

BACKGROUND AND RATIONALE

Environmental effects of Aquaculture

Aquaculture has received considerable attention from both private and public sectors as a means to further diversify the agricultural and fisheries economies of both developed and developing nations, while satisfying the increasing demand and continued shortage of aquatic species for human consumption. The continued expansion of aquaculture has sparked concerns about the environmental impact of the discharge of nutrient rich wastes into the environment as a by-product of fish production. The constituents of most concern in wastewater discharged from fish farms are suspended solids, phosphorous and nitrogen, which when released into the environment can cause hypereutrophication, triggering phytoplankton blooms (eutrophication) (Smith et al. 1999). Algae blooms can be damaging to aquatic ecosystems as they eventually consume the available nutrients and die in large numbers. Their subsequent degradation can drastically reduce the oxygen concentrations in the local ecosystem leading to the potential death of fish and other organisms (Goldburg and Triplett 1997). Eutrophication of marine waters has been highlighted in recent years by the occurrence of toxic algal blooms in estuarine and coastal ecosystems (Smith et al. 1999). Rosenburg et al. (summarized by Smith et al. 1999) describes the occurrence of a toxic algae bloom in the Baltic Sea in 1988 that resulted in die-offs of macroalgae, invertebrates and fish.

In most cases all of the nitrogen and phosphorous within the effluent stream of an aquaculture facility originates in the feed. It has been estimated that fish assimilate 20-30% of their feed, the remainder of which is lost as uneaten food, feces and dissolved nutrients. Even

when fish consume 100% of the feed they are offered, approximately 50 to 80% of the nitrogen and 35 to 85% of the phosphorous end up in the effluent (Schneider et al. 2005).

Recirculating Aquaculture Systems

The necessity for mitigating the environmental impacts of waste discharge from agricultural activities and the need for the conservation of high value land and water resources has increased the focus on the development of recirculating aquaculture systems (van Rijn 1996). Closed recirculating aquaculture systems (RAS) reduce water consumption and provide the potential for reducing nutrient discharge by treating and reusing >90% of their total daily water volume (Van Gorder 2000). While traditional aquaculture systems, extensive (ponds and cages) and semi-intensive (flow-through), are characterized as having a high volume, low concentration discharge, intensive systems (RAS) are characterized as having low volume, high concentration discharge which can be localized to a point source for discharge or further treatment. A RAS typically includes fish holding tanks supported by a water treatment process that incorporates solids removal techniques and biological filtration for dissolved constituents, such as ammonia-nitrogen via, nitrification, a process which leads to the conversion of ammonia-nitrogen to less toxic nitrate-nitrogen (Masser 1999). These systems may also have temperature control, water sterilization and oxygen input depending upon the requirements of the species being cultured.

Recirculating aquaculture systems are an important technological approach that allow for reducing water consumption. In addition, if waste treatment is designed into the system, they may also allow for the reduction of discharge of nutrients into the environment. However, the small volume of effluent they generate during routine management is nutrient rich and requires

further treatment prior to discharge to prevent environmental harm. Methods of treating the effluent from a recirculating system include thickening and composting for land application as fertilizer, or draining through a constructed wetland (Timmons et al. 2001); however, such techniques are only applicable to freshwater systems.

Currently, aquaculture provides twenty-five percent of the world's seafood supply, a figure that should increase to fifty percent by the year 2030. However, due to diminishing availability of freshwater, most of this growth will likely take place in seawater (Neori et. al. 2005). Alternative methods of desalinization of waste and/or municipal treatment are cost prohibitive for the aqua culturist involved in culturing marine species. It is imperative, therefore, that efficient methods be developed for the reduction of nutrients in the effluents produced by marine RAS for the continued growth of mariculture as a clean and sustainable industry.

Characterization of aquaculture effluent wastes

Knowing the characteristics of effluent is fundamental to assessing their potential impact on the environment and in developing methods for management or treatment. The quantities of total suspended solids, nitrogen and phosphorous generated in an aquaculture system is directly proportional to feed input, although the concentrations vary based on the degree of water recycling vs. replacement (Van Gorder 2000; Twarowska et al. 1997).

