Nitrogen removal and reuse in land-based aquaculture

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Abstract

Land-based prawn and barramundi farms produce large volumes of dilute wastewater containing both nitrogen rich suspended solids and dissolved organic and inorganic nitrogen. Settlement ponds are used to treat aquaculture wastewater by removing total suspended solids (TSS) through settling. Transformation of soluble nitrogen is also facilitated by the microbial community in the sediments of the settlement ponds but the prevailing transformation pathways and rate processes are largely unknown in these systems. Denitrification and anaerobic ammonium oxidation (anammox) are two transformation pathways which permanently remove fixed nitrogen from the system by converting it to gaseous nitrogen (N_2). Potential rates of denitrification and anammox were measured in the sediments of four settlement ponds using isotope tracer techniques in homogenised sediment. 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. Potential N_2 production rate did not correlate with any of the measured sediment variables (total organic carbon, total nitrogen, iron, manganese, sulphur and phosphorous) and was not stimulated by the addition of an exogenous carbon source. A simple mass balance model demonstrated that only 2.5% of added (through wastewater inputs) fixed nitrogen was removed in these settlement ponds through denitrification and anammox.

Denitrification and anammox are outcompeted in some tropical ecosystems by transformation pathways which retain nitrogen within the system. Manipulative intact core experiments were conducted using sediment collected from one settlement pond to elucidate the entire suite of soluble nitrogen transformation pathways and to ascertain the potential role of competing pathways in limiting N_2 production. Indeed

denitrification was slower 39 ± 9 µmol m^{-2} h^{-1} than nitrate (NO_3^-) uptake (89 ± 63 µmol m^{-2} h^{-1}). Denitrification also occurred at slightly lower rates than dissimilatory nitrate reduction to ammonium (DNRA). Additional retention pathways of dissolved organic nitrogen (DON) and ammonium (NH_4^+) uptake (747 ± 40 and 22 ± 22 µmol m^{-2} h^{-1}, respectively) and release (20 ± 3 and 12 ± 2 mmol m^{-2} h^{-1}, respectively) were also rapid. Understanding the transformation of DON in aquaculture settlement ponds is particularly important as it is the dominant nitrogen species in the dissolved fraction but has rarely been studied. Following the rapid uptake and release of DON, it was subsequently transformed to NH_4^+ (remineralisation) and to NO_3^- (nitrification) and a small proportion (0.7%) was transformed to N_2 after 17 h, indicating that DON removal occurred, albeit at slow rates. Taken together, results from the homogenised sediment experiments and the intact core experiments indicate that the majority of the added nitrogen is conserved within a settlement pond system and that sludge removal is essential to prevent water quality degradation through mineralisation and subsequent release of soluble nitrogen.

Accordingly, the potential of enhancing wastewater treatment by capturing and converting nitrogen rich TSS to a secondary product was investigated. TSS were characterised and subsequently harvested. Particle sizes ranged from 0.04-563 µm with the majority of particles residing in the 11-20µm size fraction. Microalgae constituted a large portion of the TSS (26.1 ± 2.7%), and the nitrogen and carbon content of the TSS was high (3.9 ± 0.3% and 20.2 ± 1.8%, respectively). The microalgal community was comprised predominantly of cyanobacteria and diatoms and was rich in fatty acids (28.5-42.0 mg FAME g^{-1} DW of TSS). 60% of the TSS were captured during harvest using an Evodos (centrifugal force). Diatoms were selectively removed with cyanobacteria and chlorophytes remaining in the water post processing. TSS were

pyrolysed and the resulting biochar was high in nitrogen (2.5-3.5%) and potassium (1.4-2.0%). However, carbon content, cation exchange capacity and surface area were moderate to low. It was estimated that biochar production, on a large prawn farm (~100 ha) could capture and reuse 940 tonnes of waste TSS per annum. This equates to annual sequestration of 226 tonnes of carbon and 28 tonnes of nitrogen.

This thesis culminates with a review to identify technologies originally developed for the treatment of municipal wastewaters and intensive recirculating aquaculture systems which could be transferred to land-based aquaculture systems to enhance wastewater treatment. I present a conceptual model with recommendations for treatment steps which focuses on value adding outputs. Initially wastewater should be treated in a set of deep anaerobic ponds that can be easily managed and desludged. Resulting sludge (from anaerobic ponds and from culture ponds) should be digested anaerobically and power generation through methane conversion is possible. Non-settled colloidal and supracolloidal solids and dissolved nutrients can then be removed through biological treatment in algal treatment ponds. Algal cultivation has potential to produce 146 tonnes of valuable biomass per annum and available conversion options include pyrolysis to biochar, inclusion into aquaculture feeds, application as fertilisers, and refining to biofuel or bioenergy. Constructed wetlands should be used as a polishing stage to assimilate residual waste nutrients into biomass or convert them to N_2, with concomitant benefits in the form of ecosystem services.

This study is the first to evaluate tropical settlement ponds in terms of soluble nitrogen cycling. It provides evidence to support upgrades to farm management and system design, with a focus on optimising nutrient cycling to enhance sustainability and increase profit margins.

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1.1. Aquaculture

Aquaculture is a diverse global industry, with origins dating back thousands of years to early China (Lucas, 2003). Modern aquaculture has been transformed over the last 50 years into a biotechnology based primary production platform with ever increasing growth in volume and value (FAO, 2010). This rapid growth has been driven by the demand for protein from an escalating world population, predicted to reach 9.1 billion by 2050 (FAO, 2009). Consequently, aquaculture will soon be the major provider of aquatic protein as capture fisheries reach a plateau, decline or crash (Pinsky et al., 2011; Subasinghe et al., 2009; Worm et al., 2009). The rapid expansion of aquaculture raises important environmental issues around the sustainability of high feed inputs as they reach new scales of intensification and output (Hall et al., 2011; Naylor et al., 2000). The level of environmental impact from individual aquaculture systems is highly variable and ranges from little or no impact when culturing extractive organisms such as seaweeds (Neori et al., 2004) and shellfish (Jelbart et al., 2011), to potentially high impact when culturing intensively reared and fed organisms such as prawns (shrimp) and finfish (Hall et al., 2011; Vaiphasa et al., 2007). Consequently, intensification is a key driver for new and improved management technologies that are specifically targeted to mitigate the environmental impacts from high-density, high feed-input aquaculture. Notably, these intensive culture systems are either land-based or located in coastal waters using cage production systems. Land-based systems, by having a single controllable discharge point, are able to ameliorate environmental impacts through wastewater treatment, efficient water quality and pond biota control systems, efficient feeding regimes, high level of biosecurity and disease control and the prevention of stock escapes. In most cases land-based production systems utilise settlement ponds as

the primary basis to treat water prior to discharge. These ponds are expansive (1-12 ha) and shallow (typically <1m) and typically retain wastewater for periods of one day to one month prior to discharge. Treatment is achieved through physical settling of particulate waste and ideally biological transformation of dissolved waste. This thesis focuses on the quantification of biological nitrogen transformation and nitrogen removal in settlement pond systems, the production of biochar from waste suspended solids and how wastewater treatment processes can be developed and improved to enhance sustainable land-based aquaculture production.

1.2. The environmental impact of wastewater from land-based aquaculture

The major concerns in terms of the environmental sustainability of land-based aquaculture systems are the dependence on fishmeal as a feed supply in intensive culture (Naylor et al., 1998; Naylor et al., 2000), mangrove deforestation to provide coastal space for land-based production (Primavera, 2006; Thu & Populus, 2007) and the consequent disturbance of acid sulphate soils (Powell & Martens, 2005), antibiotic use (Gräslund & Bengtsson, 2001), the introduction of exotic species through stock escapes (Toledo-Guedes et al., 2012), the collection of wild seedstock for production (Ahmed & Troell, 2010), and the release of chemicals and nutrients to the coastal environment (Costanzo et al., 2004; Gräslund & Bengtsson, 2001). Reducing nutrients discharges to receiving ecosystems has become a focus for industries in developed, high income, non-food deficient economies because all other impacts have, to a large degree, been managed through targeted legislation. For example, land-use and the siting of aquaculture infrastructure are closely regulated in many developed countries. Consequently, the most recent focus of legislative change is designed to improve aquaculture management to minimise the discharge of nitrogen and phosphorus into

receiving environments. This provides impetus to recapture and reuse waste nutrients, particularly in Australia where current discharge loads for TSS, total nitrogen and total phosphorus are 12, 1 and 0.15 kg ha^{-1} day^{-1}, respectively (EPA, 2005). These regulations are being upgraded and any new farm will have to implement offsets which ensure that there is no annual net-increase in nutrients and TSS in the receiving water body (Australian Government, 2011). Since nitrogen is the limiting nutrient in most marine systems (Howarth & Marino, 2006) any discharge can stimulate algal growth in receiving waters. Phosphorous abatement, although important in marine systems, has not received as much attention as it is primarily limiting within freshwater systems (Sarà et al., 2011). Concerns around the discharge of nitrogenous wastewater from ponds and cages stem from impacts of nitrogen discharge in marine systems (Burford et al., 2003; Costanzo et al., 2004; Mente et al., 2006). For example, prawn farm discharge causes increased NH_4^+ and chlorophyll *a* concentrations within discharge creeks and amplified $\delta^{15}N$ signatures of both mangrove leaves and macroalgae beyond the discharge creek (Costanzo et al., 2004). Even under strict regulation, land-based aquaculture farms can add a quarter of the annual nitrogen supply to receiving creeks which is subsequently assimilated through the food web, as evidenced by stimulated bacterial metabolism, primary production and zoo-plankton grazing rates (Trott et al., 2004). Nitrogenous wastewater, from land-based aquaculture requires treatment before release if environmental sustainability is to be ensured (Hall et al., 2011; Thomas et al., 2010).

1.3. Nitrogen constituents in aquaculture systems

The successful removal of nitrogen from land-based aquaculture systems requires a fundamental understanding of the composition of discharge water and the associated

nutrient load. The discharge composition and nutrient load are site and time specific and dependent on farm management regimes, intake water quality and the prevailing climatic conditions (Jackson et al., 2004). Weekly sampling over three years and multiple farms demonstrated that TSS load ranged from 5 to 86 kg ha^{-1} day^{-1} and associated total nitrogen load ranged from 1 to 2 kg ha^{-1} day^{-1} (Jackson et al., 2004). Over half of the total nitrogen in the wastewater occurred in the dissolved form with dissolved organic nitrogen (DON) comprising 37-43% and total ammonia nitrogen comprising 12-21% of the total nitrogen load (Jackson et al., 2003a). Mass balance models demonstrate that more than 70% of the nitrogen load in prawn ponds originates from the input of protein rich feeds(Briggs & Funge-Smith, 1994; Casillas-Hernandez et al., 2006; Jackson et al., 2003a), either as excreted wastes or uneaten feed. Even under efficient management regimes, where the majority of the added feed is ingested by the target culture animals (Casillas-Hernandez et al., 2006) nitrogenous wastes remain high (Burford & Longmore, 2001). This is because not all of the nitrogen in the ingested feed is assimilated into animal biomass, with a proportion (15% and 48%) being excreted through the gills, body mucus or faeces in prawns and rainbow trout, respectively (Burford & Williams, 2001; Dalsgaard & Pedersen, 2011). Dissolved nitrogen, released through gill excretions and leaching from faeces or un-ingested pellets, is present primarily as dissolved inorganic nitrogen (DIN) in the form of ammonium (NH_4^+) (Dalsgaard & Pedersen, 2011), or dissolved organic nitrogen (DON) in the form of urea, dissolved primary amines (DPA) (including amino acids), and other unknown DON compounds (Burford & Williams, 2001; Kajimura et al., 2004). DON typically prevails over DIN as the major constituent of the dissolved nitrogen fraction because leaching of DON from faeces and pellets during decomposition is high (Burford & Williams, 2001). DON, in the form of urea (26%), is the main constituent

leached from faeces, whereas DON in the form of DPA (22%) is the main constituent of the dissolved nitrogen leached from pellets (Burford & Williams, 2001).

To improve water quality and reduce feeding costs, nitrogen leaching from pellets has been reduced significantly, by optimising the formulation of the binder used to hold the ingredients of the pellet together. This increases the stability of the pellet in the water column without compromising digestibility (Carvalho & Nunes, 2006; Mihalca et al., 2010). Despite the stability of modern feed pellets, water quality still deteriorates under semi-intensive and intensive culture conditions and requires the removal of nitrogen prior to discharge (Burford & Longmore, 2001).

Water quality is a critical factor across all land-based infrastructure. The speciation of nitrogen, the cycling of nitrogen, and its subsequent removal from either the water column or sediment is controlled by a complex interaction of abiotic (pH, salinity, dissolved oxygen, temperature, nutrient load) and biotic (microbial community, algal community, culture species) components. The focus of this study is on the biotic pathways by which nitrogen is transformed in settlement ponds. These pathways are nitrification, denitrification, anammox, DNRA, mineralisation and assimilation (Fig. 1.1), all of which are mediated by the microbial community.

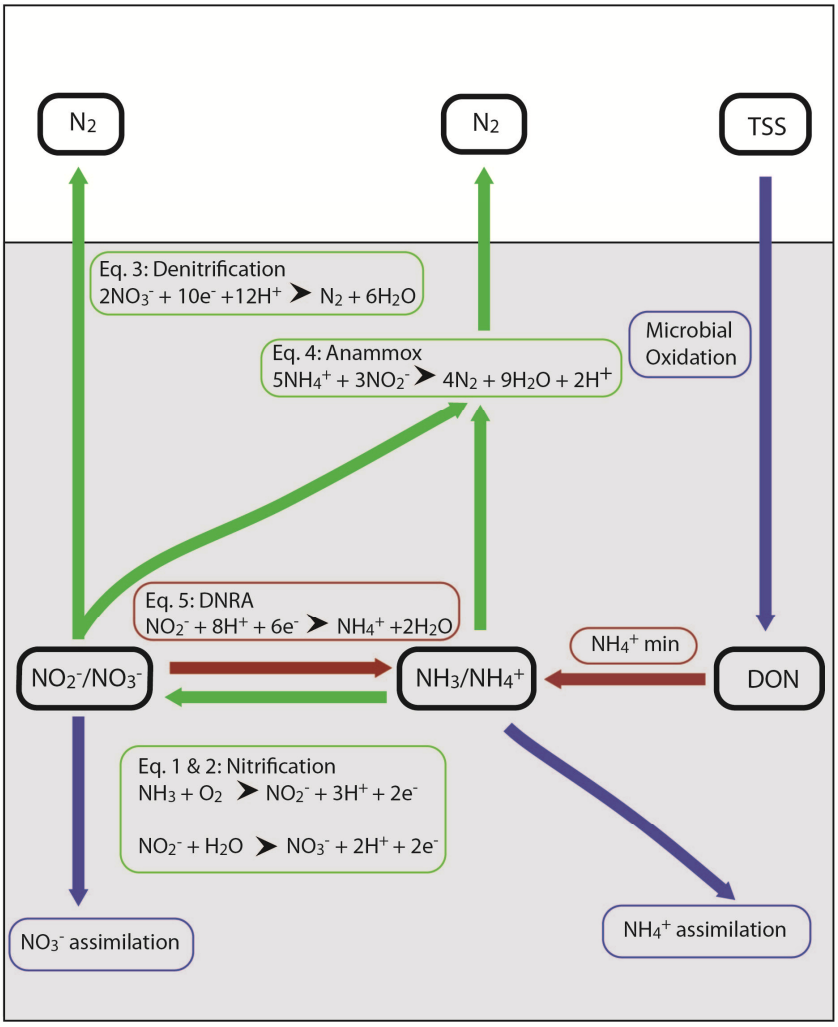

Fig. 1.1 Biological transformation of nitrogen during the wastewater treatment process. Different nitrogen species are displayed within the black boxes. Green arrows demonstrate the beneficial biological transformation of one nitrogen species to another, while red arrows demonstrate detrimental biological transformation of one nitrogen species to another and blue arrows demonstrate additional pathways of biological nitrogen transformation. Note: Eq. = equation; DNRA = dissimilatory nitrate reduction to ammonium; NH_4^+ min = mineralisation of ammonium; assimilation = incorporation of nitrogen into microbial biomass.

1.4. Microbial communities, nitrogen biogeochemistry and nitrogen removal

The microbial community of bacteria, archaebacteria, microscopic fungi and eukaryotes and viruses, inhabit the water column, suspended flocs and biofilms on every surface, living or non-living, including those of macroalgae, sediment particles, and pond

infrastructure. The majority of our understanding of nitrogen cycling in tropical prawn farms is the result of extensive work in prawn grow-out ponds (Burford & Glibert, 1999; Burford & Longmore, 2001; Burford & Lorenzen, 2004; Burford & Williams, 2001) and to a lesser extent in settlement ponds (Jackson et al., 2003b). Microbes actively sequester, transform and release nutrients, thus influencing biogeochemical cycles and dissolved nutrient concentrations in aquaculture production systems (Burford & Glibert, 1999; Burford & Longmore, 2001). The range of metabolic pathways (internal chemical reactions) displayed by microbes is extremely diverse and includes both assimilatory (biomass creating) and dissimilatory (energy creating) transformations (Burgin et al., 2011). The beneficial microbial transformations which permanently remove nitrogen from an aquaculture system are the dissimilatory pathways of nitrification, denitrification and anammox (green arrows; Fig. 1.1). Conversely, mineralisation and dissimilatory nitrate reduction to ammonium (DNRA) are both detrimental to wastewater treatment as they retain nitrogen within the system (red arrows; Fig. 1.1). Assimilation of dissolved nitrogen is only beneficial if the resulting biomass is removed (Fig. 1.1). Every process in the nitrogen cycle is closely linked to the other processes within the cycle and they cannot be investigated or discussed in isolation. In addition, the rate of each process and the mechanisms driving those rates with influence all other aspects of the nitrogen cycle. For clarity each of the key processes discussed in this thesis are described in separate sections in detail below.

1.5. Nitrification

Nitrification is a two-step, autotrophic, aerobic process in which ammonia (NH_3) is oxidised to nitrite (NO_2^-) by ammonia-oxidising bacteria (AOB) or ammonia-oxidising

Archaea (AOA) (Equation 1.1). During the second step NO_2^- is oxidised by nitrite-oxidising bacteria (NOB) to nitrate (NO_3^-) (Canfield et al., 2005) (Equation 1.2).

Equation 1.1: $$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^-$$

Equation 1.2: $$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$

As nitrification is often tightly coupled to denitrification through the supply of NO_3^- as a substrate in the denitrification process (see section 1.6), nitrification is a vital, and often rate limiting step in the removal of nitrogen (through N_2 production by denitrifiers) from eutrophied environments such as aquaculture grow-out and settlement ponds (Burford & Lorenzen, 2004; Terada et al., 2011). Slow or stifled nitrification is attributed to a range of factors within the pond environment including the high activation energy required to utilise NH_3. This activation energy is supplied by free electrons from activated O_2 (van de Leemput et al., 2011). O_2 concentration fluctuates in pond systems with dense microbial communities (Kayombo et al., 2000) so during periods of low O_2 there are less free electrons available to subsidise nitrification. Additional extreme environmental conditions in settlement ponds, such as large and rapid fluctuations in salinity, high concentrations of NH_4^+, and abundant free sulfides are also unfavourable for nitrification. Despite this, nitrification occurs in environments which are presumed unsuitable for nitrifying bacteria, indicating that they have developed adaptations to extreme conditions (Geets et al., 2006). For example, AOB can switch from nitrification to denitrification to sustain maintenance requirements under anoxic conditions and produce N_2 bubbles to enhance motility for movement from harsh O_2 depleted conditions (i.e. in the sediments) to O_2 rich conditions (i.e.

water column) (Philips et al., 2002). Microbial species typically found in O_2 poor environments include *Nitrosomonas oligotropha* and closely related strains (Gieseke et al., 2001), and it is possible that these are also common in tropical aquaculture settlement ponds.

1.6. Denitrification

Denitrification is a multi-step pathway, often tightly coupled to nitrification, that transforms NO_3^- to N_2 and involves a phylogenetically diverse group of microorganisms (Equation 1.3).

Equation 1.3: $$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$$

It is a facultative anaerobic process, often occurring at the oxic-anoxic interface where NO_3^- is available (Knowles, 1982). The definitions of denitrification differ, but the reduction of NO_3^- to NO_2^- and subsequently to gaseous oxides such as nitric oxide (NO) then nitrous oxide (N_2O) is sometimes classified as incomplete denitrification (Knowles, 1982). Depending on the environmental conditions and microbial consortia, these oxides may be further reduced to N_2, defining complete denitrification (Equation 1.3). The denitrification pathway may be terminated at any stage, and either N_2O or N_2 is released to the atmosphere (Herbert, 1999). The balance between the release of N_2O or N_2 is important because unlike inert N_2, N_2O is ~300 times more harmful than CO_2 as a greenhouse gas (IPCC, 2001). Fluctuations in DO, NH_4^+ and NO_2^- concentrations control the balance between N_2 and N_2O release (Rassamee et al., 2011). Also important to nitrogen removal from ponds are the factors controlling the rate of denitrification such as the redox state of the sediments, the presence of free sulphides,

the decomposition of labile carbon, and the influence of benthic productivity and burrowing macrofauna on the oxygenation status of sediments (Eyre & Ferguson, 2005; Joye & Hollibaugh, 1995). Furthermore, the rate of denitrification is affected by the competing biochemical pathways of dissimilatory nitrate reduction to ammonium (DNRA) and NO_3^- assimilation (see below). However, there are additional pathways by which N_2 can be produced from fixed nitrogen and which are able to withstand conditions in settlement ponds. These are nitrite-dependent anaerobic methane oxidation (N-DAMO) (Raghoebarsing et al., 2006), nitrate reduction to N_2 by foraminifera (Risgaard-Petersen et al., 2006) and importantly anaerobic ammonium oxidation (anammox) (van de Graaf et al., 1995). Anammox is the only other N_2 production pathway discussed in detail given its potential impact on nitrogen removal in tropical land-based aquaculture.

1.7. Anaerobic ammonium oxidation

Anaerobic ammonium oxidation (anammox) is a key additional nitrogen removal pathway. The bacteria responsible for anammox are within the order *Planctomycetales* and grow by fixing carbon dioxide, and produce N_2 by oxidising NH_4^+ using NO_2^- as an electron acceptor (Dalsgaard & Thamdrup, 2002; Strous et al., 1999; Strous et al., 1998) (Equation 1.4).

Equation 1.4: $$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+$$

Given first confirmation of anammox as a pathway for the removal of nitrogen in 1995 (van de Graaf et al., 1995), denitrification is traditionally identified as the sole biologically mediated nitrogen removal pathway. Denitrification was proposed as the

largest sink for nitrogen entering the marine ecosystem, being responsible for 78% (or 129×10^{12} g N y^{-1}) of all nitrogen lost compared to 22% lost through physical pathways such as burial and export (Christensen, 1994). However, it is now proposed that 30-50% of the nitrogen removed from marine systems occurs through the anammox pathway (Kuypers et al., 2006). Anammox is a powerful tool in municipal wastewater treatment (Terada et al., 2011), and one which could be harnessed for the treatment of land-based aquaculture wastewater. However, to date there is no information regarding anammox in tropical aquaculture settlement ponds. In the only relevant study, in semi-intensive prawn ponds and the associated discharge channel in subtropical Vietnam, maximum anammox activity was 0.7 nmol N_2 cm^{-3} h^{-1} (Amano et al., 2011). However, this contribution to N_2 production was 50-100 fold less than that from denitrification (Amano et al., 2011).

Unlike denitrifying bacteria, anammox bacteria are not functionally dependent on organic matter as an electron donor and can, in fact, be inhibited by high concentrations of organic carbon (Engström et al., 2005). It is, however, unlikely that organic carbon is the controlling factor of anammox in settlement ponds because, despite the highly productive nature of settlement ponds (dense phytoplankton communities), the organic carbon content of aquaculture sediments is low at 0.85 ± 0.52% in the beginning of the production cycle and 1.88 ± 0.46% at the end of the production cycle (Burford et al., 1998). It is probable that high temperatures in tropical settlement ponds limit anammox rates which are normally optimal at 15ºC (Dalsgaard & Thamdrup, 2002; Dong et al., 2011). Furthermore, anammox requires consistently high concentrations of NO_3^- and/or NO_2^- which is unlikely in the reduced sediments of settlement ponds (Trimmer et al., 2005). It is hypothesised then, that anammox activity will be negligible. Nevertheless, to develop an understanding of the nitrogen removal

capacity of settlement ponds, anammox needs to be quantified and the mechanisms regulating anammox in tropical land-based aquaculture systems defined.

1.8. DNRA

In terms of nitrogen transformations which retain nitrogen within the settlement pond system, DNRA is common in shallow tropical sediments (Dong et al., 2011). DNRA transforms NO_3^- to NH_4^+ (Equation 1.5) and therefore is in direct competition with denitrification for NO_x (NO_x constitutes NO_2^- and NO_3^- as they are readily converted from one compound to the other) as a substrate (Equation 1.3).

Equation 1.5: $$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O$$

DNRA prevails over denitrification in some environments because it proceeds either through electron flow from organic matter during fermentation, or through sulphur oxidation (Burgin & Hamilton, 2007). Dominance of DNRA over denitrification favours nitrogen retention within the settlement pond system, recycling nitrogen between pelagic and benthic systems. Microbial genera capable of DNRA such as *Beggiatoa, Thioploca* and *Thiomargarita* are adapted to thrive under harsh nutrient gradients such as those found in sediments (Jørgensen, 2010). The dominance of DNRA over denitrification occurs in environments with relatively high labile carbon and reduced sulphur and iron (Burgin & Hamilton, 2007). Furthermore, the adaptations of large size, efficient electron transfer, motility and nitrate storage ability allow dominance of DNRA over denitrification in many shallow tropical coastal regions (Jørgensen, 2010).

1.9. Mineralisation and assimilation

Mineralisation and assimilation are additional processes that play key roles in determining the nitrogen biogeochemistry of aquaculture systems (Burford & Longmore, 2001; Burford & Lorenzen, 2004). Mineralisation is the release of dissolved nitrogen due to the oxidation of organic matter and occurs post settlement of faeces, uneaten feed pellets and dead microalgae and microbial biomass. DON (mineralisation) and NH_4^+ (remineralisation) are released back to the water column after organic matter has been decomposed. Burford and Williams (2001) quantified the release of dissolved nitrogen from prawn feed and faeces in a tank system and demonstrated that >10% is released as DIN while the remainder is released as DON. It is likely that a large proportion of the dissolved nitrogen released from the settlement pond sediments will be DON.

Mineralisation hampers nitrogen removal by making DON and NH_4^+ available for assimilation by the microalgal community which creates a feedback loop, stimulating algal growth, increasing sedimentation rates (of dead algae) and further increasing rates of remineralisation and mineralisation (Ferguson & Eyre, 2010). This has been demonstrated in prawn grow-out ponds previously and is likely to occur in settlement ponds (Burford & Glibert, 1999). As sedimentation rates increase so too does benthic oxygen demand, resulting in anaerobic metabolism. Consequently, the anaerobic waste metabolites of hydrogen sulphide (H_2S) and methane (CH_4) accumulate from the activation of sulphate reduction and methanogenesis pathways (Jördening & Winter, 2005). H_2S has detrimental consequences for nitrogen removal as it inhibits the nitrification and denitrification pathways (Joye & Hollibaugh, 1995). An obvious solution to the assimilation-sedimentation-mineralisation feedback loop is the removal of nutrient rich biomass prior to settlement to the pond floor. However, wastewater

treatment systems require tailoring to ensure that the cost of implementing biomass removal technologies is off-set through the creation of secondary products with value. The success of such a system relies on consistent and high productivity of biomass, and a marketable end product for this biomass.

1.10. Algal biomass production

Algae (micro- and macro-) transform residual and difficult to manage dissolved nutrients into valuable biomass through assimilation (Burford & Glibert, 1999). Macroalgae, in particular, produce large quantities of biomass per unit area and time due to high rates of productivity as a function of growth and density. As an extreme example the red alga *Asparagopsis armata* has a productivity of 100 g (dry weight) m^{-2} d^{-1} and therefore assimilates 90 μmol TAN L^{-1} h^{-1} under optimised conditions with high (500 μmol L^{-1} h^{-1}) TAN flux regimes (Schuenhoff et al., 2006). However, not all species are this efficient especially under *in situ* conditions. For example, macroalgal (*Gracilaria edulis*) treatment of prawn farm effluent under laboratory conditions resulted in a decrease in NH_4^+ concentration from 51 to 1.3 μM (Jones et al., 2001). However, in a similar experiment conducted in the field, NH_4^+ concentration increased in all macroalgal treatments by ~6-10 μM compared to the control treatment where NH_4^+ decreased by ~1.5 μM (Jones et al., 2002). This highlights the importance of careful species selection and the benefits of using algal species which naturally thrive in aquaculture systems. Ideally, the species of algae selected for simultaneous biomass production and nutrient abatement should have high growth and nutrient assimilation rates, be robust to fluctuations in environmental conditions and have commercial value. Commercial applications for algal biomass are traditionally human consumption and phycocolloid production (Chopin & Sawhney, 2009; Paul & Tseng, 2012). However,

strict regulations on intensive aquaculture discharge have caused a shift in integrated algal culture towards species with high bioremediation efficiency, rather than high value. For example, large floating mats of naturally occurring green-tide algae are common in settlement ponds associated with land-based aquaculture farms in the tropics (Paul & de Nys, 2008). These algal species have fast growth rates, efficient nutrient uptake (3.3 kg N day^{-1} ha^{-1}) and are resilient to adverse conditions associated with dynamic tropical settlement ponds (de Paula Silva et al., 2008). However, these species have no traditional market or commercial value. To facilitate cost recovery, novel commercial uses of biomass from bioremediation systems are being developed ranging from aquaculture feeds (Bolton et al., 2009; Hasan & Charkrabarti, 2009) to biochar (Bird et al., 2011a; Bird et al., 2011b), biofuels, and bioenergy (Ross et al., 2008). Of these, biochar offers a novel and expansive application for both mirco and macroalgal biomass.

