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Denitrification and Anammox in Tropical Aquaculture Settlement Ponds: An Isotope Tracer Approach for Evaluating N_2 Production

Sarah A. Castine[1]*, Dirk V. Erler[2], Lindsay A. Trott[3], Nicholas A. Paul[4], Rocky de Nys[4], Bradley D. Eyre[2]

1 AIMS@JCU, School of Marine and Tropical Biology, Australian Institute of Marine Science, Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville, Queensland, Australia, **2** School of Environmental Science and Management, Centre for Coastal Biogeochemistry, Southern Cross University, Lismore, New South Wales, Australia, **3** Australian Institute of Marine Science, Townsville, Queensland, Australia, **4** School of Marine, Tropical Biology and Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville, Queensland, Australia

Abstract

Settlement ponds are used to treat aquaculture discharge water by removing nutrients through physical (settling) and biological (microbial transformation) processes. Nutrient removal through settling has been quantified, however, the occurrence of, and potential for microbial nitrogen (N) removal is largely unknown in these systems. Therefore, isotope tracer techniques were used to measure potential rates of denitrification and anaerobic ammonium oxidation (anammox) in the sediment of settlement ponds in tropical aquaculture systems. Dinitrogen gas (N_2) was produced in all ponds, although potential rates were low (0–7.07 nmol N cm^{-3} h^{-1}) relative to other aquatic systems. Denitrification was the main driver of N_2 production, with anammox only detected in two of the four ponds. No correlations were detected between the measured sediment variables (total organic carbon, total nitrogen, iron, manganese, sulphur and phosphorous) and denitrification or anammox. Furthermore, denitrification was not carbon limited as the addition of particulate organic matter (paired *t*-Test; $P = 0.350$, $n = 3$) or methanol (paired *t*-Test; $P = 0.744$, $n = 3$) did not stimulate production of N_2. A simple mass balance model showed that only 2.5% of added fixed N was removed in the studied settlement ponds through the denitrification and anammox processes. It is recommended that settlement ponds be used in conjunction with additional technologies (i.e. constructed wetlands or biological reactors) to enhance N_2 production and N removal from aquaculture wastewater.

Citation: Castine SA, Erler DV, Trott LA, Paul NA, de Nys R, et al. (2012) Denitrification and Anammox in Tropical Aquaculture Settlement Ponds: An Isotope Tracer Approach for Evaluating N_2 Production. PLoS ONE 7(9): e42810. doi:10.1371/journal.pone.0042810

Editor: Jacqueline Mohan, Odum School of Ecology, University of Georgia, United States of America

Received April 9, 2012; **Accepted** July 12, 2012; **Published** September 4, 2012

Funding: This research was funded by an AIMS@JCU research scholarship awarded to Sarah Castine at the beginning of her PhD. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: sarah.castine@my.jcu.edu.au

Introduction

The release of anthropogenic N to the coastal zone poses a threat to many shallow marine ecosystems [1]. Discharge of aquaculture wastewaters has contributed to N enrichment of some coastal regions [2] and settlement ponds have been established as a remediation strategy from aquaculture wastewater prior to release to the environment [3,4]. Settlement pond technologies are widely implemented as a low cost option for treating municipal [5], fish farm [6] and dairy farm wastewater [7]. However, the nutrient removal efficiency of settlement ponds associated with land-based tropical aquaculture systems is unclear. Generally, newly established (<1 yr old) settlement ponds, with a basic design, provide significant reductions in total suspended solids, but are less efficient in the remediation of dissolved nutrients [3,8]. Furthermore, given that the efficiency of wetland wastewater treatment systems can decrease with age [9], it is likely that the performance of settlement ponds, which act as brackish water constructed wetlands, will decrease over time unless they are actively managed. Methods to improve the long term performance of tropical aquaculture settlement ponds include the use of extractive organisms such as algae, which can be cultured and subsequently harvested [10], and also the removal of settled organic rich particulates (sludge) which prevents remineralization of dissolved N back into the water column [3,11]. Microbial nutrient transformation, which is largely un-quantified, also presents a potentially significant mechanism to reduce dissolved inorganic nitrogen (DIN) in aquaculture wastewater.

Denitrification and anammox are the major microbial processes removing fixed N from wastewater through the production of dinitrogen gas (N_2). During denitrification, nitrate (NO_3^-) is reduced to nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O), before eventually being converted to N_2. Anammox also directly removes fixed N and couples NO_2^- reduction with ammonium (NH_4^+) oxidation to produce N_2 [12,13]. Denitrification and anammox are also important for the removal of N from natural system such as intertidal flats [14], marsh sediments [15], deep anoxic waters [16] and sediments from the continental shelf (50 m) and slope (2000 m) [17]. Denitrification and anammox in natural systems can remove up to 266 mmol m^{-2} d^{-1} and 61 mmol m^{-2} d^{-1} of N, respectively [16]. These processes may be active in the treatment of aquaculture effluent water and could be exploited to enhance treatment. However, to date there has been no published quantification of denitrification and anammox in settlement pond systems treating waste from tropical aquaculture farms.

The first step in optimizing the removal of fixed N through the denitrification and anammox pathways is to quantify their activity in settlement ponds and relate this to the environment of the ponds. Accordingly, the aim of this study was to determine if denitrification and anammox occur in sediments collected from tropical settlement ponds that are used to treat effluent from commercial production of prawns (shrimp) and fish. We used sediment slurry assays to investigate potential N_2 production in multiple zones of four settlement ponds on three farms (two prawn farms and one fish farm). We also investigated the relationship between the potential rates of N_2 production with the geochemical characteristics of the ponds. Additionally, the effect of carbon on N_2 production was tested since intensive aquaculture systems have N rich wastewaters where microbial N removal is typically limited by the supply of carbon as an electron donor [18]. Together these data provide new insight into N cycling processes in shallow tropical eutrophic marine systems in the context of N management.