While there have been a number of studies that have characterized wastes produced by traditional pond and cage culture systems (Davies and Slaski 2003; McIntosh and Fitzsimmons 2002), limited information is available on the nature of wastewater generated in marine RAS. Most previous studies have reported the efficiencies of specific components of the RAS

treatment system (Twarowska et al. 1997; Easter et al. 1996; Singh et al. 1999), or for specific types of organisms cultured (Zeimann et al. 1992). The waste characterization studies of RAS systems typically focus on those parameters most implicated in environmental degradation; solids, biochemical oxygen demand, total nitrogen, total phosphorous, ammonia, nitrate, nitrite, and orthophosphate.

A study was conducted on a freshwater RAS for the culture of tilapia fingerlings (*Oreochromis niloticus*, *Oreochromis niloticus* x *Oreochromis aureus*) to determine efficiencies of system components based on total water volume, biomass and feed input (Twarowska et al. 1997). These authors examined the particle trap /sludge collector, drum screen filter and the biofilter for the removal of waste constituents, including total solids (TS), volatile solids (VS), suspended solids (SS), total Kjeldahl nitrogen (TKN), total ammonia nitrogen (TAN = $\text{NH}_4 + \text{NH}_3$), nitrite (NO_2), nitrate (NO_3), total phosphorous (TP), and chemical oxygen demand (COD) (Table1). However, the wastewater volume and concentration of these constituents were reported for the sludge collectors and the drum screen separately and did not include the quantities of dissolved nutrients acted upon by the biofilter. The biofilter was evaluated by examining TAN, NO_3 and NO_2 concentrations in the influent and effluent of the biofilter, and the results were reported as TAN reduction in mg/L over a 24 h period. The particulate removal rate efficiencies were examined separately for the sludge collectors and the drum screen filter by analyzing total solids, volatile solids and suspended solids from their respective influent and effluent streams. These analyses were reported as percentage solids removed as a percentage of feed input. This study focused on the performance of system components and did not include data on the waste flowing through all system components. The authors conclude that the

combinations of components utilized in this freshwater system provided good environmental conditions for the growth of tilapia.

In another study, waste was characterized from an intensive freshwater RAS for the culture of hybrid striped bass (*Morone saxatilis x M. chrysops*) to determine the efficiency of a sedimentation basin (Easter et al. 2000). These authors also examined solids (TSS), volatile suspended solids (VSS), fixed suspended solids (FSS), COD, BOD, TKN, TAN, NO₂, NO₃, TP and dissolved phosphates (DP) (Table1). Constituent concentrations, in the effluent stream of the RAS in this study, however, were determined only for the multi-tube settling basin and omitted analyses of parameters within the system biofilter, a rotating biological contactor.

A survey of water quality characteristics of effluent generated by four types of organisms (freshwater fish, freshwater prawn, marine fish and marine shrimp), were reported (Zeimann et al. 1992). Although the study included marine animals raised in a RAS, the data was combined with that of differing systems (earthen ponds and raceways) to obtain a composite quantification of effluent characteristics.

Information is available on characterization of wastewater in four independent freshwater RAS configurations for the culture of hybrid striped bass fingerlings, *M. saxatilis x M. chrysops* (Singh et al. 1999). The four configurations included 2,000 L tanks and different combinations of two types of biofilters (trickling and bead) and two types of solids removal filters (rotating screen filter and multi-tube settling basin). The analyses in this study focused on total organic carbon (TOC), BOD, TAN and NO₂-N and compared removal efficiencies between the different configurations (Table1). Little information exists regarding the characterization of wastewater from an intensive marine RAS.

Biological treatment alternatives

The integration of biological species occupying different ecological niches within an aquaculture system is considered to be the most economically feasible approach to treat waste from intensive production systems (Neori et al. 2005, van Rijn 1996). The metabolism of plants and animals counteract one another with respect to the environmental parameters such as dissolved oxygen, pH, ammonia and CO₂. Plants generally have a net production of oxygen during the day, consume CO₂ and inorganic nitrogen and increase pH while animals consume oxygen, create CO₂ and inorganic nitrogen and decrease pH (Neori and Shpigel 1999).