1.11. Biochar production from algal biomass

Capturing and removing micro- and macroalgal biomass from land-based aquaculture systems enhances nitrogen removal and wastewater treatment efficiency (Bolton et al., 2009; de Paula Silva et al., 2008; Mata et al., 2010). However, it is an expensive process unless nutrients are processed and reused through conversion to a secondary product with value. The production of biochar from algal biomass converts recovered biomass in to a soil conditioner which sequesters carbon, thereby returning it to the soil (Lehmann & Joseph, 2009b). Biochar contains recalcitrant (stable) carbon created through burning organic feedstock at high temperatures (i.e. > 300ºC) under a low O_2 atmosphere (Jeffery et al., 2011; Lehmann et al., 2006). Its production has concomitant benefits to global carbon budgets (Lehmann et al., 2006), soil productivity (Jeffery et

al., 2011) and waste processing (Chen et al., 2011). Importantly, the properties of macro- and micronutrient content, heavy metal content, cation exchange capacity, pH and electrical conductivity of the biochar must be evaluated to determine its potential for sequestering carbon and use as a soil conditioner. Biochars based on both microalgal (Grierson et al., 2011) and macroalgal (Bird et al., 2011a; Bird et al., 2011b) feedstocks were high in nitrogen and extractable inorganic nutrients. The potential for the production, harvesting and processing of algal biomass, from tropical land-based aquaculture systems, into biochar is extensive (Bird et al., 2011a), and provides a clear opportunity for carbon capture, recycling of carbon, and carbon sequestration.

1.12. Aims and chapter summaries

The aims of this study are therefore to (1) evaluate the nitrogen removal capacity, through N_2 production of current settlement pond systems used to treat wastewater from land-based prawn and barramundi farms, (2) to elucidate the competing and additional biogeochemical nitrogen transformation pathways to determine the partitioning between nitrogen removal and nitrogen retention within the settlement pond, and (3) to investigate the potential of biochar as a value-adding secondary product produced using waste solids from an aquaculture discharge stream.

In Chapter 2, the potential rates of denitrification and anammox are measured using isotope tracer techniques in homogenised sediment samples collected from multiple settlement ponds associated with land-based aquaculture systems. A carbon source (either particulate organic matter or methanol) is added to a parallel set of samples to quantify the effect on N_2 production rate and to determine if sediment microbial communities are carbon limited.

In Chapter 3, intact sediment cores are collected from one settlement pond and amended with ^{15}N-labeled compounds to elucidate the cycling of nitrogen through all major biochemical pathways. The rate of nitrogen removal through coupled nitrification-denitrification and anammox are compared to the rate of nitrogen retention through dissimilatory nitrate reduction to ammonium, mineralisation and assimilation. Importantly, a focus of this chapter is the cycling of DON as it is a major component of aquaculture wastewater and has rarely been elucidated in any system.

In Chapter 4, waste suspended solids are stripped from 6000 L of commercial prawn farm discharge to explore options for the production of a valuable secondary product. The fatty acid and nutrient content of the suspended solid biomass is determined and biochar is subsequently produced. The biochar is evaluated based on the chemical composition (C, N, P, and micronutrients) and physical attributes (BET surface area, cation exchange capacity and electrical conductivity).

In Chapter 5, a desktop study is conducted to identify technologies which are suitable for transfer from highly developed municipal wastewater treatment plants and intensive recirculating aquaculture wastewater treatment plants to upgrade the rudimentary single-step settlement pond systems currently used by land-based aquaculture operations. Initially, the characteristics of the three types of wastewaters (municipal, intensive recirculating and land-based aquaculture) are compared to identify technologies which target the saline and comparatively dilute wastewater generated by land-based aquaculture farms. Subsequently, a range of treatment options which enhance the profitability of the farm by capturing and converting waste nutrients into a valuable secondary product are discussed.

Chapter 2: Denitrification and Anammox in Tropical Aquaculture Settlement Ponds: An Isotope Tracer Approach for Evaluating N_2 Production[1]

2.1. Introduction

The release of anthropogenic nitrogen to the coastal zone poses a threat to many shallow marine ecosystems (Galloway et al., 2008). Discharge of aquaculture wastewaters contributes to nitrogen enrichment of some coastal regions (Thomas et al., 2010) and settlement ponds have been established as a remediation strategy from aquaculture wastewater prior to release to the environment (Bartoli et al., 2005; Jackson et al., 2003b). Settlement pond technologies are widely implemented as a low cost option for treating municipal (Archer & Mara, 2003), fish farm (Porrello et al., 2003) and dairy farm wastewater (Craggs et al., 2004a). However, the nutrient removal efficiency of long-established settlement ponds associated with commercial 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 (Bolan et al., 2009; Jackson et al., 2003b). Furthermore, given that the efficiency of wetland wastewater treatment systems can decrease with age (Tanner & Sukias, 2003), 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 bivalves and algae, which can be cultured and subsequently harvested (de Paula Silva et al., 2008; Jones et al., 2001; Jones & Preston, 1999), and

[1]Chapter 2 was adapted from Castine SA, Erler DV, Trott LA, Paul NA, de Nys R, Eyre BD (2012) Denitrification and anammox in tropical aquaculture settlement ponds: An isotope tracer approach for evaluating N_2 production. PLoS ONE 7(9): e42810.

also the removal of settled organic rich particulates (sludge) which prevents remineralisation of dissolved nitrogen back into the water column (Erler et al., 2007; Jackson et al., 2003b). Microbial nutrient transformation, which is largely un-quantified in settlement ponds, 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 nitrogen 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 nitrogen and couples NO_2^- reduction with ammonium (NH_4^+) oxidation to produce N_2 (Jetten et al., 2001; Strous et al., 1999). Denitrification and anammox are also important for the removal of nitrogen from natural system such as intertidal flats (Nicholls & Trimmer, 2009), marsh sediments (Koop-Jakobsen & Giblin, 2009), deep anoxic waters (Dalsgaard et al., 2003) and sediments from the continental shelf (50m) and slope (2000 m) (Trimmer & Nicholls, 2009). Denitrification and anammox in natural systems can remove up to 266 mmol m^{-2} d^{-1} and 61 mmol m^{-2} d^{-1} of nitrogen, respectively (Dalsgaard et al., 2003). 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 nitrogen 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

and fish. I 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). I 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 nitrogen rich wastewaters where microbial nitrogen removal is typically limited by the supply of carbon as an electron donor (Avnimelech, 1999). Together these data provide new insight into nitrogen cycling processes in shallow tropical eutrophic marine systems in the context of nitrogen management.

2.2. Materials and Methods

2.2.1. 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; Fig. 2.1). Additionally, sediment was collected from the only settlement pond at Farm 2 (Pond C) and the only settlement pond at Farm 3 (Pond D) (Fig. 2.1). The three farms spanned the wet and dry tropics which allowed comparison of N_2 production in different environments. Each pond was split into 3 zones (Z1, Z2 and Z3) (Fig. 2.1). 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 had 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 were diurnal pH

fluctuations (1-1.5 pH). According to farm records, salinity fluctuated 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 was 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).

Fig. 2.1 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.

2.2.2. 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) (Fig. 2.1).

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 nitrogen 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 (Loring & Rantala, 1992).

2.2.3. 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) (Fig. 2.1) with a 30 mm i.d. corer (Erler et al., 2008). The top 0-3 cm was collected because this included the oxic and suboxic layers where NO_x is reported to present or being reduced (denitrification) (Canfield et al., 1993) and the anoxic layer below the interface, where NO_x is reported to penetrate but O_2 does not, making conditions favorable for anammox

(Thamdrup & Dalsgaard, 2002). 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 these 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. (2003) and Thamdrup and Dalsgaard (2002) with modifications (artificial seawater of the same salinity as site water) according to Erler et al. (2008). Artificial seawater was used to preclude the potential interference of ambient NO_3^- in the isotope assay. A known volume of sediment (3-6g) 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. (2003) and 17 and 24 h were modified from Erler et al. (2008). 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-N_2 and ^{15}N-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 (2002). 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 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.

2.2.4. 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 (Roy et al., 2010). 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 stimulated or correlated with N_2 production in some systems previously (Adav et al., 2010; Avnimelech, 1999; Trimmer & Nicholls, 2009). 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 stimulated denitrification but inhibited anammox in some circumstances (Güven et al., 2005; Jensen et al., 2007). MeOH additions were carried out by adding MeOH at a concentration of 3 mM (based on Jensen et al. (2007)) to a parallel set of samples from Pond C prior to amendments.

2.2.5. Modeling N removal

A simplistic model was constructed to estimate the mean dry season nitrogen removal (NR) capacity (%) of the four settlement ponds (Equation 2.1). NR was estimated using the potential N_2 production rates calculated in the present study, and nitrogen inputs into the pond through the wastewater. Given the substantial contribution of nitrogen remineralized from sludge in prawn grow-out ponds, often exceeding inputs of nitrogen originating from feeds (Burford & Lorenzen, 2004), a variable to account for remineralisation inputs was also added (N_{imin}). Equation 2.1 was used to calculate nitrogen removal and the parameters are further defined in Table 2.1:

Equation 2.1:

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

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 2.1). I 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 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 (Bartoli et al., 2005), 1.5-4 mm in sediments below fish cages and associated reference sites and up to 20 mm in a muddy macrotidal estuary (Dong et al., 2000). This active zone was subsequently extrapolated to estimate rates for the entire area of the settlement pond. The remaining parameters were 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 nitrogen input via mineralisation (deduced from Table 3 in Burford and Longmore (2001); NH_4^+ and DON fluxes).

Table 2.1. An estimate of nitrogen inputs and microbial removal from settlement ponds, note TN = total nitrogen, WW = wastewater, min = mineralisation

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}	Burford and Lorenzen 2001
Mean net DON min	0.6	mmol m^{-2} h^{-1}	Burford and Lorenzen 2001
Mean N_2 production	2.9	nmol N cm^{-3} h^{-1}	Slurry assay
Net N removal	2.5	%	Model

2.2.6. Calculations and statistical analysis

The sediment characteristics data were analysed as a 2-factor nested design, pond and zone(pond) using permutational multivariate analysis of variance (PERMANOVA) (Anderson et al., 2008). 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 performed a rank correlation of the two similarity matrices (described above) and tested every combination of sediment characteristics to determine which set of variables best explains the observed N_2 production rates (Clarke & Warwick, 2005). 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).

2.3. Results

2.3.1. Pond characteristics and abiotic factors

Surface water temperature (25.8°C ± 1.0) and pH (7.6 ± 0.2) varied little 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 2.2) due to its location in the wet tropics where precipitation was high (Fig. 2.1).

Table 2.2 Mean surface water salinity (n = 3 ± 1 SE) and abiotic sediment characteristics (n = 9 ± 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

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 2.2) 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 2.3; PERMANOVA; pond; *Pseudo F* = 2.06, P = 0.028) and between zones within ponds (Table 2.3; 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%).

Table 2.3 A summary of statistical analyses; PERMANOVAs based on the Bray-Curtis similarities of transformed (4^{th} 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

2.3.2. Denitrification and anammox potential

There was also a significant difference in the potential rate of N_2 production between ponds (Table 2.3; 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 2.4) and lowest in sediments collected from pond C, where again denitrification was responsible for 100% of the N_2 produced (0.004 ± 0.003 nmol N cm^{-3} h^{-1}; Table 2.4). 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, Fig. 2.2A & 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 (Fig. 2.2A). This was highlighted in the vector loadings for which the vectors for anammox and denitrification were clearly negatively correlated (Fig. 2.2B).

Table 2.4. 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

Highly positive or negative loadings of the sediment characteristics appeared to have little influence on total N_2 production or denitrification (Fig. 2.2B) as these were perpendicular to the positive loadings of all the sediment characteristics. Anammox clustered near sediment variables (Fig. 2.2B), 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; $\rho = 0.134$, $P = 0.730$).

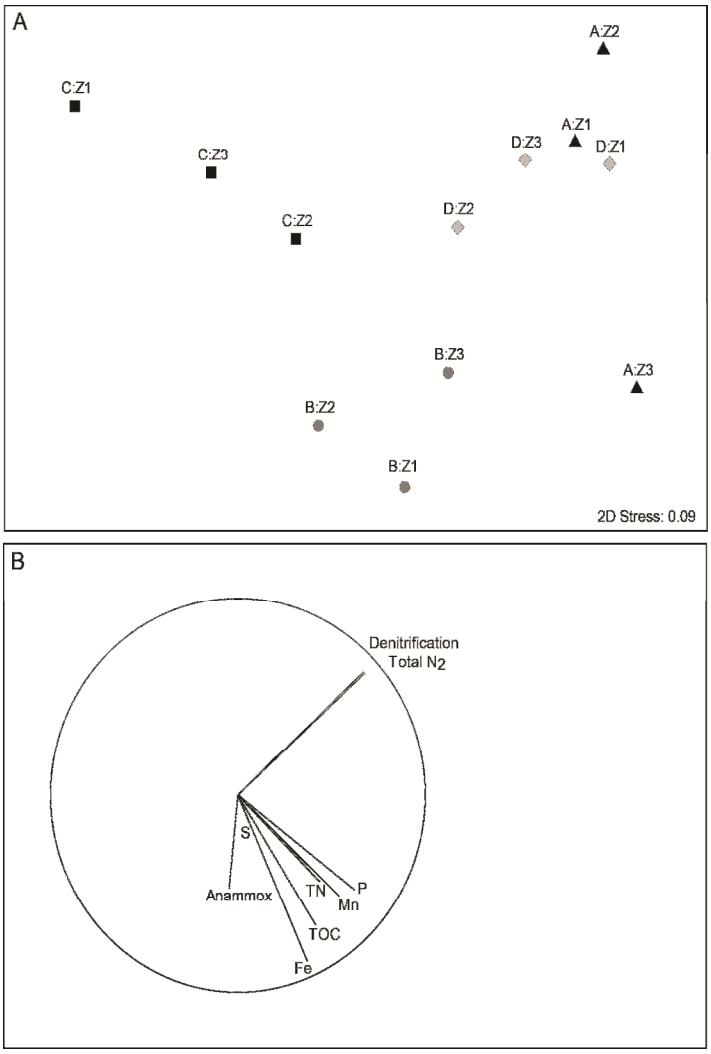

Fig. 2.2 Similarity between N_2 production rates and sediment characteristics in the four settlement ponds. A) nMDS ordination; 2-D stress = 0.09. B) The same nMDS as A), with vectors superimposed, the length and direction of which indicated the strength of the correlation and direction of change between the two nMDS axes.

In incubations with ^{15}N labeling of nitrate only, the majority of $^{15}N\text{-}NO_3^-$ converted to N_2 was found in $^{30}N_2$ (Fig. 2.3). 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 $^{30}N_2$ (Fig. 2.3). 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 2.5). 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 2.5).

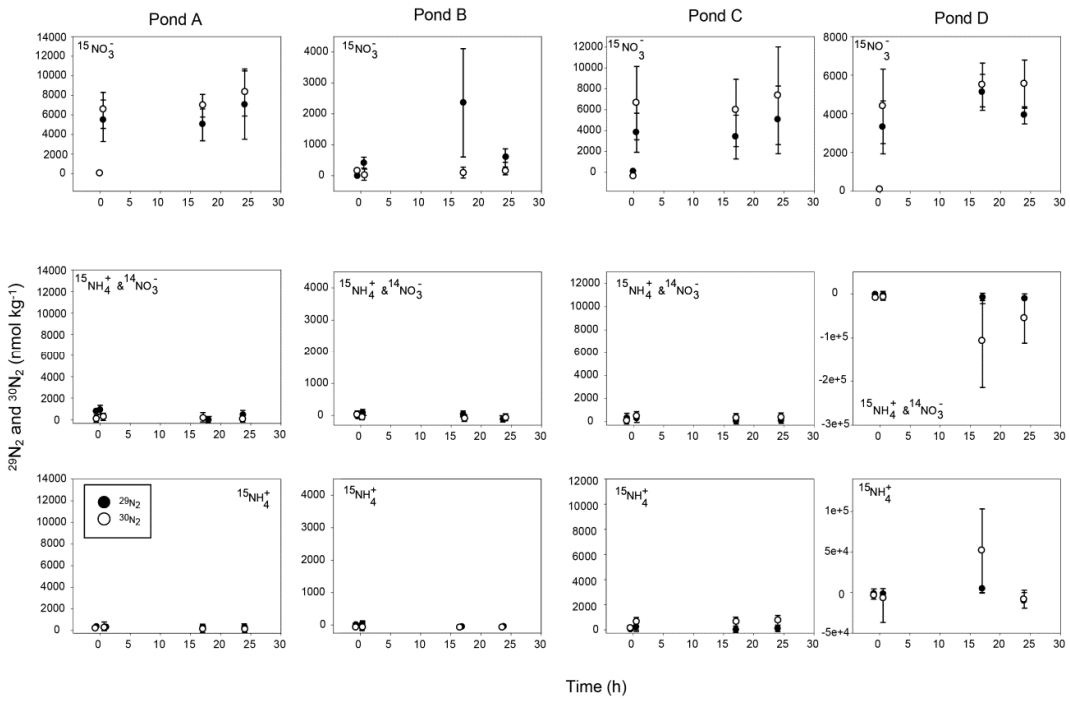

Fig. 2.3 Production of $^{29}N_2$ (black circles) and $^{30}N_2$ (white circles) over 24 h. $^{15}N\text{-}N_2$ production in the presence of $^{15}N\text{-}NO_3^-$ is represented in row 1, $^{15}N\text{-}N_2$ production in the presence of $^{15}N\text{-}NH_4^+$ and $^{14}N\text{-}NO_3^-$ is represented 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.

Table 2.5 The 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

2.3.3. Slurry assay with carbon manipulation

There was no significant difference in the rate of N_2 production when either POM (Table 2.3; Pond A; paired *t*-Test; $P = 0.350$, $n = 3$) or methanol (Table 2.3; Pond C, paired *t*-Test; $P = 0.744$, $n = 3$) was added to the experimental sediment slurries (Table 2.4; 24 h incubation compared to carbon incubation).

2.3.4. Nitrogen removal capacity

I estimated that 2.5% of the total nitrogen inputs to the settlement pond were removed through denitrification and anammox (Table 2.1).

2.4. Discussion

2.4.1. 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}) (Dalsgaard & Thamdrup, 2002), but lower than those reported for subtropical mangrove and prawn grow-out pond sediments (21.5-78.5 nmol N cm^{-3} h^{-1}) (Amano et al., 2011). 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 nitrogen removal. To achieve this, an understanding of the mechanisms controlling N_2 production is required. I 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. Nitrogen removal can be enhanced through the addition of an exogenous carbon source, for example glucose and cassava meal (Avnimelech, 1999) or molasses (Roy et al., 2010) were added to prawn 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} (Adav et al., 2010). However, in the present study N_2 production was not enhanced through the addition of carbon, suggesting that there were 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 (Bulow et al., 2010). Instead, Bulow et al. (2010) 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 the present study. NO_3^- concentration also regulates anammox activity in estuarine sediments (Trimmer et al., 2005), 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 stimulated nitrate ammonifiers (DNRA) and therefore enhanced competition for NO_x as a substrate (Yin

et al., 2002). 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 have been rapidly consumed by competing pathways such as DNRA or assimilation. The prevalence of DNRA or assimilation over denitrification determines the balance between nitrogen being removed from the system through gaseous N_2 production, or conserved within the system (Brunet & Garcia-Gil, 1996; Burgin & Hamilton, 2007; King & Nedwell, 1984). Furthermore, although dominance of DNRA over denitrification and anammox has been demonstrated in tropical estuaries (Dong et al., 2011) and under fish cages (Christensen et al., 2000), DNRA has never been quantified in tropical settlement ponds and warrants further investigation.

Another potential controlling factor may have been the presence of free sulfides. Sulfur is cycled rapidly in tropical sediments (Madrid et al., 2006), 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 (Alongi et al., 2000; Meyer-Reil & Köster, 2000). Free sulfides inhibit nitrification and therefore may have reduced N_2 production in the present study by reducing the amount of NO_3^- available to denitrifiers (Joye & Hollibaugh, 1995). Additionally, DNRA may have been stimulated in the presence of sulfur, increasing competition with denitrifiers for NO_3^- (Jørgensen, 2010). 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 nitrogen removal in settlement ponds.

2.4.2. Denitrification verses anammox

In my 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^- (Dang et al., 2010; Risgaard-Petersen et al., 2005). Further controlling factors for anammox are NH_4^+, total kilojoule nitrogen, TN, TP, salinity, redox state, and an inverse relationship with TOC (Li et al., 2011). Given these controlling factors, anammox potential varies seasonally (Li et al., 2011) and reported anammox contribution to N_2 production is highly variable with values of 1-8% (Trimmer et al., 2003), ≤ 3% (Koop-Jakobsen & Giblin, 2009), 10-15% (Hietanen & Kuparinen, 2008), 19-35% (Dalsgaard et al., 2003), up to 65% (Trimmer & Nicholls, 2009), 2-67% (Thamdrup & Dalsgaard, 2002) and 4-79% (Engström et al., 2005).

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 (Trimmer & Nicholls, 2009). Bulow et al. (2010) demonstrated that 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 (Dong et al., 2011).

The apparent detection of anammox in the presence of MeOH in sediments collected from pond C was unusual given that anammox is normally inhibited by

MeOH (Güven et al., 2005). It was possible that during the 24 h incubation $^{15}NH_4^+$ was transformed through anoxic nitrification (Hulth et al., 1999), producing $^{15}NO_3^-$ and the resulting $^{15}N_2$ was produced as the result of denitrification.

2.4.3. Settlement pond functioning and implications

Microbial N_2 production has the potential to play a major role in removing nitrogen from aquaculture wastewater. However, I estimated that only 2.5% of total nitrogen added to the settlement pond via wastewater inputs and mineralisation was removed through N_2 production. This is similar to prawn grow-out ponds where denitrification efficiency was <2% (Burford & Longmore, 2001). 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 nitrogen from wastewater. In addition, H_2S accumulation causes a shift in the species of gaseous nitrogen produced from N_2 to N_2O due to the inhibition of the last step of denitrification (Brunet & Garcia-Gil, 1996). 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 (IPCC, 2001). 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. I recommend that sludge be extracted at this point to prevent H_2S release and to prevent the recycling of soluble nitrogen through mineralisation, DNRA or assimilation and subsequent senescence, as has been recommended for grow-out ponds previously (Burford & Lorenzen, 2004). 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. (2008) from a constructed wetland of 965 μmol N m^{-2} d^{-1}, then 100% of total daily nitrogen inputs could be removed from settlement ponds every day. However, the estimates in the present study were based on a fundamental understanding of the settlement pond functioning estimated under laboratory conditions. 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 nitrogen 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 (Giblin et al., 2010). Denitrification is further stimulated during periods of heavy precipitation due to increased NO_3^- concentrations from land run-off (Christensen et al., 2000). 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 I acknowledge that homogenizing sediments disrupts the sediment profile and can result in different nutrient availability than that which occurs *in situ* (Minjeaud et al., 2009). Additionally, an understanding of the rates of competing biogeochemical pathways, such as DNRA and assimilation, would enhance the accuracy of the model by including nitrogen retention rates in the model.

3.1. Introduction

Coastal eutrophication caused by anthropogenic nitrogen discharge is a worldwide environmental problem (Cloern, 2001). Land-based aquaculture, although important for food production, has been criticized for mismanaging nitrogen rich wastewater and potentially contributing to coastal eutrophication (Vaiphasa et al., 2007; Vizzini & Mazzola, 2004). Strict guidelines require that aquaculture farms use large shallow settlement ponds to treat wastewater and meet environmental compliance (Bartoli et al., 2005; Jackson et al., 2003b). Settlement ponds rely mainly on the sedimentation of particulate organic matter (POM) to clarify wastewaters (Jackson et al., 2003b). However, mineralisation of settled POM releases soluble (defined as compounds <0.45 µm) forms of nitrogen (i.e. dissolved organic nitrogen (DON) and dissolved inorganic nitrogen (DIN)) back to the water column (Burford & Lorenzen, 2004). While the removal of POM through settlement has been estimated (Jackson et al., 2003b), the subsequent mineralisation of this material into soluble forms is poorly defined in eutrophic systems such as aquaculture settlement ponds. Few studies have quantified the production and transformation of soluble nitrogen from aquaculture settlement pond sediments. Furthermore, there is a paucity of information relating to the transformation of soluble nitrogen following POM diagenesis in tropical settlement ponds. In particular, the loss of nitrogen as gaseous N_2 is poorly quantified in aquaculture systems, despite the fact that N_2 production is the primary biological process responsible for permanent nitrogen loss in aquaculture settlement ponds, albeit at low rates (Castine et al., 2012).

A large proportion of the nitrogen within aquaculture settlement ponds exists as DON (Burford & Williams, 2001). This large amorphous pool of nitrogen has been poorly studied in natural systems and was long thought to be a recalcitrant "black box" of nitrogen because net flux measurements showed little movement of DON (Bronk et al., 2010). More recently, stable isotope tracer techniques revealed that some components of the DON fraction are cycled rapidly, but that the magnitude of the uptake and production of DON is similar, resulting in low net flux (Bronk et al., 2010). The role of DON in nitrogen cycling is, therefore, being increasingly recognized (Berman & Bronk, 2003). In aquaculture settlement ponds, as in other eutrophic systems (i.e. prawn grow-out ponds), it is likely that DON is the major remineralisation product of sediment organic nitrogen (Burford & Williams, 2001). To date, no studies in aquaculture ponds have tracked the movement of DON through the NH_4^+ pool to NO_3^- and to N_2. DON can be taken up by microalgae and bacteria, with estimates that 30-50% of the nitrogen requirements of some phytoplankton can be provided by the constituents of the DON pool (Benner et al., 1997). Other sinks for DON include adsorption to small particles (Schuster et al., 1998), transformation of recalcitrant DON to labile DON such as dissolved primary amines (DPA) through a photochemical reaction (Berman & Bronk, 2003; Bronk et al., 2010), and, most commonly, mineralisation by bacteria to ammonium (NH_4^+) (Herbert, 1999).

If DON is mineralised to NH_4^+, nitrification transforms NH_4^+ to NO_3^- and denitrifiers subsequently oxidise NO_3^- to N_2. Alternatively, NO_3^- is reduced to NH_4^+ through dissimilatory nitrate reduction to ammonia (DNRA) (Tiedje, 1988). The latter two processes (i.e. DNRA and denitrification) compete for NO_3^- and govern the partitioning between nitrogen lost as N_2 or nitrogen retained as NH_4^+. To date there have been no comparisons of denitrification and DNRA in aquaculture settlement

ponds. Christensen (2000) measured DNRA in sediments underlying fish farms and found that DNRA prevailed under sulphidic, high carbon conditions. It is possible that DNRA dominates in settlement ponds because of the presence of hydrogen sulfide (H_2S). However, organic carbon concentrations are typically low in prawn farm sediments (Burford et al., 1998) and this favours denitrification as the major nitrate reduction pathway (Burgin & Hamilton, 2007).

In this study I address three key questions that will help better manage eutrophic settlement ponds; 1) what are the main soluble products from benthic mineralisation (i.e. DON or NH_4^+), and what are their rates of production, 2) what is the fate of these soluble nitrogen products, and 3) what is the partitioning of soluble nitrogen between the processes that retain nitrogen (i.e. DNRA and microbial uptake) and permanently remove nitrogen (i.e. denitrification) in these settlement ponds.

3.2. Materials and Methods

3.2.1. Study site

Sediment nitrogen transformation was studied within a settlement pond used to treat wastewater from a commercial prawn (*Penaeus monodon*) farm (~32 ha) in far north Queensland, Australia (Fig. 3.1). The farm consisted of 38 prawn production ponds fed with water from a tropical estuary (Fig. 3.1). The farm operated as a partial flow through system and each production pond received an average of ~0.2 ML d^{-1} of inlet water (Farm proprietor, pers. comm.). The production ponds were operated with a phytoplankton dominated water column and were stocked with prawns at a density of ~20 m^{-2}. Prawns were fed daily with commercial feed pellets (Ridley Aquafeed) until surface feeding activity ceased.