Methods

Study site

The presence of denitrification and anammox and their potential rates were measured in sediment collected from four settlement ponds across two operational prawn (*Penaeus monodon*) farms and one barramundi (*Lates calcarifer* Bloch) farm. At Farm 1 sediment was collected from the two functional settlement ponds, this allowed comparison of N_2 production over small spatial scales (A and B; Figure 1). Additionally, sediment was collected from the only settlement pond at Farm 2 (Pond C) and the only settlement pond at Farm 3 (Pond D) (Figure 1). The three farms spanned the wet and dry tropics allowing comparison of N_2 production in different environments. Each pond was split into 3 zones (Z1, Z2 and Z3) (Figure 1). In all ponds Z1 was near the inlet, Z2 was near the middle of the settlement pond, and Z3 was near the outlet of the settlement pond. Ponds have diurnal fluctuations in dissolved oxygen (DO) concentration; from <31.2 µM at night to supersaturation (>312.5 µM) during the day, indicating rapid water column productivity. Similarly, there are diurnal pH fluctuations (1–1.5 pH). According to farm records, salinity fluctuates seasonally, with dramatic decreases from 35‰ to 5‰ caused by heavy precipitation over the summer wet season. During the wet season access to the farms by road is limited. All assays were, therefore run within the same dry season, although salinity at Farm 2 was still reasonably low due to particularly heavy rainfall over the 2009/2010 wet season (see results section).

Geochemical characteristics

To investigate the spatial variation of sediment characteristics within and between settlement ponds, and their role in driving N_2 production, sediments were collected at Z1, Z2 and Z3 in each of the four settlement ponds (total of 12 zones) (Figure 1). Sampling was conducted in March 2010 for Ponds B and C and August 2010 for Ponds A and D. Directly before taking sediment samples, surface water salinity, temperature and pH were also measured at each zone within each pond using specific probes (YSI-Instruments). Probes were calibrated 24 h before use. They were submerged directly below the surface and left to stabilize for 5 min before recording data. A known volume of sediment (30–60 mL) was subsequently collected in intact sediment cores ($n = 3$ per zone). The sediments were extruded, weighed and subsequently oven dried (60°C) and reweighed for porosity (ø) determination ($n = 3$). Dried sediment was then milled (Rocklabs Ring Mill) for total N determination (LECO Truspec CN Analyzer). TOC was determined on a Shimadzu TOC-V Analyzer with a SSM-5000A Solid Sample Module. Solid phase S, P, Fe and Mn were also analyzed from milled sediment samples subjected to strong acid digestion. A THERMO Iris INTREPID II XSP ICP_AES was used to determine element content in triplicate sediment samples from each zone [19].

Denitrification and anammox potential

Slurry assays were conducted to test for the presence of N_2 (inclusive of both N_2 and N_2O) production through denitrification and anammox in March 2010 (Ponds B and C) or August 2010 (Ponds A and D). At the time of abiotic sample collection (see above), approximately 500 g of the most reactive sediments were collected from each zone in the four settlement ponds ($n = 1$ from each zone within each pond) (Figure 1) with a 30 mm i.d. corer [20]. The top 0–3 cm was collected because this includes the oxic and suboxic layers where NO_x is present or being reduced (denitrification) [21] and the anoxic layer below the interface, where NO_x penetrates but O_2 does not, making conditions favorable for anammox [22]. Each sediment sample was placed into sterile plastic bags with minimal air and subsequently homogenized by hand and doubled bagged before transportation to the laboratory. Sediments remained in initial plastic bags at room temperature for up to five days until the start of the experiment. Standard anammox assays were run according to Trimmer et al. [23] and Thamdrup and Dalsgaard [22] with modifications (artificial seawater of the same salinity as site water) according to Erler et al. [20]. Artificial seawater was used to preclude the potential interference of ambient NO_3^- in the isotope assay. A known volume of sediment (3–6 g) was loaded into Exetainers (Labco Ltd, High Wycombe, UK) and ~5 mL of degassed (flushed for 1 hr with ultra pure He), artificial seawater was added to form a slurry. Sediments were pre-incubated (overnight) under anoxic conditions to ensure all residual NO_3, NO_2^- and O_2 were consumed. Three different enrichment treatments (100 µM $^{15}N\text{-}NH_4^+$, 100 µM $^{15}N\text{-}NH_4^+$ plus 100 µM $^{14}N\text{-}NO_3^-$ or 100 µM $^{15}N\text{-}NO_3^-$) were added to the slurries. After the isotope amendment, the Exetainers were filled with the degassed seawater, capped without headspace and homogenized by inverting 2–3 times. Triplicate samples were sacrificed from each treatment at 0, 0.5, 17 h and 24 h by introducing 200 µL 50% w/v $ZnCl_2$ through a rubber septum ($n = 3$). The 0 and 0.5 h time periods were chosen based on rapid turnover rates determined by Trimmer et al. [23] and 17 and 24 h were modified from Erler et al. [20]. Sacrifice of the slurry samples involved the addition of 2 mL He headspace to the samples through the septum. Samples were stored inverted and submerged in water at 4°C until analysis to ensure there was no diffusion of N_2 into or out of the Exetainers. A gas chromatograph (Thermo Trace Ultra GC) interfaced to an isotope ratio mass spectrometer (IRMS, Thermo Delta V Plus IRMS) was used to determine $^{29}N_2$ and $^{30}N_2$ content of dissolved nitrogenous gas (includes $^{15}N\text{-}N_2$ and $^{15}N\text{-}N_2O$, collectively referred to as N_2). Varying volumes (3–10 µL) of air were used as calibration standards.