Seaweed biofilters have been under study in Israel since the mid 80's and have been shown to solve major water quality problems, including dissolved oxygen, pH and ammonia, associated with intensive marine fish ponds, while improving upon effluent quality (Neori and Shpigel 1999, Ryther et al. 1981). It has been reported that biological mitigation of effluents from recirculating aquaculture systems could be achieved by incorporating macrophytes and mollusks into the treatment system (van Rijn 1996). A study on the integration of the seaweed *Ulva lactuca* or *Gracilaria conferta*, abalone *Haliotis tuberculata* and the sea bream *Sparus aurata* in a recirculating system reported a significant reduction of the TAN from 45% of the total feed nitrogen in the fish effluents to 10% of total feed nitrogen in the discharge from the seaweed culture tanks (Amir Neori et al. 1996). In another study, the synchronization of fish and photosynthetic metabolism was achieved by paying close attention to feeding regimes as they correlated to peak photosynthetic periods in an integrated recirculating system for the culture of abalone *H. discus hannai*, sea urchin *Paracentrotus lividus*, sea bream *S. aurata* and the seaweed *U. lactuca* (Schuenhoff et al. 2002). In this study, the authors evaluated the

performance of a commercial scale system with respect to nutrient budgets and found a reduction of 70% total ammonia nitrogen and 20% available phosphate as a result of increased plant biomass. A variation on this theme may be seen in land-based integrated aquaculture, in which microalgae and bacterial systems together mitigate wastes and in turn become food for herbivores and filter feeders (Richmond 2004).

Early studies have reported that secondary-treated sewage effluent provides excellent enrichment for the maintenance of mixed natural populations of phytoplankton (Dunstan and Menzel 1971, Goldman et al. 1975, Goldman 1975, Goldman and McCarthy 1978). In this study the authors report the production of large quantities of plant carbon with a concomitant removal of nitrogen and phosphorous from the sewage effluent. To date, no studies have reported the integration of microalgae and fish production in a marine finfish recirculating aquaculture system.

HYPOTHESES

My general hypothesis is that the effluent from an intensive marine recirculating system for the culture of finfish can be used to produce selected or natural phytoplankton to reduce nutrient loads, specifically ammonia and orthophosphate, mitigate environmental impact and increase economic returns via the culture of a valuable food source for potential filter feeders such as oysters or clams.

The specific objectives of my study are as follows:

1. Characterize the effluent from an intensive marine recirculating system for the culture of finfish.
2. Based upon the characterization of these wastes, estimate an optimal concentration of effluent for the growth of the microalgae *Isochrysis galbana* by comparing effluent nutrient constituents with those of commercially available nutrient stocks used for phytoplankton production.
3. Culture the microalgae *I. galbana* in the laboratory comparing different concentrations of effluent from the intensive marine RAS as a nutrient base, and determine the effect of the phytoplankton growth on nutrient concentrations.
4. Based on the results of laboratory studies, scale up the cultivation of *I. galbana* from flask (2000 mL), to carboy (20 L) to cylinder (140 L) and ultimately to outdoor bioreactor using optimal dilutions of effluent, and determine the effects of phytoplankton growth on the nutrient concentration.
5. Cultivate indigenous phytoplankton colonies in the laboratory using different concentrations of effluent, and determine the effects of phytoplankton growth on nutrient concentrations.

6. Scale up the cultivation (see 4) of indigenous phytoplankton colonies by using optimal dilutions of effluent, and determine the effects of phytoplankton growth on nutrient concentrations.

Specific hypotheses to be tested in the study are as follows:

- H_n: Nutrient concentrations in the effluent stream of an intensive marine finfish recirculating aquaculture system do not vary with feed input.
- H_{2n}: Nutrients in the effluent from an intensive marine recirculating system for the culture of finfish will not support monoculture microalgae growth under laboratory conditions.
- H_{3n}: Monoculture microalgae cultivated with nutrients from the effluent of an intensive marine recirculating system for the culture of finfish cannot be grown in outdoor bioreactors.

H_{4n}: Monoculture microalgae grown under laboratory conditions will not reduce the concentration of nutrients in the effluent of an intensive marine recirculating system for the culture of finfish.

H_{5n}: Monoculture microalgae grown at commercial scale will not reduce the concentration of nutrients in the effluent of an intensive marine recirculating system for the culture of finfish.

