but overall negative fluxes. In comparison, the moderately impacted BC2 cores had positive fluxes (i.e. NH_4 emission into the water column) which could be due to ammonification, nitrogen fixation or dissimilatory nitrate reduction to ammonia (DNRA) processes. NH_4 fluxes were zero or very low at most mesotrophic and oligotrophic sites which suggests low available NH_4 in the water column and balance in ammonification and assimilation, and/or ammonification due to oxic breakdown of organic matter coupled with complete nitrification.

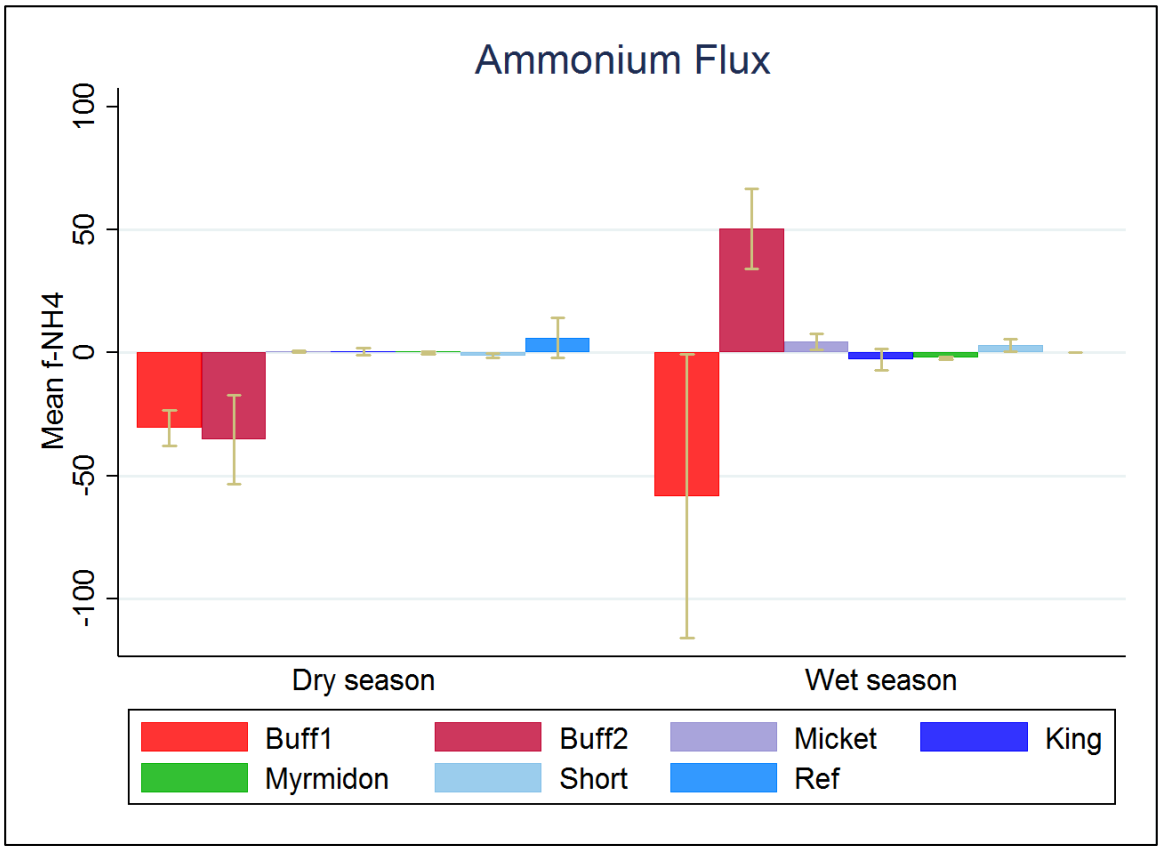


Figure 9: Average ammonium flux for each site and season (dark treatment). Positive flux marks NH_4 emission such as due to ammonification, DNRA or nitrogen fixation processes while negative flux indicates NH_4 draw-down such as due to assimilation, nitrification or anammox. Each bar consists of 3 samples. The error bars mark standard errors. Flux units are mmol NH_4 m^{-2} d^{-1}.

3.1.2 Net N_2 Flux or gross Denitrification

Positive N2 fluxes are considered to be indicative of net denitrification, while negative N2 fluxes indicate net N fixation rates. There were large seasonal differences in N2 fluxes (Figure 10), with much higher rates during the wet season. Dry season fluxes were close to zero at all sites. During the wet season, there was a net denitrification at all Shoal Bay sites as well as the mesotrophic Myrmidon site. Highly impacted Buff-1 showed the lowest positive flux while the reference site in Micket Creek had the highest positive flux. In contrast, N2 fluxes were negative for the control sites in East Arm indicating overall nitrogen fixation at these oligotrophic sites.

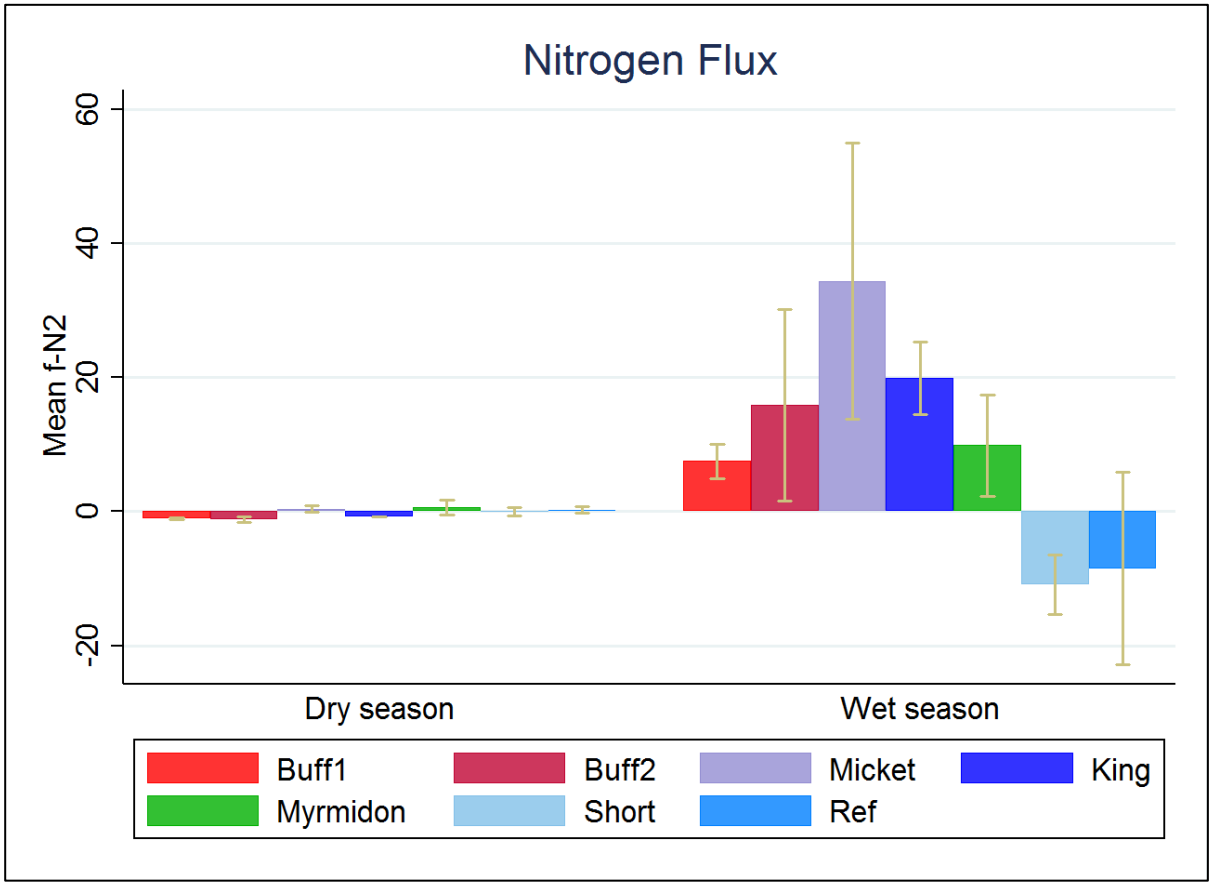


Figure 10: Average N_2 flux for each site and season (dark treatment). Positive flux marks N2 emission such as due to denitrification while negative flux indicates N2 draw-down such as due to nitrogen fixation. Each bar consists of 3 samples. The error bars mark standard errors. Fluxes are in measured in mmol N_2 m^{-2} d^{-1}.

3.1.3 Denitrification efficiency

DE% is expressed as the proportion of total inorganic nitrogen released as N_2, where:

$$DE = N_2 / (N_2 + DIN) \times 100$$

The DE% was highly variable between cores from the same site and time point (Figure 11). Similar to N2 fluxes, though less pronounced, denitrification efficiencies (DE%) were higher overall in the wet season. King and Myrmidon Creek sites had DE% > 90% at this time. Lowest DE% (<10%) were observed in cores from Buff-1 and the site in King Creek during the dry season.

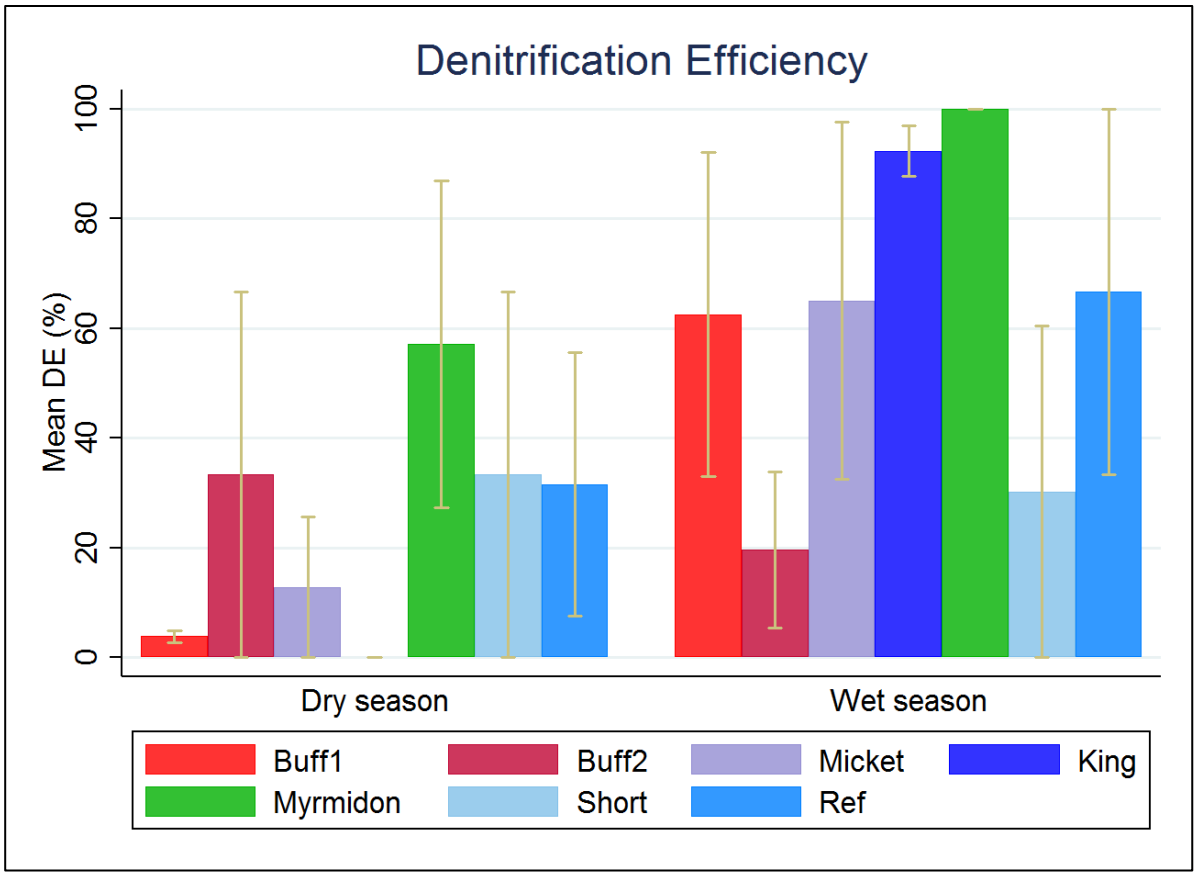


Figure 11: Average denitrification efficiency for each site and season. Each bar consists of 3 samples. The error bars mark standard errors.

3.1.4 Dissolved inorganic carbon flux

Dissolved inorganic carbon (DIC) mainly consists of dissolved carbon dioxide (CO_2) and (bi)-carbonates. DIC fluxes (fDIC) measure rates of carbon decomposition, which are a proxy for carbon loading to the seafloor (Henrichs, 1992; Eyre and Ferguson, 2009). Highest fDIC's (and apparent carbon loading) (Figure 12) were observed during the wet season which is likely due to higher primary production rates and influx of particulate organic matter.