The rate of N_2 production in the 24 h incubation trials (above) was calculated from the slope of the regression over the incubation period (0, 0.5, 17, 24 h) based on Dalsgaard and Thamdrup [24]. However, in some cases the production of $^{29}N_2$ and $^{30}N_2$ was non-linear and rates were calculated based on the first two production points. Therefore a subsequent slurry assay was run to investigate N_2 production rates over short, regular time intervals (15 min) to

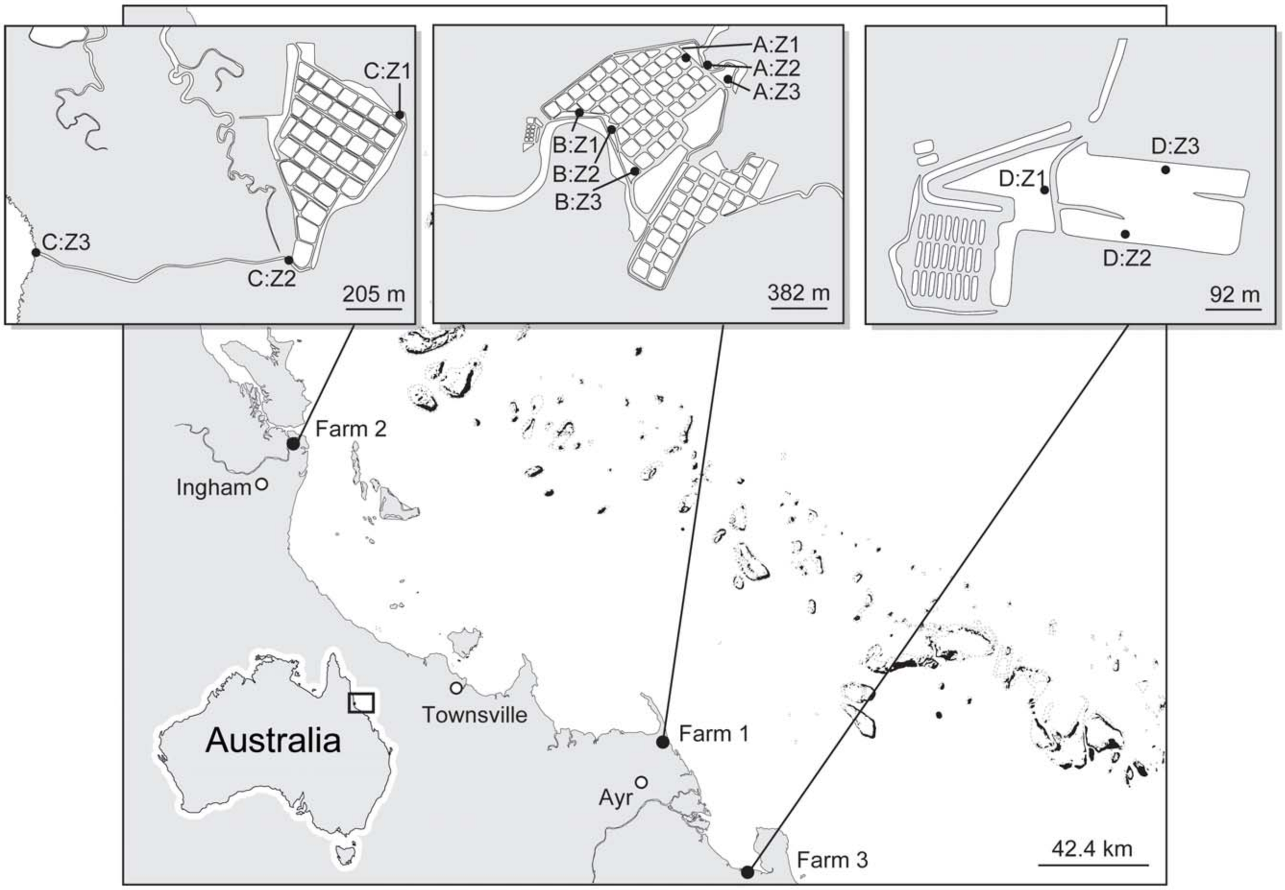

Figure 1. The location of three flow-through aquaculture farms along the North Australian coastline. The inset figures show the layout of each farm, the location of the settlement ponds and the 3 zones within each pond.
doi:10.1371/journal.pone.0042810.g001

gain a more accurate insight into potential process rates. Sediment for the additional assays was collected from settlement Pond D, Zones 1 ($n=1$) and 3 ($n=1$) in October 2010. These zones were chosen because the production of N_2 was non-linear during the 24 h incubation assay (see results section). Assays were run as described above, following the same sediment collection, pre-incubation, amendment and analysis techniques. However, samples were sacrificed at 0, 15, 30, 45 and 90 min.

Slurry assay with carbon manipulation

The effect of an additional carbon source on the occurrence of denitrification and anammox was tested with a separate set of slurry assays because organic carbon limits N_2 production in some aquaculture systems [25]. Extra sediment was collected in March and August (2010) in the sampling described above. Sediments from Ponds A (August) and C (March) were assayed with and without addition of a carbon source because organic carbon has stimulated or correlated with N_2 production in some systems previously [17,26,27]. Concentrated particulate organic matter (POM) was used to test the effect of an *in situ* carbon source collected from Pond A. POM was collected by transporting settlement pond-influent water to the laboratory at the same time that sediments were collected. Suspended solids in influent water were concentrated by centrifugation (10 min at 3000 rpm). 400 μL aliquots of concentrated (~100 mg L^{-1}) POM were added to Exetainer vials prior to the addition of amendments. However, in the absence of a high total suspended solid load at Pond C, methanol (MeOH) was used as the carbon source as it stimulates denitrification but inhibits anammox in some circumstances [28,29]. MeOH additions were carried out by adding MeOH at a concentration of 3 mM (based on Jensen et al. [29]) to a parallel set of samples from Pond C prior to amendments.