In addition to *I. galbana* monoculture, hypotheses #1 - #4 above will be tested with indigenous phytoplankton colonies.

MATERIALS AND METHODS

Experimental recirculating grow-out system for marine finfish:

This study was conducted at the University of North Carolina at Wilmington's Center for Marine Science aquaculture facility located in Wrightsville Beach, North Carolina where a commercial scale marine RAS for the grow-out of finfish such as summer flounder *Paralichthys dentatus*, southern flounder *P. lethostigma* and black sea bass, *Centropristis striata* is currently in operation. The system design is based on the North Carolina State University Fish Barn (Losordo et al. 2000) and consists of a group of two 11.3 - m³ covered tanks supported by an independent recirculating system (Fig. 1). The components of the RAS are the dual center drain, a sludge collector for settleable solids, a 60 um micro screen drum filter for the removal of

suspended solids and a foam fractionator for removal of fines (< 30 um) and dissolved solids. The recycled water enters the 4.57 m diameter circular tanks through a vertical manifold with the flow directed parallel to the tank wall, creating a rotational velocity of the water column. This design was chosen as it employs the principles of centrifugal sedimentation, i.e. the particles in the tank are subjected to centrifugal forces in proportion to the rotational velocity of the water column thereby affecting an increase on their density, which facilitates the transport of the solids to the bottom center of the tank. These solids are drained at approximately 5% of the total tank flow rate through the lower portion of the dual drain to the sludge collector, which also operates on the principles of centrifugal sedimentation. The solids are separated out and collect in the bottom of the sludge collector while the clarified water overflows a weir and continues on to the micro screen drum filter. It has been reported that up to 98% of the total solids in the tank are removed from this lower drain (Timmons et al. 2001, Twarowska 1997). These solids are manually discharged from the system on a daily basis. The remainder of the flow is drained through the upper portion of the dual drain center standpipe of the fish tank at approximately 95% of the flow rate and sent directly to the micro screen drum filter. The micro screen drum filter removes all of the solids greater than 60 um from the effluent stream and drains them out of the system by an automated backwash system. The solids in a recirculating system's effluent contain most of the phosphorous exiting the system (50-85%), but very little of the effluent nitrogen (Timmons et al. 2001).

Nitrogen is excreted through the gills of the fish as dissolved ammonia and must undergo nitrification via biological filtration. The filtered water from the micro screen is distributed over a biological filter with 3 mm polystyrene micro beads. This media has a specific surface area of $1135 \text{ m}^2/\text{m}^3$ (Carroll and Watanabe 2005). A portion of the flow from the biological filter is

circulated through a foam fractionator for additional solids reduction to <30 μm . The rest of the flow is sent through a heat pump for temperature control, a UV sterilizer to destroy pathogens, an oxygen cone to restore oxygen levels, and then back into the fish tanks. The flow from the manual discharge of the sludge collector and the automatic backwashing of the micro screen and foam fractionator combine to form the wastewater discharged from the system at design rate of 10% of the system volume per day. This wastewater is discharged at a single source point into a 1,800 L collection tank (sump) (Fig.2). During the 24 h collection periods the system was operated at an exchange rate of 10%, however, during the intervals between sampling the exchange rate was increased approximately by 10 to 20%.

Waste characterization

Fish are fed on a diurnal (24 h) cycle, and effluent volumes and concentrations of constituents are not uniform throughout the day, therefore, effluent was characterized in terms of the type and amount of wastes discharged during a 24 h period collection. For each 24 h collection, the volume of waste in the holding tank was measured. In addition, the water quality parameters of the waste in the sump were recorded, including dissolved oxygen, pH, temperature and salinity. A sub-sample of 1,000 mL of waste effluent was collected and stabilized by filtering through a 0.20 μm glass fiber Whatman filter to remove the bacteria associated with metabolic conversion of metabolites (Dosdat et al. 1995). The filter was weighed, dried for 24 h at 105⁰C and reweighed to quantify filterable solids.