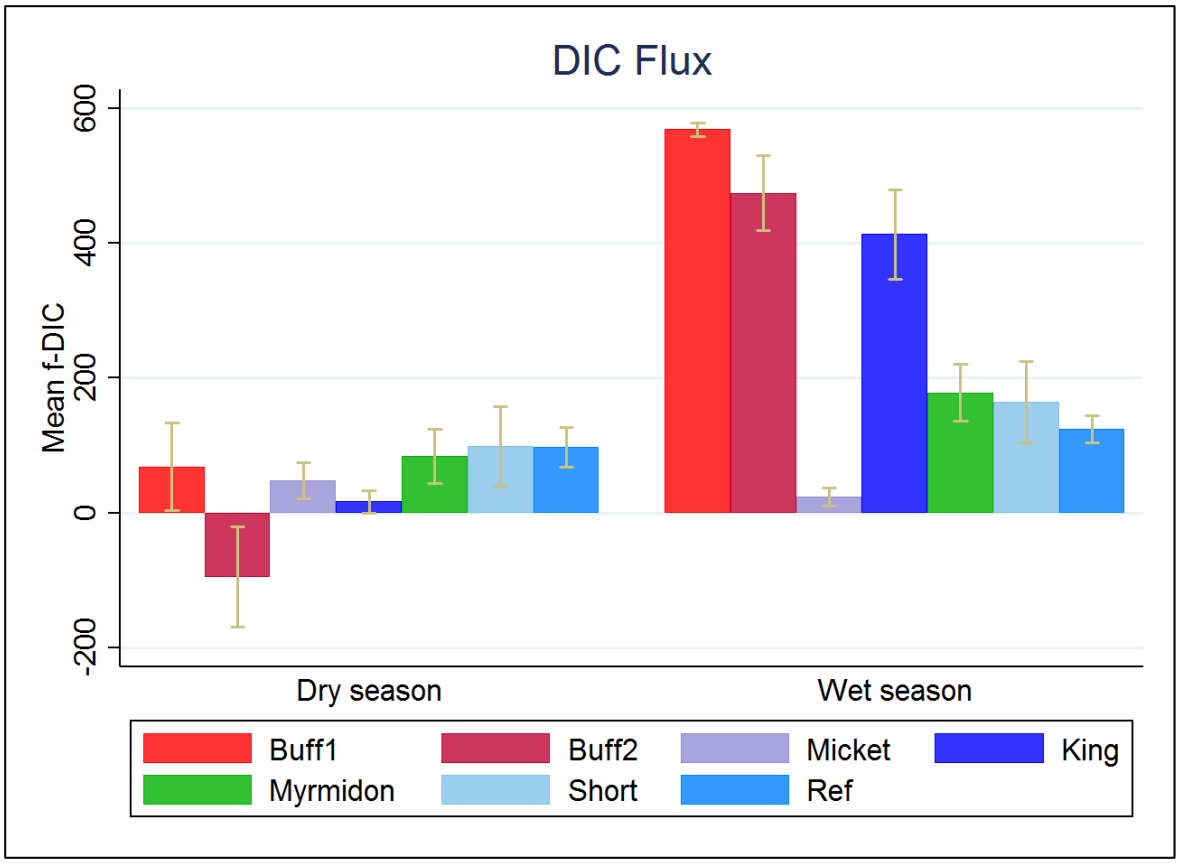


Figure 12: Average DIC flux for each site and season (dark treatment). Positive flux marks DIC (or CO_2) emission such as due to respiration, while negative fluxes indicate DIC draw-down such as through benthic photosynthesis. Each bar consists of 3 samples. The error bars mark standard errors. Fluxes are measured in mmol DIC m^{-2} d^{-1}.

3.2 Desktop Analysis for qPCR Assay Design

3.2.1 Statistical Analysis of N-cycle gene microarray probes

An Indicator Value and negative binomial statistical analysis were conducted on the N-cycle gene microarray probes of the ARC-LP water and sediment samples to identify N-cycle gene targets which were significantly associated with effluent impacted, urban run-off or control samples (Figure 13).

The majority of *amoA* gene signals in the microarray were archaeal and there were fewer *amoA* gene signals for water than sediment. No *amoA* genes were detected in effluent samples and only low levels in neap-water or sediment samples close to the outfall. Accordingly, ammonia oxidizing archaea AOA –probes AamoA.01 and .26 were associated with low impacted and control samples in sediment. AamoA.01 (or also labelled as AOA.1) has been found in the genus *Nitrosopumilus*, a common marine Crenarchaeota which is well adapted to highly nutrient limiting conditions (Martens-Habbena et al., 2009). AOA.26 was also associated with runoff water (Figure 13).

Denitrification probes nosZ.038, 055 or 057 were associated with effluent or highly impacted sediment. These probes have mainly been described in nitrogen fixing bacteria associated with plants such as rhizobia or the genus *Herbaspirillum* (Figure 13).

A bacteria probe 'OTU606' that targets the order of nitrite oxidizing Nitrospirales was associated with control samples. The probe was a 16S rRNA gene but we retained it as a potentially valuable test for nitrite oxidation since this is an important precursor to denitrification.

N-cycle gene or OTU Target			AamoA.01	AamoA.26	nosZ.038	nosZ.055	nosZ.057	nosZ.159	OTU9496	OTU3665	OTU1426797	OTU15	OTU4606
Water	Group most associated with		Mod Imp	Drain	Drain	OF	OF	Con	OF	High Imp	OF	OF	Con
	IndVal max		0.20	0.87	0.33	0.48	0.59	0.36	0.13	0.06	0.81	0.64	0.09
	P value (corrected)		0.458	0.002	0.057	0.005	0.004	0.139	0.603	0.856	0.014	0.023	0.856
	Occurred in n samples ARCLP		59	19	19	94	63	122	13	3	16	27	14
	Rel freq within group	Runoff	60%	100%	60%	60%	40%	60%	0%	0%	0%	0%	0%
		OF	8%	0%	50%	100%	100%	75%	25%	0%	92%	83%	0%
		High Imp	40%	4%	28%	80%	64%	100%	19%	8%	4%	19%	8%
		Mod Imp	59%	13%	6%	75%	63%	97%	12%	3%	12%	30%	9%
		Control	46%	16%	2%	63%	23%	96%	2%	0%	0%	3%	16%
	Rel abund across groups	Runoff	17%	87%	55%	17%	4%	10%	0%	0%	0%	0%	0%
		OF	1%	0%	32%	48%	59%	6%	50%	0%	88%	77%	0%
		High Imp	13%	1%	12%	15%	11%	22%	29%	72%	3%	13%	18%
		Mod Imp	34%	10%	2%	12%	21%	25%	18%	28%	8%	10%	21%
		Control	35%	3%	0%	9%	5%	37%	3%	0%	0%	0%	61%
Sed	Group most associated with		Low Imp	Low Imp	OF	OF	OF	High Imp	OF	OF	High Imp	OF	Con
	IndVal max		0.26	0.21	0.09	0.48	0.42	0.34	0.84	0.87	0.05	0.74	0.54
	P value (corrected)		0.762	0.217	0.364	0.007	0.018	0.551	0.012	0.012	0.970	0.012	0.012
	Occurred in n samples ARCLP		73	26	4	64	48	96	33	20	5	22	62
	Rel freq within group	OF	67%	7%	13%	80%	80%	80%	93%	93%	0%	80%	0%
		High Imp	64%	5%	0%	68%	50%	95%	41%	14%	9%	18%	32%
		Mod Imp	81%	41%	4%	56%	41%	96%	29%	6%	3%	3%	68%
		Control	69%	33%	3%	56%	36%	95%	3%	3%	5%	13%	87%
	Rel abund across groups	OF	13%	16%	68%	61%	52%	8%	90%	94%	0%	93%	0%
		High Imp	30%	9%	0%	12%	29%	36%	6%	4%	52%	3%	4%
		Mod Imp	32%	52%	19%	13%	13%	27%	4%	2%	18%	1%	34%
		Control	24%	23%	13%	14%	6%	29%	0%	0%	29%	3%	62%
Reasoning for assay design			Neg bin model	IndVal	IndVal Neg bin model	IndVal Neg bin model	IndVal	Neg bin model	IndVal	IndVal	IndVal	IndVal	IndVal
Taxonomy or previous description in			marine Crenarchaea Nitrosopumilus		rhizobia N-fixing plants	Herbaspirillum	Agricultural soil	Coastal Marine Sediment	f__Chromatiaceae	c__[Pedosphaerae]	f__Ruminococcaceae	f__Ruminococcaceae	o__Nitrosphirales

Figure 13: N-cycle gene microarray probes which were associated with impacted or control samples. P values were corrected for multiple testing using the False Discovery Rate (FDR) method. The relative frequency rows indicate the percentage of samples within each subgroup which contained these targets while the relative abundance shows the relative distribution of the targets between the subgroups (the latter sums to 100%).

Only one nitrogen fixing (*nifH*) array probe (nifH.005) showed a significant association with water outfall samples (data not shown).

None of the other N-cycle gene array probes (i.e. AOB, *nxrB*, *nrfA* or *hzsA*) showed any significant associations with particular sample groups. Only sediment close to the outfall showed *nxrB* gene signals in the microarray. This gene codes for an enzyme which transforms nitrite to nitrate and is a marker for Nitrobacter

bacteria. These bacteria are important in wastewater plants removing excess nitrogen (Internat J Environ, Chem, Ecol, Geol Geophysic Engineer Vol 9, 2015).

Anammox is the anaerobic oxidation of ammonia and nitrite to dinitrogen gas, which is another important pathway to reduce nitrogen levels in wastewater. We included the microbial *hzsA* gene in the N-cycle microarray as a marker for anammox presence and diversity. We mainly found anammox genes in sediment at Myrmidon creek, in particular at the outfall which reflects the particularly high abundance of ammonia at that site.

3.2.2 Phylogenetic tree analysis of microarray N-cycle gene probes

A phylogenetic tree of all *nosZ* microarray probes showed the relatedness of the probes (Figure 14). This tree revealed clusters of related probes such as *nosZ*-55, 56, 61 or 79 (labelled red) which were mainly associated with impacted sediment and water. Probes which were significant in the IndVal analysis and belonged to one of these red clusters (or pink for runoff) were primarily chosen as a target for the qPCR tests.

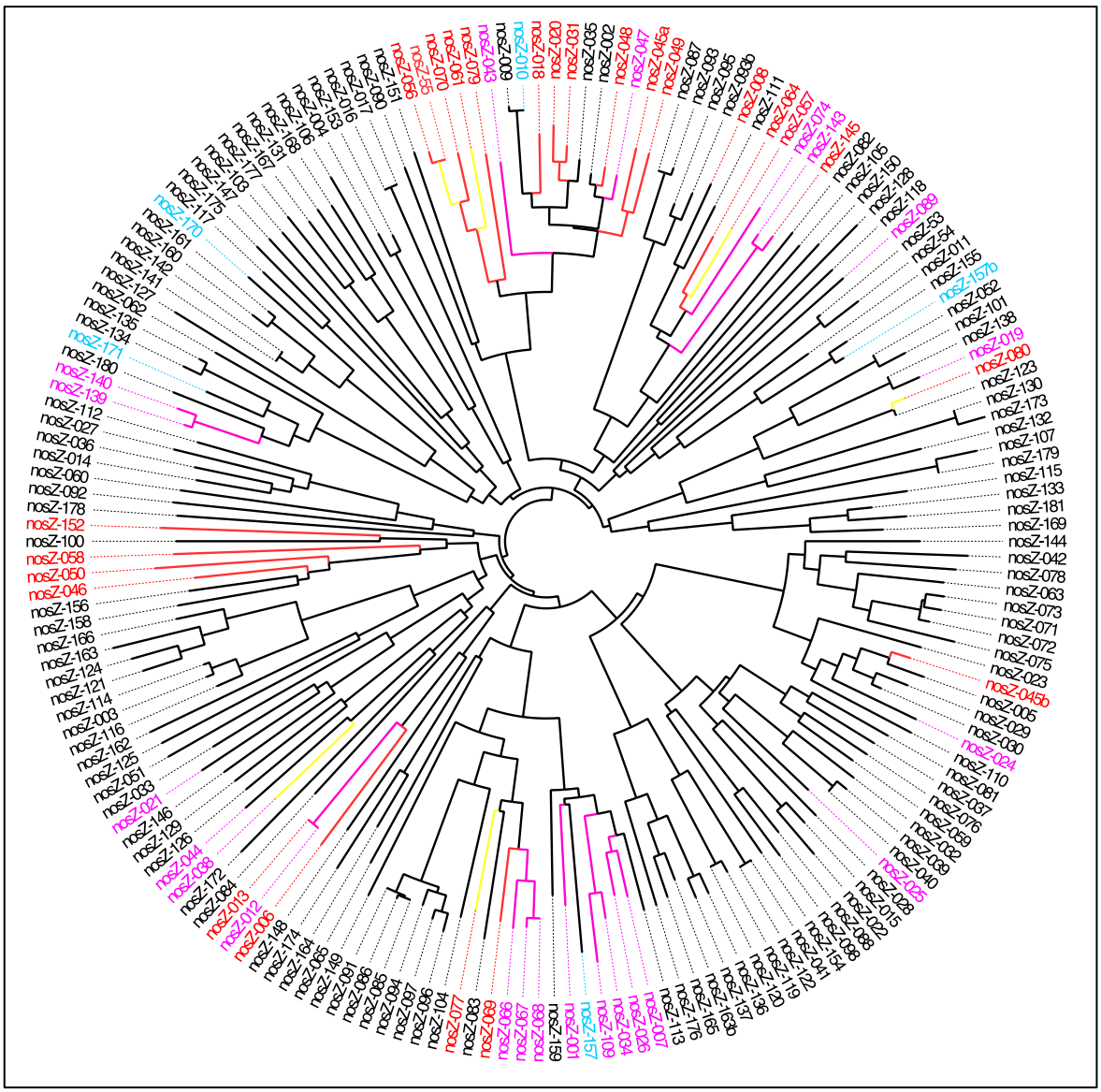


Figure 14: Phylogenetic tree showing the relatedness of *nosZ* array probes. Red colours indicate nosZ array probes which were associated with impacted sediment for both, sediment and water; pink with drain water; blue with control samples for both, sediment and water – yellow branches indicate high IndVal scores.

3.2.3 N-cycle gene target selection based on statistical analysis

Based on the IndVal results, we designed 15 qPCR tests using bioinformatics tools (see Appendix A1-3 for details) and tested them with DNA extracted from effluent impacted and reference sediment of the technical validation sample set. The initial tests included the evaluation of the PCR efficiency, limit of detection and semi-quantifiable range. Five assays were considered promising for further test development.

1. **Denitrification - *nosZ* qPCR (SYBR green based)**

   This assay measured the abundance of all *nosZ* genes in a sample (Henry et al., 2006)

2. **Denitrification - *nosZ*.38 qPCR (probe based)**

   This N-gene was associated with urban runoff or outfall sediment in the IndVal analysis

3. **Ammonia oxidation - AOA.1a qPCR (probe based)**

   This N-gene was more abundant at sites of low impact and control water and sediment samples in the IndVal analysis.

4. **Nitrite oxidation - otu606 qPCR (probe based)**

   This N-gene is specific for the nitrite oxidizing Nitrospirales that oxidizes NO_2^- to NO_3^-. It was associated with control water and sediment samples.

5. **16s qPCR (SYBR green based)**

   This assay was used to measure the bacterial load in the samples and all other qPCR results were normalized by the results of this assay in order to get the target gene abundance in reference to bacterial load.

In all tests, the qPCR results are shown in terms of an x fold change in the target gene abundance compared to a highly impacted sediment from upper Buffalo Creek and normalized by the bacterial load in that sample.

3.3 Technical validation of qPCR assays

3.3.1 qPCR assay for all *nosZ* genes

Figure 15 shows the qPCR results of the *nosZ* gene qPCR test.