Modeling N removal

A simplistic model was constructed to estimate the mean dry season N removal (NR) capacity (%) of the four settlement ponds. NR was estimated using the potential N_2 production rates calculated in the present study, and N inputs into the pond through the wastewater. Given the substantial contribution of N remineralized from sludge in shrimp grow-out ponds (often exceeding inputs of N originating from feeds [30]), a variable to account for remineralization inputs was also added (N_{imin}). The following equation was used to calculate N removal and the parameters are further defined in Table 1:

Equation 1.

$$NR=\frac{N_2 \times A \times t \times A_r}{(N_{iww}+N_{i\,\min})} \times 100$$

where N_2 = the mean total (inclusive of anammox) N_2 production rate measured during the 24 h incubation (nmol N cm^{-3} h^{-1}; Table 1). We adopted a conservative approach and assumed that N_2 production, driven by denitrification, only occurs in the top 1 cm of the sediment. Denitrification occurs at the oxic-anoxic

Table 1. An estimate of nitrogen inputs and microbial removal from settlement ponds, note TN = total nitrogen, WW = wastewater, min = mineralization.

Parameter	Value	Unit	Reference
Pond area	6000	m^2	Farm proprietors Pers. comm.
Mean TN WW input	14.8	kg N d^{-1}	EPA monitoring data
Mean net NH_4^+ min	27.8	mmol m^{-2} h^{-1}	[30]
Mean net DON min	0.6	mmol m^{-2} h^{-1}	[30]
Mean N_2 production	2.9	nmol N cm^{-3} h^{-1}	Slurry assay
Net N removal	2.5	%	Model

doi:10.1371/journal.pone.0042810.t001

interface so the depth at which it occurs is dependent on O_2 penetration into the sediments. O_2 penetration is estimated at <0.5 mm in fish farm wastewater treatment ponds [4], 1.5–4 mm in sediments below fish cages and associated reference sites and up to 20 mm in a muddy macrotidal estuary [31]. This active zone is subsequently extrapolated to estimate rates for the entire area of the settlement pond. The remaining parameters are defined as follows: A = mean area of the settlement pond (m^2); $t = 24$ (h d^{-1}); A_r = atomic weight of N; N_{iww} = mean rate of TN input (inclusive of particulates and dissolved) via the wastewater (environmental protection agency (EPA) monitoring data, quantified monthly by Farm 1; kg N d^{-1}); N_{imin} = mean rate of N input via mineralisation (deduced from NH_4^+ and DON fluxes in Burford and Longmore [32]; Table 1; kg N d^{-1}).

Calculations and statistical analysis

The sediment characteristics data was analysed as a 2-factor nested design, pond and zone(pond) using permutational multivariate analysis of variance (PERMANOVA) [33]. PERMANOVA calculated *p*-values from 9999 permutations based on Bray-Curtis distances. A 1-factor PERMANOVA was subsequently used to compare differences in N_2 production rate data (three variables; denitrification, anammox and total N_2 production) between ponds with zones as replicates ($n = 3$). 9999 permutations were again used to calculate *p*-values based on Bray-Curtis distance. PRIMER version 6 and PERMANOVA+ version 1.0.4 were used to conduct both analyses.

The relationship between N_2 production rate (three variables: denitrification, anammox and total N_2 production) and sediment characteristics was subsequently investigated using the BIOENV procedure in PRIMER. This procedure performs a rank correlation of the two similarity matrices (described above) and tests every combination of sediment characteristics to determine which set of variables best explains the observed N_2 production rates [34]. A Bray-Curtis similarity matrix comprised of both N_2 production rate data and sediment variable data was also used to conduct a hierarchical agglomerative cluster analysis which was superimposed on a multidimensional scaling (nMDS) plot. The nMDS plot provided a 2-D visualization of the relationship between sediment characteristics and N_2 production rates.

The effect of carbon addition on potential N_2 production rate in sediments was analyzed with paired *t*-Tests for each carbon source (POM and MeOH).

Results

Pond characteristics and abiotic factors

Surface water temperature (25.8°C±1.0) and pH (7.6±0.2) varied little similar across all ponds and zones. Surface water salinity in Pond C (Farm 2) was lower (17–18‰) than the other three ponds (31–35‰; Table 2) due to its location in the wet tropics where precipitation is high (Figure 1).

Sediment at all zones was uniformly dark black with minor color variation shown in a narrow lighter band (~3 mm oxic zone) at the surface of the sediment. The porosity ranged between 41–72% (Table 2) and sediments produced a rich hydrogen-sulfide smell and gaseous bubbles (presumably consisting of a mix of biogases) at the water surface when the sediment was disturbed. Very little bioturbation by burrowing organisms or flora was evident. There was significant variability between ponds (Table 3; PERMANOVA; pond; *Pseudo* $F = 2.06$, $P = 0.028$) and between zones within ponds (Table 3; PERMANOVA; zone (pond); *Pseudo* $F = 33.83$, $P < 0.001$). The variance in sediment characteristics at the finer scale (i.e. meters) between zones within ponds (52.4%) was greater than the variance between settlement ponds located kilometers apart (31.6%).

Denitrification and anammox potential

There was also a significant difference in the potential rate of N_2 production between ponds (Table 3; PERMANOVA; pond; *Pseudo* $F = 3.91$, $P = 0.001$). The potential rate was highest in sediments collected from pond A, with denitrification the sole producer of N_2 (7.07±2.99 nmol N cm^{-3} h^{-1}; Table 4) and lowest in sediments collected from pond C, where again denitrification was the responsible for 100% of the N_2 produced (0.004±0.003 nmol N cm^{-3} h^{-1}; Table 4). However, there was no correlation between the potential production of N_2 in zones within ponds and different sediment characteristics that defined each pond (nMDS, Figure 2a & b). For example, pond B zone 3 had the highest anammox rates and low denitrification, whereas pond A, zones 2 and 3 had the opposite trend (Figure 2a). This is highlighted in the vector loadings for which the vectors for anammox and denitrification are clearly negatively correlated (Figure 2b).