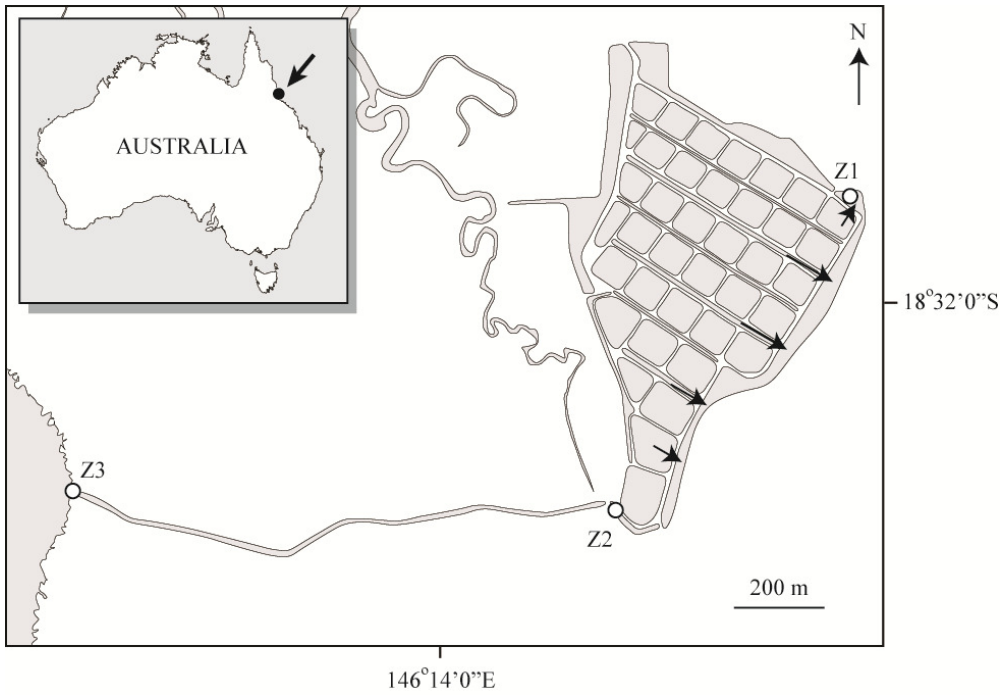

Fig. 3.1 The location of a commercial prawn farm on the North Australian coast and the position of the three sampling zones (Z1, Z2 and Z3) in the settlement pond treating wastewater from the farm. The location of the discharge channels into the settlement ponds are indicated with arrows.

Outlet water from the production ponds was channelled to a 12 ha settlement pond which, at the time of sampling, had been in operation for 10 years without being de-sludged (Farm proprietor, pers. comm.). The residence time in the settlement pond was 26.7 d and a fraction of the settlement pond water was recycled back to the production ponds (~5%). The depth of water in the settlement pond was ~1.9 m in the deepest sections, and was much shallower at the time of sampling than when it was first established, due to sludge build up (Farm proprietor, pers. comm.). Approximately 8.3 ML d^{-1} was discharged from the settlement pond to a nearby river.

For the purpose of this study I designated three zones within the settlement pond (Fig. 3.1). Zone 1 (Z1) was close to the first inlet of the settlement pond, zone 2 (Z2) was the midpoint between the main bioremediation pond and narrow mangrove lined channel and zone 3 (Z3) was located at the end of the mangrove channel, directly

adjacent to the discharge monk drain (Fig. 3.1). Grow-out ponds are discharged into channels which are then released to the settlement pond. There are multiple channel inlets into the settlement pond (indicated on Fig. 3.1 by arrows) and Z1 is located prior to the majority of the inlets.

3.2.2. Abiotic factors

To investigate the spatial variance of sediment characteristics between zones, settlement pond sediment was collected at Z1, Z2 and Z3 of the settlement pond. Sediments (30-60 mLs) were collected in intact cores (*n*=3 per zone), extruded and weighed. Sediments were subsequently oven dried (60°C) and reweighed for porosity (ø) determination (*n*=3). Dried sediments were then milled for total organic carbon and nitrogen (Shimadzu C/N Analyser), sulfur (inductively coupled plasma analysis (ICP)), phosphorus (ICP), iron (ICP) and manganese (ICP) determination (*n*=3). Surface water salinity, temperature and pH (YSI probes) were also measured at each zone within the pond. A10 mL sample of surface water was filtered, using Minisart syringe filters (0.45 µm) and frozen for later DIN determination (see 'sample analysis' section).

3.2.3. Core experiments

Six sediment cores were collected at each of the three zones on 5th March 2010. Cores were collected by pushing 9 cm i.d. PVC tubing (30 cm length) into the sediments and capping with a rubber bung. Approximately 25-30 cm of sediment was collected in each tube. The sediment cores were transported to the laboratory in the sealed PVC tubes before being transferred into Perspex incubation tubes (50 cm length x 9 cm i.d.) and submerged in 150 L incubation chambers containing low NO_3^-, estuarine water of the same salinity (18 ppt). Cores were left uncapped to pre-incubate at *in situ* temperature

and salinity (28°C and 18 ppt) for 72 h. The incubation chambers were exposed to 65-75 μE m^{-2} s^{-1} of light from a sodium vapour lamp (Growlux) operated at a 11:13 L:D regime. The chamber and all cores were covered with shade cloth (~90% light reduction) to mimic the *in situ* turbidity of the settlement pond. The water column in each core was gently mixed with a rotating magnet, driven by a larger rotating external magnet (30 rpm), placed ~10 cm off the surface of the sediment. Sediment and water heights in each core were measured to calculate their volumes within the Perspex tubes.

After the pre-incubation period the cores were sealed with Perspex lids containing two access tubes with valves, and another sampling port that was closed with a rubber bung. One access tube led to a reservoir filled with incubation water and the other access tube was reserved for collecting water samples. The water sampling access tube extended to ~10 cm above the sediment surface. Two cores from each site (n=2) were then amended with stock solutions of 10% ^{15}N labeled Urea ($(^{15}NH_2)_2CO$), 10% ^{15}N labeled KNO_3 or 10% ^{15}N labeled NH_4Cl to achieve final concentrations of 100, 50 and 50 μmol L^{-1}, respectively. The amendments were injected directly into the overlying water through one of the access tubes. The reservoir bags were also amended with labeled ^{15}N at the same concentration as the water overlying the sediment cores. A control core, without sediment, was also incubated for each amendment (3 in total).

3.2.4. Sample Collection

Water samples for DIN, total dissolved N (TDN = DON + DIN), ^{15}N-N_2 content, alkalinity, $\delta^{15}N$-NH_4^+, $\delta^{15}N$-NO_3^-, $\delta^{15}N$-NO_2^- and $\delta^{15}N$-DON were taken before the addition of ^{15}N. At 0.5, 8, 17, and 22 h after the addition of ^{15}N, water samples were also collected. Water was collected from the cores by attaching a 60 mL syringe to the access sample tube, opening the valve to the reservoir bag and to the syringe, and

carefully withdrawing 60 mL. This was repeated a number of times until sufficient water for all analyses was withdrawn (up to 200 mL, or ~8% of the overlying water volume). Dissolved oxygen (DO) concentration and pH were measured at each sampling occasion by removing the rubber bung and inserting DO and pH sensors (Hach) into each core. These measurements were made before water samples were withdrawn.

Immediately after withdrawal, extracted water (12 mL) was added to an Exetainer vial (Labco) until overflowing, 50 µL of saturated $HgCl_2$ was added and the vial was capped without headspace for later $\delta^{15}N\text{-}N_2$ measurement. Triplicate 50 mL water samples were filtered into separate acid-rinsed high-density polyethylene (HDPE) bottles. Samples were frozen immediately until analysis of $\delta^{15}N\text{-}NH_4^+$, $\delta^{15}N\text{-}NO_3^-$ (including $\delta^{15}N\text{-}NO_2^-$) or $\delta^{15}N$-DON.

Two 10 mL samples of water were filtered, using Minisart syringe filters (0.45 µm) for DIN and TDN determination. Samples were frozen immediately. Lastly, 10 mL of sample water was collected and refrigerated for analysis for alkalinity determination. Samples for particulate organic nitrogen in the overlying water were initially collected by filtering 60 mL of sample through 25 mm GFF filter discs, however, no visible material was observed on the disc, hence I assumed that this fraction of nitrogen would be negligible. At the end of the incubation period three sediment sub-cores were taken from each core using a 20 mm Perspex tube inserted 10 cm into the sediment. The three sub-cores were combined and frozen for later determination of the partitioning between adsorbed and assimilated ^{15}N (collectively ^{15}N sediment uptake).

3.2.5. Sample analysis

DIN concentration was determined using standard colorimetric methods on a segmented flow analyzer (Braune and Lubbe) in un-oxidised water samples. Parallel sets of water samples for TDN analysis were subjected to oxidation by alkaline persulphate digestion under high temperature (110°C) and pressure in an autoclave, and the resulting NO_3^- was subsequently determined (Solórzano & Sharp, 1980). The concentration of DON was calculated as TDN minus the DIN (DON = TDN – DIN).

The ^{15}N-N_2 content of dissolved N_2 was determined via gas chromatography isotope ratio mass spectrometry (GC-IRMS) after the addition of a 2 mL He headspace to the Exetainers. This measurement includes dissolved $^{15}N_2O$. Varying volumes (3-10 µL) of lab air were used to determine the ratio of peak area to N_2 concentration. This was used to calculate the total concentration of N_2 in the samples. Excess concentrations of $^{29}N_2$ and $^{30}N_2$ were calculated as the difference in the ratio of peak area $^{29}N_2$, or $^{30}N_2$ to $^{28}N_2$ between the sample and the chamber water multiplied by the total N_2 concentration in the sample.

The $\delta^{15}N$ signature of combined NO_3^- and NO_2^- was determined following the VCl_3-azide method (Lachouani et al., 2010). Briefly, 1.6 mL of fresh 2 M acidified sodium azide was injected through the Teflon lined rubber septa of a 60 mL vial containing enough sample to give 0.1 µmol of nitrogen. Sample volumes less than 20 mL were made up to 20 mL using a 30 g L^{-1} NaCl solution. 10 mL of 0.05 M vanadium(III) chloride (VCl_3) was then added to the vial, and a vent needle was used to release excess pressure. Vials were inverted and placed in an oven at 37°C for 18 h after which time the reaction was stopped by injecting 2.5 mL of 10 M NaOH through the septa. The $\delta^{15}N$ signature of N_2O produced during the reaction was measured via GC-IRMS following cryogenic focusing on a custom built purge and trap (PT) system. Our

PT-GC-IRMS system was based on the design of McIlvin and Casciotti (2010) with the exclusion of the automated components. The IRMS was set to measure m/z 44, m/z 45 and m/z 46 (representing $^{14}N^{14}N^{16}O$, $^{14}N^{15}N^{16}O$ and $^{15}N^{15}N^{16}O$, respectively) and return the isotope signature of the sample N_2O relative to a standard gas (BOC gases). A set of standards with known $\delta^{15}N\text{-}NO_3^-$ (IAEAN-NO-3, USGS 32 and USGS 34) were analysed alongside the samples and used to calculate the final $\delta^{15}N$ signature of the sample relative to N_2 in air (Lachouani et al., 2010).

The $\delta^{15}N$ signature of TDN was determined after persulfate oxidation of TDN to NO_3^- (Lachouani et al., 2010) using double recrystalised $K_2S_2O_8$ (Grasshoff et al., 1999) in order to reduce the $\delta^{15}N$ blank. The $\delta^{15}N\text{-}NO_3^-$ was determined using the procedure already described. The $\delta^{15}N$ signature of NO_2^- was determined after azide conversion to N_2O (McIlvin & Altabet, 2005). The $\delta^{15}N$ signature of NH_4^+ was determined after hypobromite (BrO^-) oxidation to NO_2^- followed by azide reduction to N_2O (Zhang et al., 2007). Briefly, 2 mL of BrO^- working solution was reacted with each sample for 30 min followed by the addition of 0.4 mL arsenite reagent used to consume excess BrO^- and stop the reaction. The resulting NO_2^- was converted to N_2O using the azide conversion already described. The $\delta^{15}N\text{-}NH_4^+$ relative to N_2 in air was determined using standards of known $\delta^{15}N$ content (USGS 25 and USGS 26) which were run in parallel with the samples. Both the $\delta^{15}N\text{-}NH_4^+$ and $\delta^{15}N\text{-}NO_3^-$ were corrected for interference with $\delta^{15}N\text{-}NO_2$. The ^{15}N content of DON was calculated using a simple mixing model (Equation 3.1).

Equation 3.1:

$$^{15}DON = \frac{(^{15}\text{TDN} \times [\text{TDN}] - {}^{15}NH_4^+ \times [NH_4^+] + {}^{15}NO_3^- \times [NO_3^-] + {}^{15}NO_2^- \times [NO_2^-])}{\text{DON}}$$

where ^{15}DON is the δ^{15}N-DON, ^{15}TDN is the measured δ^{15}N-TDN, [TDN] is the measured concentration of TDN in the water samples, $^{15}NH_4^+$ is the measured δ^{15}N-NH_4^+, $[NH_4^+]$ is the measured concentration of NH_4^+ in the water samples, $^{15}NO_3^-$ is the measured δ^{15}N-NO_3^-, $[NO_3^-]$ is the measured concentration of NO_3^- in the water samples, $^{15}NO_2^-$ is the measured δ^{15}N-NO_2^-, $[NO_2^-]$ is the measured concentration of NO_2^- in the water samples, DON is the calculated concentration of DON (i.e. TDN – DIN).

For sediment analysis, each collected sediment portion was thawed and homogenized. Weighed 10 g sub-samples (n = 3) were added to 40 mL of 2 M KCl and shaken for 6 h (100 rpm) to extract loosely adsorbed DIN. A further set of sub-samples were dried at 60°C until a constant weight was reached. After KCl extraction the sediment subsamples were centrifuged and the supernatant kept for analysis of DIN content, mainly to verify if adsorbed nitrogen was in the form of NH_4^+. Sediments were rinsed and centrifuged three more times with distilled water and dried to constant weight. Dried sediments were ground in a ball mill and weighed into tin capsules (100 mg) for ^{15}N isotope analysis via elemental analyser-IRMS (EA-IRMS). The nitrogen content of the sediments was calculated by multiplying the sediment dry weight by the measured %N. The ^{15}N content of the sediments were determined by multiplying the amount of sediment nitrogen in the sediments that were not subject to KCl extraction, by the measured Atom %. This is referred to as the total retained sediment nitrogen.

The KCl extracted sediments, i.e. sediments for which the adsorbed nitrogen component had been removed, yielded bulk sediment nitrogen and ^{15}N content, hereafter referred to as assimilated nitrogen. Adsorbed values were calculated by subtracting the bulk values from the retained sediment nitrogen and ^{15}N content (i.e. non KCl extracted values). The fraction of assimilated ^{15}N was calculated as the assimilated ^{15}N divided by the total retained ^{15}N, the fraction of ^{15}N adsorbed to sediments was calculated as the adsorbed ^{15}N divided by the total retained ^{15}N.

3.2.6. Calculations and statistical analysis

Net fluxes - Oxygen flux was calculated using the slope of DO concentration over time by fitting a linear regression. To account for the rapid consumption of DO between 0-0.5 h an initial conservative DO concentration of 252 μM was assumed for all experimental cores based on the mean concentration of DO in control cores at 0.5 h. The net flux of the different nitrogen species (N-DON, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$) during the core incubation (in units of μmol m^{-2} h^{-1}) was calculated by fitting a linear regression of the mass of a species per unit area (i.e. concentration multiplied by overlying water volume divided by the sediment area) versus time. The replacement of nitrogen from overhead reservoirs and removal of nitrogen during sample extraction at each sampling period was taken into account for all flux calculations.

Net fluxes of ^{15}N species were calculated by firstly converting the measured isotope ratio ($\delta^{15}N$) into a fraction of ^{15}N ($F^{15}N$) (Equation 3.2).

Equation 3.2:

$$F^{15}N = \frac{\delta^{15}N + 1000}{\delta^{15}N + 1000 + (\frac{1000}{0.0036765})}$$

The concentration of ^{15}N in each species ($C^{15}N$ in µmol L^{-1}) was then calculated as the concentration of nitrogen multiplied by $F^{15}N$. The net flux of a particular ^{15}N species (µmol m^{-2} h^{-1}) was calculated as the difference in concentration between time 0.5 and 17 h multiplied by the water volume and divided by the sediment area. The water column above the sediment cores collected at Z2 and Z3 had reached hypoxia at 17 h, and *in situ* DO conditions were no longer reflected, hence the net fluxes of ^{15}N were based on the changes occurring between 0.5 to 17 h only.

Mass balance - To describe the movement of added ^{15}N within the cores and the fate of soluble nitrogen I calculated mass balance models for all three zones and for the whole settlement pond (average of rates for the 3 zones). For the mass balance I firstly calculated the change in concentration of the ^{15}N species (^{15}N-NH_4^+, ^{15}N-NO_3^-, ^{15}N-DON and ^{15}N-N_2) within each amendment over the 16.5 h time step and expressed this as a ratio of the added amendment ^{15}N concentration (Equation 3.3). In other words, I calculated the fraction of ^{15}N in the amendment that was transformed into another ^{15}N species, ($^{15}N^i$) over the time step. Note that $^{15}N^i$ could also be the amount of amendment ^{15}N that remained in the water column between the time steps 0.5 to 17 h.

Equation 3.3:

$$^{15}N^i = \frac{(C^{15}N^i_{ty} - C^{15}N^i_{tx})}{C^{15}N^a_{tx})}$$

where *i* is a particular ^{15}N species (or the amendment ^{15}N species), $C^{15}N^{i}$ is the concentration of that particular nitrogen species (including the amendment), tx and ty are the initial and final times (0.5 and 17 h respectively), and $C^{15}N^{a}$ is the concentration of ^{15}N in the amendment. For instance in the DON amendment I calculated the fraction of ^{15}N-DON that was transformed to ^{15}N-NH_4^+, ^{15}N-NO_3^-, and ^{15}N-N_2, as well as the amount that remained as ^{15}N-DON between 0.5 and 17 h. The fraction of added ^{15}N that was not recovered in the soluble nitrogen fraction was assumed to be taken up by the sediment. The ^{15}N taken up into sediment was further distinguished into that assimilated into sediment microbial biomass and that adsorbed to sediment particles. Assimilation was calculated as the fraction of assimilated ^{15}N (see above) multiplied by the calculated fraction of added amendment ^{15}N in the sediment. Similarly the amount of adsorbed ^{15}N was calculated as the fraction of adsorbed ^{15}N multiplied by the calculated amount of added amendment ^{15}N in the sediment.

The values for $^{15}N^{i}$ were multiplied by the total concentration of the amendment nitrogen (i.e. labelled and unlabelled N) to give the gross rate of transformation for that particular species. The amount of amendment nitrogen that was assimilated or adsorbed was calculated the same way. The rate of gross production, also referred to as gross mineralisation, of an nitrogen species was calculated as shown in Equation 3.4. Equation 3.4 was also used to determine the main soluble nitrogen product from benthic mineralisation.

Equation 3.4:

$$MinN = [N_{17}^{a}] - {}^{15}N^{i} \times [N_{0.5}^{a}]$$

where MinN is the rate of gross mineralisation of a species, N^{a}_{17} is the total concentration of that species at 17 h, $^{15}N^{i}$ is the fraction of amendment ^{15}N remaining at 17 h and $N^{a}_{0.5}$ is the total concentration of amendment at time 0.5 h. The gross rate of uptake was calculated as the net flux, calculated earlier, subtract gross mineralisation.

All transformation rates were normalised to μmol m^{-2} h^{-1} by multiplying by the water volume and dividing by the sediment surface area and time step (16.5 h). In the NO_3^- amendment the amount of NH_4^+ produced from added NO_3^- was taken as the rate of DNRA. The amount of adsorbed nitrogen was added to this value (see discussion) because all adsorbed nitrogen was found to be in the form of NH_4^+. The amount of ^{15}N-N_2 recovered in the NH_4^+ amendment was assumed to the rate of coupled nitrification/denitrification based on water column NH_4^+. It is important to note that the mass balance looks at the transformation of added nitrogen, not necessarily the processing of pore water nitrogen produced *in situ*. In order to identify whether *in situ* transformation was important, particularly for processes such as coupled nitrification-denitrification which rely on pore water NH_4^+, I used the traditional isotope pairing technique (IPT) to calculate the rate of coupled nitrification-denitrification based on pore water ^{15}N-NH_4^+ (Nielsen, 1992). This was done using production values of ^{29}N-N_2 and ^{30}N-N_2 in the NO_3^- amended cores.

Nitrogen removal – Nitrogen load on the settlement pond was estimated from past monitoring data at this farm (Table A.1; Appendix 1). The fraction of ^{15}N recovered in each N pool (Equation 3.3) was multiplied by the nitrogen load for each species to estimate the *in situ* cycling of each nitrogen species in kg N d^{-1} (Equation 3.5).

Equation 3.5:

$$NT = N_{ww}^{i} \times ({}^{15}N^{i} \div 100)$$

Nitrogen exported from the discharge of the settlement pond was also determined from monitoring data (Table A.1; Appendix 1) and was then compared to the estimated exported nitrogen, calculated by the sum of all nitrogen sources minus the sum of all nitrogen sinks for each nitrogen species. I assumed that 100% of the particulate nitrogen (PN) entering the settlement pond was oxidised and therefore the partitioning between DON and NH_4^+ production (64 to 36%, respectively) during gross mineralisation (Equation 3.4) was used to calculate this additional source of DON and NH_4^+. Net DON and NH_4^+ fluxes were also taken into account and added as a sink in the estimated exported DON calculation (negative flux) and a source for the estimated exported NH_4^+ calculation (positive flux). Assimilation of dissolved nitrogen into the pelagic phytoplankton community was not measured during the incubation so PN release from the settlement pond was not calculated. Total N_2 production was calculated as the sum of N_2 produced from the fraction of ^{15}N recovered in the N_2 pool after each ^{15}N amendment (Equation 3.5) and this was compared to the total nitrogen load on the settlement pond (sum of PN, DON, NH_4^+ and NO_3^- inputs) to estimate the nitrogen removal capacity of the pond, and the partitioning between processes that retain nitrogen and processes that permanently remove nitrogen from the system.

Statistics - The sediment characteristics data was analysed as a 1-factor design using permutational multivariate analysis of variance (PERMANOVA) (Anderson et al., 2008). PERMANOVA calculated *p*-values from 999 permutations based on Bray-Curtis distances. PRIMER version 6 and PERMANOVA+ version 1.0.4 was used to conduct the analysis (Anderson et al., 2008). To determine if the DO concentration

varied significantly over time a repeated measures analysis of variance (ANOVA) was conducted, with zone (i.e. Z1, Z2 or Z3) and nitrogen amendment (i.e. $DO^{15}N$, $^{15}NO_3^-$ or $^{15}NH_4^+$) as fixed factors. I used Hotelling's Trace test statistic to determine the significance of this variation and SYSTAT version 13 was used to conduct the analysis.

3.3. Results

3.3.1. Pond characteristics and abiotic factors

Surface water temperature, pH, salinity, and NO_3^- concentration were similar across zones (Table 3.1). Surface water NH_4^+ was higher in Z2 than the other two zones (Table 3.1). The sediment at each zone consisted of fine black mud. Of the solid phase elements measured, most (Fe, P, S, TOC and TN) were more concentrated in Z2 (Table 3.1). Mn was the only solid phase element which increased in concentration along the length of the settlement pond, being lowest at Z1 and highest at Z3 (Table 3.1). There was significant variability in the concentration of solid phase elements and porosity between zones (PERMANOVA; *Pseudo F* = 22.88, *P* = 0.004). Sediment porosity was similar at Z1 (41 ± 1) and Z2 (44 ± 1) and higher at Z3 (49 ± 1) (Table 3.1). Mean sediment C:N ratio at Z3 (14.4 ± 1.5) was higher than the mean C:N ratio at Z1 (7.1 ± 0.2) or Z2 (7.2 ± 0.2) (Table 3.1). TOC:TP ratios ranged between 7.6 and 14.0 and were also highest at Z3 (Table 3.1).

Table 3.1 Water column and sediment characteristics (mol cm^{-3}, unless stated) in the three zones (Z1, Z2 and Z3) of the studied aquaculture wastewater settlement pond.

	Zone 1	Zone 2	Zone 3
Temperature (°C)	29.1	30.0	27.8
pH	7.53	7.07	6.85
Salinity (‰)	17.1	17.6	18.2
NH_4^+ (µM)	28.1	63.6	29.5
NO_3^- (µM)	0.7	0.7	0.7
Porosity (%)	41 ± 1	44 ± 1	49 ± 1
TOC	12 ± 2	27 ± 0	18 ± 4
TOC (%)	0.3 ± 0	0.7 ± 0	0.5 ± 0
TN	2 ± 0	3 ± 0	1 ± 0
TN (%)	0.05 ± 0	0.10 ± 0	0.04 ± 0
TP	1 ± 0	3 ± 0	2 ± 0
TOC:TN	8 ± 0	8 ± 0	17 ± 2
TOC:TP	8.4 ± 0	7.8 ± 0	11.6 ± 1
S	4 ± 1	10 ± 0	3 ± 0
Fe	5 ± 1	12 ± 0	8 ± 0
Mn	0.6 ± 0.1	1.2 ± 0.1	1.5 ± 0.1

3.3.2. Benthic metabolism

DO concentration in the water column decreased significantly (repeated measures ANOVA and Hotelling's trace test; $F_{3,7} = 922.52$, $p = 0.000$) to 5-139 µM at the end of the incubation (22 h) in all cores except the control cores, which remained above 219 µM (Fig. 3.2). At 17 h the water column above sediment cores sampled from Z2 and Z3 had reached hypoxia (defined as between 1-30% DO saturation i.e. ~66 µM under this temperature and salinity regime). DO concentration in the water column above the cores collected at different zones of the settlement pond was also significantly different over time (repeated measures ANOVA and Hotelling's trace test; $F_{6,12} = 26.48$, $p = 0.000$) with Z2 and Z3 demonstrating consistently lower DO levels than Z1 over the duration of the incubation (Fig. 3.2).

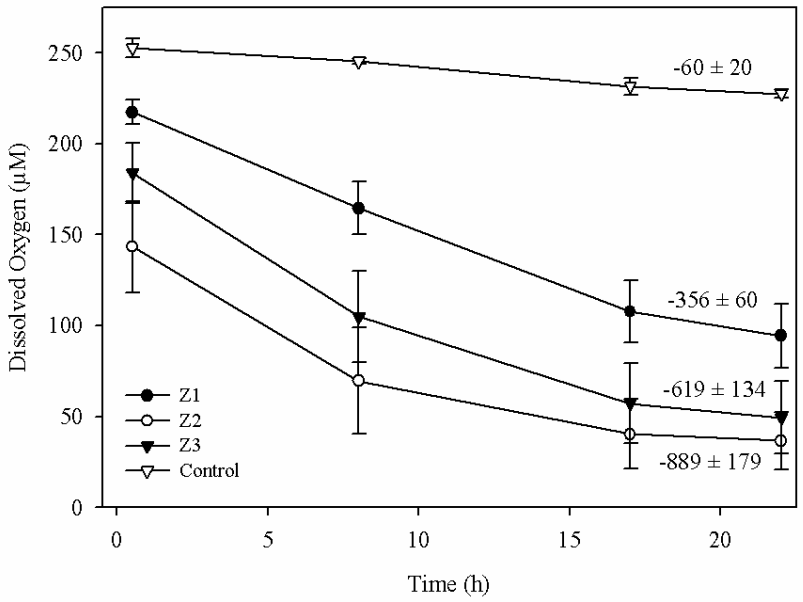

Fig. 3.2 Mean (n = 6 cores from each zone or n = 3 control cores) dissolved oxygen concentration at each zone over the 22 h incubation period. Mean (n = 6 cores from each zone or n = 3 control cores) O_2 flux rate (µmol m^{-2} h^{-1}) is noted next to each line.

The rate of O_2 consumption ranged from 146 to 1371 µmol m^{-2} h^{-1} in experimental cores, and was slow towards the end of the incubation period as DO dropped to hypoxic (~66 µM) or even anoxic levels (~16 µM) in some cores (i.e. 5 out of 6 cores from Z2 were anoxic at 22 h) (Fig. 3.2). CO_2 flux was positive (Fig. 3.3), and the concentration increased on average from 1.5 to 1.7 mM over the incubation period, which indicated a state of net heterotrophy.

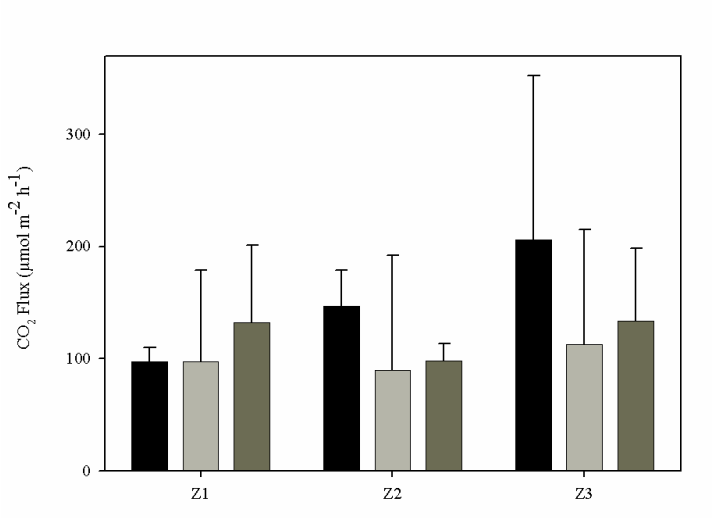

Fig. 3.3 Mean (n = 2) CO_2 flux rate at zone 1 (Z1), zone 2 (Z2) and zone 3 (Z3) of the settlement pond. Black bars represent the treatment in which DON was added, grey bars are treatments where NO_3^- was added and dark grey bars are where NH_4^+ was added.