A multiple regression with standard errors clustered for sites showed an average 67% more *nosZ* genes in the control sediment (P=0.001) and 64% more genes in the low impacted sediment (P=0.004) compared to the highly impacted sediment. This trend was not seen in water.

There were also an average 76 % less *nosZ* genes in water compared to sediment (P=0.004) and an average 21% less *nosZ* genes in the wet compared to the dry season (P=0.038).

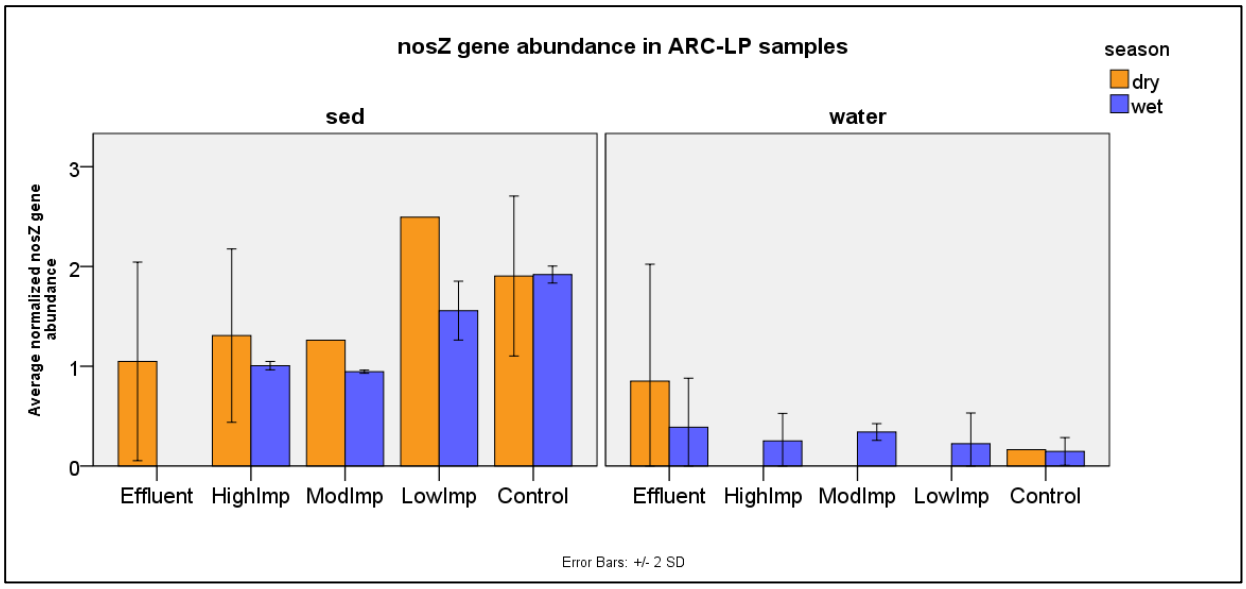


Figure 15: Technical validation of *nosZ* gene qPCR assay. Each bar shows the average abundance of nosZ genes in the corresponding group of samples (see above for details). Each group consists of 1 to 6 samples with 40 samples in total. The error bars mark two standard deviations.

3.3.2 *nosZ*.38 qPCR assay

Figure 16 shows the nosZ.38 qPCR test results on the technical validation set. It shows that the gene was mainly detected in effluent related sediment or water samples albeit with a large variability. A multiple regression indicated no significant trends between level of impact nor between seasons or type of samples.

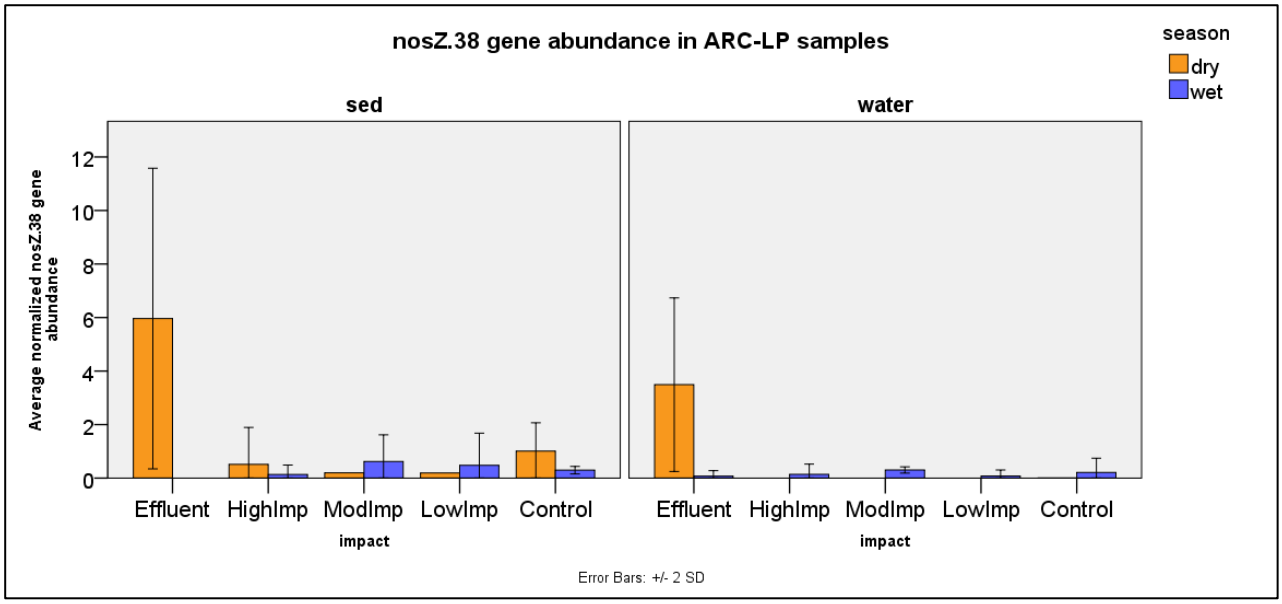


Figure 16: Technical validation of *nosZ.38* gene qPCR assay. Each bar shows the average abundance of *nosZ.38* genes in the corresponding group of samples (see methods or Section 3.2 for details). Each group consists of 1 to 6 samples with 40 samples in total. The error bars mark two standard deviations.

3.3.3 AOA.1a qPCR assay

There were on average 82 % less AOA.1a genes in effluent-related sediment (P=0.013) and 91 % less AOA.1a genes in highly impacted sediment (P=0.041) compared to control sediment. This difference in abundance

between effluent and control samples was even more apparent for water samples with no AOA.1a genes detected in effluent.

There were also on average 85 % less AOA.1a genes in water compared to sediment and a weak trend for less genes in the wet compared to the dry season; however, the latter was not significant showing large variability (P=0.057).

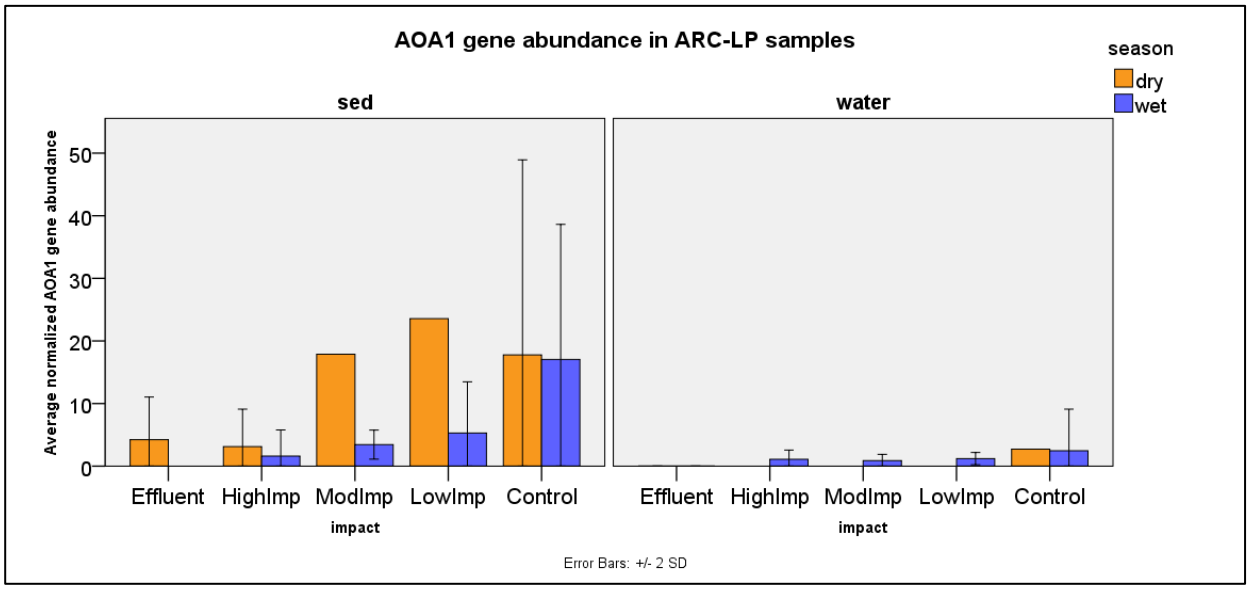


Figure 17: Technical validation of *AOA.1a* gene qPCR assay. Each bar shows the average abundance of *AOA.1a* genes in the corresponding group of samples (see methods or Section 3.2 for details). Each group consists of 1 to 6 samples with 40 samples in total. The error bars mark two standard deviations.

3.3.4 otu606 qPCR assay

This assay was strongly associated with control samples and showed the most promising results in terms of differentiating between the different impact levels and replicating the microarray results (Figure 18). Compared to control sediment and for the wet season, there were on average 99 % less otu606 genes in effluent-related sediment (P<0.001), 89 % less in highly impacted sediment (P<0.001) and 51 % less in moderately impacted sediment (P=0.018).

On average there were 56 % less genes in the wet compared to the dry season (P=0.012). There was a significant interaction effect between seasons and level of impact i.e. the above trends were even stronger for the dry season and there was also a 37 % reduction in otu606 genes in low impacted sediment compared to control sediment during that time (P=0.042).

This assay is only useful for sediment and close to no signals were obtained from water samples.

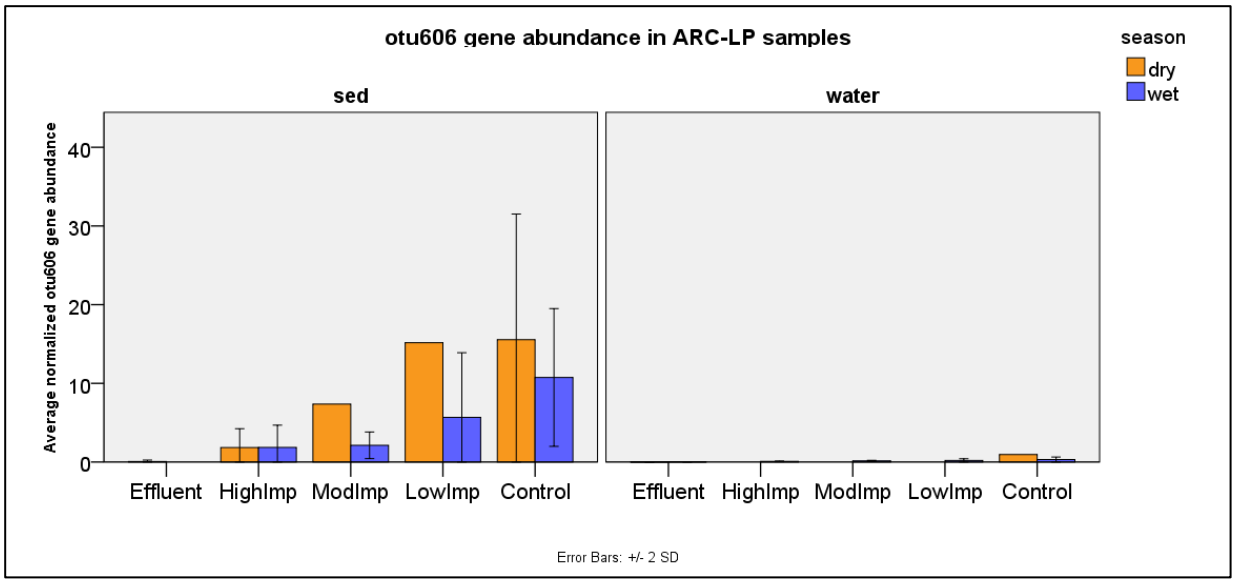


Figure 18: Technical validation of *otu606* gene qPCR assay. Each bar shows the average abundance of otu606 genes in the corresponding group of samples (see methods or Section 3.2 for details). Each group consists of 1 to 6 samples with 40 samples in total. The error bars mark two standard deviations.

3.4 Biological validation of qPCR assays

3.4.1 qPCR assay for all *nosZ* genes

Similar to the technical validation, the *nosZ* gene abundance was higher in the sediment from the oligotrophic sites compared to the hypereutrophic sites (Figure 19). A multiple regression showed that compared to hypereutrophic Buff1, there were on average 2.7 times more *nosZ* genes at Buff2 ($P<0.001$), 1.9 times more at the Myrmidon site ($P=0.003$) and between 2.4 and 3.8 times more at the oligotrophic control sites ($P<0.001$ for all).

In contrast to the technical validation there were 1.5 times more *nosZ* genes in the wet season samples compared to the dry season. The technical validation sediment subset was collected between Apr 2013 and Jul 2014 while the sediment of the biological validation was from July-15 (dry) or Mar-16 (wet season samples).