Highly positive or negative loadings of the sediment characteristics appeared to have little influence on total N_2 production or denitrification (Figure 2b) as these are perpendicular to the positive

Table 2. Mean surface water salinity ($n = 3 \pm 1$ SE) and abiotic sediment characteristics ($n = 9 \pm 1$ SE) in the four settlement ponds (A, B, C and D) used to treat aquaculture wastewater (μmol g^{-1} unless stated).

	Pond A	Pond B	Pond C	Pond D
Salinity (‰)	31±0	34±0	18±0	35±0
Porosity (%)	0.5±0.0	0.5±0.0	0.5±0.0	0.6±0.0
TOC	61±13	62±6	43±5	63±4
TOC (%)	0.7±0.9	0.8±0.1	0.5±0.1	0.8±0.1
TN	5±1	6±1	4±1	8±1
TN (%)	0.1±1.0	0.1±0.4	0.1±0.8	0.1±0.6
TP	18±4	14±2	5±1	14±3
S	9±1	9±2	12±2	9±0
Fe	43±6	52±5	18±2	25±1
Mn	8±2	6±1	1±0	2±0

doi:10.1371/journal.pone.0042810.t002

Table 3. A summary of statistical analyses; PERMANOVAs based on the Bray-Curtis similarities of transformed (4th root) sediment characteristic data and potential N_2 production rate data.

Sediment characteristics				
Test				**PERMANOVA**
Factors	**df**	**MS**	**Pseudo-*F***	***P***
Pond	3	39	2.06	0.028
Zone (Pond)	8	19	33.83	0.000
N_2 production rate				
Test				**PERMANOVA**
Factors	**df**	**MS**	**Pseudo-*F***	***P***
Pond	3	4029	3.91	0.001

doi:10.1371/journal.pone.0042810.t003

loadings of all the sediment characteristics. Anammox did cluster near sediment variables (Figure 2b), however there was no correlation between the N_2 production matrix (inclusive of total N_2, denitrification and anammox) or the sediment variable matrix (BIOENV analysis; $\rho = 0.134$, $P = 0.730$).

In incubations with ^{15}N labeling of nitrate only, the majority of $^{15}N\text{-}NO_3^-$ converted to N_2 was found in $^{30}N_2$ (Figure 3). Only in pond B was more of $^{15}N\text{-}NO_3^-$ that was converted to N_2 found in $^{29}N_2$ than in $^{30}N_2$ (Figure 3). Anammox was detected in pond B sediments as indicated by the higher percent recovery (0.67±0.28%) of $^{15}N\text{-}N_2$ in treatments where $^{15}N\text{-}NH_4^+$ and unlabelled $^{14}N\text{-}NO_3^-$ were added compared to treatments where $^{15}N\text{-}NH_4^+$ was added (0.28±0.09%; Table 5). However, in this pond total recovery of $^{15}N\text{-}NO_3^-$ as $^{15}N\text{-}N_2$ was extremely low (0.20±0.07; Table 5).

Slurry assay with carbon manipulation

There was no significant difference in the rate of N_2 production when either POM (Table 3; Pond A; paired *t*-Test; $P = 0.350$, $n = 3$) or methanol (Table 3; Pond C, paired *t*-Test; $P = 0.744$, $n = 3$) was added to the experimental sediment slurries (Table 4; 24 h incubation compared to carbon incubation).

Nitrogen removal capacity

We estimate that 2.5% of the total N inputs to the settlement pond are removed through denitrification and anammox (Table 1).

Table 4. The rate (nmol N cm^{-3} h^{-1}) of N_2 production in three incubations (i.e. 24 h, 1.5 h and in the incubation with carbon additions).

Pond	24 h incubation		1.5 h incubation		Carbon Incubation	
	DNT	ANA	DNT	ANA	DNT	ANA
A	7.07±2.99	ND			7.97±3.35	ND
B	0.06±0.06	0.22±0.12				
C	0.004±0.003	ND			0.004±0.003	0.03±0.02
D	4.36±2.01	ND	6.32±4.16	0.48±0.48		

DNT = denitrification; ANA = anammox.
doi:10.1371/journal.pone.0042810.t004

Discussion

Total N_2 production and controlling mechanisms

Isotope tracer techniques confirmed the production of N_2 in sediment collected at all three zones within each of the four settlement ponds used to treat wastewater from commercial prawn and barramundi farms. The potential rates (0–7.07 nmol N cm^{-3} h^{-1}) were within the range of those reported for a subtropical constructed wetland (1.1±0.2 to 13.1±2.6 nmol N cm^{-3} h^{-1}) [20], but lower than those reported for subtropical mangrove and shrimp grow out pond sediments (21.5–78.5 nmol N cm^{-3} h^{-1}) [35]. Nevertheless, it can be assumed that both denitrifying bacteria and *Planctomycetes* (anammox bacteria) are present in the ponds and that there is potential to stimulate N_2 production rates and enhance N removal. To achieve this, an understanding of the mechanisms controlling N_2 production is required. We therefore investigated the effect of carbon additions on N_2 production rate and the relationship between the concentration of sediment elements and N_2 production rates. However, there was no significant change in the rate of N_2 production under carbon loading and there was no correlation between any of the measured sediment variables and N_2 production rate via denitrification or anammox.