The filtrate was analyzed in triplicate for the following parameters: orthophosphate, nitrate/nitrite, ammonia, total phosphorous, total nitrogen, suspended solids and biological

oxygen demand. Total nitrogen and total phosphorous were determined by the ascorbic acid method following persulfate digestion. Following digestion total nitrogen and total phosphorous were analyzed on the Braun-Luebe continuous flow auto analyzer. Nitrate/nitrite, ammonia and orthophosphate were also analyzed on the auto analyzer. Biological oxygen demand was determined using dark incubation over a five-day period (BOD₅) and measuring dissolved oxygen differences. All of the parameters were analyzed according to the methods outlined in the “Methods of Seawater Analysis” (Grasshoff et.al. 2002). This characterization reports the amount of suspended and dissolved constituent levels as a fraction of total feed input. Three 24 h collections were made each month and mean values (\pm standard error = se) for each parameter were determined. Waste characterization studies were conducted for a period of one year and at different levels of fish biomass (hence feed input) to determine seasonal influence on constituent parameters as well as to ascertain the relationships between nutrient waste characteristics and level of feed input.

Laboratory-scale effluent-based microalgae culture

The second phase of the study began in May 2004 to determine the appropriate dilutions of effluent for the optimal growth of the microalgae *I. galbana*. This microalgae was chosen for its physical and chemical characteristics that make it optimal for consumption by shellfish (Walne 1974) and because rearing requirements for this species have been well documented (Hoff et al. 1987; Walne 1974). Specific formulas (Table 2) for the dietary needs of the microalgae were compared to effluent nutrient concentrations (particularly nitrogen) from the intensive marine recirculating finfish aquaculture system. These formulas included similar

amounts of NO₃ and PO₄, suggesting optimal concentration of these constituents for phytoplankton growth. Based on this information, a dilution ratio of effluent to support phytoplankton growth was estimated.

An experiment was conducted to evaluate growth of the marine microalgae *I. galbana* on RAS effluent in 2-L flasks under controlled laboratory conditions. Four treatment media were compared: RAS effluent at 100% and 50% dilutions (100-Efl; 50-Efl.), Guillard's f/2 media (f/2), and seawater (control). Media were autoclaved and then inoculated with *I. galbana* at 1x10⁶ cells/mL. Phytoplankton growth was determined at each sampling by measuring the cell densities with a hemacytometer over the growth period. In addition, nutrient concentrations in the culture media were measured every other day to determine the effect of microalgae growth on nutrient concentration. The cultures were grown until a plateau in cellular densities was achieved.

Pilot scale effluent based microalgae culture

In a second experiment *I. galbana* was grown in a pilot scale bioreactor consisting of three 2000L tanks receiving the effluent at a concentration determined to be optimal from the laboratory experiments above. During pilot scale trials, effluent was not filtered, as it is impractical to treat such high volumes on a continual basis. *I. galbana* was monitored for growth via hemacytometer and the effect of phytoplankton production on nutrient reduction was assessed by analyzing the nutrient concentrations entering and exiting the bioreactors (Fig. 3).

In addition to monoculture of *I. galbana*, experiments were conducted using an indigenous colony of phytoplankton collected by plankton tow from the Wrightsville Beach

intracoastal waterway. These experiments also progressed from the laboratory to pilot scale to evaluate the effects of phytoplankton growth on nutrient reduction.

RESULTS

Waste characterization

Over the course of the 12 month study period the biomass in the culture tanks ranged from 13 to 73 kg/m³. The fish were fed an average of 3.7 kg feed/day (range = 2.5 to 5.3 kg/day). The average daily volume of effluent collected over the 12-month study period was 1,357 L (range = 1,178 to 1,452 L) (Fig. 4).

The dissolved oxygen in the collection sump averaged 0.06 mg/L (range = 0.00 to 0.29 mg/L) during the 12 month study period. The pH averaged 6.62 (range = 6.19 to 7.05). The temperature averaged 22.8⁰ C (range = 18.0 to 28.5⁰ C). The salinity averaged 30.3 g/L (range = 27.0 to 34.0 g/L).