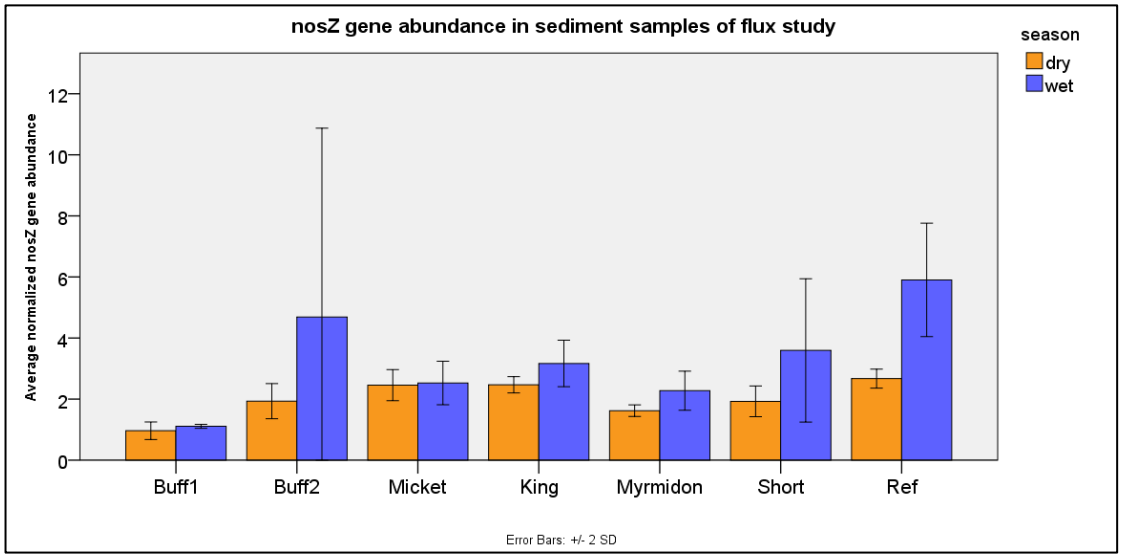


Figure 19: Biological validation of the *nosZ* gene qPCR assay. Each bar shows the average abundance of *nosZ* genes in the corresponding group of sediment samples (see methods or Section 3.3 for details). Each group consists of duplicate sediment samples with 28 samples in total. The error bars mark two standard deviations. The creek condition status's (Table 1) were hypereutrophic (Buff1), eutrophic (Buff2), mesotrophic (Myrmidon) and oligotrophic/control (Micket, King, Short and Ref).

Figure 20 shows a scatter plot comparing the N_2 flux (or gross denitrification) with the *nosZ* gene abundance of these sediment samples. There was no apparent association.

Figure 21 shows a scatter plot comparing the denitrification efficiency with the *nosZ* gene abundance. There is a positive trend; however, a multiple regression accounting for season and standard errors clustered for sites showed no significant association between the DE% and *nosZ* gene abundance.

The nosZ gene abundance was not correlated with fDIC, fNOx or fDO flux (Appendix A8) (not shown).

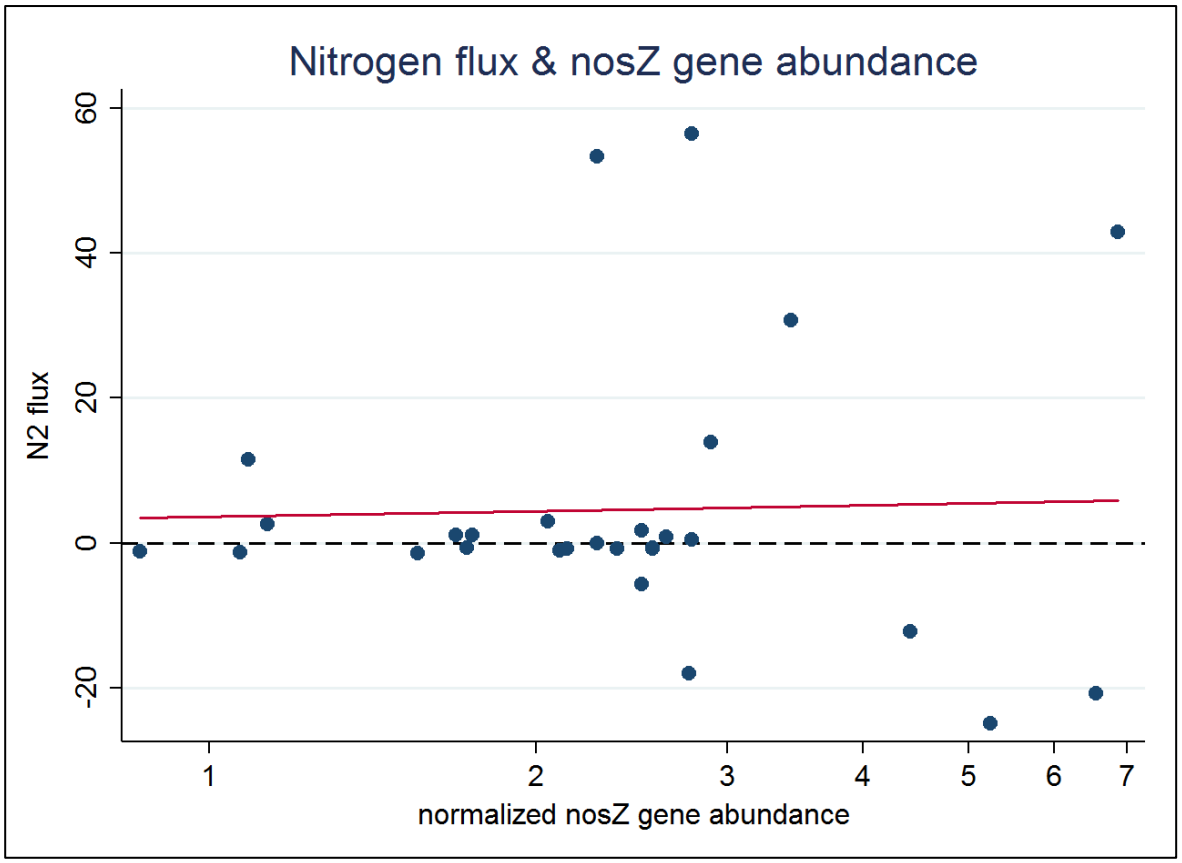


Figure 20: Scatter plot of N_2 flux and nosZ gene abundance. The nosZ gene abundance is in natural log. The dashed line indicates a flux of zero. The red line marks the linear fit ($R^2 < 0.01$).

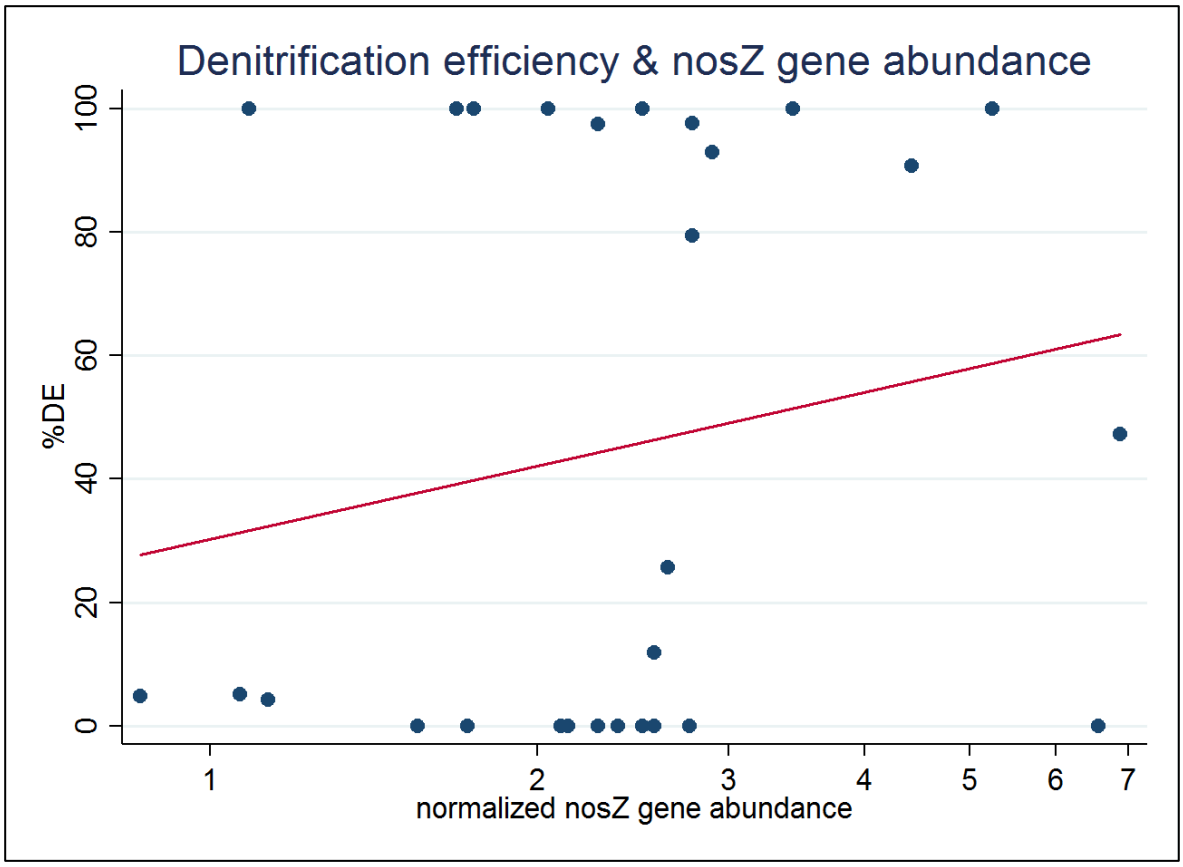


Figure 21: Scatter plot of % DE and nosZ gene abundance. The nosZ gene abundance is in natural log. The dashed line indicates a flux of zero. The red line marks the linear fit (R^2 0.034).

There was a positive association between ammonium flux and the *nosZ* gene abundance (Figure 22). A multiple regression adjusted for season with robust standard errors clustered for sites estimated an 8.2 times increase in fNH_4 flux for every 10 % increase in the *nosZ* gene abundance (P=0.016). Moreover, negative fNH_4 fluxes such as due to assimilation or nitrification, were associated with lower *nosZ* gene abundance. The ammonium flux showed a distinctively negative flux for the eutrophic site Buff1 (Figure 9) which was matched by the low *nosZ* gene abundance in these samples. A conservative, rank-based Spearman correlation provided evidence for a weak correlation between fNH_4 and *nosZ* gene abundance (P=0.057).

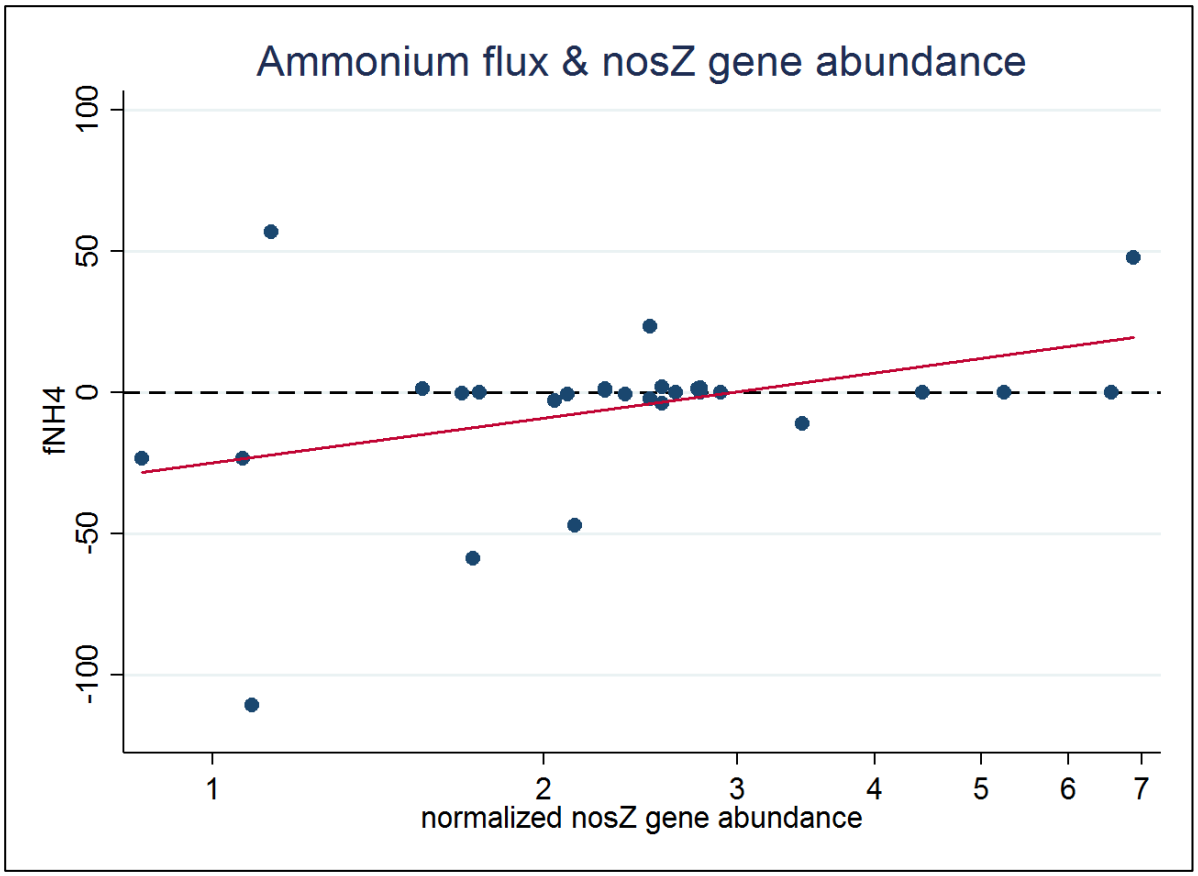


Figure 22: Scatter plot of ammonium flux and *nosZ* gene abundance. *NosZ* gene abundance is in natural log. The dashed line indicates a flux of zero. The red line marks the linear fit (R^2 0.148).

3.4.2 *nosZ.38* qPCR assay

In contrast to the technical validation, nosZ.38 was detected in control sediment (Figure 23). There were no statistically significant associations between the *nosZ.38* gene abundance and the sites used in the biological validation (data not shown).