Denitrification is often limited by carbon in aquaculture ponds, as carnivorous marine species require high inputs of protein rich feeds. N removal can be enhanced through the addition of an exogenous carbon source, for example glucose and cassava meal [26] or molasses [25] have been added to shrimp farm wastewater treatment processes, resulting in up to 99% removal of NH_4^+, NO_3^- and NO_2^-. Similarly, methanol is a common additive to enhance denitrification for municipal wastewater treatment, increasing degradation of NO_2^- in activated sludge from 0.27 mg NO_2^- g^{-1} volatile suspended solids (VSS) h^{-1} to 1.20 mg NO_2^- g^{-1} VSS h^{-1} [27]. However, in the present study N_2 production was not enhanced through the addition of carbon, suggesting that there are additional controlling mechanisms driving N_2 production. This concurs with the lack of significant correlation between measured sedimentary TOC and N_2 production. The lack of stimulation of N_2 production after the addition of carbon has also been demonstrated in the oxygen minimum zone of the Arabian Sea, where denitrification (and anammox) was only enhanced at one out of 11 depths [36]. Instead, Bulow et al. [36] highlighted a correlation between denitrification and NO_2^- concentration, a factor which likely also plays a role in controlling denitrification in settlement pond systems but was not measured in

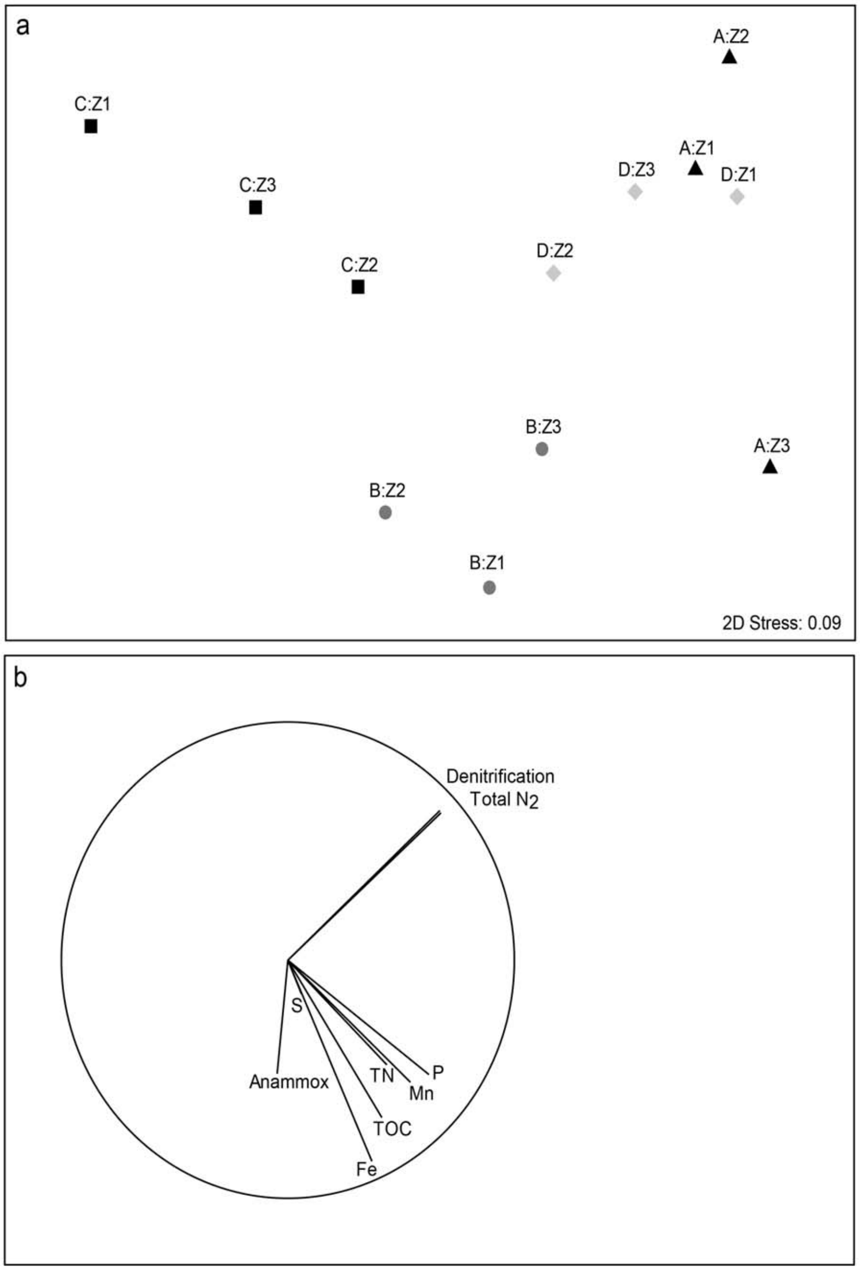

Figure 2. Similarity between N_2 production rates and sediment characteristics in the four settlement ponds. a) nMDS ordination; 2-D stress = 0.09. b) The same nMDS as a), with vectors superimposed, the length and direction of which indicated the strength of the correlation and direction of change between the two nMDS axes.

doi:10.1371/journal.pone.0042810.g002

the present study. NO_3^- concentration also regulates anammox activity in estuarine sediments [37], so future work should aim to correlate extractable NO_3^-, NO_2^- and NH_4^+ with denitrification and anammox potentials to determine if these are driving process rates in settlement ponds.

It is also possible that the exogenous carbon source is instead stimulating nitrate ammonifiers (DNRA) and therefore competition for NO_x as a substrate [38]. Of the added $^{15}NO_3^-$ only 7.9±2.7% was recovered as $^{15}N_2$, so a large portion (i.e. ~90%) of added $^{15}NO_3^-$ could be rapidly consumed by competing pathways such as DNRA or assimilation. The prevalence of DNRA or assimilation over denitrification will determine the balance between N being removed from the system through gaseous N_2 production, or conserved within the system [39–41]. Furthermore, although dominance of DNRA over denitrification and anammox has been demonstrated in tropical estuaries [42] and under fish cages [43], DNRA has never been quantified in tropical settlement ponds and warrants further investigation.