3.3.3. Soluble N and ^{15}N fluxes

DON was the major soluble nitrogen species produced during benthic mineralisation (Fig. 3.4). The maximum rate of gross DON mineralisation (25.8 µmol m^{-2} h^{-1}) occurred in sediments collected from Z3. The mean rate of gross NH_4^+ production was 11.8 ± 2.4 µmol m^{-2} h^{-1} (Fig. 3.4) and the maximum rate occurred in sediments sampled from Z3 (16.5 µmol m^{-2} h^{-1}). Gross uptake of DON was large and consequently there was only a slight net influx of DON to the sediments (Fig. 3.4). Conversely, gross uptake of NH_4^+ was slow, resulting in a net efflux of NH_4^+ from the sediments (Fig. 3.4). Gross production of NO_3^- (nitrification) also occurred (2.9 ± 0.8 µmol m^{-2} h^{-1}) although uptake occurred at a similar rate, resulting in no net flux of this nitrogen species (Fig. 3.4).

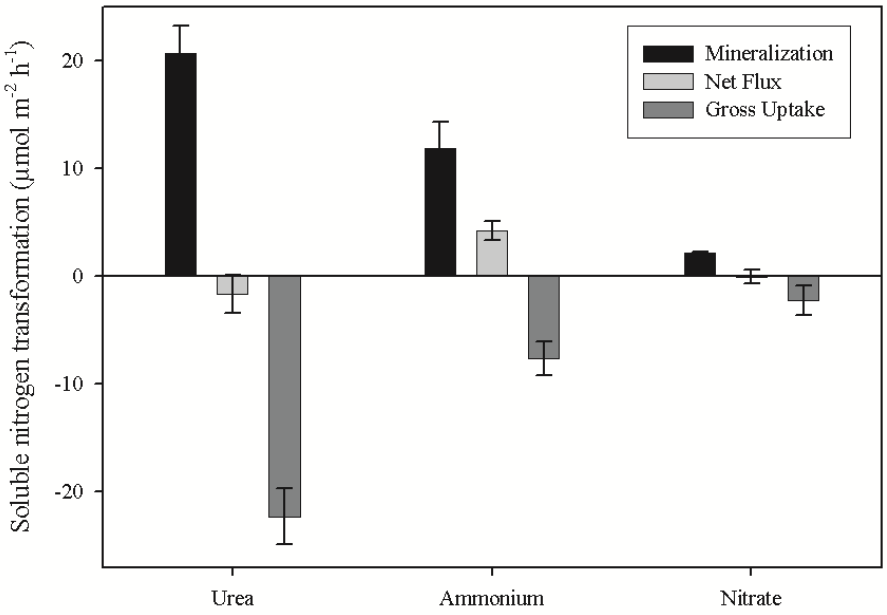

Fig. 3.4 The rate (µmol m^{-2} h^{-1}) of mineralisation (gross production), gross uptake and net flux of DON, NH_4^+ and NO_3^- in the prawn farm settlement pond.

3.3.4. Transformation of ^{15}N tracer

The fate of soluble nitrogen was investigated by quantifying the transformation of ^{15}N tracer. Rapid transformation of ^{15}N occurred between 0.5 and 17 h. A large portion (57%) of ^{15}N-DON (added as labelled urea) was taken up by the sediment microbial community in the first 17 h (Fig. 3.5A). Nitrogen uptake to the sediments was divided into assimilation and adsorption. Of the 57% of the ^{15}N-DON tracer which was taken up, the majority of it (73 ± 14% or 3.0 ± 0.5 µmol g^{-1} dry sediment; *n*=3; mean of all zones) was assimilated, with a smaller portion being adsorbed as ^{15}N-NH_4^+ (27 ± 14% or 1.2 ± 0.6 µmol g^{-1} dry sediment; *n*=3; mean of all zones %). Of the added ^{15}N-DON, 41% (includes the adsorbed portion) was transformed to ^{15}N-NH_4^+ (Fig. 3.5A). After 17 h, 0.7% of the labelled ^{15}N-DON was recovered as ^{15}N-N_2 (Fig. 3.5A).

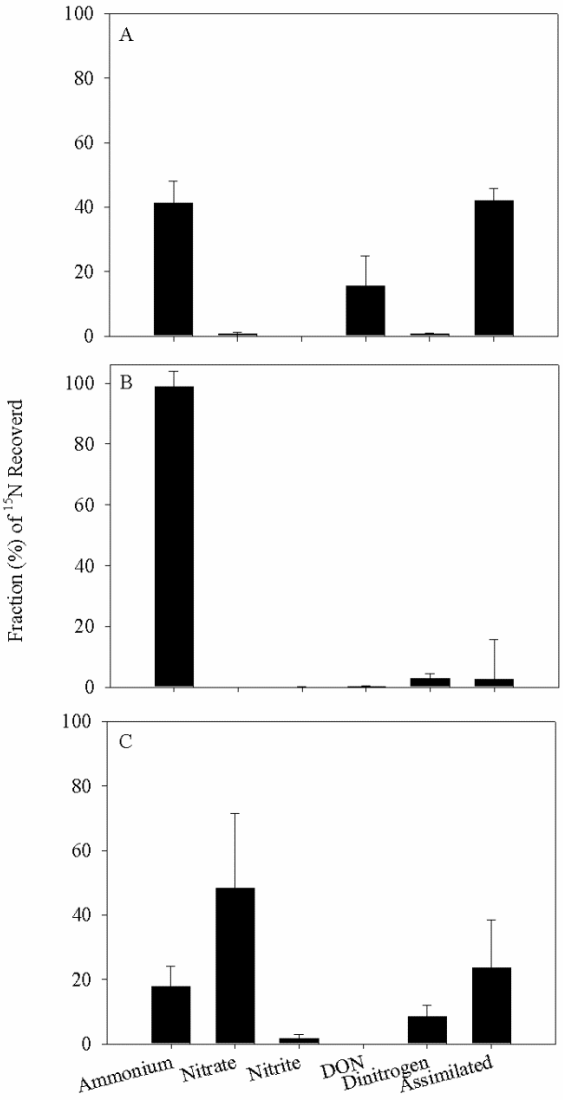

Fig. 3.5 Movement of added tracer (A) ^{15}N-DON, (B) ^{15}N-NH_4^+ and (C) ^{15}N-NO_3^- in various N pools during the first 17 h of the incubation, values represent the fraction of added ^{15}N recovered in an particular pool.

The transformation of ^{15}N-NH_4^+ was negligible and 99% of the added ^{15}N-NH_4^+ remained in the water column during the incubation period (Fig. 3.5B). Of the small portion transformed, distribution was split almost evenly between ^{15}N-N_2 production (coupled nitrification-denitrification) and sediment uptake (Fig. 3.5B).

Of the added ^{15}N-NO_3^-, only half was transformed over the incubation period with the sediment being the largest sink (33%) (Fig. 3.5C). The distribution between

assimilated and adsorbed $^{15}N\text{-}NO_3^-$ (as $^{15}N\text{-}NH_4^+$) was split 75 ± 5% to 25 ± 5%, respectively. This equated to 1.1 ± 0.7 and 0.3 ± 0.3 µmol g^{-1} dry sediment assimilated and adsorbed, respectively. Some of the $^{15}N\text{-}NO_3^-$ was transformed to $^{15}N\text{-}NH_4^+$ (0.1% of the added) and some was recovered as $^{15}N\text{-}N_2$ (0.1%) (Fig. 3.5C).

The zonal trends for ^{15}N transformation are shown in Table 3.2. Generally the transformation of added ^{15}N was faster in sediment collected from Z2 compared to sediments collected from the other two zones. ^{15}N-DON (as urea) was rapidly consumed at Z2 and subsequently transformed to $^{15}N\text{-}NH_4^+$ (684.2 µmol m^{-2} h^{-1}) (Table 3.2). In sediment collected from Z1 and Z3, ^{15}N-DON transformation to $^{15}N\text{-}NH_4^+$ occurred more slowly than transformation in sediment collected at Z1 (445.0 and 476.7 µmol m^{-2} h^{-1}, respectively; Table 3.2). Similarly, the rate of DNRA was highest in Z2, (82.3 µmol m^{-2} h^{-1}) (Table 3.2). Rapid $^{15}N\text{-}NO_3^-$ assimilation, DNRA and ^{15}N-DON oxidation in Z2 corresponded to maximum concentrations of TOC, TN, TP, S, and Fe in this zone (Table 3.1). Coupled nitrification-denitrification, determined by the mass balance and the IPT approaches, was most rapid in Z3 (54.4 µmol m^{-2} h^{-1} and 11.5 µmol m^{-2} h^{-1}, respectively), where porosity, Mn, C:N and C:P were highest (Table 3.1).

Table 3.2 The rate (µmol m^{-2} h^{-1}) of transformation of added ^{15}N in the cores as determined by the mass balance approach. The added N species is indicated in bold next to the arrows and the resulting N species is indicated in the column heading.

$DO^{15}N$ ➡	$^{15}NH_4^+$	$^{15}NO_3^-$	$^{15}NO_2^-$	$DO^{15}N$	$^{15}N_2$	^{15}N-Assimilated
Z1	445.0	25.4	0.0	N/A	2.9	490.2
Z2	684.2	0.0	0.0	N/A	8.5	560.5
Z2	476.7	0.0	0.2	N/A	13.3	587.3
$^{15}NH_4^+$ ➡	$^{15}NH_4^+$	$^{15}NO_3^-$	$^{15}NO_2^-$	$DO^{15}N$	$^{15}N_2$	^{15}N-Assimilated
Z1	N/A	0.0	0.0	3.9	2.0	49.1
Z2	N/A	0.0	3.1	0.0	16.3	0.0
Z2	N/A	0.0	0.0	0.0	54.4	0.0
$^{15}NO_3^-$ ➡	$^{15}NH_4^+$	$^{15}NO_3^-$	$^{15}NO_2^-$	$DO^{15}N$	$^{15}N_2$	^{15}N-Assimilated
Z1	31.7	N/A	11.7	0.0	46.9	11.5
Z2	82.3	N/A	2.2	0.0	8.7	126.5
Z3	25.2	N/A	1.4	0.0	20.5	27.3

3.3.5. Nitrogen loss verses nitrogen retention

^{15}N dynamics in the prawn farm settlement pond were dominated by ^{15}N-DON (urea) uptake (546 ± 29 µmol m^{-2} h^{-1}; pooled across zones), and transformation of ^{15}N-DON to ^{15}N-NH_4^+ (535 ± 75 µmol m^{-2} h^{-1}; pooled across zones) which are both nitrogen retention pathways (Table 3.2). Permanent nitrogen loss did occur in every zone through the production of N_2 (Table 3.2), although it only removed 0.7, 2.8 and 8.5% of the added ^{15}N-DON, ^{15}N-NH_4^+ and ^{15}N-NO_3^-, respectively (Fig. 3.5). Where ^{15}N-NO_3^- was added, sediments from Z1 dominated N_2 production (46.9 µmol m^{-2} h^{-1}; Table 3.2). However, the highest rate of N_2 production occurred in Z3, after the addition of ^{15}N-NH_4^+ (54.4 µmol m^{-2} h^{-1}; Table 3.2), demonstrating a tight coupling between nitrification and denitrification at this zone. This was supported by calculating denitrification using the IPT method (Nielsen, 1992). IPT demonstrated that coupled

nitrification-denitrification occurred at 11.5 μmol m^{-2} h^{-1} in Z3 but that coupled nitrification-denitrification was not occurring at Z1 or Z2. Overall, denitrification rates calculated using IPT (38.5-52.0 μmol m^{-2} h^{-1}) agreed with rates calculated using the mass balance approach (8.9-46.9 μmol m^{-2} h^{-1}; Table 3.2). DNRA (25.2-82.3 μmol m^{-2} h^{-1}) was faster than denitrification (8.7-46.9 μmol m^{-2} h^{-1}), particularly in sediments collected from Z2 (Table 3.2).

I predicted that 0.15 kg N d^{-1} can be removed from the settlement pond through microbial N_2 production, which was 1.1% of the total daily nitrogen load to the settlement pond (Fig. 3.6). Using the transformation rates that I measured during the core incubation I also estimated that 11.45 kg N d^{-1} is exported from the settlement pond (Table 3.3), compared to the measured export of 12.45 kg N d^{-1} (Table 3.3). Despite my estimated nitrogen export (11.45 kg N d^{-1}) being similar to the measured nitrogen export (12.45 kg N d^{-1}), my estimated exports of DON and NH_4^+ (4.77 and 6.28 kg N d^{-1}) from the settlement ponds were much higher than the measured exports of DON and NH_4^+ (3.92 and 2.81 kg N d^{-1}) (Table 3.3). I did not estimate PN export from the pond, or DON and NH_4^+ assimilation into phytoplankton biomass. Nitrogen remaining in the NH_4^+ fraction was high (2.25 kg N d^{-1}) compared to nitrogen remaining in the DON (0.65 kg N d^{-1}) and NO_3^- (0.36 kg N d^{-1}) fractions (Fig. 3.6). DON dominated nitrogen dynamics in the settlement pond with high import (4.18 kg N d^{-1}), release during PN mineralisation (4.12 kg N d^{-1}), and assimilation into the sediments (1.75 kg N d^{-1}) and fast transformation to NH_4^+ (1.72 kg N d^{-1}) (Fig. 3.6).

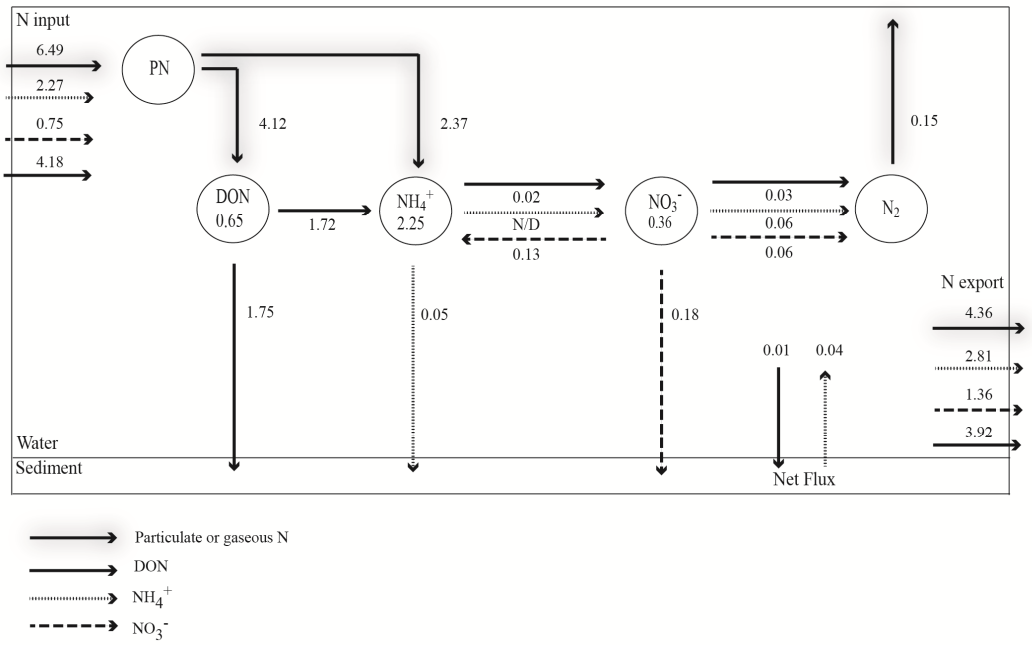

Fig. 3.6 Nitrogen dynamics in the prawn farm settlement pond. The type of arrow denotes the nitrogen species with shadowed arrows denoting particulate or gaseous nitrogen, solid arrows denoting the DON pool, dotted arrows from the NH_4^+ pool and dashed arrows from the NO_3^- pool. The rate of each transformation process (kg N d^{-1}, over the whole settlement pond) is given adjacent to each arrow. The input and export of nitrogen to and from the settlement pond is demonstrated by the arrows on the left and right hand sides, respectively. See Tables 3 and 4 for details of calculations.

Table 3.3 Estimated and measured particulate nitrogen (PN), dissolved organic nitrogen (DON), ammonium (NH_4^+) and nitrate (NO_3^-) release from the settlement pond (kg N d^{-1}).

	Estimated N export	Measured N export
PN	Not determined	4.36
DON	4.77	3.92
NH_4^+	6.28	2.81
NO_3^-	0.40	1.36
Total	11.45	12.45

3.4. Discussion

3.4.1. Benthic production of soluble nitrogen

Understanding the partitioning between the production of DON and NH_4^+ during organic matter oxidation (mineralisation) is important as the two compounds vary in their bioavailability and subsequent uptake and transformation pathways. The dominance of DON as the prevailing product of mineralisation in shallow tropical ecosystems has only been recognized by a handful of other studies (Eyre & Ferguson, 2002; Eyre et al., 2011), partly because isotopic analysis of ^{15}N-DON incurs some complexities (Erler et al., 2010). Where mineralisation has been studied, ^{15}N-DON measurements were usually omitted or assumed. Burford and Lorenzen (2004) and Christensen et. al (2000) demonstrated that remineralisation of organic material and release of NH_4^+ was rapid in prawn grow-out ponds (6% of sedimented material d^{-1}), and under fish cages (up to 12 mmol m^{-2} d^{-1}), respectively. However, they did not quantify production of DON during organic matter oxidation. I built on their findings and confirmed that NH_4^+ is produced in the sediments of aquaculture settlement pond systems. In addition, I demonstrated considerable production of DON. I used the uptake of ^{15}N-urea to estimate the gross uptake rate of DON. While urea is a dominant nitrogen species in aquaculture systems (Burford & Williams, 2001) it is not the only species of DON present. The DON pool consists of urea, dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA), proteins, nucleic acids, amino sugars and humic substances, many of which are recalcitrant (Berman & Bronk, 2003). The calculated rates of gross production were based on the assumption that the available DON behaves as urea and hence the rates were most likely overestimated. However, I observed that the net change in total DON concentration (including urea) during the incubation was small and negative, whereas the uptake of the added ^{15}N-urea was large.

Therefore, there must have been a large positive flux of total DON from the sediments to the water column. Hence my conclusion remains valid, that the production of DON in these sediments is considerable.

3.4.2. The fate of soluble nitrogenous compounds in settlement ponds

The fate of ^{15}N-urea (DON) and ^{15}N-NH_4^+, produced by the sedimentary microbial community, was determined. The sediments played a critical role in the transformation of DON as benthic uptake was rapid in sediment collected in all zones of the settlement pond, indicating that the urea component of the DON fraction was rapidly removed. Conversely, uptake of NH_4^+ only occurred in sediment collected from Z1 and occurred at a much slower rate. This suggests that NH_4^+ would accumulate in the water column and would likely be assimilated by the phytoplankton community as has been demonstrated in prawn grow-out ponds previously (Burford & Glibert, 1999).

The preference for sedimentary uptake of urea over NH_4^+ as a nitrogen source in the settlement pond sediments probably reflects the fact that carbon was limiting and urea provided a source of both labile carbon and nitrogen. This was particularly observable in Z3 where the sediments appeared most recalcitrant (high C:N) and have the highest uptake rate of urea. The fate of urea in prawn farm settlement ponds has been poorly investigated in the past. In this study I demonstrated that a large fraction of added urea was rapidly transformed to NH_4^+, some of which was adsorbed to the sediment (15%) and some of which was lost to the water column (26%). In addition I have, for the first time, mapped the transformation of urea to N_2. Although this was only a very small fraction of the added urea, the study only tracked this transformation for 17 h before cores became anoxic. Given that the bulk of the added urea was taken up by the sediment microbial community and detected in the NH_4^+ pool, I suspect that

coupled mineralisation-nitrification-denitrification was indeed occurring in prawn farm settlement ponds, but that slow nitrification was hindering nitrogen loss in the settlement pond system, as occurs in prawn grow-out ponds (Burford & Lorenzen, 2004). York et al. (2010) estimated that 11-48% of remineralised NH_4^+ underwent coupled nitrification-denitrification in a temperate eutrophic estuary. However, there are few additional studies demonstrating movement of DON to NH_4^+ and subsequently to N_2. It is likely that a larger proportion of the DON pool would be released as N_2 under more stable O_2 regimes and a longer incubation period.

Nitrification appeared to be the rate limiting step in transformation of ^{15}N-DON to ^{15}N-N_2 with a buildup of NH_4^+ rather than NO_3^- after urea addition. The major limitations on nitrification in settlement ponds are sulfide inhibition, high NH_3 concentrations and low DO. Under anaerobic regimes, organic matter oxidation proceeds primarily through sulfate (SO_4^{2-}) reduction and free sulfide is consequently released (Alongi et al., 2000). Free sulfides inhibit nitrification and also inhibit the final reductive steps of denitrification (An & Gardner, 2002; Gardner et al., 2006; Joye & Hollibaugh, 1995), so transformation of DON to N_2 is impeded under sulfidic conditions stimulated by high NH_4^+ concentrations. High ammonia (NH_3) concentration can limit nitrification because, despite being a necessary substrate, excessive NH_3 inhibits the final step of the nitrification process (Chen et al., 2010a). This causes accumulation of NO_2^- in the water column. Noticeably, at Z2 in the present study, where TAN was most concentrated (63.6 μM; Table 3.1), conversion of $^{15}NH_4^+$ to $^{15}NO_2^-$ was detected (Table 3.2), indicating a build-up of NO_2^- and potential inhibition of the last step of nitrification.

In terms of the fate of ^{15}N-NH_4^+, the effects of reduced nitrification were again demonstrated, with only a small portion of ^{15}N-NH_4^+ being transformed to ^{15}N-NO_3^-

and subsequently to ^{15}N-N_2 through coupled nitrification-denitrification (D_n) (mass balance approach). However, the rate of D_n was faster (2.0-54.4 µmol m^{-2} h^{-1}) than stand-alone nitrification which was not detected. This was likely because any NO_3^- produced by nitrification was rapidly consumed by denitrifiers. Tight coupling of these processes is common (York et al., 2010), especially in reduced environments where water column NO_3^- concentration is low. Given the low rates of denitrification based on nitrate existing in the water column (D_w), D_n is a critical process leading to nitrogen abatement. For example, under fish cages D_w only contributed to nitrogen removal when land run-off increased water column NO_3^- concentration, during the summer months, D_n was otherwise responsible for 90% of all denitrification activity (Christensen et al., 2000). Again, this highlights the keystone role of nitrification in the nitrogen removal efficacy of settlement pond systems.

Unlike ^{15}N-NH_4^+, ^{15}N-NO_3^- was primarily transformed by sediment uptake. Some of the added NO_3^- was removed via dissimilatory reduction of NO_3^- to NH_4^+ (DNRA) and to N_2 (denitrification), although DNRA was the dominant NO_3^- reduction pathway. DNRA and denitrification occurred at almost equal rates. Noticeably, 48% of the added ^{15}N-NO_3^- remained in the water column after 17 h, despite O_2 levels dropping to hypoxic or near hypoxic levels. Hypoxia typically results in rapid consumption of NO_3^- as it is the next thermodynamically favorable election acceptor (after oxygen) for organic matter oxidation and is readily utilised by denitrifiers, nitrate ammonifiers and the benthic microbial communities. A possible explanation for the slow consumption of ^{15}N-NO_3^- is that denitrifiers were carbon limited in these ponds, however, previous work in this pond demonstrated that the addition of an exogenous carbon source did not significantly stimulate N_2 production (Castine et al., 2012). Alternatively, NO_3^- diffusion into the fine grained sediments might be slow.

Dominance of DNRA over denitrification has been reported previously in nutrient rich, shallow tropical systems (An & Gardner, 2002) and tropical estuaries with moderate nutrient loads (Dong et al., 2011). The prevalence of DNRA over denitrification is a product of many controlling factors, including high concentrations of organic electron donors and low concentration of NO_3^- (Christensen et al., 2000), a higher affinity for NO_3^- by nitrate ammonifiers than denitrifiers at higher temperatures (Dong et al., 2011), the presence of sulfides which inhibits nitrification and partially inhibits denitrification (Gardner et al., 2006), and the presence of solid phase sulfur which stimulates DNRA (Brunet & Garcia-Gil, 1996; Jørgensen, 2010). My data suggests that solid phase sulfur is an important controlling mechanism in these ponds. For example, in Z2 sulfur is concentrated (10 ± 0 mol cm^{-3}), DNRA is fast (82.3 µmol m^{-2} h^{-1}) and denitrification in slow (8.7 µmol m^{-2} h^{-1}). Conversely, in Z3, sulfur concentration is lower (3 ± 0 mol cm^{-3}), DNRA is slower (25.2 µmol m^{-2} h^{-1}) and denitrification is faster (20.5 µmol m^{-2} h^{-1}). Prevalence of DNRA results in NH_4^+ accumulation and nitrogen retention in the settlement ponds.

3.4.3. Nitrogen retention and nitrogen loss

The efficiency of the settlement pond system to denitrify and permanently remove nitrogen from the prawn farm wastewater was estimated in relation to three nitrogen input sources; 1) from the measured import of nitrogen with the wastewater entering the settlement pond, 2) from net NH_4^+ flux from the sediments (net flux; Fig. 3.4) and 3) from mineralized nitrogen (DON and NH_4^+) produced during PN oxidation (mineralisation; Fig. 3.4). Subsequently, the transformation rates which I measured during the core incubation were used to determine nitrogen sinks and export from the settlement pond for each nitrogen species. This provided important insight into *in situ*

nitrogen cycling but must be interpreted with some caution given that the rates were measured under laboratory conditions. I predicted that 0.15 kg N d^{-1} can be removed from the settlement pond through microbial N_2 production, which was 1.1% of the total daily nitrogen load to the settlement pond. The settlement pond was not an efficient producer of N_2 and the majority of nitrogen was being recycled within or exported from the settlement pond with release of the wastewater to the environment. This has implications for both the environmental sustainability and the profit margin of the farm and highlights the opportunity to enhance nutrient use efficiency through improved wastewater technologies. DON dominated nitrogen dynamics in the settlement pond with high import, high release of DON during PN mineralisation, high assimilation into the sediments and high transformation to NH_4^+. However, once DON had been oxidised to NH_4^+, further transformation was inhibited. The concentration of nitrogen remaining in the NH_4^+ fraction was high compared to nitrogen remaining in the DON and NO_3^- fractions and it was likely that slow rates of bacterial NH_4^+ transformation (nitrification) was limiting N_2 production. Competition with phytoplankton for NH_4^+ assimilation may have been limiting bacterial NH_4^+ transformation. This is supported by the fact that I overestimated the amount of NH_4^+ exported from the settlement pond by 3.47 kg N d^{-1}. This discrepancy is likely attributed to *in situ* NH_4^+ assimilation into phytoplankton biomass, which I did not measure in the core incubation, but which would have resulted in the export of PN out of the settlement pond instead of the export of NH_4^+. An additional reason for accumulation of nitrogen in the NH_4^+ fraction is limited nitrification. DNRA also produced 0.13 kg of NH_4^+ d^{-1} adding to the accumulation of NH_4^+, and competing with denitrifiers for NO_3^-. 12.45 kg N d^{-1} was exported from the settlement pond primarily in the form of PN, DON and NH_4^+. Targeted management

and treatment technologies are required to remediate these nitrogen species before wastewater is released to the environment.

3.4.4. Shifting N retention to N loss through improved management

To improve nitrification, integrated management approaches could be employed whereby sludge is removed from ponds, coupled nitrification-denitrification is enhanced though biofiltration, and nighttime aeration is used to stabilise O_2 regimes and enhance water column mixing, (Bartoli et al., 2005). Additionally, integration of mangrove wetlands, which improve sediment aeration, has proven benefits for nitrogen abatement, with indications that 40.8 ± 8.3% of the added nitrogen is lost through N_2 production (Erler et al., 2010).

The positive effect of burrowing organisms on nutrient cycling, and specifically nitrification is also well documented (Bertics et al., 2010; Mayer et al., 1995; Wang et al., 2010). The mechanism being that, oxic zones are created deep into the sediments through the irrigation behavior (import and export of water to and from the burrow) of the burrowing organism (Mayer et al., 1995). NH_4^+ produced as a metabolic waste from the resident macrofauna and bacterial biomass is subsequently deposited and made available to nitrifying community (Mayer et al., 1995). In organically perturbed sediments around fish cages, polychaete-microbe interactions had positive effects on nitrogen cycling (Kunihiro et al., 2008; Wada et al., 2008). These could be added to settlement ponds to increase bioturbation. Wada et al. (2008) used sediment microcosms to demonstrate a significant increase in the degradation of organic matter by co-habiting polychaetes, *Capitella* sp., and bacterial iolates, *Vibrio* spp. compared to either organism on their own. A large-scale, field based study, in which 9.2 million polychaetes were released in the sediments under fish cages, then highlighted an

increase in bacterial biomass particularly of the *a-Proteobacteria,* and a suggested link between these two organisms and organic matter degradation (Kunihiro et al., 2008).