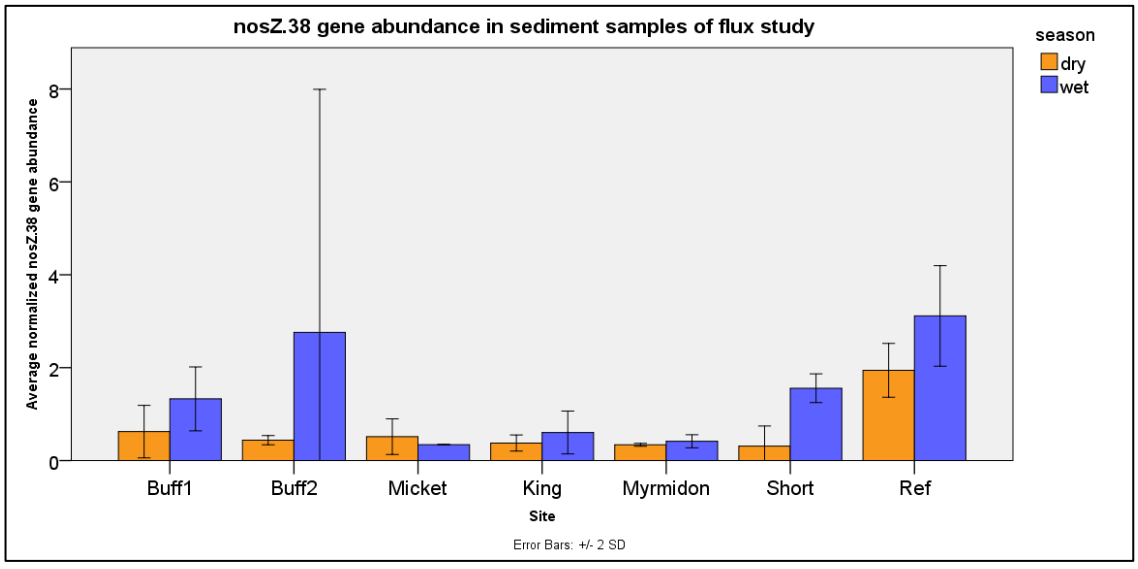


Figure 23: Biological validation of the nosZ.38 gene qPCR assay. Each bar shows the average abundance of nosZ.38 genes in the corresponding group of sediment samples (see methods or Section 3.3 for details). Each group consists of duplicate sediment samples with 28 samples in total. The error bars mark two standard deviations. The creek condition

status's (Table 1) were hypereutrophic (Buff1), eutrophic (Buff2), mesotrophic (Myrmidon) and oligotrophic/control (Micket, King, Short and Ref).

3.4.3 AOA.1a qPCR assay

The archaeal ammonia oxidizer AOA.1 gene abundance was highly variable. Gene abundance peaked in the wet season in the controls and moderately impacted sediment at Buff2 which had an average 14 times more AOA.1 genes compared to Buff1 (P<0.001)(Figure 24). Apart from Micket Creek, AOA.1 was significantly more abundant at all oligotrophic sites compared to Buff1 ranging from 3.7 times more genes at King creek (P=0.018) to 22 times more at Short creek (P<0.001). Similar to the technical validation results there were very low AOA.1 signals for high impact samples at Buff1. The distinct trend of more AOA.1 genes in the control samples was less evident in the dry season when only the sites Buff2 and Ref creek showed significantly more AOA.1 genes compared to Buff1.

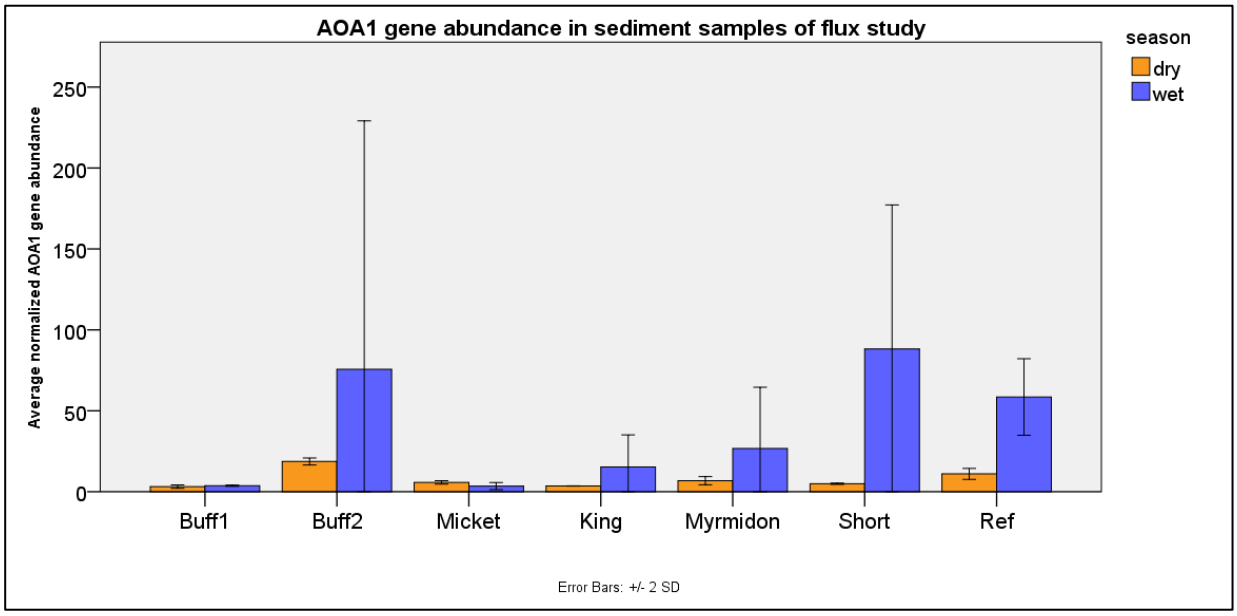


Figure 24: Biological validation of the AOA1 gene qPCR assay. Each bar shows the average abundance of AOA1 genes in the corresponding group of sediment samples (see methods or Section 3.3 for details). Each group consists of duplicate sediment samples with 28 samples in total. The error bars mark two standard deviations. The creek condition status's (Table 1) were hypereutrophic (Buff1), eutrophic (Buff2), mesotrophic (Myrmidon) and oligotrophic/control (Micket, King, Short and Ref).

There was no statistically significant association between the AOA.1 gene abundance and any flux measurements (data not shown).

3.4.4 otu606 qPCR assay

Similar to the technical validation, the wet season otu606 gene abundance peaked in the control samples with all sites showing a significantly higher abundance compared to Buff-1 ranging from 9 times more at King Creek (P<0.001) to 40 times more at the Short Creek site (P<0.001) (Figure 25). Trends were less distinct during the dry season.

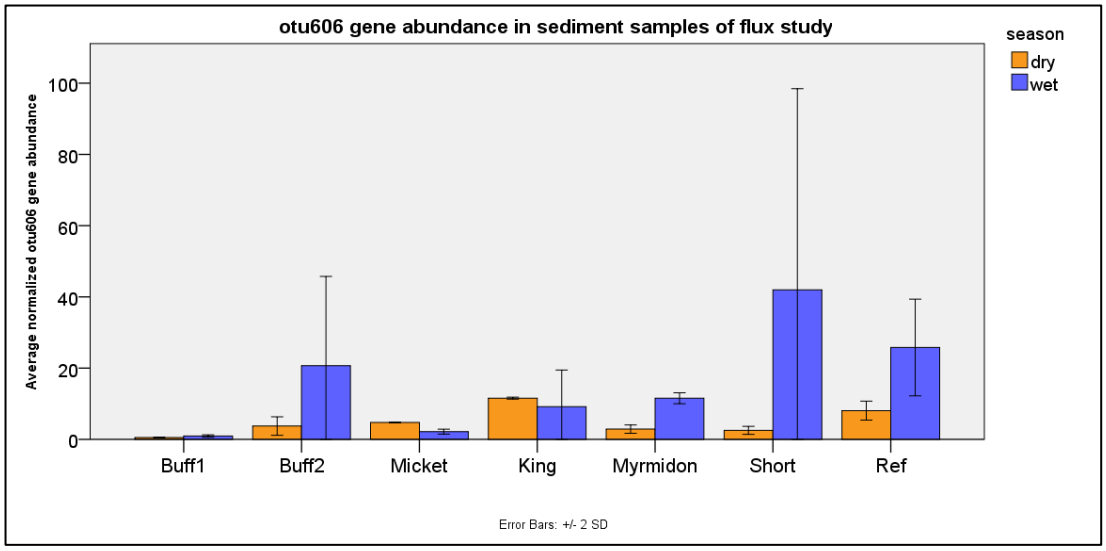


Figure 25: Biological validation of the otu606 gene qPCR assay. Each bar shows the average abundance of otu606 genes in the corresponding group of sediment samples (see methods or Section 3.3 for details). Each group consists of duplicate sediment samples with 28 samples in total. The error bars mark two standard deviations. The creek condition status's (Table 1) were hypereutrophic (Buff1), eutrophic (Buff2), mesotrophic (Myrmidon) and oligotrophic/control (Micket, King, Short and Ref).

There was no significant correlation between the otu606 gene abundance and any of the fluxes (data not shown).

3.4.5 The explanatory power for location of the composite of qPCR assays vs flux

Figure 26 shows the results of all the qPCR assays combined (excluding the nosZ.38 test). Figures 26A) and B) show a clear clustering of samples according to site and impact level. In contrast, there is no clear clustering according to season (Figure 26C). Figure 26D) shows the partial correlation vectors of the PCR assays. The otu606 assay separates samples best along the 1st PCO axis and in particular Buff1 or the highly impacted samples from the control creeks while the *nosZ* and AOA1 assays separate the samples also along the 2nd PCO axis and mainly Buff2 and Myrmidon from the Shoal Bay control samples.

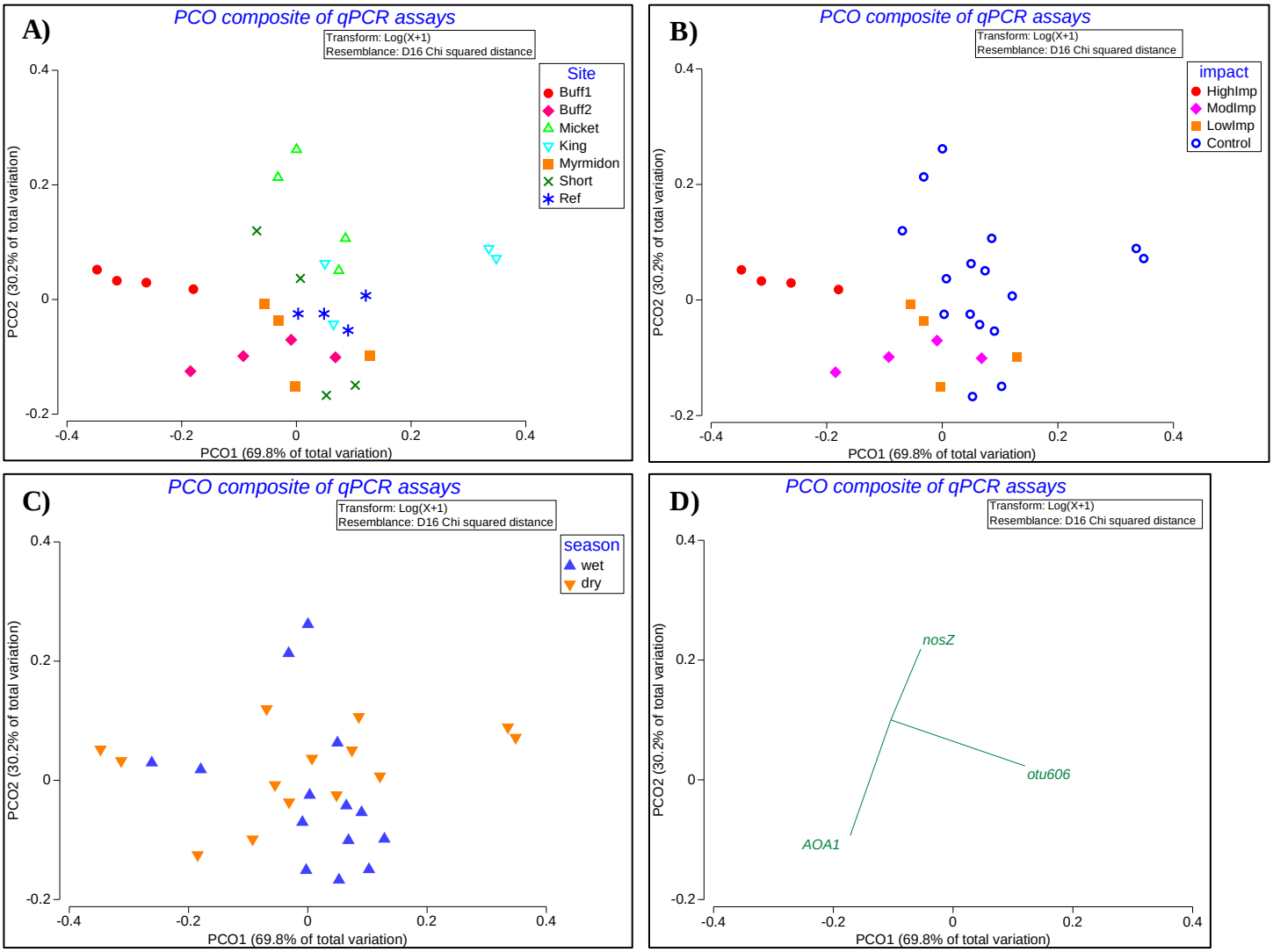


Figure 26: PCO of the composite of qPCR assays i.e. the nosZ, AOA.1 and otu606 assays (excluding the nosZ.38 assay). Each dot is a sample and the closer the dots, the more related the samples in terms of the qPCR assay results. The same ordination is shown 4 times with A) labelled according to site, B) level of human impact, C) season and D) showing the partial correlation vectors of each qPCR assay with the direction and strength of correlation of each assay with the PCO axes.

A PERMANOVA analysis for site and season confirmed the visual inspection of Figure 26. There was strong evidence that the composite of qPCR assays differed between sites (P=0.001) while there was only weak evidence for a difference between seasons (P=0.057). There were significant differences in 16 of 21 pairwise site comparisons (P<0.05), and in particular for comparisons that included Buff1 and the Shoal Bay sites. There were no significant differences between the East Arm sites i.e. between Myrmidon, Short and Reference Creek's (data not shown).

Figure 27 shows a PCO ordination of the sediment samples from biological validation (as in Figure 26) using the composite dark treatment flux data (fN_2, fNH_4, fNOx, fDIC and fDO). There was no clear clustering according to sites (Figure 27A), and a large amount of variability in the mesotrophic (Myrmidon) and eutrophic (Buff2 and Buff1) sites (Figure 27B). There was some clustering according to season especially for the impacted sites (Figure 27C). The vectors in Figure 27D reflect the highly variable fNOx data. The fN_2 and fNH_4 mainly differentiate the samples according to season, while fDO is the main vector separating the impacted from the control sediment. The fDIC vector differentiates the seasons and separates Buff-1 from the other sites.