Another potential controlling factor may be the presence of free sulfides. Sulfur is cycled rapidly in tropical sediments [44], and is the most important anaerobic decomposition pathway in tropical benthic systems, occurring at rates of 0.2–13 mmol S m^{-2} d^{-1} and releasing free sulfides [45,46]. Free sulfides inhibit nitrification and therefore may be reducing N_2 production in the present study by reducing the amount of NO_3^- available to denitrifiers [47]. Additionally, DNRA may be stimulated in the presence of sulfur, increasing competition with denitrifiers for NO_3^- [48]. Again, the effect of sulfur on N_2 production in tropical settlement ponds is largely unknown and further studies are needed to elucidate the potential of this factor on stifling N removal in settlement ponds.

Denitrification verses anammox

In our study denitrification was the dominant N_2 production pathway. In coastal, hyper-nutrified sediments, low N_2 production through anammox has been attributed to the limitation of NO_2^- [49,50]. Further controlling factors for anammox are NH_4^+, total kilojoule nitrogen, TN, TP, salinity, redox state, and an inverse relationship with TOC [51]. Given these controlling factors anammox potential varies seasonally [51] and reported anammox contribution to N_2 production is highly variable with values of 1–8% [23], ≤3% [15], 10–15% [52], 19–35% [16], up to 65% [17], 2–67% [22] and 4–79% [53].

Anammox was detected in sediment collected in ponds B (24 h incubation), C (carbon incubation) and D (1.5 h incubation), notably, where overall N_2 production was exceptionally low. For example, during the 24 h incubation with sediment collected in pond B, N_2 production was lower than in sediment collected from all other ponds, but anammox contributed 95% to N_2 production. Low carbon oxidation rates and correspondingly low denitrification (and thus competition for substrate) have been proposed as the reason anammox contribution is high in environments where denitrification is low [17]. Bulow et al. [36] demonstrated that

Table 5. The percent recovery of added ^{15}N as labelled N_2 in three treatments.

	$^{15}N\text{-}NO_3^-$	$^{15}N\text{-}NH_4^+$ & $^{14}N\text{-}NO_3^-$	$^{15}NH_4^+$
A	11.8±1.17	0.00±0.00	0.00±0.00
B	0.20±0.07	0.67±0.28	0.28±0.09
C	10.92±1.99	0.26±0.08	0.43±0.07
D	8.79±0.61	0.01±0.32	0.00±0.00

doi:10.1371/journal.pone.0042810.t005

high anammox rates corresponded with low denitrification rates at one site in the oxygen minimum zone in the Arabian Sea. At this site both anammox and denitrification were stimulated by the addition of organic carbon. This suggests that N_2 production was carbon limited giving anammox the competitive advantage. In tropical estuary systems where high temperatures, low sediment organic content and low water column NO_3^- concentrations prevail, the order of NO_x reduction pathways is proposed to be DNRA>denitrification>anammox [42].

The apparent detection of anammox in the presence of MeOH in sediments collected from Pond C is unusual given that anammox is inhibited by MeOH [28]. It is possible that during the 24 h incubation $^{15}NH_4^+$ was transformed through anoxic