I provide the first elucidation of soluble nitrogen cycling in tropical aquaculture settlement ponds and build on previous studies regrading nitrogen cycling in prawn grow-out ponds. N_2 production efficiency was low (1.1%), limited by low nitrification rates and this is in agreement with Burford and Longmore (2001) and Jackson et al. (2003a) who demonstrated denitrification efficiencies of <2% and 3%, respectively in prawn grow-out ponds. Low nitrification rate (15% of the nitrogen pool day^{-1}) have also been demonstrated in grow-out ponds (Burford & Lorenzen, 2004). In the present study DON (urea) cycling was fast, relative to inorganic nitrogen transformation, with DON being both produced during gross mineralisation and assimilated by the sediment microbial community. In accordance, the majority (>70%) of the total dissolved nitrogen mineralised from prawn feed and faeces was organic nitrogen and urea was rapidly assimilated by the microbial community (Burford & Williams, 2001). DON was also transformed to NH_4^+in the present study. It is likely that the phytoplankton community plays an active role in nitrogen cycling through assimilation of NH_4^+ but that this hinders nitrogen removal by stimulating pelagic-benthic nitrogen cycling instead of N_2 production pathways (Burford & Lorenzen, 2004). This research highlights the importance of addressing excess sludge and nutrient rich suspended solids to improve wastewater treatment.

Chapter 4: Algal bioproducts derived from suspended solids in intensive land-based aquaculture

4.1. Introduction

Intensive land-based aquaculture farms primarily operate under 'biofloccculation' management regimes where the control of pH, and carbon addition, produces an organic rich biofloc composed of a suspended solid biomass of algae, bacteria or zooplankton (Schryver et al., 2008). This biofloc provides the benefit of additional nutrition for culture species, above that of formulated feed inputs and progressive improvements in biofloc technology have enabled improved nutrition and improved discharge water quality (Burford et al., 2004; Ray et al., 2010). However, suspended solids which are not consumed by the culture species are subsequently released as a discharge and are considered a waste product. Suspended solids in aquaculture are traditionally treated in settlement ponds prior to release to the environment. However, the settlement efficacy of solids within these ponds is low in saline systems, with up to 40% of solids remaining in suspension (Jackson et al., 2004; Jackson et al., 2003b). In addition, unless removed those solids that settle produce a nutrient rich sludge which releases dissolved nitrogen back to the water column through microbial decomposition (Burford & Lorenzen, 2004; Castine et al., 2012; Preston et al., 2001). An alternative to the release or internal recycling of suspended solids is their capture and re-use (Jones et al., 2001). Previous works in prawn aquaculture systems investigated nutrient capture and re-use through combinations of settlement ponds, oyster filtration and macroalgal nutrient assimilation (Jones et al., 2001; Jones & Preston, 1999; Jones et al., 2002). These studies demonstrated the potential for reductions in suspended solids, phytoplankton,

bacteria and dissolved nutrients but noted the difficulties, mainly due to fouling, of trialling these technologies in commercial operations (Jones et al., 2001).

The effective capture (harvest) of suspended solids is dictated by the concentration and particle size distribution of the suspended solids in the discharge water (Levine et al., 1991). Suspended solid concentration and particle size distribution were determined in prawn farm effluent (Jones et al., 2002), and have been well documented in temperate aquaculture, including freshwater salmon (Cripps, 1995; Kelly et al., 1997) and trout farms (Maillard et al., 2005), and are dilute in concentration (1.5-8.0 mg L^{-1}) and predominantly small in size (< 30 µm) (reviewed by Cripps and Bergheim (2000). However, particle size distribution warrants further investigation in wastewater from tropical land-based aquaculture farms as operations intensify and wastewater treatment techniques advance. .

Importantly, within tropical land-based systems a large proportion of suspended solids can be microalgae, a rich source of lipids and extractable fatty acids. Microalgal derived fatty acids have applications in aquaculture feeds and nutraceuticals (Spolaore et al., 2006), and as a biofuel through trans-esterification to biodiesel (Mata et al., 2010). The quality and quantity of fatty acids varies greatly between classes and phyla of microalgae (Hu et al., 2008; Huerlimann et al., 2010) and quantifying the yield and quality of microalgal biomass, including fatty acid composition, is a critical first step in evaluating the potential of suspended solids as a bioresource.

An alternative to the extraction and utilisation of fatty acids is the *in-toto* utilisation of lipid rich (high calorific value) microalgal biomass through thermo-chemical conversion by pyrolysis. Slow pyrolysis of biomass produces energy and biochar (Panwar et al., 2012), and is an established waste mitigation tool (Lehmann & Joseph, 2009a). Biochar production sequesters carbon, and can mitigate CO_2, CH_4 and

N_2O emissions on large scales (Read, 2008; Yanai et al., 2007). The addition of biochar to soils improves soil structure and fertility, and enhances agricultural production (Rondon et al., 2007; Steiner et al., 2007). Biochar produced from macroalgal biomass has high ash, nitrogen and extractable inorganic nutrient content, and excellent agricultural properties (Bird et al., 2011a; Bird et al., 2011b). Consequently, quantifying the yield and quality of biochar from microalgal biomass is an important additional assessment in evaluating the potential of suspended solids harvested from intensive land-based aquaculture as a bioresource.

Therefore, the first aim of this study is to quantify, characterise, and subsequently harvest the suspended solids from two discharge waste streams in pond-based intensive aquaculture. The first discharge is the water from the culture ponds. The second discharge is the water from the settlement ponds. The second aim is to quantify the fatty acid profile of harvested biomass (suspended solids) and evaluate its potential as a bioproduct in aquaculture feeds and nutraceuticals. The third aim is to convert the harvested biomass (suspended solids) to biochar and evaluate its potential as a soil ameliorant. Finally, I discuss the scale of this bioresource and scope for its application.

4.2. Materials and Methods

4.2.1. Study Site

All experiments were conducted at Pacific Reef Fisheries Ltd. in North Queensland, Australia (S19˚28’46”, E147˚29’18”). This 73 ha farm was situated in the dry tropics, and cultured black tiger prawns (*Penaeus monodon*). At the time of sampling (May 2012), feeding rates were high with culture ponds at peak biomass production. Discharge water from the culture ponds was treated in traditional settlement ponds

before being released to the environment. Water was collected from the discharges of three operational culture ponds and the three operational settlement ponds (Fig. 4.1).

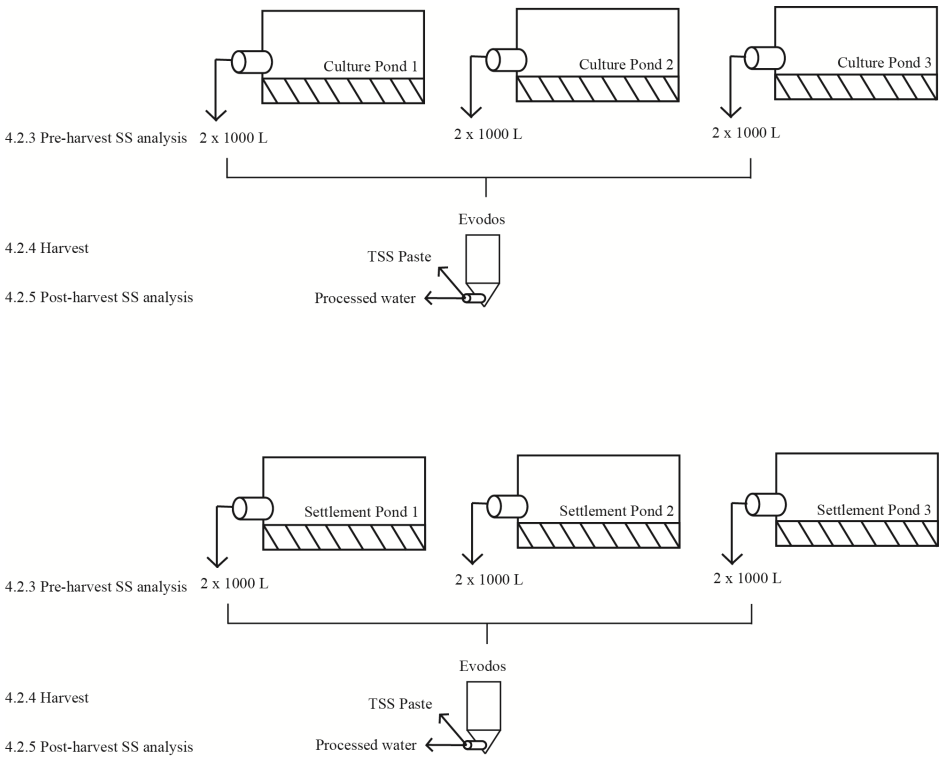

Fig. 4.1 A schematic of the protocol for analysing and harvesting suspended solid (SS) from the discharge of prawn farm culture and settlement ponds. The numbers denote the sub-headings in the methods section in the text.

4.2.2. Initial assessment of suspended solid characteristics

Preliminary characterisation of suspended solids was conducted with particle size distribution and suspended solid concentration being quantified for three culture and settlement ponds on three days within one week (2-8th May 2011; see below for methods). Particle size distribution ranged between 0.03-400 µm across both pond types and the mean suspended solid load was 90.8 ± 8.2 mg L^{-1} in culture pond discharge water and 65.3 ± 0.7 mg L^{-1} in settlement pond discharge water. From this preliminary data it was estimated that 6000 L of water from each pond type was required to provide

sufficient harvested biomass for the characterisation of physico-chemical properties of the resulting algal pastes, the quantification of the fatty acid content, as well as the production and characterisation of biochar made from these pastes (Fig. 4.1).

4.2.3. Pre-harvest analysis of suspended solid

Water was collected from the discharge streams of three operational culture ponds (3 x 2000 L) on 16th May 2011 and from three settlement ponds (3 x 2000 L) on 18th May 2011 (Fig. 4.1). The water from each pond was collected and transported in 2 x 1000 L intermediate bulk containers (IBC) (Fig. 4.1). Each IBC was used as a duplicate, and water samples were collected from each IBC on filling for the characterisation of suspended solids. Suspended solid concentration, particulate nitrogen, particulate carbon, chlorophyll *a*, microalgae community composition and particle size distribution were quantified and characterised for each water sample.

Suspended solid concentration (mg L^{-1}) was quantified by filtering 250 mL of discharge water through a pre-weighed, 0.4 µm membrane filter and drying to a constant weight at 60°C. To quantify particulate nitrogen and particulate carbon (as mg L^{-1} and converted to % of suspended solids) a known volume of water was filtered through pre-ashed (450ºC for 5 h) Whatman glass fibre filters (GFF), and stored frozen until analysis. Once thawed, filters were subjected to high temperature combustion in a Shimadzu TC-5000 fitted with a solid sample inlet. Chlorophyll *a* (determined as mg L^{-1} and converted to % of suspended solids) content was quantified by filtering a separate water sample through GFF filters with the filters frozen until analysis. Subsequently, filters were ground in 90% acetone and chlorophyll *a* concentration was quantified fluorometrically in extracts. Chlorophyll *a* constitutes approximately 1-2% of a microalgal cell (APHA, 1980) and the proportion of the suspended solids comprised of

microalgae was estimated based on an average value of 1.5% chlorophyll *a* (mg) per unit of microalgae.

The community composition of the suspended solids was grouped into four broad categories: chlorophytes, cyanobacteria, diatoms and unknown microalgae. The relative proportion of each group was determined by categorising between 150-250 cells from 20 µL sub-samples using preserved material (1% Lugol's solution) at 1000x magnification under oil immersion (Leica DMLB light microscope).

Particle size distribution was determined with Malvern Mastersizer 2000 laser particle sizer using a 0.02 - 2000 µm lens (Malvern Instruments). The detector was calibrated and aligned before each batch of samples using water as the background correction.

4.2.4. Industrial harvest of suspended solids

Suspended solids were harvested from each culture and settlement pond discharge using a centrifugal algal harvesting system at the MBD Energy Research Facility, James Cook University, Townsville (Evodos BV – SPT325; serial number: 609805; operated at 3800 L hr^{-1} and ambient temperature). The post-harvest effluent water was collected from the harvesting of each of the culture and settlement discharges for analysis of remaining suspended solids (see section 4.2.5). To ensure that sufficient biomass was harvested for the quantification of fatty acids and physico-chemical parameters of the paste, and the production and assessment of biochar, the biomass from all six samples (6x 1000 L) from the culture ponds were combined, as were all six samples (6x 1000 L) from settlement ponds. This provided a single biomass paste of harvested suspended solids (paste) from each source. Once harvested, each paste was re-suspended in freshwater and re-processed through the Evodos centrifuge to ensure that any residual

salt associated with the suspended solids was removed. Subsequently, three sub-samples (~2g each) from both the culture pond paste, and the settlement pond paste, were lyophilised (VirTis 2K) for analysis of fatty acids (see section 4.2.5). The remaining paste was then spread on a Teflon baking tray and dried at 60°C for three days. Once dried, a representative sub-sample was collected by dividing the dry paste 4-6 times using a riffle splitter. The sub-sample was homogenised using a SRM-standard ring mill (ROCKLABS Ltd.) to provide ~20 g of ground sample for physico-chemical characterisation (see section 2.5.3). The paste was packaged in a vacuum-sealed bag and stored frozen prior to the production of biochar (see section 4.2.6).

4.2.5. Post-harvest analysis of suspended solids

Post-harvest analysis of effluent water - Post-harvest water was analysed for suspended solid concentration, particulate nitrogen, particulate carbon, chlorophyll *a*, microalgae community composition and particle size distribution (as above) to determine the quality and quantity of the suspended solids removed by the centrifuge.

Fatty acid analysis of harvested solids - Fatty acid analysis was conducted using lyophilised pastes. Direct trans-esterification was used to simultaneously extract and esterify the fatty acids from both samples, following methods adapted from Cohen et al. (1988) and Rodriguez-Ruiz et al. (1998) as described in Gosch et al. (2012). Gas chromatography was carried out in scan-mode on an Agilent 7890 GC (DB-23 capillary column) equipped with a flame ionization detector (FID) for quantification and connected to an Agilent 5975C Electron Ionisation Turbo Mass Spectrometer (EI-MS) (Agilent Technologies Australia Pty Ltd) for identification of fatty acid methyl esters (FAMEs). Oven program and instrument settings followed David et al. (2002). The quantity of FAMEs was determined by comparison with FID peak areas of authentic

standards (Sigma Aldrich), and corrected for recovery of internal standard. Total fatty acid content was calculated from the sum of all FAMEs.

Physico-chemical characterisation of harvested solids - The physico-chemical properties of the dried culture pond and settlement pond pastes were characterised prior to conversion to biochar. Loss on ignition (LOI) was used to estimate the organic and carbonate contents. LOI was calculated by combusting a small quantified amount of material (100 – 500 mg ± 0.1 mg) at 550°C for 2 h, cooling the material to room temperature and weighing. Samples were subsequently re-combusted at 1000°C for 1 h, cooled and re-weighed to determine the carbonate content (Heiri et al., 2001). Total nitrogen (TN) and total organic carbon (TOC) were determined following PN and PC methods as described above. Sulphur (S), phosphorus (P), iron (Fe), manganese (Mn), magnesium (Mg), potassium (K), calcium (Ca), and sodium (Na) were determined by inductively coupled plasma mass spectrophotometry (ICP-MS). Electrical conductivity (EC) and pH were determined in 10:1 water:sample mixtures according to Australian standard methods for soil analysis (Rayment & Higginson, 1992). Cation exchange capacity (CEC) was determined using silver thiourea extracts (Rayment & Higginson, 1992), and BET (Brunauer, Emmet, and Teller) surface area was determined by nitrogen adsorption (Particle and Surface Sciences Pty Ltd., in Gosford, New South Wales, Australia).

4.2.6. Biochar production and characterisation

Biochar was produced from both the dried culture pond paste, and the settlement pond paste, using slow pyrolysis under conditions previously optimised by Bird et al. (2011a). Approximately 200 g of dried paste was weighed (to 3 decimal places), loaded into a wire mesh basket and suspended in a sealed 2 L stainless steel vessel inside a

muffle furnace. The stainless steel vessel was constantly purged with dry nitrogen gas at 3.5 L min^{-1} and heated for over 1 hr to a final hold temperature of 450 ± 5°C (Bird et al., 2011a). The furnace was maintained at 450 ± 5°C for 2 hrs after which time the vessel was removed from the muffle. The resulting biochar was cooled to room temperature and weighed to determine weight loss accompanying pyrolysis. Subsequently, the physico-chemical properties (section 4.2.5) were determined for both biochars.

4.2.7. Statistical Analysis

The pre-harvest water quality characteristics and microalgal community composition data (the proportions of each group) were compared between pond types (culture and settlement) and within pond types (duplicates at three ponds nested within each pond type) in a nested design using multivariate permutational analysis of variance (PERMANOVA). Particle size distribution data was included as a single variable by selecting the size class that gave the maximum value (mode) for the particle volume distribution. The PERMANOVA was run on a Bray-Curtis similarity matrix using fourth root transformed data (PRIMER version 6 and PERMANOVA+ version 1.0.4) (Anderson et al., 2008). A principal component analysis (PCA) plot was used to interpret the PERMANOVA results by relating the main vector loadings to both between and within pond type variation.

4.3. Results

4.3.1. Pre-harvest analysis of suspended solids

The characteristics of the suspended solids from the culture pond and settlement pond discharge sources were significantly different (PERMANOVA; *Pseudo F* = 10.28, *P* =

0.008). There was also significant variability in suspended solid characteristics within pond types (PERMANOVA; *Pseudo F* = 4.231, *P* = 0.002). The concentration of suspended solids was 66% higher in the culture pond discharge stream (131.8 ± 8.8 mg L^{-1}; *n* = 3) than in the settlement pond discharge stream (87.6 ± 24.7 mg L^{-1}; *n* = 3), although one settlement pond (SP1) had notably higher TSS than the remaining two (Fig. 4.2A). The suspended solid results were reflected for all metrics as particulate nitrogen (Fig. 4.2B), particulate organic carbon (Fig. 4.2C) and chlorophyll *a* (Fig. 4.2D) concentration also tended to be higher in culture ponds, again with some variation within pond type. Conversely, the quality of the suspended solids, in terms of the content of particulate nitrogen, particulate organic carbon and chlorophyll *a* (as a percentage of suspended solid biomass) tended to be higher in the settlement pond discharge streams (4.0 ± 0.8, 24.8 ± 4.7, 0.3 ± 0.0%, respectively; *n* = 3) compared to the culture pond discharge streams (3.8 ± 0.6, 22.7 ± 3.1, 0.2 ± 0.1%, respectively; *n* = 3).

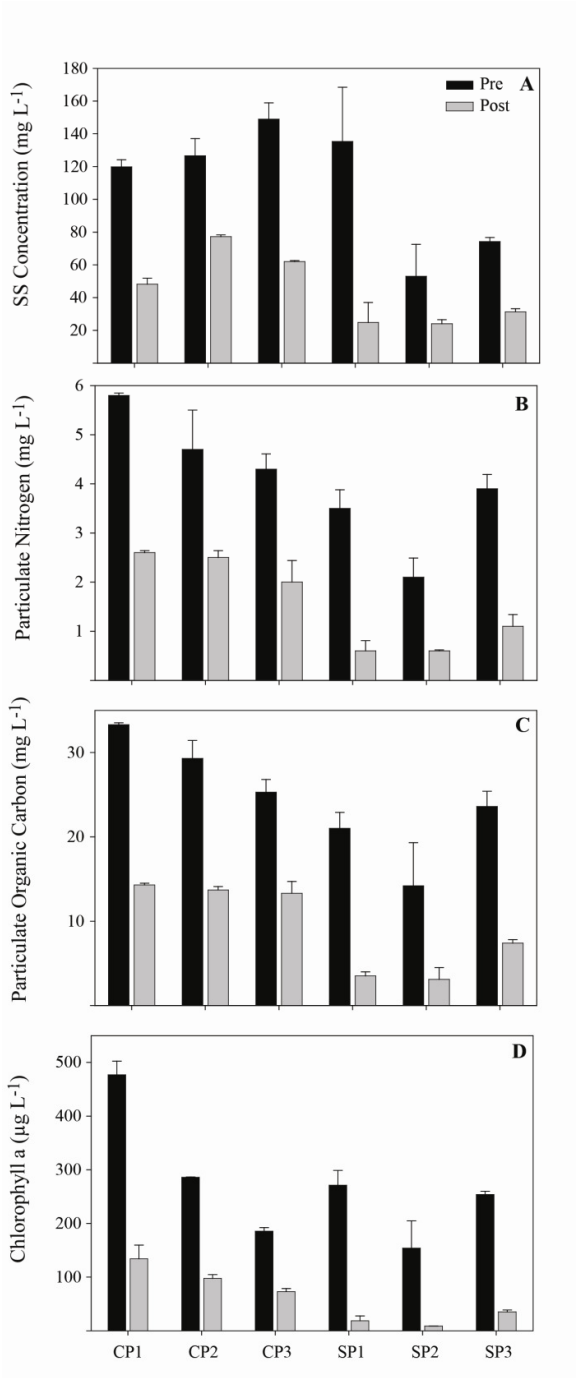

Fig. 4.2 Mean of A) suspended solids (SS) (mg L^{-1}), B) particulate nitrogen (mg L^{-1}), C) particulate organic carbon (mg L^{-1}), and D) chlorophyll *a* (µg L^{-1}) concentrations in discharge water from three prawn farm culture ponds (CP) and three prawn farm settlement ponds (SP) (*n*=2). Black bars represent the mean concentration of each constituent before water has been processed in an Evodos centrifuge and grey bars represent mean concentrations after water has been processed.

Using chlorophyll *a* as a proxy for microalgal biomass (Fig. 4.2D), it was estimated that 24.6 ± 7.8% (n = 3,) of the suspended solids in the culture pond water was microalgae compared to 27.5 ± 4.0% (n = 3, means of three ponds) of the suspended solids in the settlement pond. Filamentous cyanobacteria dominated in culture pond discharge streams, making up between 24-89% of the microalgal community (Fig. 4.3) whereas diatoms (mainly centric *Cyclotella* spp. and *Chaetoceros* sp., and pennate *Cylindrotheca* sp.) were more common in the settlement pond discharge streams, making up between 16-52% of the microalgal community (compared with 2-9% in culture pond) (Fig. 4.3).

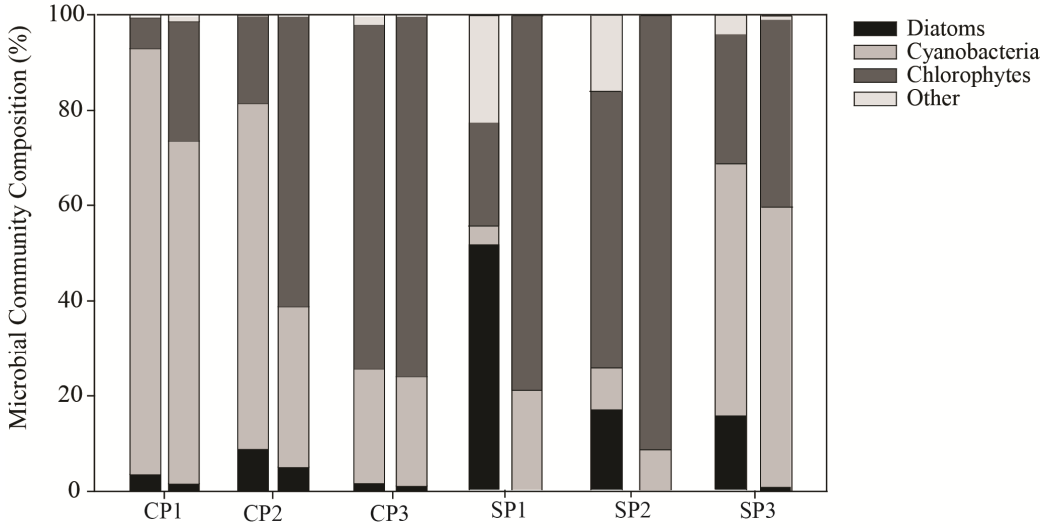

Fig. 4.3 Microbial community composition (% cells) of the suspended solids in discharge streams from the culture ponds (CP1, CP2 and CP3) and the settlement ponds (SP1, SP2 and SP3) from a commercial prawn farm. The first bar for each pond represents the community composition in the water pre-harvest and the second bar for each pond represents the community composition in the water post-harvest.

Particle size ranged from a minimum of 0.04 µm up to a maximum of 563 µm across all ponds, and was more variable in the settlement pond discharge water than in the culture pond discharge water (Fig. 4.4A and B). The size fraction with the

maximum value for particle volume distribution was 11-20 µm in both the culture pond and settlement pond discharge water.

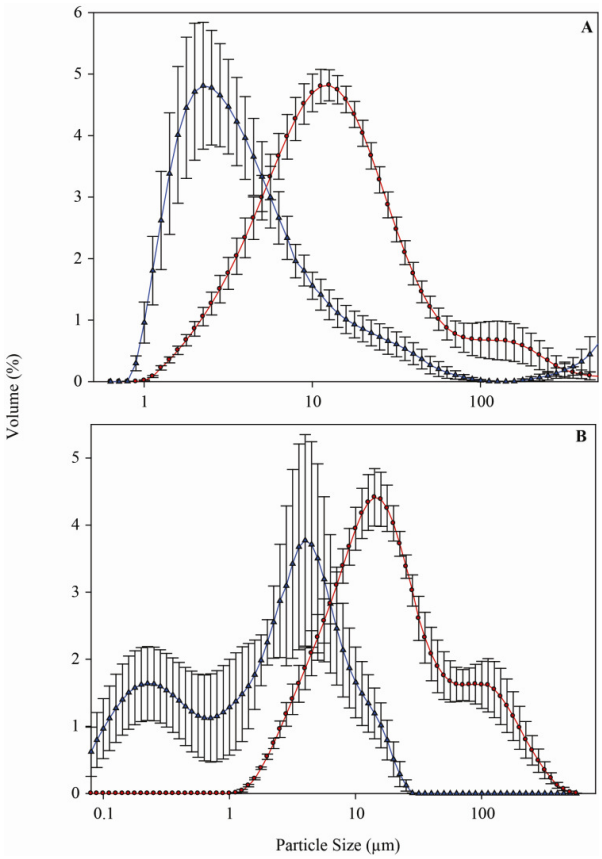

Fig. 4.4 Particle size distribution (logarithmic scale) of the suspended solids in the culture pond (A) and settlement pond (B) discharge water. Red lines represent the suspended solids in discharge water before it has been processed and blue lines represent the remaining suspended solids post-processing.

4.3.2. Post-harvest analysis of suspended solids

Post-harvest analysis of effluent water - The quality of water improved after processing through the Evodos (post-harvest) for both culture and settlement ponds (Fig. 4.2). Approximately 60% of the suspended solids were captured using centrifugal technology, thereby reducing suspended solids in post-harvest water from a mean of 131.8 ± 8.8 mg L^{-1} to 62.5 ± 5.3 mg L^{-1} for culture ponds, and 87.6 ± 24.7 mg L^{-1} to 26.7 ± 2.7 mg L^{-1} for settlement ponds (Fig. 4.2A). Particulate nitrogen (Fig. 4.2B),

particulate organic carbon (Fig. 4.2C) and chlorophyll *a* (Fig. 4.2D) followed the same trend as the suspended solid concentration, with the concentration of each variable being lower in the water post-processing. There was also a significant effect of processing on the phytoplankton community (Fig. 4.3). In both the culture pond and settlement pond discharge water, the community composition of post-harvest water was dominated by small chlorophytes (1-5 μm) with almost 100% of the diatoms removed from the settlement pond water (Fig. 4.3).

The centrifuge selectively removed larger suspended solids, specifically targeting diatoms and cyanobacteria over chlorophytes and fine sediment. This is demonstrated by a reduction in the proportion (as a % of total) of suspended solids in the larger size fractions (> 10 μm) in water post-harvest in both the culture pond (63 ± 5%) and the settlement pond (94 ± 1%) water (red line compared to blue lines; Fig. 4.4).

Fatty acid analysis of harvested solids - The harvested suspended solids from both the culture and settlement ponds were rich in fatty acids (measured as fatty acid methyl esters, FAMEs) with a mean total of 28.491 ± 0.257 and 41.990 ± 0.340 mg FAME g^{-1} DW suspended solids in the culture pond and settlement pond harvested solids, respectively (Table 4.1). The fatty acid profiles of the suspended solids harvested from both discharge streams were similar and within each harvest the polyunsaturated fatty acid portion in the culture pond and settlement pond comprised 45.3% and 44.2% of the fatty acid profiles, respectively (Table 4.1). Furthermore, the harvested biomass from the culture ponds had 9.0% ω-6 and 23.5% ω-3 fatty acids, while the settlement ponds had 3.1% ω-6 and 26.6% ω-3 (Table 4.1). The ω-6:ω-3 fatty acids ratios were low at 0.4 and 0.1 in the culture pond and settlement pond harvests, respectively (Table 4.1).

Table 4.1 Fatty acid composition (mg FAME g^{-1} DW suspended solid) of waste harvested suspended solids collected from an aquaculture culture pond and settlement pond discharge. Data are mean (± 1 standard error).