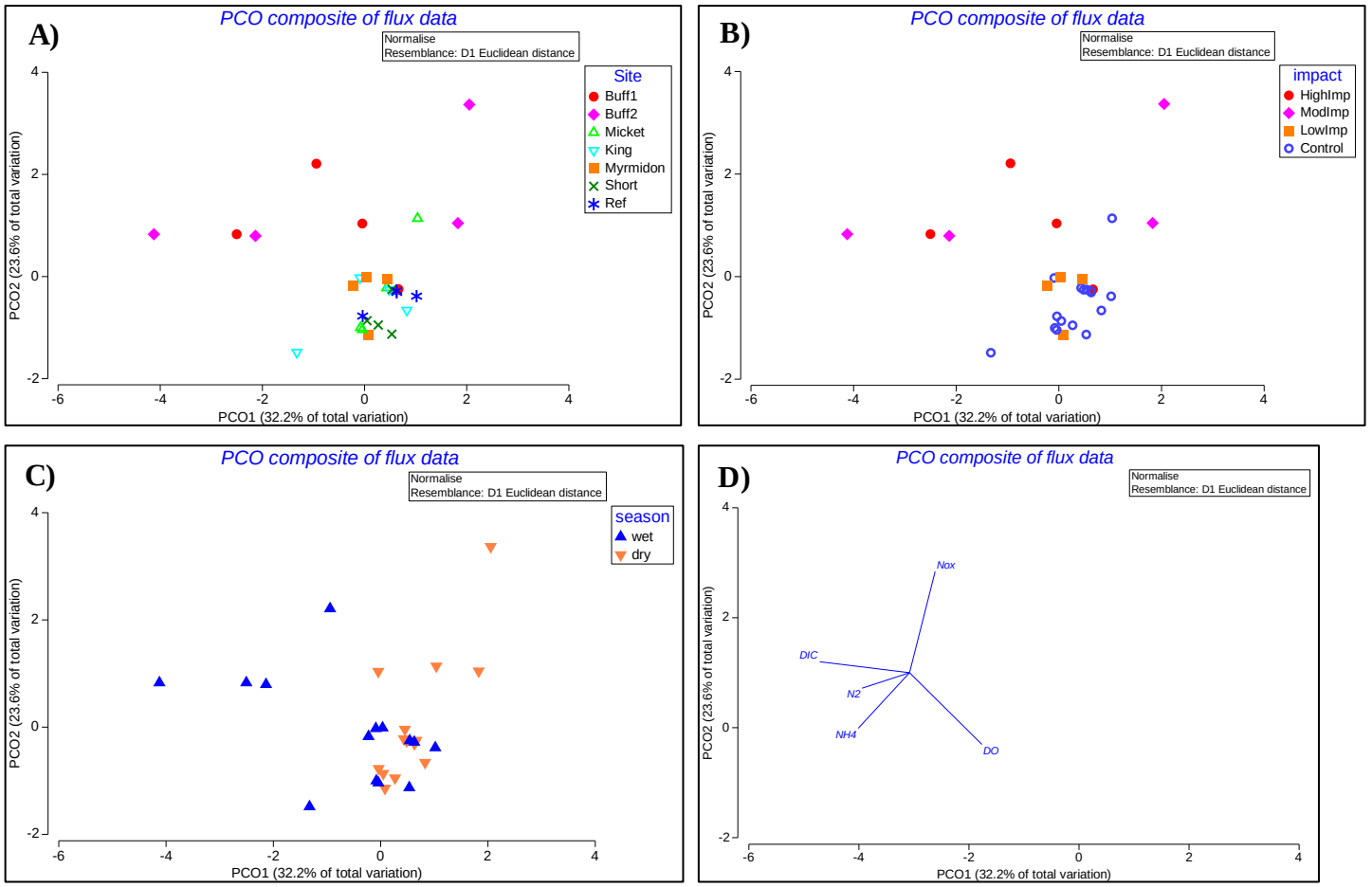


Figure 27: PCO of the composite of fluxes (i.e. fDIC, fDO, fN_2, fNH_4 and fNOx) excluding fNO_3 and fNO_2. Each dot is a sample and the closer the dots, the more related the samples i.e. their flux data. The same ordination is shown 4 times with A) labelled according to site, B) level of human impact, C) season and D) showing the partial correlation vectors of the fluxes with the direction and strength of correlation of each flux with the PCO axes.

A PERMANOVA analysis also revealed an overall significant difference in the composite flux data between sites (P=0.001) but in contrast to the qPCR composite data, there was also strong evidence for a difference in the flux data according to season (P=0.002). A pairwise comparison of the flux data between sites showed that the flux composite only significantly differed between 8 of 21 site pairs (as compared to 16 of 21 pairs for the qPCR composite data). It was of interest that these 8 pairs did not include the hypereutrophic Buff-1 but were mainly between Buff-2 and the Shoal Bay control sites and all East Arm sites (data not shown). There was also a significant difference in the flux data between the Micket site and all East Arm sites.

4. Discussion

4.1 N-cycle microarray - a source of candidate N-gene markers

In this study we used an N-cycle gene microarray to identify promising microbial N-genes based on their signal in human impacted vs reference sites. Array signals relating to denitrification (*nosZ*) genes from sediment at the outfall and from highly impacted sites along Buffalo Creek were different from all other samples.

For archaeal ammonia oxidation (AOA), there were fewer signals for water than sediment which supports the focus on sediment as the most appropriate sample type for N function genes. No AOA signals were detected in effluent and only low levels were found in neap-water samples close to the outfall. This result suggests reduced nitrification (ammonia oxidation) by AOA near the outfall and is consistent with previous research showing that AOA thrives under nutrient-limiting conditions (Martens-Habbena et al., 2009).

A bacteria probe 'OTU606' for nitrite oxidation was selected because it was associated with control samples in the microarray.

4.2 Denitrification vs nosZ gene abundance

Denitrification is an important step for nitrogen removal and DE% is commonly used as an indicator of change associated with nutrient over-enrichment (Cook et al., 2004; Eyre and Ferguson, 2009; Smith et al., 2012). DE% is closely related 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 NO_3 levels. In comparison, low carbon loadings can lead to the dominance of sediment by oxic zones and thus a lack of denitrification (Eyre and Ferguson 2009). The presence of sulphides also inhibits nitrification and denitrification, while in the presence of high N loading, other processes might compete for NO_3 such as DNRA or assimilation by benthic algae (Dong et al., 2011).

Dinitrogen and DIC fluxes were 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 (and generally higher %DE) 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 2005 study by Burford et al. (2008).

The DE%, N2 and NOx fluxes were highly variable not only between seasons and sites but also between replicates from the same site. The variability was most evident at hypereutrophic sites. The high variability 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. Dry season DE% was particularly low at the hypereutrophic Buff1 site confirming previous reports which suggest that sediment function is compromised in Buffalo Creek (Burford et al., 2008; Smith et al., 2012).

The considerable temporal and within-site variability was not observed for the *nosZ* gene abundance with the exception of the Buff2 site which showed a large variability across all flux and qPCR results. This discrepancy may be due to technical differences; the qPCR approach measured the N-cycle microbial gene abundance from the upper 10 cm of bulk sediment whereas unknown depths of sediment encapsulated in core barrels contributed to the benthic fluxes. However, it is known that much of the decomposition of organic matter occurs near the sediment-water interface (Henrichs, 1992). This result is also in line with previous findings that the sediment microbiota reflects current and past conditions and is subject to only slow change (Kaestli et al., 2017; Reed and Martiny, 2013).

The *nosZ* gene abundance in sediment was lowest at hypereutrophic Buff-1 site with a long history of receiving treated effluent and highest at oligotrophic sites in East Arm. There was no statistically significant association between the *nosZ* gene abundance and N_2-flux or the DE%. This is in line with a published report showing no correlation between the *nir* gene abundance in sediment encoding the nitrite-reductase enzme used for denitrification and absolute rates of denitrification (Graham et al., 2010). Instead, the denitrification in that report was associated with water-column nitrate and soluble-reactive phosphorus levels while the *nir* gene abundance correlated with the intrinsic denitrification efficiency, i.e. the coefficient of denitrification, K_{den}, which represents the long-term optimal denitrification rates at given environmental conditions.

There was a positive association between the *nosZ* gene abundance and fNH_4 flux. Mainly negative fNH_4 fluxes were associated with less *nosZ* genes. Negative fNH_4 fluxes were mainly observed at hypereutrophic sites in Buffalo creek with large positive fDIC flux and fDO draw-down. These negative fNH_4 fluxes might point to nitrogen assimilation by benthic algae or anammox.

A qPCR assay was also designed for the single *nosZ* gene marker (*nosZ.38*) because this marker was associated with urban runoff and outfall sediment in the microarray experiment and technical validation. This marker has mainly been described in nitrogen fixing bacteria associated with plants such as rhizobia or the genus *Herbaspirillum.* However, the biological validation showed no association of the qPCR assay with highly impacted samples which suggests that the qPCR assay also amplifies N-cycle genes other than the nosZ.38 related genes. We suggest to design new assays targeting this and similar microarray probes nosZ.038, 055 or 057 which showed promise in the microarray experiment.

4.3 N-gene qPCR tests for archaeal ammonia oxidation - AOA.1

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).

We designed a qPCR assay targeting the AOA.1 gene which we found to be associated with control sediment in the microarray experiment and which has been described in the genus *Nitrosopumilus*, a common marine Crenarchaeota which is well adapted to highly nutrient limiting conditions (Martens-Habbena et al., 2009).

The biological validation showed more AOA.1 genes in the control sediment compared to the hypereutrophic Buff1 site but also at the moderately impacted Buff2 site. Conditions at Buff2 might have been favourable for AOA with a combination of less nutrients than at the highly impacted Buff1 site while being less saline than the control samples particular during the dry season. AOA abundance has been found to be highest at intermediate salinity levels in an estuary (Bernhard et al., 2010).

Similarly to the *nosZ* gene abundance and N_2 flux, we found no association between the AOA.1 gene abundance and NH_4 flux. AOA abundance has been found to not necessarily correlate with nitrification rates (Bernhard et al., 2010).

4.4 N-gene qPCR tests for nitrite oxidation - otu606

The Nitrospirales otu606 qPCR test for nitrite oxidation performed well. Low to no otu606 genes were detected at Buff1 and there was a significantly higher abundance in control samples. These nitrifying bacteria have been found to decrease under anoxic conditions in marine sediment (Devereux et al., 2015).

High nitrite flux can 'feed' high denitrification - conditions that tend to occur at low impact and reference sites and high otu606 signals might be indicative of good ecosystem function.

We recommend the otu606 nitrite oxidation test be included in routine monitoring over time and space to generate baseline data as an additional functional measure of a 'healthy ecosystem'. It is a natural partner for the denitrification *nosZ* tests, and as with denitrification, it would be useful to include additional sites subject to different nutrient loadings – i.e. further validation.

4.5 Are the N-gene qPCR tests a useful measure for human impact?

The qPCR assays successfully allowed us to differentiate between eutrophic and oligotrophic sediment. This location-specific clustering was less evident for the flux measurements which were highly variable. This variability is a function of the dynamic harbour with strong macrotidal and seasonal forces and interacting factors including large variations in turbidity, light, salinity etc. Flux sample numbers were also low per site and season (n=3) reflecting the labour intensive procedure of flux measurements.

The combination of the best performing 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 in the biological validation. 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).

Thus, findings in this study suggest that a composite of qPCR assays could be a valuable tool to map zones of long-term impact in Darwin Harbour while the flux measurements provide the actual N-cycle rates dependent on the conditions at the time of sampling.

5. Conclusions and Outlook

Our ARC-LP data showed that when measuring the whole bacteria community (16s rRNA genes), water was a superior medium to sediment to reveal spatial and seasonal patterns for tracking impact (Kaestli et al., 2017). The microbiota in the top 10 cm layer of sediment was found to reflect current and past conditions and was less subject to short-term or seasonal change. This study also found that the sediment N-cycle bacteria in this layer reflected long term conditions at a site while flux measurements were more variable over time and depended on conditions encountered at the time of sampling. The top 10 cm layer of sediment is a product of years of sedimentation and thus, also explains the slower temporal change in the microbiota.

We found N-cycle bacteria to be far 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 the best performing 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.

The tests developed (qPCR assays) were faster and less labour intensive than the conventional nutrient flux assays. Further work can be done to refine these assays. However, indications are that a composite of these qPCR assays are a valid and robust method for routine assessment of sediment health that have the advantage over benthic flux measurements in that many more samples can be processed to map zones of 'influence' (such as potentially from stormwater) in Darwin Harbour.

Work on this project will continue. Based on these encouraging results, further qPCR assays will be designed. Activity of these N-cycle genes will also be assessed by screening a small subset of the best performing qPCR assays on valuable reverse transcribed RNA samples to confirm the N-cycle gene abundance data. A publication in an international peer-reviewed scientific journal is planned for 2018.

Furthermore, we suggest to conduct a biological validation on the N-cycle gene abundance of just the near-surface layer to capture the bacteria likely responsible for measured fluxes and avoid mixing N-cycle gene abundance from different sediment layers along the top-10 cm with potentially different oxic and redox profiles.

Ultimately, we anticipate using the best qPCR assays for routine monitoring of ecosystem health of sediment in Darwin Harbour or other macrotidal estuaries in the wet-dry tropics.

6. Acknowledgements

We thank Alea Rose (CDU) for technical assistance in the molecular laboratory and provision of effluent DNA samples and the ECMU team (CDU) namely Dionisia Lambrinidis for assistance in the chemistry laboratory. We thank the Aquatic Health Unit (NTG) for field assistance and Anna Padovan (CDU) for general advice for molecular laboratory matters. We thank Lev Bodrossy and Jodie Van de Kamp (CSIRO, Hobart) for the design of the N-cycle array and conducting the microarray experiment for the ARC-LP.

7. Reference list

Aquatic Health Unit (2015). Darwin Harbour Region Report Card 2015. Department of Land Resource Management, Northern Territory Government.

Aquatic Health Unit (2016). Darwin Harbour Region Report Card 2016. Department of Land Resource Management, Northern Territory Government.

Alongi, D. (2002). Present state and future of the world's mangrove forests. Environmental Conservation 29(3):331-349

Bernhard, A.E., Landry, Z.C., Blevins, A., de la Torre, J.R., Giblin, A.E., and Stahl, D.A. (2010). Abundance of Ammonia-Oxidizing Archaea and Bacteria along an Estuarine Salinity Gradient in Relation to Potential Nitrification Rates. Applied and Environmental Microbiology 76(4), 1285-1289. doi: 10.1128/Aem.02018-09.

Borges, A.V., S. Djenidi, G. Lacroix, J. Théate, B. Delille, and M. Frankignoulle (2003), Atmospheric CO2 flux from mangrove surrounding waters, Geophys. Res. Lett., 30, 1558.