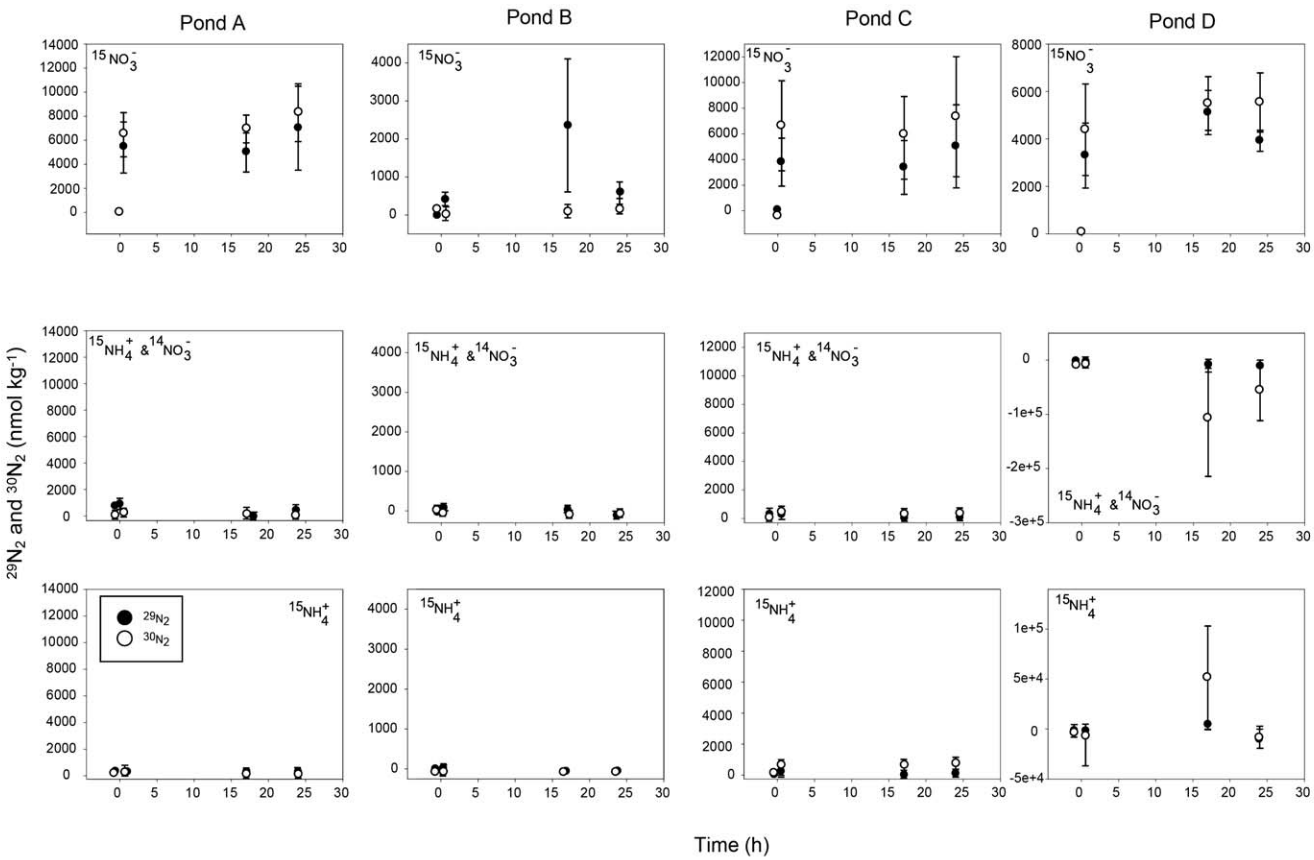

Figure 3. Production of $^{29}N_2$ (black circles) and $^{30}N_2$ (white circles) over 24 h. $^{15}N\text{-}N_2$ production in the presence of $^{15}N\text{-}NO_3^-$ is represented in row 1, $^{15}N\text{-}N_2$ production in the presence of $^{15}N\text{-}NH_4^+$ and $^{14}N\text{-}NO_3^-$ is represented in row 2 and $^{15}N\text{-}N_2$ production in the presence of $^{15}N\text{-}NH_4^+$ is represented in row 3. Column 1 represents $^{15}N\text{-}N_2$ production in sediments collected from pond A, column 2 represents $^{15}N\text{-}N_2$ production in sediments collected from pond B, column 3 represents $^{15}N\text{-}N_2$ production in sediments collected from pond C and column 4 represents $^{15}N\text{-}N_2$ production sediments collected from pond D.
doi:10.1371/journal.pone.0042810.g003

nitrification [54], producing $^{15}NO_3^-$ and the resulting $^{15}N_2$ was produced as the result of denitrification.

Settlement pond functioning and implications

Microbial N_2 production has the potential to play a major role in removing N from aquaculture wastewater. However, we estimated that only 2.5% of total N added to the settlement pond via wastewater inputs and mineralization is removed through N_2 production. It is likely that the noxious compounds of H_2S and NH_4^+ are produced in settlement ponds when they are left unmanaged with no removal of settled particulate organic matter (sludge). These compounds have significant consequences for the inhibition of microbial processes that remove N from wastewater. In addition, H_2S accumulation causes a shift in the species of gaseous N produced from N_2 to N_2O due to the inhibition of the last step of denitrification [41]. This has detrimental consequences for global warming as N_2O is ~300 times more potent than CO_2 as a greenhouse gas whereas N_2 is relatively inert [55]. Future research should determine the concentration of H_2S at which the last reductive step of denitrification is inhibited and relate this to the amount of sludge that has built up in the settlement pond. We recommend that sludge be extracted at this point to prevent H_2S release and to prevent the recycling of soluble N through mineralization, DNRA or assimilation and subsequent senescence, as has been recommended for grow out ponds previously [30]. Innovative technology, such as anaerobic digesters and biogas capture, is required to convert the large volumes of sludge to a saleable product once removed from the pond. The simple management approach of removing sludge could have the added benefit of decreasing the incidence of competition between DNRA and denitrification thereby optimizing the denitrification and anammox processes for N_2 production. If N_2 production could be enhanced to the mean rate reported by Erler et al. [20] from a constructed wetland of 965 μmol N m^{-2} d^{-1}, then 100% of total daily N inputs would be removed from settlement ponds every day. However, the estimates in the present study are based on a very simplistic understanding of the settlement pond functioning and the model requires better definition of the parameters. For example, accurate rates of NH_4^+ and DON production from the sediments are required to estimate N inputs accurately. Additionally, N_2 production was measured in the dry season in the present study when rates are likely lower than in the wet season. Wet season precipitation lowers the salinity in the ponds to 5‰ in some cases, which favors higher denitrification, lower DNRA and lower NH_4^+ fluxes [56]. Denitrification is further stimulated during periods of heavy precipitation due to increased NO_3^- concentrations from land run-off [43]. An increased understanding of the temporal and spatial variability in N_2 production rates measured using intact core assays, instead of slurry assays, would also allow accurate predictions of N_2 production rates. Slurry assays only generate potential rates of N_2 production and we acknowledge that homogenizing sediments disrupts the sediment profile and can result in different nutrient availability than that which occurs *in situ* [57]. Additionally, an understanding of the rates of competing biogeochemical pathways such as DNRA and assimilation would enhance the accuracy of the model by including N retention rates into the model.

Acknowledgments

We thank all proprietors, managers and staff at Pacific Reef Fisheries (Farm 1), Coral Sea Farm (Farm 2) and Farm 3 for access to the settlement ponds, water quality data and interest in our study. In particular, thank you to Pacific Reef Fisheries for access to records including EPA load calculations. Thank you to Tim Simmonds (AIMS) for assistance with the graphics and to David McKinnon (AIMS) for advice, support and guidance.

Author Contributions

Conceived and designed the experiments: SC DE LT NP. Performed the experiments: SC DE. Analyzed the data: SC DE NP. Contributed reagents/materials/analysis tools: SC DE BE. Wrote the paper: SC DE LT RdN.

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