Fatty acid	Grow-out	Settlement
C12:0	0.08 (0.00)	0.08 (0.00)
C14:0	1.74 (0.16)	2.81 (0.01)
C14:1	0.17 (0.01)	0.15 (0.01)
C15:0	0.20 (0.01)	0.28 (0.01)
C15:1	0.33 (0.04)	0.38 (0.01)
C16:0	6.49 (0.85)	8.04 (0.03)
C16:1 (7)	0.26 (0.04)	0.14 (0.00)
C16:1 (9)	3.79 (0.43)	3.63 (0.02)
C16:2 (7,10)	0.70 (0.15)	0.20 (0.00)
C16:2 (9,12)	0.59 (0.05)	1.74 (0.01)
C17:0	0.00 (0.00)	0.11 (0.01)
C17:1	0.62 (0.06)	0.76 (0.02)
C16:3 (6,9,12)	0.77 (0.14)	0.26 (0.01)
C16:4 (4,7,10,13)	0.70 (0.09)	0.85 (0.02)
C16:4 (6,9,12,15)	0.60 (0.25)	1.24 (0.04)
C18:0	0.29 (0.02)	0.39 (0.10)
C 18:1 (cis 9)	1.04 (0.17)	1.37 (0.01)
C 18:1(trans 9)	0.32 (0.03)	0.34 (0.00)
C 18:2 (9,12)	2.40 (0.49)	1.10 (0.01)
C18:3 (6,9,12)	0.18 (0.01)	0.24 (0.01)
C 18:3 (9,12,15)	2.82 (0.44)	2.32 (0.01)
C18:4 (6,9,12,15)	0.61 (0.05)	2.30 (0.02)
C20:0	0.10 (0.01)	0.24 (0.01)
C20:1	0.0 0 (0.00)	4.59 (0.12)
C20:3 (8,11,14)	0.19 (0.02)	0.05 (0.00)
C 20:4 (5,8,11,14)	0.17 (0.01)	0.22 (0.01)
C 20:5 (5,8,11,14,17)	2.42 (0.23)	4.13 (0.03)
C22:0	0.16 (0.01)	0.00 (0.00)
C24:0	0.00 (0.00)	0.15 (0.02)
C 22:6 (4,7,10,13,16,19)	0.76 (0.04)	3.89 (0.04)
Total	28.49 (0.26)	41.99 (0.34)
Total saturated	9.06 (0.71)	12.10 (0.89)
Total monounstaturated	6.52 (0.44)	11.35 (0.61)
Total polyunsaturated	12.91 (0.25)	18.54 (0.39)
ω-3	6.71 (0.55)	11.19 (0.76)
ω-6	2.57 (1.12)	1.32 (0.44)
ω-6/ω-3	0.38	0.12

Physico-chemical characterisation of harvested solids - The ash content of the culture pond suspended solids was 44.5% compared to 35.2% in the settlement pond suspended solids (Table 4.2). Similarly, LOI at 500˚C, which is an indicator of organic content, and organic carbon were both lower in the culture pond suspended solids (51.4 and 25.9%, respectively) than in the settlement pond suspended solids (61.3 and 30.3%, respectively) (Table 4.2).

4.3.3. Biochar production and characterisation

The organic carbon content of the culture and settlement pond biochar was low at 14.5 and 22.7%, respectively (Table 4.2). Conversely, biochar from both discharge sources was rich in nitrogen (2.5% and 3.5% in the culture and settlement pond suspended solids, respectively; Table 4.2). Given the high nitrogen content, the C:N ratio of the biochars produced from the suspended solids harvested from the culture ponds and settlement ponds was low (5.8 and 6.5, respectively; Table 4.2).

The biochars from the harvested biomass are also rich in beneficial micronutrients, particularly K (2.0 and 1.4%), Mn (0.058 and 0.136%), Na (2.0 and 1.4%) and Fe (3.8 and 3.5%) in culture pond and settlement pond biochars, respectively (Table 4.2). However, the biochars are also relatively high in the heavy metals, Cu (0.010 and 0.013%) and Ni (0.007 and 0.063%; Table 4.2), in culture pond and settlement pond biochars, respectively (Table 4.2).

Cation exchange capacity (CEC) for the biochars was low with values of 3.5 and 0.5 cmol(+) kg^{-1}, in culture pond and settlement pond biochars, respectively (Table 4.2). The EC of the biochar is also relatively low at 8.9 and 6.9 mS cm^{-1} (Table 4.2).

Table 4.2 Composition of suspended solids and biochar which was collected from prawn farm culture pond and settlement pond discharge water. Note: ''LOI" = loss on ignition; ''CEC" = cation exchange capacity. Total and organic carbon and nitrogen data derived from non-acidified and acidified samples, respectively.

	Unit	Culture pond feedstock	Settlement pond feedstock	Culture pond biochar	Settlement pond biochar
LOI @ 500ºC	%	51.4	61.3	21.1	31.7
LOI @ 1000ºC	%	4.1	3.6	24.7	34.4
Pyrolysis loss	%	NA	NA	45.7	37.3
Ash	%	44.5	35.2	54.2	33.9
C:N		5.4	6.2	5.8	6.5
Organic carbon	%	25.9	30.3	14.5	22.7
Organic nitrogen	%	4.8	4.8	2.5	3.5
P	%	0.9	0.8	1.0	1.0
Ca	%	1.0	0.4	1.4	0.7
Mg	%	0.8	0.6	1.3	1.0
K	%	1.3	0.9	2.0	1.4
Zn	mg kg^{-1}	164.0	185.0	235.0	274.0
Mn	mg kg^{-1}	386.0	861.0	575.0	1355.0
Na	%	1.3	0.8	2.0	1.4
Fe	%	2.5	1.9	3.8	3.5
S	%	0.7	0.8	0.4	0.4
Cu	mg kg^{-1}	62.6	69.1	101.0	133.0
Pb	mg kg^{-1}	18.7	12.3	61.0	35.0
Ni	mg kg^{-1}	51.7	138.0	71.0	626.0
pH		6.2	5.9	7.9	7.2
BET surface area	m^2 g^{-1}	30.3	28.3	10.7	20.7
EC	mS cm^{-1}	5.9	3.3	8.9	6.9
Exchangable cations					
Ca	cmol(+) kg^{-1}	NA	NA	18.3	9.4
K	cmol(+) kg^{-1}	NA	NA	13.9	12.2
Na	cmol(+) kg^{-1}	NA	NA	58.8	36.6
Mg	cmol(+) kg^{-1}	NA	NA	10.7	6.0
CEC	cmol(+) kg^{-1}	NA	NA	3.5	0.5

[a]Potassium and sodium are only partially extracted with the acid digestion procedure used. All other element data are complete extractable values.

The pH of biochar from culture pond and settlement pond harvests was 7.9 and 7.2, respectively (Table 4.2). Finally, BET surface areas are relatively low for the culture pond and settlement pond biochars, at 10.7 and 20.7 m^{-2} g^{-1}, respectively (Table 4.2).

4.3.4. Bioresource scale

The scale of the bioresource of harvestable suspended solids from intensive land-based aquaculture of prawns is significant but will differ depending on the discharge stream from which suspended solids are harvested (Jackson et al., 2004). For example, I estimate that a 100 ha prawn farm releases more than 2 GL yr^{-1} (based on industry data; EPA monitoring at Pacific Reef Fisheries; 2008-2009 grow-out season). The release of this water from the settlement ponds, from where water is discharged to the environment, would contain 2084 tonnes of suspended solids (based on a mean of 87.6 mg L^{-1}) (Fig. 4.2A). In this study ~70% of suspended solids from the settlement ponds were removed through harvesting (Fig. 4.2A) and therefore 1271 tonnes could be captured per annum from the settlement ponds. Utilising biochar as an example, 63% of the harvested suspended solid biomass can be converted to a valuable bioproduct (i.e. 481 tonnes from the settlement ponds). The carbon and nitrogen contents of the settlement pond biochar were 22.7% and 3.5%, respectively. This equates to 109 tonnes of carbon and 17 tonnes of nitrogen being sequestered per annum, although this estimate is based on triplicate samples of biochar produced under laboratory conditions and must be tested more rigorously to gain an accurate understanding of nutrient sequestration at scale. Taking this approach to a larger scale, with caution, there are more than 900 ha of intensive prawn aquaculture in Australia which could produce 8460 tonnes of biochar, sequestering 2034 tonnes of carbon and 252 tonnes of nitrogen.

Expanding this simple example to a global scale, where there are estimated to be more than 0.5 million ha of crustacean culture (FAO, 2010), the bioresource scales to 15,930,000 tonnes of harvestable solids, which, at 63% efficiency, would produce 10,035,900 tonnes of biochar, 2,278,149 tonnes of sequestered carbon and 351,256 tonnes of sequestered nitrogen. The extrapolation acknowledges the variation in discharge concentration and load across farms and seasons (Jackson et al., 2004),and further investigations are required to consolidate any modelled estimates.

4.4. Discussion

4.4.1. Pre-harvest analysis of suspended solids

Despite the culture ponds and the settlement ponds being intrinsically linked through discharge of the former to the latter, the characteristics of the suspended solids from the two discharge sources were different, with higher concentrations of suspended solids, particulate nitrogen and carbon and chlorophyll *a*, in the culture pond discharge stream than in the settlement pond discharge stream. Conversely, the quality of the suspended solids, in terms of the content of particulate nitrogen, particulate organic carbon and chlorophyll *a* (as a percentage of suspended solid biomass) tended to be higher in the settlement pond discharge streams. The higher organic carbon content of settlement pond suspended solids compared to the culture pond suspended solids is likely explained by the presence of inorganic solids which are eroded from the floor of the culture pond by the movement of the aerators (Preston et al., 2001). These later settle in the settlement pond and were therefore not present in the suspended solids in the discharge stream of the settlement ponds. This implies that for the production of a high quality secondary product, the harvest of waste suspended solids should occur from the stream discharging from the settlement ponds.

Particle size ranged from 0.04 µm to 563 µm with the majority of particles residing in the 11-20 µm size fraction in both the culture pond and settlement pond discharge water. This is supported by particle size distribution data in waste streams from land-based prawn farms and recirculating farms where the majority of suspended solids are < 30 µm (Cripps, 1995; Cripps & Bergheim, 2000; Jones et al., 2002). Given this similarity, suspended solids capture technologies such as rotating micro-screens, bead filters and flotation columns, which are used in advanced recirculating farms could be adapted to flow-through land-based farms and used to capture suspended solids > 60 µm (Cripps & Bergheim, 2000). Cost effective technologies to harvest smaller biological solids (< 60 µm) is a priority for the capture of energy rich microalgae for bioproducts.

4.4.2. Post-harvest analysis of suspended solids

Post-harvest analysis of effluent water - Water quality improved after processing the culture and settlement pond discharge streams through the Evodos. Over half of the total suspended solids were captured, with the Evodos selectively removing larger particles such as diatoms. Given the high energy and beneficial fatty acid content of diatoms, a diatom capture rate approaching 100% is beneficial for the production of bioproducts from suspended solids. Furthermore, a 60% reduction in total suspended solid load provides opportunity for water re-use and improved water security.

Fatty acid analysis of harvested solids - Microalgae are rich in fatty acids, including beneficial omega 3 (ω-3) and omega 6 (ω-6) polyunsaturated fatty acids which have applications in aquaculture feeds and nutraceuticals (Spolaore et al., 2006). Correspondingly, the harvested suspended solids from both the culture and settlement ponds were rich in fatty acids with polyunsaturated fatty acids comprising over 40% of

the fatty acid profiles. Furthermore, the harvested biomass had ω-6 and ω-3 contents comparable to species within the genus *Nannochloropsis* which have 5.8 ± 0.8% ω-6 and 20.9 ± 3.7 ω-3 and are cultured specifically for their polyunsaturated ω-3 and ω-6 fatty acids (Huerlimann et al., 2010). The low ω-6:ω-3 fatty acids ratios makes the harvested biomass, particularly from the settlement pond, preferable for inclusion into nutraceuticals, because western diets are deficient in ω-3 fatty acids due to the industrial production of ω-6 rich cereal grains (Simopoulos, 2002). A higher proportion of DHA, an ω-3 fatty acid, occurred in the settlement pond harvest than in the culture pond harvest. The abundance of diatoms, which are rich in DHA (Huerlimann et al. 2010) most likely drives the difference in ω-6:ω-3 ratio between culture and settlement pond harvested solids.

Physico-chemical characterisation of harvested solids - The primary driver of differences in physico-chemical properties of harvest suspended solids (paste) between the two discharge sources is the higher inorganic content of suspended solids harvested from the culture ponds. This is likely driven by large inorganic particles which are eroded from the culture pond floor by the action of the aerators (Preston et al., 2001).

4.4.3. Biochar production and characterisation

Biochar, produced from harvested suspended solids from the culture pond and settlement pond discharge streams, reflect the physico-chemical properties of the solids in their low organic carbon content (14.5 and 22.7%, respectively). This is comparable to organic carbon found in macroalgal biochars (8.2-33.8%; (Bird et al., 2011a) and microalgal biochar (16%) (Grierson et al., 2011). However, it is low relative to biochar based on ligno-cellulosic feedstocks, which range from 62 to 80% (Brewer et al., 2011).

The nitrogen content of the biochars were also within range (1.6-5.3%) reported for other algal biochars (Bird et al., 2011a) albeit higher than biochar produced from municipal wastewater sludge (0.02-0.22%) (Hossain et al., 2010) and ligno-cellulosic sources (0.3 and 0.8%) (Brewer et al., 2011). The C:N ratio of the biochars produced from the suspended solids harvested from the culture ponds and settlement ponds was low (5.8 and 6.5, respectively) even relative to microalgal biochar which had a ratio 10:1 (Grierson et al., 2011). This is reflective of the high nitrogen content in the harvested biomass which is a positive attribute, given that many agricultural systems are nitrogen limited.

The biochars from the harvested biomass were also rich in beneficial micronutrients, particularly K, Mn, Na and Fe. These cations are beneficial for plant growth because plants must maintain neutral charge and balance nitrogen uptake, which occurs predominantly through uptake of the anion NO_3^- (Chan et al., 2007). However, the biochars were also relatively enriched in the heavy metals, Cu and Ni. The nickel content of the settlement pond biochar exceeded levels deemed safe for normal land application of biosolids (Ang & Sparks, 2000), although the stability and leaching of heavy metals from biochar is largely unknown and not considered in the biosolid application guidelines. A possible explanation for the high nickel content in the settlement pond biochar is the mobilisation of this heavy metal from acid sulphate soils regimes (Gröger et al., 2011) with the subsequent uptake into algal biomass (Saunders et al., 2012).

CEC for the biochars was among the lowest of any values reported in the literature, and the EC of the biochar was also relatively low compared to biochar from other microalgae (Grierson et al., 2011). Notably, the exchangeable cation, Na, was lower in this study (58.8 and 36.6 cmol(+) kg^{-1}; Table 2) than in Grierson et al. (2011)

(110 cmol(+) kg^{-1}), which is likely attributed to the freshwater wash removing Na^{+} and other exchangeable cations.

pH of biochar ranges from 4-12 (Lehmann, 2007) and can be manipulated by adjusting pyrolysis conditions. Given the pH of biochar in this study (7.9 and 7.2, in the culture and settlement pond biomass, respectively) the biochar would aid in the stabilisation of soil pH to near neutral and be beneficial for application to both acidic and alkaline soils.

Finally, the low BET surface area of biochar in this study is likely due to the high ash content and concentrated inorganic compounds which are hypothesised to block micro-pores and reduce the surface area of a biochar (Bruun et al., 2012). Accordingly, BET surface area (10.7 m^{-2} g^{-1}) was lower in biochar from the culture pond where ash content was high, with higher BET surface area (20.7 m^{-2} g^{-1}) in the biochar from the settlement pond where ash content was lower. The low ash content of the biochar produced from the harvested suspended solids from the settlement pond (33.9%) was similar to that of other microalgal biochars (Grierson et al., 2011), while the higher ash content of culture pond biochar (54.2%) reflected a lower microalgal content and a higher content of inorganic particles.

4.4.4. Bioresource scale

The scale of the bioresource of harvestable suspended solids from intensive aquaculture of prawns is potentially significant. For example, harvesting the suspended solids at the settlement pond discharge of a 100 ha prawn farm is estimated to produce enough raw biomass for the production of 481 tonnes of biochar and generate significant energy during pyrolysis (Panwar et al., 2012). In Australia there are more than 900 ha of intensive prawn aquaculture which could produce 8460 tonnes of biochar, and globally

there are estimated to be more than 0.5 million ha of crustacean culture (FAO, 2010), which has potential to produce 10,035,900 tonnes of biochar, sequestering 2,278,149 tonnes of carbon and 351,256 tonnes of nitrogen. There is also opportunity to harvest from multiple discharge points with a land-based aquaculture system, which allows for further optimisation of harvesting and the quality of the harvested biomass. However, these estimates should be interpreted with caution and they are provided to exemplify the potential scale of this bioresource. The variable nature of aquaculture discharge and associated constituents means that intensive site-specific sampling is required to develop more general models.

Harvestable suspended solids from intensive land-based aquaculture such as prawn production offer a new and potentially valuable bioresource for conversion to bioproducts and bioenergy. Harvested suspended solids, ranging in concentration (16.2-158.8 mg L^{-1}) and size (0.04-563 μm), were efficiently harvested from both culture and settlement pond discharge. The harvested solids were rich in microalgae and while the quantity of harvested solids was higher from culture ponds, the quality was lower. Consequently, the quantity and quality of fatty acids was higher in the biomass harvested from the settlement pond. However, both bioresources were rich in ω-3 and ω-6 fatty acids. The harvested biomass was also an excellent bioresource for the production of biochar and potentially alternative thermo-chemical conversions to biofuels (Biller & Ross, 2011). A cost-benefit analysis of biochar or biofuel production would add rigor to these estimates, especially given conflicting uses for suspended solids to enhance food conversion ratios using bioflocculation technology in prawn aquaculture (Xu & Pan, 2012).

Chapter 5: Improving wastewater treatment for land-based aquaculture: Existing technology and value-adding alternatives

5.1. Introduction

Land-based aquaculture is an integral part of global aquaculture production for fishes (28.8 million tonnes), molluscs (13.1 million tonnes), and crustaceans (5.0 million tonnes) (FAO, 2010; Hall et al., 2011). Irrespective of whether land-based systems support the intensive culture of fresh, brackish and marine water organisms, a unifying feature of these systems is the addition of high protein feeds to sustain the rapid growth of intensively farmed animals (Alam et al., 2009). However, the assimilation of this protein is inefficient and can result in up to 80% of the nutrients being lost in wastewaters (Briggs & Funge-Smith, 1994; Karakassis et al., 2005). This wastewater is typically rich in both suspended solids (particulates) and dissolved nutrients, and if untreated can cause sediment loading and eutrophication in receiving waters (Burford et al., 2003; Costanzo et al., 2004; Gräslund & Bengtsson, 2001; Hall et al., 2011; Preston et al., 2001; Tello et al., 2010; Vaiphasa et al., 2007). In some systems, rapid production within the water column facilitates assimilation of waste nutrients (McKinnon et al., 2002). Tidal flushing can also mitigate the impacts of the wastewater and effects on the receiving environment, for example through mangrove creeks (McKinnon et al., 2002; Trott & Alongi, 2001). However, the potential impacts of wastewater released from intensive land-based aquaculture systems have prompted strict legislation for the treatment and release of waste streams from land-based aquaculture and the development of Best Management Practices in the USA (Tucker & Hargreaves, 2008), Australia (Donovan, 2001; PIMC, 2005) and the Mediterranean (IUCN, 2009), all of which are largely based on treatment in settlement ponds. Policies and legislation

provide the imperative to improve the design and management of marine and brackish settlement pond systems, with the goal to better manage the critical water quality variables of suspended solids and dissolved nutrients (Erler et al., 2004).

Large-scale municipal wastewater treatment (MWWT) is an analogous wastewater system for which similar drivers lead to ongoing improvements to treatment processes and can provide significant insight into treating the large volumes of water that are characteristic of many land-based systems (Zakkour et al., 2002). More recently, intensive recirculating aquaculture systems (IRAS) have also adapted technology from freshwater MWWT systems to achieve effective low-cost water treatment in saline systems (Couturier et al., 2009). Both MWWT and intensive recirculating aquaculture wastewater treatment (IRAWWT) technologies provide an opportunity to redefine wastewater management for land-based aquaculture systems (Couturier et al., 2009; Dereli et al., 2010; Deviller et al., 2004; Gray, 2005). They offer the opportunity to treat large volumes of wastewater to an acceptable standard. Specifically, these technologies must target wastewater with comparatively low total suspended solid (TSS) and dissolved nutrient concentrations.

The aims of this review are to firstly identify technologies that can be transferred from MWWT or IRAWWT, and secondly, to synthesise these into a wastewater treatment model applicable to land-based aquaculture wastewater treatment (LBAWWT) and which also optimises opportunities for value-adding. The focus of this review is primarily targeted at marine (30-50‰) and brackish (0.5-30‰) water LBAS with particular emphasis on the crustacean (shrimp and prawn) industry. It enforces the concept of a multi-stage treatment process to concentrate and convert suspended and dissolved wastes into manageable biomass. However, in instances where technologies

are applicable across a broad salinity range, transfer to and from freshwater (< 0.5‰) systems is also highlighted.

5.2. Land-Based Aquaculture and Settlement Systems

Flow-through land-based aquaculture systems generally consist of large earthen ponds or raceways and range in size from one or two hectares to 100s of hectares. Large water exchanges have typically been used to control nutrient levels in grow-out ponds (Boyd & Tucker, 1998) but as culture intensifies and scale increases the cost of exchanging large volumes becomes unsustainable, both economically and environmentally. Furthermore, this strategy dilutes wastewater and makes the removal of solids difficult, especially in saline systems where solid settling efficacy is reduced (Mesquita et al., 2011). Consequently, aquaculture wastewater is unique in the properties that determine treatment technologies as it is brackish or saline, comparatively dilute in suspended solids, and rich in dissolved organic nutrients.

5.2.1. Land-Based Aquaculture Wastewater Characteristics

Solid constituents - Understanding the unique characteristics of land-based aquaculture wastewater (LBAWW) is fundamental to treatment, in particular, the particle (suspended solids) density and composition, and attributes of the "sludge" - the particulate matter which settles on the pond floor. Suspended solids are categorised into two groups; supracolloidal (1-100µm) and settleable (>100µm) particulates (Levine et al., 1985). While these categories are similar to those in municipal and intensive aquaculture waste streams, the key difference is that suspended solid concentration is low in LBAWW (5-119 mg L^{-1}) compared to IRAWW (5-390 mg L^{-1}) and MWW (93-800 mg L^{-1}) (Table 5.1). This, and the large temporal and spatial variation in suspended

solid concentration, impacts on treatment technologies for LBAS primarily because the low concentration of suspended solids results in slower settling, as particles make minimal contact with each other and do not form larger, heavier aggregates that naturally settle faster than individual particles (Fornshell, 2001; Jackson et al., 2004).

Table 5.1 Examples of wastewater characteristics in municipal wastewater, intensive recirculating aquaculture wastewater and land-based aquaculture wastewater (mg L^{-1})

Characteristic	MWW	Reference	IRAWW	Reference	LBAWW	Reference
TSS	800	Hammer and Hammer (2008)	5 to 50	Cripps and Bergheim (2000)	5 to 30	Henderson and Bromage (1988)
	100-200	Morari and Giardini (2009)	390	Piedrahita (2003)	76.8 ± 7.8	Castine (unpublished data)
	93	Woertz et al. (2009)			1.3	Piedrahita (2003)
	340	Dereli et al. (2010)			40-119	Jackson et al. (2004)
Total ammonia nitrogen	39	Woertz et al. (2009)	6.8-25.6	Chen et al. (1993)	0.41	Castine (unpublished data)
	36	Dereli et al. (2010)				
	100-800	Chen et al. (1993)				
Nitrate	<0.01	Woertz et al. (2009)	11.7 ± 1.5	Summerfelt et al. (2009)	0.19	Castine (unpublished data)
	2	Dereli et al. (2010)			0.091	Jackson et al. (2003a)
Nitrite	<0.01	Woertz et al. (2009)	0.06 ± 0.025	Summerfelt et al. (2009)	0.23	Castine (unpublished data)
					0.004	Jackson et al. (2003a)
Total nitrogen	52	Dereli et al. (2010)	18.00	Piedrahita (2003)	0.2	Piedrahita (2003)
					1.5 to 3	Jackson et al. (2003a)
					2.1-3.1	Jackson et al. (2004)
Total phosphorus	10	Dereli et al. (2010)	2.10	Piedrahita (2003)	0.02	Piedrahita (2003)
					0.22-0.28	Jackson et al. (2004)

Particles in LBAWW are primarily comprised of unutilised or degraded formulated feed, and excrement from culture species. However, heavy inorganic particulates (>100 µm) from earthen LBAS are eroded from the floor and banks of culture ponds and can make up a large portion (60-90%) of the settleable load in wastewaters from grow-out ponds (Preston et al., 2001). This particulate fraction is best

divided into two groups based on size and mode of remediation. Settleable solids are >100 µm (group 1; Fig. 5.1) and are remediated during pre-treatment, which is largely effective and has little scope for improvement. Supracolloidal particles (1-100 µm; group 2; Fig. 5.1) are remediated during primary treatment which is more complex. Notably, particulates in LBAWW are typically 1.5-210 µm (Maillard et al., 2005), with the majority being < 30 µm (Cripps, 1995; Jones et al., 2002). Broadly speaking, the larger fraction of this size range (i.e. > 100 µm) will settle in grow-out ponds (pre-treatment), while approximately 50% of the supracolloidal particles (1-100 µm) are settled in settlement ponds (primary treatment) (Fig. 5.1).

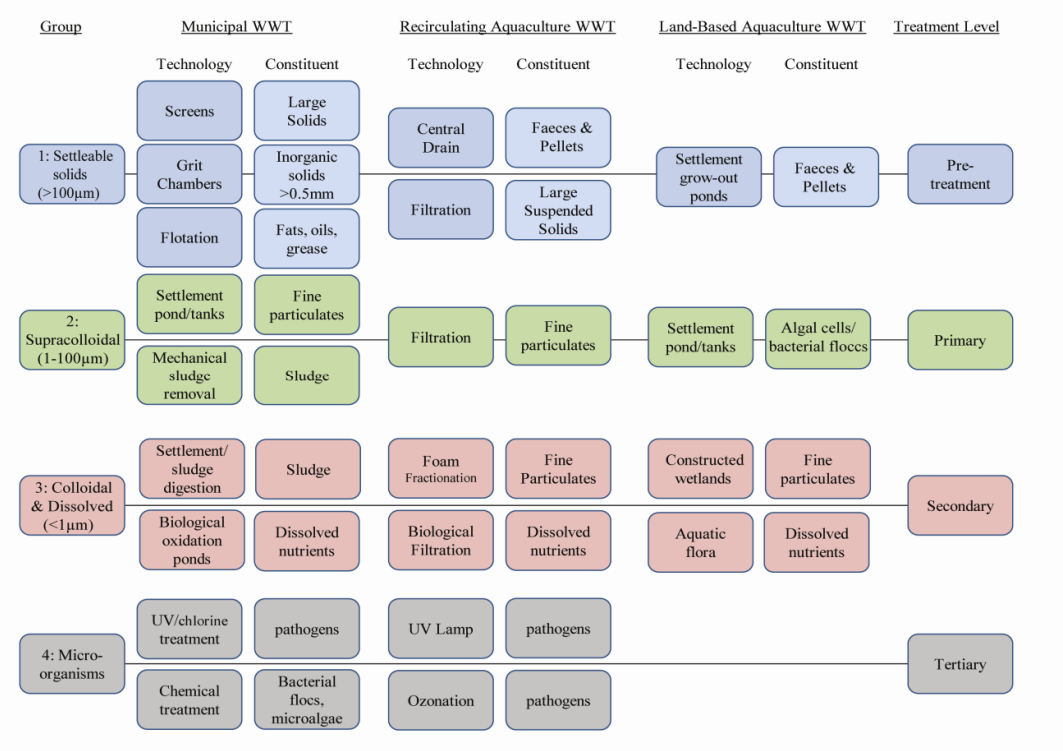

Fig. 5.1 The principal treatment steps in municipal, intensive recirculating and land-based aquaculture wastewater treatment systems. Blue boxes represent pre-treatment options to treat group 1 constituents, green boxes represent primary treatment options to treat group 2 constituents, red boxes represent secondary treatment options to treat group 3 constituents and grey boxes represent tertiary treatment options to treat group 4 constituents. Based on Gray (2005) and Hammer and Hammer (2008).