Bork, P., de Vargas, C., Gorsky, G., Karsenti, E., Wincker, P., and Tara Oceans (2015). Tara Oceans studies plankton at planetary scale. Introduction. Science 348:873. doi: 10.1126/science.aac5605

Burford, M.A., Alongi, D.M., McKinnon, A.D and Trott, L.A. (2008). Primary production and nutrients in a tropical macrotidal estuary, Darwin Harbour, Australia. Estuarine, Coastal and Shelf Science 79: 440–448.

Butler, E. C. V., Streten-Joyce, C., Tsang, J. J., Williams, D. K., Alongi, D.M., Furnas, M. J., et al. (2013). A Procedure for Evaluating the Nutrient Assimilative Capacity of Darwin Harbour. Report for Aquatic Health Unit, NT Department of Land Resource Management. Australian Institute of Marine Science (Darwin).

Chen, B., Yang, Y., Liang, X., yu, K., Zhang, T., and Li, X. (2013). Metagenomic profiles of antibiotic resistance genes (ARGs) between human impacted estuary and deep ocean sediments. Environ. Sci. Technol. 47, 12753–12760.doi: 10.1021/es403818e

Cook PLM, Eyre BD, Leeming R, Butler ECV (2004) Benthic fluxes of nitrogen in the tidal reaches of a turbid high nitrate subtropical river. Estuar Coast Shelf Sci 59: 675–685.

Dalsgaard, T (Ed), Nielsen, L.P, Brotas, V., Viaoli, P., Underwood,G.,Nedwell, D.B., Sunback, K., Rysgaard, S., Miles, A., Bartoli, M., Dong, L., Thornton, D.C.O., Ottosen, L.D.M., Castaldelli, G. and Rysgaard-Petersen, N. (2000). Protocol handbook for NICE – Nitrogen Cycling in Estuaries: a project under the EU research programme: Marine Science and Technology (MASTIII). National Environmental Research Institute, Silkeborg, Denmark

Devereux, R., Mosher, J.J., Vishnivetskaya, T.A., Brown, S.D., Beddick, D.L., Yates, D.F., et al. (2015). Changes in northern Gulf of Mexico sediment bacterial and archaeal communities exposed to hypoxia. Geobiology 13(5), 478-493. doi: 10.1111/gbi.12142.

Dong, LF., Sobey, Smith, Rusmana, Phillips, Stott, Osborn, Nedwella (2011) Dissimilatory reduction of nitrate to ammonium, not denitrification or anammox, dominates benthic nitrate reduction in tropical estuaries Limnol. Oceanogr., 56(1):279–291

Drewry, J., Fortune, J. and Majid, M. (2010). Chlorophyll-a spatial distribution and water quality in urban affected estuaries in the Darwin Habour region. Report No 17/2010D. Aquatic Health Unit, Department of Land Resource Management, the Arts and Sport. NT.

Duffy, J. E., Amaral-Zettler, L. A., Fautin, D. G., Paulay, G., Rynearson, T. A., Sosik, H. M., et al., (2013). Envisioning a marine biodiversity observation network. Bioscience 63, 350–361. Doi: 10.1525/bio.2013.63.5.8

Eyre, B.D and Ferguson, A.J.P (2009) Denitrification efficiency for defining critical loads of carbon in shallow coastal ecosystems. Hydrobiologia 629:137–146.

Fortune, J.V. (2015) Supplement to the 2014 Darwin Harbour Region Report Card. Report No. 01/2015D. Aquatic Health Unit, Department of Land Resource Management, Palmerston, NT.

Gilbert, J. A., Jansson, J. K., and Knight, R. (2014). The Earth Microbiome Project: successes and aspirations. BMC Biol. 12:69. doi: 10.1186/s12915-014-0069-1

Graham, D.W., Trippett, C., Dodds, W.K., Obrien J.M., Banner, E., Head, I., Smith, M., Yang R., Knapp, C (2010) Correlations between in situ denitrification activity and *nir*-gene abundances in pristine and impacted prairie streams. Env Pollution 158:3224-3229.

Henrichs, S.M. (1992). Early diagenesis of organic matter in marine sediments: progress and perplexity. Marine Chemistry 39: 119-149.

Henry, S., Bru, D., Stres, B., Hallet, S., and Philippot, L. (2006). Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. Appl Environ Microbiol 72(8), 5181-5189. doi: 10.1128/AEM.00231-06.

Johnson, G., Sarker, Nannini, Ferrini, Taylor, Lass-Flörl, Mutschlechner, Bustin, Agrawal1 (2015). Aspergillus Lateral-Flow Device and qPCR testing in Broncho-Alveolar Lavage Fluid: A Combination Biomarker Approach for the Clinical Diagnosis of Invasive Pulmonary Aspergillosis. J Clin Microbiol, doi: 10.1128/JCM.00110-15

Kaestli M, Skillington A, Kennedy K, Majid M, Williams D, McGuinness K, Munksgaard N, Gibb K. (2017). Spatial and Temporal Microbial Patterns in a Tropical Macrotidal Estuary Subject to Urbanization. Frontiers in Microbiology. doi: 10.3389/fmicb.2017.01313

Kana TM, Sullivan MB, Cornwell JC, Groszkowski K. (1998). Denitrification in estuarine sediments determined by membrane inlet mass spectrometry. Limnol Oceanogr 43: 334–339.

Karsenti, E., Acinas, S. G., Bork, P., Bowler, C., De Vargas, C., Raes, J., et al., (2011). A holistic approach to marine eco-systems biology. PLoS Biol. 9:e1001177. doi: 10.1371/journal.pbio.1001177

Kopf, A., Bicak, M., Kottmann, R., Schnetzer, J., Kostadinov, I., LehmannK, et al., (2015). The ocean sampling day consortium. GigaSci. 4:27.doi: 10.1186/s13742-015-0066-5

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25(4), 402-408. doi: http://dx.doi.org/10.1006/meth.2001.1262.

Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R., and Stahl, D.A. (2009). Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature 461(7266), 976-979. doi: http://www.nature.com/nature/journal/v461/n7266/suppinfo/nature08465_S1.html.

Muller-Karger, F. E., Kavanaugh, M. T., Montes, E., Balch, W. M., Breitbart, M., Chavez, F. P., et al., (2014). A framework for a marine biodiversity observing network within changing continental shelf seascapes. Oceanography 27, 18–23. doi: 10.5670/oceanog.2014.56

Nadkarni, M.A., Martin, F.E., Jacques, N.A., and Hunter, N. (2002). Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148(1), 257-266. doi: doi:10.1099/00221287-148-1-257.

Nicholson, G.J., Longmore, A.R and Berelson, W.M. (1999) Nutrient fluxes measured by two types of benthic chamber. Mar Freshw Res 50:567–572.

Potts, J., Blakey, C. and Watson, R.(2005) Nutrient fluxes in 5 lagoons along the Illawarra coast. Report for Wollongong City Council, Department of Environment and Conservation, Sydney.

Reed, H.E., and Martiny, J.B.H. (2013). Microbial composition affects the functioning of estuarine sediments. ISME J 7(4), 868-879. doi: http://www.nature.com/ismej/journal/v7/n4/suppinfo/ismej201215451.html.

Santschi P, Höhener P, Benoit G, Buchholtz-ten Brink M. (1990) Chemical processes at the sediment–water interface. Mar Chem 30:269–315.

Skinner, L., Townsend, S., and Fortune, J. (2009). The Impact of Urban Land-Use on Total Pollutant Loads Entering Darwin Harbour . Report 06/2008D. Aquatic Health Unit, Northern Territory Government.

Smith, J., Burford, M., Revill, A., Haese, R., and Fortune, J. (2012). Effect of nutrient loading on biogeochemical processes in tropical tidal creeks. Biogeochemistry 108(1-3), 359-380.

Tacão, M., Correia, A., and Henriques, I. (2012). Resistance to broadspectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of blaCTX-M-like genes. Appl. Environ. Microbiol. 78, 4134–4140. doi: 10.1128/AEM.00359-12

Thureborn P, Lundin D, Plathan J, Poole AM, Sjo¨ berg B-M, et al., (2013) A Metagenomics Transect into the Deepest Point of the Baltic Sea Reveals Clear Stratification of Microbial Functional Capacities. PLoS ONE 8(9): e74983. doi:10.1371/journal.pone.0074983

Williams, D., Wolanski, E., and Spagnol, S. (2006). "Hydrodynamics of Darwin Harbour," in The Environment in Asia Pacific Harbours, ed E. Wolanski (Dordrecht: Springer Netherlands), 461–476.

Wittebolle, L., Vervaeren, H., Verstraete, W., and Boon, N. (2008). Quantifying community dynamics of nitrifiers in functionally stable reactors. Appl Environ Microbiol 74(1), 286-293. doi: 10.1128/AEM.01006-07.

Zampoukas, N., Palialexis, A., Duffek, A., Graveland, J., Giorgi, G., Hagebro,C., et al., (2014). Technical guidance on monitoring for the marine strategy framework directive, in Joint Research Centre of the European Commission. EUR 26499 (Brussels: European Commission Joint Research Centre Institute for Environment and Sustainability).

Appendix

A1. Primer and probe sequences

New.ReferenceOTU3665

Name	Sequence
F_otu665	TGCTGTGAGCGGAATCGA
P_otu665	CCGTTCGTGGCGAAGCTATCGC
R_otu665	TCCAGGCGGTGCACTTATC

OTU9496

Name	Sequence
F_otu496	TCGGGCTAGAGTGTGGTAGA
P_otu496	TGGAATTCCCGGTGTAGCGGTGAAAT
R_otu496	AGCGGATCCAACGGCTAGTTG

OTU4606

Name	Sequence
F_otu606	GAAGGCCGGTGGTGAAGAC
P_otu606	CCTCTGGGCAATGACTGACGCTG
R_otu606	TCGCCACCCACACCTAGTAC

New.CleanUp.ReferenceOTU1426797

Name	Sequence
F_otu797	AGCGGTGAAATGCGTAGATATTAGG
P_otu797	AACACCAGTGGCGAAGGCGACTA
R_otu797	GGGTCGATACCTCCTACACCTA

New.ReferenceOTU15

Name	Sequence
F_otu15	TAGGCGGGTCTGCAAGTC
P_otu15	TGTGAAATTCCCGGGCTCAACCC
R_otu15	CACTTTCCTCTCCGGTACTCAA

N-cycle gene targets

nosZ-055

Name	Sequence
F_nosZ.55	AGCCGACGGCAAGTACTACA
P_nosZ.55	TCGGGCAACAAGTTCTCGAAGGA
R_nosZ.55	CGCCGGTGATGTCGATCAG

nosZ-061

Name	Sequence
F_nosZ.61	AGCCGGGCCATACGTACAC
P_nosZ.61	CTCGATGGGCGAAACCAAGGAAGC
R_nosZ.61	AAGCGGTCCTTCGAGAACTTG

nosZ-057

Name	Sequence
F_nosZ.57	AAGACGGTCAAGCCGGTGATC
P_nosZ.57	CAAGATCGACGTGCACTATCAGCCC
R_nosZ.57	GCGGTCCTTGGAGAACTTGT

nosZ-077

Name	Sequence
F_nosZ.77	AGCCGCACGACTTCATCATC
P_nosZ.77	TTCAAGCGCGAGCTGGTCAA
R_nosZ.77	TGCCTGCGAGGTCATCTTG

nosZ-038

Name	Sequence
F_nosZ.38	GGCAAGGGCAATGGTTATACG
P_nosZ.38	CGCTGTTCCTCGACAGCCAGGT
R_nosZ.38	GGACGTCGATCTTCTGGATGA

nosZ-080

Name	Sequence
F_nosZ.80	CGATGCGGCGATCAAGTTC
P_nosZ.80	CGGCGGTGACAAGAATGCCAA
R_nosZ.80	GATTGCGAGGCGTTGATGTG

nosZ.159

Name	Sequence
F_nosZ.159	GGTGTCGCTGAACAAGTTCTC
P_nosZ.159	CAAGGACCGTTACCTGAACGTCGG
R_nosZ.159	GGCGAAGGTCGGGCTATC

AamoA-26

Name	Sequence
F_AOA.26	GCCGTAGGCAAGTTCTATAACAG
P_AOA.26	TCCAGTTGCACTCGGTGCAGG
R_AOA.26	GTCCACGTGTTCAGCTTACATC

AamoA-01a+b

Name	Sequence
F_AOA.01a	GGGTGCAGTATTGGCATGTAC
P_AOA.01a	TGCAGCATTAGGTTGCAAACTGAACA
R_AOA.01a	CAAGCGGCCATCCATCTGTA

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

A2. Example of multiple alignment to assess suitability of primers and probes

```
CLUSTAL W (1.83) multiple sequence alignment

nos-38          TGGGCTGGGGCCGCTGCACACCGCCTTCGATG-----------------
nos55           CA-------GTCA-------------------------------------
nos57           ---------G-----------------------------------------
nos61           TGGGCTGGGGCCGCTGCACACAGGTTTCGACGGCCGCGGCAACGCGTACA
nos77           ---------GTCG-------------------------------------
                         *

nos-38          --------------------------GCAAGGGCAATGGTTATACGACG
nos55           ----------------------GATCGTCAAGTGGAACGTC--GATGCCG
nos57           ------------------------TCGTCAAGTGGAACGTG--GACAAGG
nos61           CGTCGCTGTTCCTGGACAGCCAGATCGTCAAGTGGAGTGTC--GACAAGG
nos77           --------------------------TGAAGTGGAACGTG--GAAGCCG
                                           *** * *  *     *    *

nos-38          CTGTTCCTCGACAGCCAGGTCTGCAAGTGGAGCATCGATCTCGCCAAGCG
nos55           C--------GAT--CAAGCAGTTC--------------------AAGGG
nos57           C--------CAT--TGCCCAGTTC--------------------AATGG
nos61           C--------CGT--CGCCCAGTTC--------------------AAGGG
nos77           C--------GAT--CAAGTTCCAC--------------------AAGGG
                *                    *                      **  *

nos-38          CGCCTTCAAGGGCGAGAAGGTCAATCCGATCATCCAGAAGATCGACGTCC
nos55           CGA---CAAGAACG------CGAAGGTCATCCTGGATAAGGTCGACGTGC
nos57           CGA---CAAGACGG------TCAAGCCGGTGATCGACAAGATCGACGTGC
nos61           TGA---CAAGACCA------TCCAAGTCGTGCTCGACCGCCTCGATGTTC
nos77           CGA---CAAGGCGG------CCAAGTATGTCGTCGACCGCATCGACGTGC
                 *    ****            *     *  *  *     **** ** *

nos-38          ATTATCAGCCCGGCCACAACCACTCCTCGATGGGCGAGACCAAGGAGGCC
nos55           ACTACCAGCCGGGCCATGGCTATGCCTCGATGGGCGAAACCAAGGAAGCC
nos57           ACTATCAGCCCGGCCACGGCTACTCGTCGATGGGCGAAACCAAGGAAGCC
nos61           AGTACCAGCCGGGCCATACGTACACCTCGATGGGCGAAACCAAGGAAGCG
nos77           AGTACCAGCCCGGCCACCTGAACGCGAGCCAGTCCGAAACCATGGCTGCC
                * ** ***** *****     *  *      *  *** **** **  **

nos-38          GATGGCAAGTGGCTGATCTCGCTGAACAAGTTCTCCAAGGATCGCTTCCT
nos55           GACGGCAAGTACTACAACTCGGGCAACAAGTTCTCGAAGGATCGCTTCCT
nos57           GACGGCAAGTACTTCAATTCGGGTAACAAGTTCTCCAAGGACCGCTTCCT
nos61           GACGGGAAGTACCTCAATTCCGGAAACAAGTTCTCGAAGGACCGCTTCCT
nos77           GACGGCAAGTACCTGGCGGTGGGCTGCAAGTTCTCCAAGGACCGCTTCCT
                ** ** ****               ********* ***** ********

nos-38          CAACGTCGGTCCGCTCAAGCCGGAGAACGACCAGTTGATCGACATCTCGG
nos55           GCCGGTCGGCCCGCTGCATGTCGAAACCGAGCAGCTGATCGACATCACCG
nos57           GCCGGTCGGCCCGCTGCATTGCGAGACCGAGCAGCTGCTCAATCTGACGG
nos61           CCCGGTGGGGCCGCTGCATGTCGAGACCGAGCAGCTGGTCGACATCACCG
nos77           GCCCGCCGGCCCACTGCACCCCGAGAACGAGCAGTTGATCGACATCTCGG
                    *  ** ** **  *    ** * *** *** ** ** *  *  * *

nos-38          GCGACCAGATGAAGCTGGTGCATGACGGCCCGAGCTTCGCCGAGCCGCAT
nos55           GCGACAAGATGCGCGTCATCTCCGACCACACTGCGTATCCGGAACCGCAT
nos57           GCGAAAAGATGGTGCTGGTGTCGGACCACACGGCCTATCCGGAACCGCAC
nos61           GCGACAAGATGAAGCTGATCCAGGACCATGCAGCCTACCCTGAGCCGCAT
nos77           GCGACAAGATGGTGCTGCTGGCGGACCACCCGGTGCGCGGCGAGCCGCAC
                ****  *****    *  *    ***    *          ** *****

nos-38          GACGCCGTCATCGTGCATCGATCCAAGCTGGAGGGAAAGGTCACAAGCAT
nos55           GACGCCATCATCGTGCGCCGCG---ATGTCG--TCAAGACGCGCCAG-AT
nos57           GACGGCATCATCGTGCGCCGCG---ACCGTG--TGAAAACGCGCCAG-GT
nos61           GATGCCATCATCGTCCGGCGCG---ACAAGC--TCAGGACCAAGCAG-AT
nos77           GACTTCATCATCTTCAAGCGCG---AGCTGG--TCAAGCCGAAGCAG-GT
                **   * ***** *    **     *          *          **  *
```

A3. Example of qPCR Assay Design Software output

0006 **89.76**

Oligo Detail

Oligo	Tm	GC%	Length	5' Pos	3' Pos	5'-Sequence-3'
Forward	66.2	52	21	32	52	GGCAAGGGCAATGGTTATACG
Reverse	66.5	52	21	155	135	GGACGTCGATCTTCTGGATGA
Probe	70.3	64	22	54	75	CGCTGTTCCTCGACAGCCAGGT BHQ1

Identified Mis-Alignments

Self Align	Dimer	3'Align	Hairpin	Total ΔG°	Pair Align	Dimer	3' Align	Bi-ΔG°
Forward	4	3	2	-11.06	Forward/Reverse	3	3	.00
Reverse	6	3	3	-11.07	Forward/Probe	3	2	.00
Probe	5	3	5	-11.95	Reverse/Probe	5	3	.00

Amplicon Detail

Amplicon	Rank	AT Run	GC Run	GC%	Length	Variation
Allele 1	87.97	6	3	52	124	

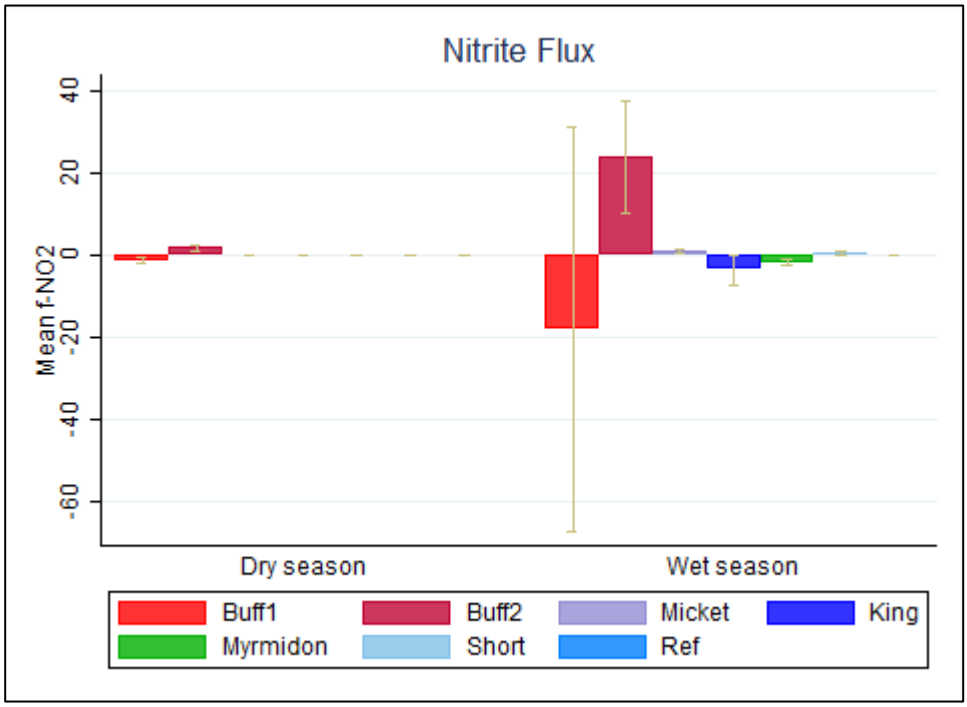
GGCAAGGGCA ATGGTTATAC GACGCTGTTC CTCGACAGCC AGGTCTGCAA GTGGAGCATC GATCTCGCCA ANNNNNNCTT
CAAGGGCGAG AAGGTCAATC CGATCATCCA GAAGATCGAC GTCC

A4. Water physico-chemistry

At each sample site, physicochemical measurements were taken before cores were extracted for the flux chamber assays. As shown in Table 2, pH ranged from 7.2-8.4 and temperature ranged from 23.5°C at Ref Ck to 32.1°C at King Creek. Salinity ranges were extremely different between seasons at some sites. For example at King and Micket Creeks salinity was very low in the wet and hypersaline in the dry, possibly due to poor mixing and inverse estuary effects. BC1 is subject to the influence of wastewater discharge from the Leanyer-Sanderson wastewater treatment plant. Dissolved oxygen was typically above 63% at most sites with the exception of the most upper site of BC1 on Buffalo Creek where DO was below 10% during the dry season. Turbidity ranged from 2.3 to 76.5 NTU. The highest value was at BC1 (76.5 NTU) during dry season sampling, turbidity was also high at Micket and King Creeks during the dry season campaign. The lowest value was at Ref Creek with 2.3 NTU measured during the dry season.

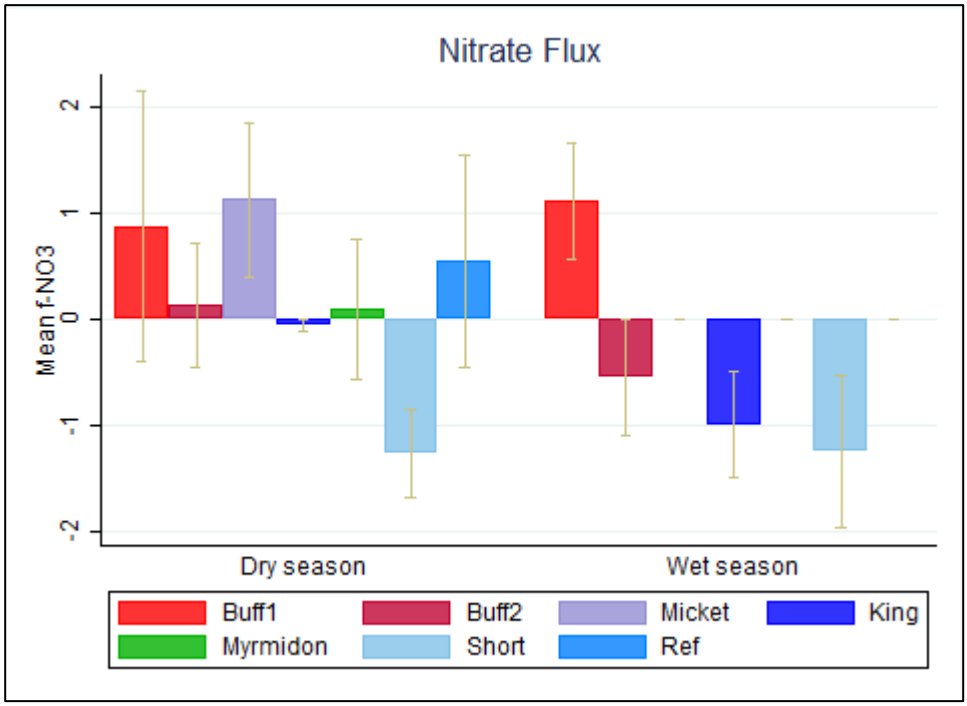
Site	Date	Time	DO (mg/L)	DO % sat	Temp Deg C	pH	Salinity (ppt)	EC (µS/cm)	Turbidity (NTU)	Zeu (m)
Micket (dry)	9/7/15	08:30	4.12	64.9	24.77	7.73	43.1	64000	60.8	0.93
Micket (wet)	17/2/16	11:32	3.44	50.1	30.94	7.25	13.9	22900	29.9	0.92
King (dry)	9/7/15	09:50	5.42	82.7	24.85	8.18	38.5	57900	55.9	0.73
King (wet)	17/2/16	12:55	4.54	65.2	32.1	7.67	7.4	12990	14.4	1.27
Myrmidon (dry)	23/7/15	10:22	6.32	94.4	24.03	8.43	37.8	57100	3.39	5.65
Myrmidon (wet)	17/3/16	09:30	4.24	66.2	30.35	7.43	27.4	42200	13.8	2.10
Short (dry)	23/7/15	11:26	6.36	96	24.17	8.4	37.9	57100	4.6	5.81
Short (wet)	17/3/16	10:29	4.05	65.7	31.04	7.51	30.2	46200	11.3	3.50
Ref (dry)	23/7/15	12:22	6.01	90.2	23.51	8.36	38.2	57600	2.39	5.66
Ref (wet)	17/3/16	11:10	4.26	72.4	31.44	7.56	31.0	47300	6.61	1.87
Buff Ck1 (dry)	28/7/15	12:43	0.76	9.4	25.34	7.67	14.8	2340	76.5	0.81
Buff Ck1 (wet)	31/3/16	09:30	2.08	31.1	31.37	7.05	12.8	21300	35.6	0.90
Buff Ck 2 (dry)	28/7/15	11:15	1.54	22.4	25.75	7.87	35.0	53100	37.3	2.32
Buff Ck2 (wet)	31/3/16	10:00	5.14	83.4	30.38	7.81	30.1	46400	37	1.06

A5. fNO_2-Flux

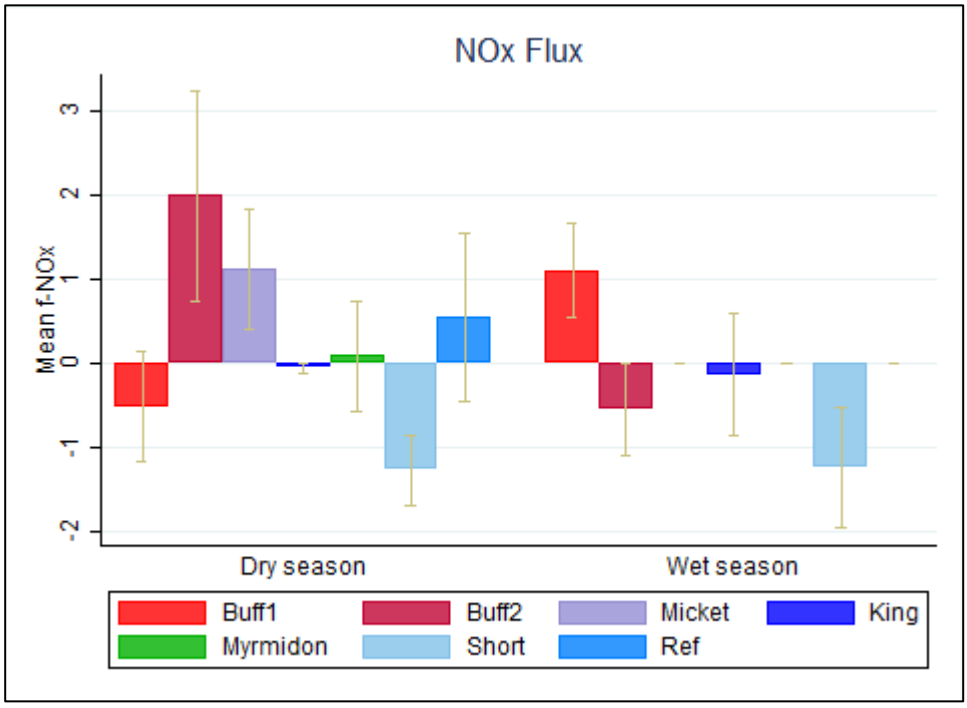


A6. fNO_3-Flux

The NO_3 flux was highly variable and likely a consequence of the highly temporal response to nitrate which might change over a matter of minutes to hours depending on tide.



A7. fNOx-Flux



A8. fDO-Flux