In either case, the settled particulates form a semi-solid sludge layer that is relatively low in organic carbon (0.85 ± 0.52% during early production cycle and 1.88% ± 0.46% during late production cycle) and total nitrogen (0.16 ± 0.09%) (Burford et al., 1998). Additional constituents of sludge include phosphorus, potassium, calcium and magnesium which make up 4.3, 0.04, 11.3 and 0.16% of the dry weight of the sludge, respectively (Bergheim et al., 1998). Sludge also contains trace metals including copper, zinc, lead, cadmium, chrome, nickel and cobalt, the most quantitatively important of which are zinc (562-608 mg kg^{-1} DM), copper (24-29 mg kg^{-1} DM) and nickel (10-19 mg kg^{-1} DM) (Bergheim et al., 1998). Sludge is viewed as a waste product that is removed from culture ponds at the end of each production cycle (Chen et al., 1993). However, sludge could become the basis of a product comprising high volatile dry matter (19.8-29.7% of dry weight) and beneficial micro- and macronutrients (i.e. calcium 11.3% of dry weight; and zinc 562-608 mg kg^{-1} DM) (Bergheim et al., 1998). In addition to the suspended solids, the wastewater is also rich in dissolved constituents that require further remediation prior to release (Jackson et al., 2003a).

Dissolved constituents - As culture species respire and excrete waste, colloidal particles (0.45-1 µm) and dissolved nutrients (<0.45 µm), in particular ammonium (NH_4^+), are expelled into the water column (collectively group 3; Fig. 5.1). There are a range of biological pathways by which these colloidal and dissolved waste compounds can be transformed to different compounds, for example, from an organic compound to an inorganic compound, or vice versa. These metabolic transformation pathways occur in bacteria, Archaea, microscopic fungi, algae, eukaryotes and viruses and are either dissimilatory (energy creating) or assimilatory (biomass creating) transformations (Burgin et al., 2011). There are both beneficial and detrimental dissimilatory pathways.

The beneficial dissimilatory pathways which permanently remove nitrogen from aquaculture systems are nitrification, denitrification and anammox (Fig. 5.2). Nitrification transforms NH_4^+ to nitrate (NO_3^-) and is often coupled to denitrification in which denitrifiers reduce NO_3^- to nitrogen gas (N_2) (Knowles, 1982) that is subsequently lost to the atmosphere (Fig. 5.2). Similarly, anammox results in the production of N_2, but it proceeds through the oxidation of NH_4^+ with NO_3^- (van de Graaf et al., 1995).

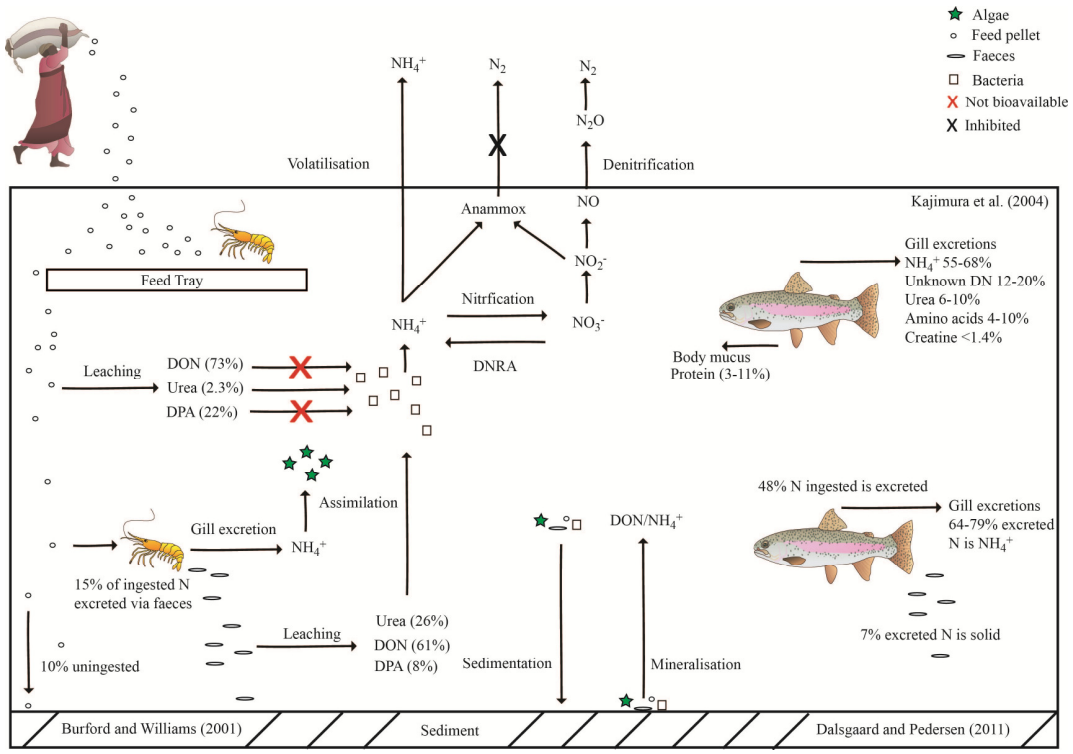

Fig. 5.2 The sources and fate of dissolved nitrogen in aquaculture ponds. Data are from Burford and Williams (2001), Kajimura et al. (2004) and Dalsgaard and Pedersen (2011). Graphics (prawn, fish and woman) are from Integration & Application Network (ian.umces.edu/symbols/)

Conversely, the detrimental dissimilatory pathways of mineralisation, remineralisation and dissimilatory nitrate reduction to ammonium (DNRA) retain nitrogen within the system (Fig. 5.2). Mineralisation (the release of dissolved organic

nitrogen; DON) and remineralisation (the release of NH_4^+) occur due to oxidation (degradation) of organic matter. These pathways typically occur in the sludge, fuelled by organic matter in the settled faeces, uneaten feed pellets, dead microalgae and microbial biomass (Burford & Lorenzen, 2004; Burford & Williams, 2001). Consequently, DON constitutes a significant proportion of the dissolved fraction (65.01 µM) of the wastewater. However, many of the DON components, with the exception of urea, are not readily bio-available (Burford & Williams, 2001) (Fig. 5.2). NH_4^+ concentration is also typically high (53.7 ± 7.4 – 61.2 ± 6.5 µM) in aquaculture wastewater (Bartoli et al., 2005) but nitrate (NO_3^-) concentration is typically much lower (3.6 ± 1.9 – 5.4 ± 1.8 µM) (Bartoli et al., 2005).

Dissolved nitrogen compounds (NH_4^+ or NO_3^-) can also be transformed through assimilation and incorporation into microbial or algal biomass that can be removed from the system by harvest (de Paula Silva et al., 2012). Remediation of dissolved nitrogen is more effective if dissolved nitrogen exists in the inorganic form (NO_3^-, NO_2^- and NH_4^+) because these compounds are essential for the nitrification, denitrification and anammox pathways occurring in the sludge and NO_3^- and NH_4^+ are preferentially assimilated by algae (de Paula Silva et al., 2012; Erler et al., 2010). Conversion from DON to dissolved inorganic nitrogen (DIN) is therefore beneficial to reducing total nitrogen (Erler et al., 2010).

5.2.2. Existing treatment technologies

Pre-treatment - The removal of settleable solids (>100 µm) is the first step in managing wastewater. Initially, particles settle efficiently because these particles (usually >100 µm: e.g. heavy faeces, particulates eroded from the pond floor and waste feed pellets, are accumulated in the centre of the grow-out pond using strategically placed aerators

(Boyd, 1998). This built-up sludge, estimated at 35-60 mt ha^{-1} $harvest^{-1}$ within a single 1 ha prawn grow-out pond (Preston et al., 2001), is removed at the end of each production cycle and is currently not further utilised.

Primary treatment - Settlement ponds – also known as settlement basins, bioremediation ponds, wastewater treatment ponds or waste stabilisation ponds – are large basins in which wastewater is retained on the principle that particulate wastes < 100 µm will settle under gravitational forces (Sperling & Chernicharo, 2005). Decreased flow and a long residence time facilitate gravitational settlement and dissolved nutrients are concurrently transformed through the unmanaged processes of nitrification, denitrification, anammox or assimilation into biomass (Fig. 5.2). This simple approach is used by more than 70% of Australian land-based aquaculture farms (Preston et al., 2001). Newly constructed settlement ponds used by marine and brackish LBAS reduce TSS by 60%, total phosphorus load by 30% and total nitrogen load by 20%, despite being rudimentary single step-ponds of relatively small area (~ 0.1-0.8 ha) and shallow depth (0.25-2 m deep) (Preston et al., 2001). Importantly, their efficacy decreases markedly over time if not maintained, and settled solids build up within the pond, forming a thick nutrient-rich sludge layer. These settled particulates decompose, releasing dissolved nutrients, hydrogen sulphide and methane into the water column (Burford & Lorenzen, 2004; Burford et al., 1998). Removal of this sludge reduces mineralisation and remineralisation of nutrients and ensure that ponds remain sufficiently deep for particulates < 100 µm to settle. LBAS currently operate on unsynchronized production cycles with heavy demand for multiple production cycles within the year, and therefore settlement ponds cannot be dried and sludge removed systematically. Consequently, pond design and management strategies need to be modified to include deep anaerobic ponds systems for enhanced efficiency of solid

transformation and to include biological filtrations for the management of the resulting nutrients (DON and NH_4^+) as the critical steps in secondary treatment.

Secondary treatment - All settlement pond systems facilitate the biological transformation of nutrients, or secondary treatment, through microbial transformation and assimilation into biomass. However, because there is little control over the prevailing abiotic variables in these environments it is difficult to enhance the beneficial microbial pathways of nitrification, denitrification and anammox. These pathways compete with detrimental pathways that recycle nitrogen in the system, such as DNRA and mineralisation, and the latter often prevails (Fig. 5.2), especially in tropical and subtropical sulfidic environments (Castine et al., 2012). However, biological transformation of nutrients by bacteria is an extremely powerful mechanism which has not yet been optimised for LBAS and represents significant opportunities for marine and brackish water LBAWWT.

Secondary treatment is also facilitated through the growth of macro- and microalgae as phototropic plant growth (assimilation) can rapidly remove nitrogen and phosphorous from wastewater (Neori et al., 2004). However, the efficacy of this approach relies on the optimisation of biomass production as nutrient remediation is proportional to algal growth. Consequently, it is necessary to actively manage algal standing stocks by removing biomass in proportion to growth to extract nutrients from the settlement pond system (de Paula Silva et al., 2008). As with the management and removal of suspended solids (sludge), the management and removal of algal biomass provides a resource of carbon, nitrogen, phosphorous, trace elements and minerals to deliver novel value adding products.

5.3. Municipal and Intensive Recirculating Aquaculture Wastewater

There is an opportunity to improve the fundamental designs of LBAWWT by utilising technologies and strategies from pre-existing industries that have evolved beyond basic remediation principles. MWWT and IRAWWT technologies are highly advanced due to the drivers of population pressure, water scarcity, environmental concerns and environmental regulation (for extensive reviews see Steicke et al. (2009), Terry and Krause (2009)). However, the wastewater characteristics within each industry vary, with higher suspended solid and dissolved nutrient loads in MWW compared to both IRAWW and LBAWW, and higher suspended solid and dissolved nutrient loads in IRAWW compared to LBAWW (Table 5.1). Therefore technology transfer needs to be optimised to target the unique characteristics of LBAWW. MWWT plants are the most evolved technology and involve three or four stages of treatment (pre-, primary, secondary and tertiary treatment stages) (Fig. 5.1), and treat highly concentrated wastewater (93-800 mg L^{-1} TSS; Table 5.1) to a level that is safe for human consumption. Similarly, multi-stage treatment systems are used in IRAWWT plants (Fig. 5.1) with many technologies optimised to saline systems. Our objective is to review and identify technologies suitable for transfer to LBAWWT, taking into consideration the characteristics of LBAWW and the capacity to engineer technologies at a scale relevant to LBAS (100s of hectares).

5.4. Lessons and Technologies from Municipal and Intensive Recirculating Aquaculture Wastewater Treatment

There are both obvious and subtle improvements for LBWWT systems using technologies from MWWT and IRAWWT. These technology transfers primarily need to address the concentration of supracolloidal and colloidal (<100 μm) particulates, the

removal and treatment of concentrated particulates, and the removal of residual dissolved nutrients in 'treated' wastewater. These technologies are synthesised in a proposed model treatment system for LBAWWT (section 5.5).

5.4.1 Concentrating, settling and removing particulates

The relatively low concentration of particulate constituents in aquaculture wastes (1.3-35.6 mg L^{-1} in LBAWW compared to 93-800 mg L^{-1} in MWW; Table 5.1) necessitates the concentration of particulates to enhance removal or settlement. Removal and settlement can be enhanced using physical processes, a combination of physical and biological processes, or multi-stage pond systems.

Physical processes utilise screens and barriers to capture or settle particulates. Tube settlers enhance settling by forcing water to flow up through a settling plate (angled at 45-60º above horizontal to facilitate self-cleaning), capturing solids on the underside of the plate (Timmons & Ebeling, 2007). They are used to settle supercolloidal (1-100 µm; 80% removal efficiency) and colloidal (0.45-1 µm; 55% removal efficiency) particulates in IRAWW (Easter, 1992). I propose this approach in preference to enhancing settlement through natural or chemical flocculation techniques. During natural flocculation, filamentous bacteria enhance the structure of the floc and protozoa grazing on non-settleable bacteria enhance the size of the floc, making it heavier and more likely to settle. However, mortality of filamentous bacteria and protozoa may increase in the presence of salt, decreasing the natural settling capability of particles (Mesquita et al., 2011). There is little evidence that chemical flocculants and coagulants (lime, iron sulphate, iron chloride, aluminium sulphate, aluminium chloride) are efficient in saline water and these have proven prohibitively expensive as treatment in freshwater systems (Cripps & Bergheim, 2000; Parsons & Smith, 2008).

Instead, rotating micro-screens, drum screens, drum filters or swirl concentrators with screen sizes ranging from 60-200 µm have been successfully implemented in land-based intensive fish farms (Cripps & Bergheim, 2000; Sindilariu et al., 2009), and 60 µm mesh has the potential to capture >80% of solids in freshwater fish farms (Kelly et al., 1997). The selection of physical filters is dependent on individual system requirements. For example, in a comparison of drum filters and swirl separators at a recirculating salmon-smolt farm, swirl separators removed 63% of TSS compared to 22% by drum filtration (Couturier et al., 2009). Granular and porous media filters provide an alternative to screen filters in IRAWWT and have the advantage of acting as both physical and biological filters, by trapping and transforming particles, respectively (Chen et al., 1993; Cripps & Bergheim, 2000).

Multi-stage baffled settlement ponds are used to control flow rates and depth regimes to enhance settlement and biological treatment for settleable (> 100 µm), supracolloidal (1-100 µm) and colloidal (<1 µm) particles in MWWT and for intensive agri-business (dairy, piggeries, feed-lot cattle). Initially, physical processes such as grit screens (> 30 mm) are also employed to remove wood, rags, grit and coarse solids. After the coarse solids are removed, a deep anaerobic pond facilitates settlement of particles < 100 µm and sludge removal (Archer & Mara, 2003; Craggs et al., 2008; Craggs et al., 2004a). Anaerobic ponds have almost been universally adopted across industries, and their design is recommended to be 4-5 m deep and twice as long as they are wide (DEC, 1996). Modifications to this design include a set of two deep anaerobic ponds, providing the flexibility to dry and remove sludge from one pond while continuing treatment in the adjacent pond (Fig. 5.3). However, LBAWWT systems do not use initial physical processes (grit screening) or designated anaerobic ponds, and the settlement ponds rarely contain baffles to direct water flow. This means that water in

LBA settlement ponds commonly short circuits, where water takes the most direct path to the outlet, resulting in low residence times irrespective of their expansive layout. This affects both particulate and dissolved nutrient remediation by reducing the residence time of wastewater available for transformation and assimilation. Retrofitting baffles using earth or high density polyethylene, as is done in the dairy industry (Craggs et al., 2004a), may provide a simple means of increasing residence time and improving the settlement and consistency of LBAWWT (Fig. 5.3).

In MWWT, settled particles form a semi-solid sludge which necessitates removal prior to the dissimilation of captured nutrients into DON (mineralisation) and NH_4^+ (remineralisation). The opportunity to concentrate and settle particles before they contribute to nutrient release through mineralisation is arguably more cost-effective than large-scale dissolved nutrient remediation. Improved solids capture will certainly reduce downstream particulate nitrogen and phosphorous concentrations, but dissolved nutrients will remain a significant waste stream in LBAWW treatment.

5.4.2 Removing Dissolved Nutrients

Reducing dissolved nutrient concentration in any type of wastewater using rudimentary single step settlement pond technology presents a significant challenge because ponds are not optimised for the assimilation of nutrients through algal growth and harvest, or for transformation by beneficial microbial pathways (Bolan et al., 2009; Craggs et al., 1996). However, three types of processes can be transferred from MWWT and IRAWWT to improve the removal of dissolved nutrients from LBAWW. The first is to use microbial processes that are enhanced through biological filters, reactors or digesters (Chen et al., 2010b; Roy et al., 2010; Sharrer et al., 2007; Summerfelt, 2006). The second is to use phototrophs (algae, cyanobacteria) to enhance assimilation (Bartoli

et al., 2005; Henry-Silva & Camargo, 2006; Mai et al., 2010). Finally, the third is to combine these processes into a functional bioremediation mesocosm comprised of multi-stage systems (Craggs et al., 2004a) that may include a constructed wetland as an end point (Lin et al., 2003).

Enhanced microbial processes in fluidised sand-bed filters can promote nitrification, removing up to 90% of the NH_4^+ in some IRAS (Heinen et al., 1996). As NH_4^+ is also a problematic nutrient in LBAS, the use of such filters would be beneficial to promote nitrification and could be readily adapted to the large volumes of LBAS. Membrane biological reactors are also efficient in promoting microbial processes (Tal et al., 2009) and NH_4^+ removal is efficient over a range of salinities, from 0 to 32‰ in IRAS (Sharrer et al., 2007). However, at higher salinity the start up time for a nitrifying reactor is extended because the nitrifying microbial community takes longer (~118 days) to acclimate and become effective (Sharrer et al., 2007).

Enhanced phototrophic processes have been a focus for the improvement of remediation in the dairy industry. Craggs et al. (2004a) overcame the fundamental issue of high dissolved nutrient content in dairy farm wastewater by upgrading traditional two stage treatment pond system to a four-stage pond system. Two-pond freshwater systems (primary and secondary treatment) consisting of a deep anaerobic settling basin and a shallower facultative pond, were traditionally recommended as they were effective in remediating biochemical oxygen demand (BOD), carbon and TSS in wastewater (Bolan et al., 2009). However, they were not optimised for dissolved nutrient removal, and the upgrade to a multi-stage pond system increased NH_4^+ removal by 37% and TSS removal by 44% (see Fig. 2 in Craggs et al., 2004a). Treatment of agricultural wastewater and MWW now includes a series of ponds with different physical characteristics, each performing a separate function in the wastewater remediation

process (Craggs et al., 2004a), and widening the opportunity for energy production and capture (Craggs et al., 2011).

Agricultural wastewater treatment comprises four stages, the first of which is a single anaerobic pond to enhance settlement of particulates. The second is a high rate microalgal pond (HRAP) with a large surface area and shallow profile (0.2-1 m deep) to increase exposure of algal cells to light for enhanced biomass production (Park et al., 2011). The third is a pair of algal settling ponds that are deeper near the inflow and become shallower, terminating in a surface outflow pipe at the discharge to ensure that solids are separated and recovered (Craggs et al., 2004a). The final stage is a maturation pond, used for disinfection through UV-radiation and removal of remaining microalgal cells through protozoan grazing (Craggs et al., 2004b). Such complexity of pond systems has not yet been adopted by marine and brackish water LBAS, due in part to the less concentrated nature of aquaculture effluents compared to that of agricultural and sewage effluent. However, it provides a working model on which to build an upgraded system for LBAWWT that is re-designed to meet the composition of saline dilute wastewater.

Primary producers play a major role in a multi-stage pond system (e.g. the HRAP in the second stage) and are employed in marine and brackish aquaculture systems (Chopin et al., 2001; Troell, 2009) and to a lesser extent in freshwater systems (Hasan & Charkrabarti, 2009). Integration of commercially-valuable microalgae into the second stage of a multi-stage pond system has many economic advantages over stand-alone algal ventures, including lower capital, water, harvesting, operational and maintenance costs (see Table 1 in Park et al., 2011). Similarly, macro flora can be integrated to manage dissolved nutrients. Macroalgal stands grown in freshwater dairy WWT ponds or steam mesocosms assimilate large quantities of dissolved nitrogen (70

mg g^{-1} dry weight m^{-2} d^{-1}) and phosphorus (13 mg g^{-1} dry weight m^{-2} d^{-1}) (Craggs et al., 1996; Kebede-Westhead et al., 2003). Other models demonstrate nitrogen removal efficiencies by filamentous green tide algae of 3.3 kg N day^{-1} ha^{-1} in a barramundi (*Lates calcarifer*) wastewater treatment pond (de Paula Silva et al., 2008). These algae have high tolerance for environmental variation and correspondingly high annual growth rates and nitrogen assimilation ability (de Paula Silva et al., 2008). Commercial seaweeds, such as *Gracilaria* spp. have also been used for small-scale mariculture as both a waste mitigation tool and for value-adding in China, Vietnam, Indonesia, India and the Philippines (for comprehensive review see (Troell, 2009). For example, *Gracilaria birdiae,* was used as a biofilter for prawn pond effluent and reduced nutrients by 34, 93.5 and 99.3% for NH_4^+, phosphate (PO_4^{3-}) and NO_3^-, respectively (Marinho-Soriano et al., 2009). Similarly, water hyacinth promoted 50% reductions in nitrogen and phosphorous from freshwater MWW (Abbasi & Abbasi, 2010). However, the selection of high value species for tropical marine and brackish water land-based aquaculture systems requires careful consideration of the variation in biotic and environmental conditions to provide a reliable treatment stage (Paul & de Nys, 2008).

As a final polishing step for residual nutrients, the use of constructed wetlands combine physical, microbial and phototrophic processes to treat municipal, aquaculture and agriculture wastewater over a broad range of salinities (Dong & Reddy, 2010; Erler et al., 2008; Kadlec et al., 2010; Yen et al., 2010). They achieve nutrient abatement by physically trapping and burying particulates, microbial assimilation and transformation of nutrients, phototrophic assimilation into plant biomass as well as volatilization and sorption (Erler et al., 2010; Erler et al., 2008; Qiu et al., 2011). Removal efficiencies of up to 98% for DIN occur in the treatment of prawn farm wastewater using constructed wetlands (Lin et al., 2002). A subsequent study demonstrated reductions of BOD

(24%), TSS (71%), chlorophyll a (88%), NH_4^+ (57%), nitrite (NO_2^-; 90%) and NO_3^- (68%), of prawn farm wastewater treatment in constructed wetlands (Lin et al., 2003). Furthermore, denitrification and anammox rates of 199.4 ± 18.7 and 965.3 ± 122.8 μmol N m^{-2} hr^{-1}, respectively have been recorded in freshwater wetlands (Erler et al., 2008), supporting the incorporation of wetlands into multi-staged treatment systems.

5.5 Land-Based Aquaculture Wastewater Treatment – A Model

I propose a multi-stage treatment system utilising the "best of" technologies to increase the removal of settled sludge and dissolved nutrients from land-based aquaculture (Fig. 5.3). The proposed system is modelled on a 100 ha farm which discharges 2000 ML of wastewater per annum containing a mean TSS load of 50 mg L^{-1}. The assumption of this model is that there is little fluctuation in the mean concentration of TSS. In reality TSS load varies both spatially and temporarily (Jackson et al., 2004), therfore scaling of the model requires testing of the likely failure rates of each step of the system under varying effluent composition. The model would begin with a set of deep anaerobic ponds with a required treatment area of 10 x 80 x 40 m (depth:length:width) (Table A.2; Appendix 2). Notably, the length to width ratio should be between 2:1 and 3:1 (Alexiou & Mara, 2003; Mara & Pearson, 1998), with the longest side perpendicular to prevailing wind to reduce wind-driven turbulence (Craggs et al., 2004a). The hydraulic retention time would be 10 d and would reduce TSS load by up to 60% (Table A.2; Appendix 2). Water would flow into the pond at depth, via an inlet pipe at the bottom of the pond, and subsequently filter up through coarse media (e.g. gravel) which would aid in the capture of particulates of all size fractions. This step would reduce the reliance on settlement of particulates in anaerobic ponds where the inlet was above the height of the bottom of the pond. A pair of anaerobic ponds would allow both to be used

simultaneously or individually, so that one pond could be dried and de-sludged while continuing treatment in the other. A de-sludging interval of approximately 3-4 years is recommended (Craggs et al., 2004a). Wastewater would subsequently pass through a pair of large-scale (0.5 x 100 x 50 m; depth:length:width) sand bed filters (Table A.2; Appendix 2). Flow in the sand filters would be optimised so that wastewater and its associated nutrients are in contact with biofilm for a sufficient length of time to ensure that particulate organic matter and DON are oxidised to inorganic nitrogen species. Resulting dissolved inorganic nitrogen would then be transformed in the next stage of treatment through assimilation into algal biomass (Fig. 5.3). Two parallel, aerated algal treatment areas, each of 2 ha (Table A.2; Appendix 2), would be tailored in depth relative to the light conditions of their location, but typically less than 0.5m. Each 2 ha treatment area would be divided into three smaller units; the first pond of 10,000 m^2, the second pond of 6,500 m^2 and the third pond of 3,500 m^2. A flow rate of 4 m^3 h^{-1} into the first treatment stage is recommended and flow rates through the smaller units would be faster, therefore increasing DIN flux despite the fact that DIN would be less concentrated by the time it reaches the smaller units (due to assimilation by algae in the first treatment unit) (Neori et al., 2003). This improves the biofilter performance of the algae (Neori et al., 2003), and would result in an annual biomass yield of 146 mt (dry weight; DW) based on a conservative productivity of 37 mt ha^{-1} y^{-1} (or 10 g DW m^{-2} day^{-1}, Table A.2; Appendix 2). The final stage of treatment would comprise a mangrove wetland (0.1 m deep and 2 ha) which would facilitate capture (through trapping) of persistent colloidal particles and would enhance beneficial microbial nutrient transformation (through sediment aeration) (Fig. 5.3). Although sediment accumulation and associated carbon and nitrogen trapping are effective almost immediately after wetland construction, heterotrophic activity and primary production take between 5-15

years to reach rates equivalent to natural marshes (Craft et al., 2003). Once fully functional, wetlands have the potential to produce polished wastewater containing only ambient TDN and TSS and could be recycled back to the culture ponds as required. This model predicts overall removal of nitrogen by 99-100% but requires simulation with farm-specific and temporal parameters. In addition, a cost-benefit model is required to ensure that construction, operation and maintenance costs are within the economic framework of the aquaculture operation.

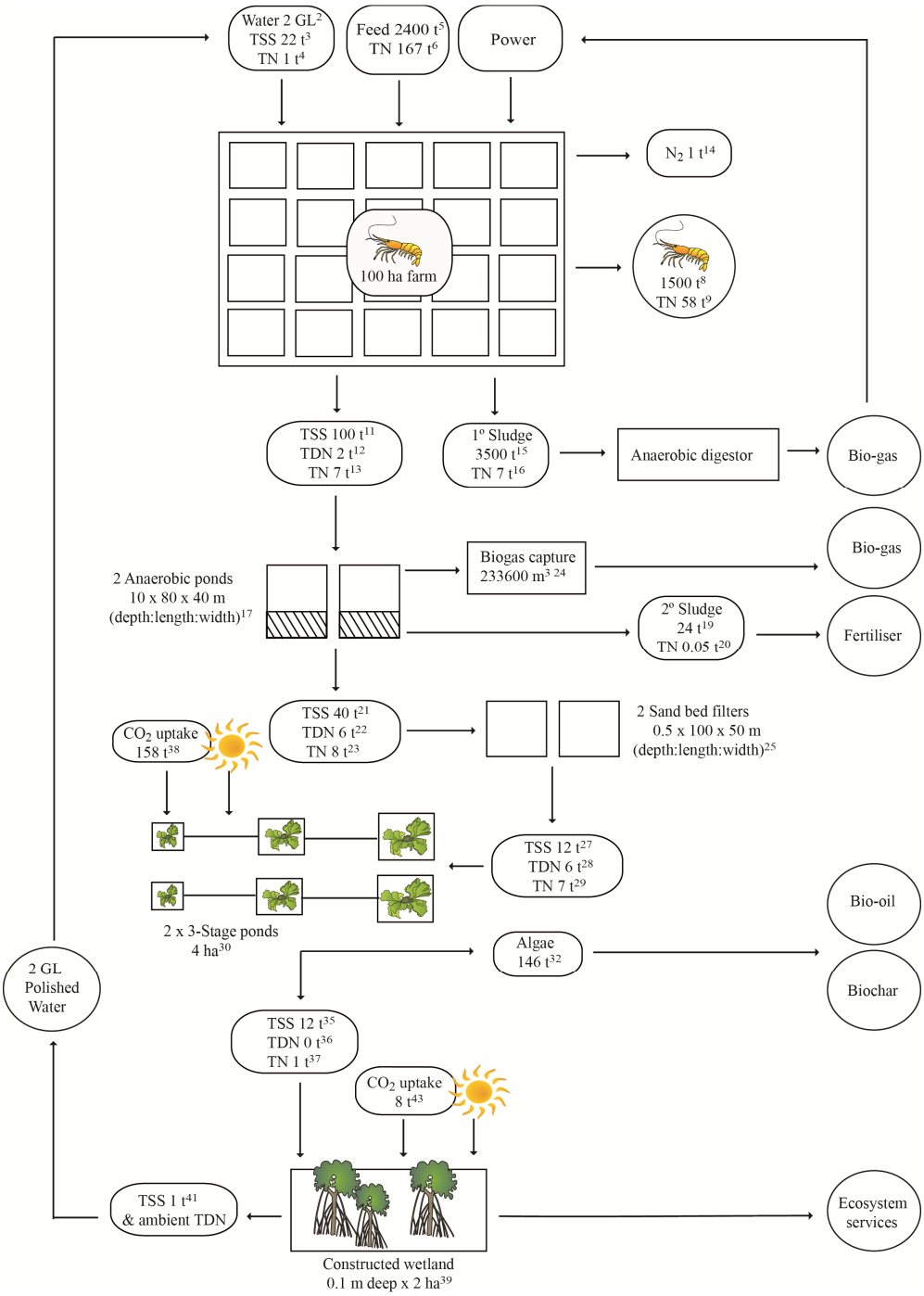

Fig. 5.3 A conceptual model of recommended treatment for land-based aquaculture wastewater. Loads are based on a 100 ha prawn farm over one year and the assumptions and working for this model are given in Table A.2. Superscripts denote the ID number for cross reference to Table A.2. Graphics are from Integration & Application Network (ian.umces.edu/symbols/)

5.6. Off-setting the Cost of Compliance

Environmental compliance incurs labour and infrastructure costs which can be off-set to ensure that farms maximise profits. One means of off-setting costs is through maximising recapture, reuse and recycling of valuable nutrients and biomass that are currently released. I have highlighted that each of the extractive steps in best practice compliance can be considered as an income stream to maximise the utilisation of input costs which are lost in traditional management regimes (Bolton et al., 2009). Specifically, in sequential order, these are sludge, digestion energy products from settled fine solids, and algal biomass.

Sludge is the first output from the waste cascade as it is collected in the primary stage of production. It is produced in large volumes (35-60 mt ha^{-1} $harvest^{-1}$ within a single 1 ha prawn grow-out pond) and potentially constitutes a valuable by-product for agriculture as it contains a range of macro-nutrients (nitrogen and phosphorous) and micro-nutrients (Ca, K, Mg, Cd, Cu, Mn) as well as organic carbon (Rosenani et al., 2004). These nutrients and trace elements can be utilised if sludge is processed to eradicate pathogens provided nutrients remain bioavailable (Hossain et al., 2011). One approach to delivering nutrients and trace elements as a defined agri-fertiliser is through production of biochar. Biochar is produced through the slow pyrolysis of biomass, and feedstocks similar to sludge have been successfully converted to biochar. The pyrolysis of sewage sludge produces a biochar (Hossain et al., 2011; Lehmann & Joseph, 2009b) and soils amended with sewage sludge biochar have enhanced nutrient availability and electrical conductivity, resulting in an increase in horticulture crops (Hossain et al., 2010). Although pyrolysis of aquaculture sludge and the use of the resulting biochar is a prospective technology, it has potential to be an avenue for off-setting the cost of environmental compliance. Notably, feedstocks such as aquaculture sludge which are

low in carbon but high in nutrients and trace elements can deliver valuable biochar through co-firing with carbon-rich lignocellulosic feedstocks.

Alternatively, suspended solids either from the culture or settlement ponds can be utilised for energy conversion through anaerobic digestion and biogas capture. Anaerobic digesters are used extensively in freshwater MWWT (Dereli et al., 2010) and are well established in the treatment of agriculture and household waste solids (Craggs et al., 2008; Krzystek et al., 2001; Larnari & Franci, 1998; Raposo et al., 2012). Anaerobic digesters have been trialled in IRAWWT (Larnari & Franci, 1998) and convert >60% of the solids from IRAWW to methane, reducing outsourced energy requirements by a small but valuable 2-5% (Gebauer & Eikebrokk, 2006; Mirzoyan et al., 2010). An experimental anaerobic digester, treating 2.8 L of trout farm faecal solids every 4 h, demonstrated high biogas yields with over 80% methane at a rate of 144 L d^{-1} (Larnari & Franci, 1998). This broader "industrial ecology" concept represents an innovation by industry in solid waste management based on an understanding of synergistic opportunities in the value-chain (Korhonen, 2005). This integrated design has become a new industrial paradigm, led by China, where 50 eco-industrial parks, including aquaculture systems, are being constructed or have been approved for construction resulting in economic benefits and waste mitigation (Mathews & Tan, 2011). Similar innovation can be expected once the co-products of intensive land-based aquaculture production are better evaluated (see chapter 4).

Alternatively, these suspended solids can be transformed to provide substrates (dissolved nutrients) for new products through assimilation. The degradation of solids and conversion of nutrients by infauna (i.e. polychaetes and associated microbial community) provides a biological technique by which complex organic molecules can be converted to simple DIN compounds (Kunihiro et al., 2008; Palmer, 2010; Wada et

al., 2008), facilitating further treatment with bacterial or algal communities. Palmer (2010) used sand beds stocked with polychaete worms to reduce suspended solid loads by >50% and produce polychaete biomass at 300-400 g m^{-2} as an aquaculture feed. This process released dissolved nutrients and provided dissolved nutrients for algal remediation.

Nutrient sequestration into algae (micro and macro) is a proven approach to deliver an income driver from the remediation of dissolved nutrients through production of bio-products and energy (Chopin & Sawhney, 2009; Park et al., 2011). Microalgae, particularly marine microalgae, contain high concentrations of fatty acids and are the target of a wide range of stand-alone aquaculture systems for nutraceuticals and biofuels (Brennan & Owende, 2010; Huerlimann et al., 2010; Mata et al., 2010; Vílchez et al., 2011). Similarly, macroalgae have already generated net positive cash flow in commercial, integrated aquaculture systems (Bolton et al., 2009; Bunting & Shpigel, 2009; Nobre et al., 2010). Algae offer economic returns through direct sales of commercial species (Chopin et al., 1999) in addition to reduction of feeding costs for herbivores and reduced pumping costs through recirculation and improvement of wastewater treatment capacity (Bolton et al., 2009; Nobre et al., 2010). More recently, there has been a renewed focus on bio-products from novel, resilient species of both microalgae (Jung & Lovitt, 2010) and macroalgae (de Paula Silva et al., 2008), demonstrating the flexibility to deliver products developed from site-specific bioremediation. For example, the production of algal biochar from green tide algae sequesters carbon and waste nutrients from aquaculture systems and results in a high-value biochar for use in soil amendments (Bird et al., 2011a). Pyrolysis of microalgal biomass for the production of both bio-oil and biochar provides beneficial fatty acids in

the resulting oil and high cation exchange capacity, nitrogen concentration and low C:N ratio in the char (Grierson et al., 2011).

The pyrolysis process also produces bioenergy (Abdullah et al., 2010), and thermal conversion of macroalgal biomass has the potential to deliver biofuels from species with high biomass productivities (Ross et al., 2008; Zhou et al., 2010). Given that dense microalgal and macroalgal communities occur naturally in LBAS, and that there is broad scope to enhance algal production to sequester waste nutrients (including the use of CO_2), these underutilised and easily implemented resources could provide an important driver for improvement of LBAWWT.

5.7 Conclusion

Solid and dissolved constituents are the two main waste sources which must be managed in LBAS. The current treatment of these waste sources in unmanaged settlement ponds is not optimised for efficient nutrient removal or reuse and I describe "best of" technologies that are tried and tested in other industries. These technologies offer off-the-shelf solutions to meet environmental compliance and enhance sustainability. By using multi-stage treatment plants with anaerobic digesters, sand filters and constructed wetlands, the difficulties associated with settling fine particles in dilute saline wastewater, and the complexities in enhancing beneficial microbial pathways for remediation of dissolved constituents, can be circumvented. Integration of algal and macrophyte cultures can also be optimised to increase WWT efficiency and profitability of the farms, and be tailored to local flora and regional requirements for specific end-products to engage with synergistic industrial ecology. My conceptual model includes specific design parameters that form the basis for environmental compliance, enable the intensification of production through increased treatment

efficiencies, and reducing water usage for LBAS. The potential for off-setting the costs of upgrading treatment systems through a suite of secondary products at each extractive stage, including bio-energy and agricultural applications should be investigated further and optimised to the specific requirements of each farm.

Nitrogen rich wastewater is produced when aquatic species are cultured intensively. Untreated wastewater released to the environment can affect phytoplankton and macro faunal communities, sediment nutrient cycling, oxygen regimes, and mangrove systems (Burford et al., 2003; Costanzo et al., 2004; Hall et al., 2011; Vaiphasa et al., 2007). Although multi-pond treatment systems are used in some flow-through, land-based aquaculture systems (Jackson et al., 2004), the majority of farms (particularly for prawn and finfish) use single-step settlement ponds to treat wastewater (Paul & de Nys, 2008; Preston et al., 2001). These are relatively efficient for removing nitrogen associated with total suspended solids (TSS) but are not optimised for removing dissolved nitrogen (Jackson et al., 2003b). Beyond this, little is known about nitrogen dynamics in settlement ponds themselves, though there are extensive studies of nitrogen cycling in prawn grow-out (culture) ponds (Burford & Longmore, 2001; Burford & Lorenzen, 2004; Burford & Williams, 2001). Grow-out ponds are subjected to different conditions than settlement ponds. Densely cultured live prawns, prawn faeces, fresh feed inputs and aerators are all present in prawn grow-out ponds and absent in settlement ponds. In addition, grow-out ponds are carefully managed through the addition of lime and molasses to control acid sulphate soils and microbial activity and sludge (and associated nutrients) is removed at the end of each production cycle. All of these factors potentially influence microbial nitrogen cycling so nitrogen dynamics in settlement ponds warranted elucidation.

There is now impetus to upgrade wastewater treatment systems for tropical land-based aquaculture. This is driven by changing discharge regulation from loads of 12 and 1 kg ha^{-1} day^{-1} of TSS and total nitrogen, respectively (EPA, 2005) to the

establishment of zero net discharge of nutrients in new aquaculture operations established within Queensland (Australia) (Australian Government, 2011). Treatment systems from municipal wastewater and intensive recirculating aquaculture wastewater are advanced compared to those currently implemented in land-based aquaculture systems. Consequently, there is a need to explore transferring developed technologies from these industries to land-based aquaculture to enhance dissolved nitrogen removal and overall treatment efficacy. However, since land-based aquaculture wastewater is comparatively dilute, appropriate technologies for dilute wastewater must first be identified.

This study adds to rigorous scientific evidence which is driving improvement in wastewater treatment management for land-based aquaculture, through recommendation of strategies to transform, capture and reuse nutrients.

The major outcomes of this study are:

Nitrogenous wastes are lost by the production of N_2 gas in land-based aquaculture settlement ponds through microbial transformation, but the production rates in existing treatment systems are low. Accumulated sludge should be removed from settlement ponds to maximise microbial denitrification **(Chapter 2)**.

Experimental evidence from homogenised sediment taken from settlement ponds on three land-based aquaculture farms demonstrates that denitrification by the microbial community is the main driver of N_2 production and that the contribution of anammox by microbes is negligible. Potential N_2 production rate does not correlate with measured sediment characteristics and is not stimulated in the presence of an exogenous carbon source.

Desludging settlement ponds through the removal of accumulated waste solids, is particularly beneficial, not only for reducing remineralisation rates (Burford &

Lorenzen, 2004), but also for enhancing denitrification. N_2 production, driven primarily by denitrification, was exceptionally low in the prawn and barramundi farm settlement ponds in this study (0-0.7 nmol N cm^{-3} h^{-1}; chapter 3), compared to rates measured using similar techniques (21.5-78.5 nmol N cm^{-3} h^{-1}) in mangrove forests and prawn ponds in Vietnam (Amano et al., 2011). In prawn culture ponds and under fish cages, low denitrification rate has been attributed to low nitrification due to unfavourable conditions for nitrifying bacteria, in particular high concentration of free sulphides and NH_3 and low O_2 (Burford & Longmore, 2001; Christensen et al., 2000). The rate of denitrification also reaches a maximum at nitrogen loading regimes of 20 mmol m^{-2} d^{-1} (Burford & Longmore, 2001), and it is possible that the nitrogen loading rate on the sediment in the settlement ponds in this study exceeds this. The removal of accumulated sludge reduces total nitrogen concentration, reduces free NH_4^+ and free sulphide release, and increases redox levels to restore O_2 balance. Therefore, a major outcome of this study is the recommendation to remove accumulated sludge from settlement ponds to improve microbial transformation of dissolved nitrogen to N_2 gas.

The permanent nitrogen removal pathways of denitrification and anammox by the microbial community occur at slower rates than the nitrogen retention pathways of mineralisation, remineralisation and DNRA. The removal of DON is problematic, and requires specific remediation strategies **(Chapter 3).**

Intact sediment core assays were implemented to identify the most important pathways of soluble nitrogen transformation in settlement ponds. Assimilation is the dominant nitrogen transformation pathway over the entire pond. The assimilation of DON (in the form of ^{15}N-urea) into microbial biomass is particularly rapid (670-803 µmol m^{-2} h^{-1}), but results in only short term removal of soluble nitrogen as the nitrogen

is subsequently returned to the water column during mineralisation of settled organic matter.

Furthermore, the nitrogen retention pathway of microbial DNRA is slightly faster than denitrification, and therefore a large portion of the added nitrogen is always going to be retained within the system. Prevalence of DNRA over denitrification is common in tropical systems (Dong et al., 2011). It is a reasonable assumption that much of the NH_4^+ produced through DNRA would be available for uptake by the phytoplankton community. NH_4^+ uptake by phytoplankton reaches a maximum when shading effects from the dense phytoplankton community limits production (Burford & Lorenzen, 2004). Equilibrium between remineralisation of organic matter and assimilation by the pelagic microalgal community is the threshold at which sludge removal should be implemented (Burford & Lorenzen, 2004). For example, in the piggery industry it is recommended that settlement ponds be desludged every 3-5 years (Craggs et al., 2004a), however, this research was based in a temperate region where mineralisation rates are likely to be significantly slower.

Two soluble nitrogen products, DON and NH_4^+, are released during organic matter degradation, but DON is released at higher rates. This study was among the first to determine the partitioning between the release of DON and NH_4^+ during organic matter mineralisation. However, urea was used as a proxy for DON cycling and urea is readily bioavailable compared to many other DON compounds in prawn ponds (Burford & Williams, 2001; Jackson et al., 2003a). Nevertheless, the present study concurs with previous studies (Burford & Williams, 2001) in highlighting the prevalence of DON in aquaculture systems and therefore the importance of implementing remediation techniques which specifically target DON, for example sand

filters or constructed wetlands. An added advantage of sand filters and constructed wetlands is that they act as mechanical filters to physically trap suspended solids.

Discharge waters from aquaculture are high in suspended solids which offer the opportunity for harvest and product development. Biochar produced from suspended solids in aquaculture waste streams is rich in nitrogen and micronutrients, and has a moderate carbon content and surface area. Biochar production is an efficient and potentially profitable means of solids and nutrient removal from aquaculture wastewater, but more efficient separation technology is desirable **(Chapter 4).**

The majority of TSS in land-based aquaculture wastewater is comprised of small particles, ranging between 11 and 20µm in size, which concurs with previous studies from recirculating aquaculture farms where TSS were established to be <30µm (Cripps & Bergheim, 2000; Kelly et al., 1997). This is beneficial for the transfer of mechanical filtration technologies from recirculating farms because previously optimised mesh sizes of 60-200µm would not need to be re-engineered. Only 61% of the TSS were captured using centrifugal technology (Evodos), suggesting more efficient removal and harvesting technologies should be trialled. However, this provided for the selective removal of diatoms which are rich in lipids (Lebeau & Robert, 2003).

Biochar produced through slow pyrolysis of the TSS removed by the Evodos centrifuge technology, and predominantly from marine diatoms, is rich in nitrogen and beneficial micronutrients as has been demonstrated from microalgal based biochars previously (Grierson et al., 2011). It is estimated that 1593 tonnes of TSS can be removed using this method and consequently 940 tonnes of biochar can be produced from an average sized prawn farm per annum. This equates to 226 tonnes of

sequestered carbon and 28 tonnes of nitrogen per annum. If left in organic form this would result in CO_2 release to the atmosphere and potential soluble nitrogen perturbation in the environment receiving wastewater due to leaching from stockpiled sludge. Biochar is an attractive option for nutrient mitigation for land-based aquaculture wastewater treatment. Some aspects require optimisation prior to implementing biochar production at a commercial scale. These include optimisation of harvest technology and pyrolysis temperature, investigation of potential blends with other feedstocks and market research to produce biochar with characteristics suited to the target market.

***Multi-stage treatment ponds, mechanical filtration, constructed wetlands, and media filters are identified as suitable technologies to target dilute aquaculture wastewater and enhance the treatment process for land-based aquaculture effluents* (Chapter 5).**

Multi-stage treatment ponds which integrate anaerobic digestion, microbial transformation of DON to DIN for subsequent algal culture and bio-oil and biochar production are potentially viable options for nutrient capture and reuse. The cost of wastewater treatment can be off-set by implementing these processes as they close the aquaculture industrial loop and ensure that valuable nutrients are utilised.

Some wastewater treatment systems associated with land-based aquaculture still comprise simplistic technologies relative to municipal and recirculating aquaculture wastewater treatment, while others have implemented recirculation technologies. This thesis confirms previous evidence that dissolved nutrients are not removed from simplistic single-step settlement ponds (Jackson et al., 2003b), rather they are recycled and retained within the system. Wastewater treatment in other industrial sectors has progressed such that rudimentary systems have been upgraded with multi-pond designs, anaerobic digesters, mechanical filtration systems, settling plates and biological

reactors. A synthesis of relevant literature (chapter 5) speculated that multi-stage treatment ponds could be particularly suitable for the treatment of dilute saline wastewater from tropical land-based aquaculture systems as they improve treatment efficiency (Craggs et al., 2004b), reduce nuisance odours (Archer & Mara, 2003), facilitate the capture of methane (Craggs et al., 2008) and production of biofuel (Park et al., 2011), and enhance algal production and harvest (Craggs et al., 2004a). However, the mechanisms controlling nutrient cycling in these systems are complex and display multifaceted interactions so future research directions should focus on optimising the pond design and operation parameters specifically suited to the characteristics of land-based aquaculture wastewater. Recommendations, based on municipal and dairy farm industries, are made for the parameters of water retention time, pond depth, width to length ratio, wet dry cycles, optimal flow regimes, pond bank slope, aeration hours, algal species selection and control and the time between desludging events (Archer & Mara, 2003; Craggs et al., 2004a; Terry & Krause, 2009).

A key finding of this research is that valuable particulate and dissolved nitrogen is lost to the environment through the release of nutrient rich suspended solids and remineralisation of settled particulates in settlement ponds releasing dissolved nitrogen with discharge water. Similar conclusions were drawn 10 years ago (Jackson et al., 2003b) and few changes have been implemented to capture and reuse waste nutrients despite this being one of the most significant costs of an aquaculture operation. The properties of secondary products produced through capture and conversion of nutrient rich suspended solids have been elucidated and this is the scientific foundation for a novel route to enhance wastewater treatment. However, further investigation is required to determine if this is the most cost effective and environmentally sustainable use for the suspended solids. In addition, the model given in Chapter 5 requires optimisation

based on site- and time-specific variation of each parameter. The adoption of the recommendations from this research has potential to significantly improve land-based aquaculture wastewater treatment provided the technologies can be implemented within the economic framework of the farm and the potential revenue generated through production of secondary products.

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Anaerobic respiration: respiration in the absence of oxygen, using a molecule other than oxygen as the final electron acceptor.

Anammox: anaerobic ammonium oxidation is a biological process where nitrite and ammonium are converted directly into dinitrogen gas.

Assimilation: the conversion of nutrient into the fluid or solid substance of an organism, by the process of digestion and adsorption.

Biochar: charcoal produced through the pyrolysis of biomass in an atmosphere void of oxygen.

Biomass: biological material typically comprising living or recently living plants or plant-based material.

Cation exchange capacity: the maximum quantity of total cations that a soil is capable of holding, at a given pH and which are available for exchange with the soil solution.

Cyanobacteria: a phylum of bacteria, sometimes known as blue-green algae, that obtain their energy through photosynthesis

Denitrification: a bacterially mediated process of nitrate reduction that may ultimately produce molecular nitrogen (N_2) through a series of intermediate gaseous nitrogen oxide products.

Diatom: a major group of unicellular phytoplankton what can exist as colonies and are encased within a cell wall.

DNRA: Dissimilatory nitrate reduction to ammonia

Fatty acid: Organic acid composed of carbon, hydrogen and oxygen that combines with glycerol to form fats.

Mineralisation: strictly mineralisation is the transformation of an organic substance to an inorganic substance but in this thesis, and many other works, it has been used to refer to the release of dissolved nitrogen due to the oxidation of organic matter

Nitrification: the biological oxidation of ammonia with oxygen into nitrite followed by the oxidation of these nitrites into nitrates

Pyrolysis: is the thermochemical decomposition of organic material at elevated temperatures under an oxygen free atmosphere

Secondary product: A commercially valuable product which is produced from the waste of the target product

Table A.1 Preliminary monitoring data of dissolved and particulate nitrogen concentrations (µg N L^{-1}) in wastewater flowing into the settlement pond at five inlet sites and wastewater flowing out of the settlement pond at two outlet sites at Coral Sea Farm.

Collection Date	Site	NH_4^+	NO_x	DON	PN
30/06/2009	Inlet (2)	205	35	409	629
30/06/2009	Inlet (3)	187	23	248	163
30/06/2009	Inlet (4)	162	32	335	769
30/06/2009	Inlet (5)	973	311	744	586
30/06/2009	Inlet (6)	661	171	621	592
15/02/2010	Inlet (2)	1	35	412	1051
15/02/2010	Inlet (3)	129	77	523	659
15/02/2010	Inlet (4)	148	91	502	832
15/02/2010	Inlet (5)	93	84	461	820
15/02/2010	Inlet (6)	36	1	516	1307
Mean (µg N L^{-1})		260	86	477	741
Mean (kg N d^{-1})*		2.27	0.75	4.18	6.49
Total (kg N d^{-1})					13.69
30/06/2009	Outlet (1)	564	246	482	660
30/06/2009	Outlet (2)	591	203	627	649
15/02/2010	Outlet (1)	110	109	354	537
15/02/2010	Outlet (2)	20	65	326	459
Mean (µg N L^{-1})		321	156	447	498
Mean (kg N d^{-1})*		2.81	1.36	3.92	4.36
Total (kg N d^{-1})					12.45

*Based on an estimated 8.76 ML d^{-1} of discharge water

Table A.2 Parameters, assumptions and calculations for the conceptual model (Fig. 5.3). ID numbers are used to explain the relationship (formula) between each parameter and for cross reference with Fig. 5.3.

Treatment Section	Parameter	ID	Value	Unit	Formula	Assumption & additional information	Reference
Farm inputs & operation	Production ponds	1	100	ha		Moderate to large prawn farm	
	Water usage	2	2000	ML y^{-1}		2.5% exchange d^{-1}	PRF farm records
	Intake TSS load	3	22	mt y^{-1}		TSS load is reported in dry weight	PRF environmental monitoring
	Intake TN load	4	1	mt y^{-1}			PRF environmental monitoring
	Feed	5	2400	mt y^{-1}	Model = 16 x (ID1 x 1.5)	16 mt ha^{-1} $crop^{-1}$ & 1.5 production cycles y^{-1}	PRF farm records
	TN in feed	6	167	mt y^{-1}	Model = (ID5 x 0.435) x 0.16	43.5% protein. N:P factor of 6.25 (16%)	Mean (starter & grower) Ridley Aqua-Feed Diets. Mariotti et al., (2008)
	Prawn yield	7	10	mt ha^{-1} $crop^{-1}$			PRF farm records
	Harvest	8	1500	mt y^{-1}	Model = (ID7 x ID1) x 1.5	1.5 production cycles y^{-1}	
	TN in harvested prawn	9	58	mt y^{-1}	Model = (ID8 x 0.24) x 0.16	Prawn flesh 24% protein. Standard N:P factor of 6.25 (16%)	PRF nutrition product label. Mariotti et al., (2008)
	FCR	10	1.6		Model = ID5/ID8		

	TSS discharge load	11	100	mt y^{-1}	Model = (50/1000) x ID2	TSS load is similar all year i.e. 50 mg L^{-1}	PRF & Castine monitoring (n = 71)
	TDN discharge load	12	2	mt y^{-1}	Model = (1/1000) x ID2	TDN load is similar all year i.e 1 mg N L^{-1}	Castine monitoring (n = 12)
	TN discharge load	13	7	mt y^{-1}	Model = (ID11 x 0.05) + ID12	TSS are 5% N	Chapter 4
	N_2 production	14	1	mt y^{-1}	Model = ((ID4 + ID6) - (ID9 + ID13)) x 0.012	Mean DNT efficiency of 1.2% & all N in sludge is bioavaliable	Burford and Longmore (2001)
Culture pond sludge	Sludge from culture ponds	15	3500	mt y-1	Model = ID1 x 35	35 mt are removed from each pond	Preston et al., (2001)
	TN in sludge	16	7	mt y-1	Model = ID15 x 0.002	0.2% of the sludge is N	Burford et al., (1998)
Anaerobic pond	Anaerobic pond volume	17	64000	m^3	Model = (10 x 80 x 40) x 2	Two ponds 10 x 80 x 40 m (depth:length:width). Length:width ratio of 2:1	Alexiou and Mara (2003); Craggs et al., (2004a); Craggs et al., (2008)
	Sludge generation from TSS settling	18	60	mt y^{-1}	Model = ID11 x 0.6	60% settlement rate	Jackson et al. (2003)
	2° produced	19	24	mt y^{-1}	Model = ID18 x 0.4	60% digested & 40% remaning	Craggs et al., (2008)
	TN in 2° sludge	20	0.05	mt y^{-1}	Model = ID19 x 0.002	0.2% of the sludge is N	
	TSS remaining	21	40	mt y^{-1}	Model = ID11 x 0.4	40% remaining if 60% settles	Jackson et al., (2003)
	TDN remaining	22	6	mt y^{-1}	Model = ID18 x 0.06 + ID12	6% of settled material is mineralised and DN is released	Burford and Lorenzen (2004)
	TN remaining	23	8	mt y^{-1}	Model = (ID21 x 0.05) + ID22	Negligable DNT. TSS are 5% N.	Castine et al., (2012); Chapter 4

	Biogas capture	24	233600	$m^3 y^{-1}$	Model = (ID17 x 0.01) x 365	Prawn AP function similalry to dairy AP i.e. 0.01 m^3 CH_4 m^{-3} d^{-1}	Craggs et al., (2008)
Sand filter	Sand filter size	25	1	ha		Two beds 0.5 x 100 x 50 m (depth:length:width).	Palmer (2010)
	TSS captured	26	28	mt y^{-1}	Model = ID21 x 0.7	70% reduction in TSS	PRF environmental monitoring
	TSS remaining	27	12	mt y^{-1}	Model = ID21 - ID26		
	TDN remaining	28	6	mt y^{-1}	Model = ID22 + (ID22 x 0.11)	11% increase in TDN	Castine monitoring
	TN remaining	29	7	mt y^{-1}	Model = (ID27 x 0.05) + ID28	TSS are 5% N. Negligable DNT due to aerobic conditions	Chapter 4
Algal Remediation	Surface area of algae pond	30	4	ha		2 x 3 ponds (1 ha, 0.65 ha and 0.35 ha). Area required to remove remaining TDN.	Neori et al., (2003)
	Algal productivity	31	37	mt ha^{-1} y^{-1}			Productivity based conservatively on Neori et al., (2003). Assuming a 5:1 wet:dry weight ratio.
	Algal production	32	146	mt y^{-1}	Model = ID30 x ID31		
	Carbon removal	33	39	mt y^{-1}	Model = ID32 x 0.27	27% Carbon	Bird et al., (2011a)
	Nitrogen removal	34	6	mt y^{-1}	Model = ID32 x 0.04	4% Nitrogen	Bird et al., (2011a)
	TSS remaining	35	12	mt y^{-1}	Model = ID27	No change in TSS concentration	
	TDN remaining	36	0	mt y^{-1}	Model = ID28- ID34	Algae would be N limited	

	TN remaining	37	1	mt y^{-1}	Model = ID27 x 0.05	TSS are 5% N	Chapter 4
	CO_2 sequestered	38	158	mt y^{-1}	Model = ID33 x (48/12)		
Constructed Wetland	Constructed wetland	39	2	ha			Erler et al., (2008), Erler et al., (2010)
	TDN removal	40	100	%		High denitrification i.e. 965 µmol N m^{-2} h^{-1}	Erler et al., (2008)
	TSS remaining	41	1	mt y^{-1}	Model = ID35 - (ID35 x 0.93)	93% removal of PON (Erler et al. 2010), therefore assume 93% removal of TSS	Erler et al., (2010)
	TN remaining	42	0	mt y^{-1}	Model = ID41 x 0.05	TSS are 5% N	Chapter 4
	CO_2 sequestered	43	8	mt y^{-1}	Model = ID39 x 4	4 t C ha^{-1} y^{-1}	Alongi et al., (2008)