Orthophosphate concentration averaged 9.91 mg/L (range = 6.54 to 12.75 mg/L or 0.013 kg/d) during the 12 month study period. Nitrate/nitrite concentration averaged 17.4 mg/L (range = 0.00 to 66.31 mg/L or 0.024 kg/d). Nitrate/nitrite concentrations were higher during the first 4 months of the collection period averaging 49.6 mg/L but declined markedly during the last 8 months averaging 1.32 mg/L. Ammonia concentration averaged 4.15 mg/L (range = 1.80 to 5.63 mg/L or 0.006 kg/d) (Fig. 5).

Total phosphorous concentration averaged 23.0 mg/L (range = 14.2 to 29.1 mg/L or 0.031 kg/d). Total nitrogen concentration averaged 111.1 mg/L (range = 68.0 to 192.51 mg/L

or 0.15 kg/d) (Fig. 6). Total nitrogen was markedly higher in the first 2 months of the study than the last 10 months and was associated with a higher feed rate initially (mean = 4,769 g) as opposed to the last 10 months (mean = 3,483 g).

The average monthly solids collected were 1,400 mg/L (range = 1,076 to 1,750 mg/L or 1.9 kg/d). The average monthly biological oxygen demand was 341 mg/L (range = 270 to 416 mg/L or 0.46 kg/d) (Fig.7).

Laboratory-scale effluent-based *I. galbana* culture (Trial 1)

In the first *I. galbana* culture trial initial cell densities were 1×10^6 cells/mL in the four treatments (100-Efl., 50-Efl., f/2, control) (Fig. 8). Cell density in the control treatment was significantly ($P < 0.05$) lower than initial by d5 (2.7×10^5) with no change thereafter. Cell density in the f/2 treatment was significantly higher than initial by d5, increasing to 1.95×10^6 cells/mL, with no change thereafter. Cell density in the 50-Efl. treatment was significantly higher than initial by d3 (3.37×10^6 cells/mL) and continued to increase (5.86×10^6 cells/mL) by d9. Cell density in the 100-Efl. treatment was significantly higher than initial by d5 (4.3×10^6 cells/mL) and continued to increase through d15 (7.9×10^6 cells/mL). On d15 cell density in the 100-Efl. treatment (7.9×10^6 cells/mL) was significantly higher than the control, f/2 and 50-Efl. treatments (Fig.9).

Initial phosphate concentrations in the culture media differed according to treatment type (Fig. 10). The control, f/2, 50-Efl. and 100-Efl. treatments had initial concentrations of 2.79, 4.37, 5.51 and 7.90 mg/L, respectively, which included residual phosphate with the microalgae inocula. The initial concentration of phosphate was significantly higher in the 100-Efl. treatment

compared to the 50-Efl., f/2 and control treatments, and higher in the 50-Efl. compared to the control treatment. Phosphate concentration in the control treatment was significantly lower than initial (2.79 mg/L) by d3 (0.00 mg/L) with no change thereafter (Fig. 10). Phosphate concentration in the f/2 treatment was significantly lower than initial (4.37 mg/L) by d3 (0.54 mg/L) with no change thereafter. Phosphate concentration in the 50-Efl. treatment was significantly lower than initial (5.51 mg/L) by d5 (1.56 mg/L) with no change thereafter. Phosphate concentration in the 100-Efl. treatment was significantly lower than initial (7.90 mg/L) by d5 (4.07 mg/L) with no change thereafter (Fig. 10).

Initial nitrate/nitrite concentrations in the culture media differed according to treatment type (Fig.11). The control, f/2, 50-Efl. and 100-Efl. treatments had initial nitrate/nitrite concentrations of 36.3, 70.4, 34.7 and 36.2 mg/L, respectively, which included residual nitrate/nitrite from the microalgae inocula. The initial concentration of nitrate/nitrite in the f/2 treatment was significantly higher than the control, 50-Efl. and 100-Efl. treatments. Nitrate/nitrite concentration in all treatments showed large and significant reductions by d3 with no change thereafter. Nitrate/nitrite concentration in the control treatment was significantly lower than initial (36.3 mg/L) by d1 (30.61 mg/L) with a large reduction by d3 (3.92 mg/L) and no change thereafter. Nitrate/nitrite concentration in the f/2 treatment was significantly lower than initial (70.4 mg/L) by d3 (23.8 mg/L) with no change thereafter. Nitrate/nitrite concentration in the 50-Efl. treatment was significantly lower than initial (34.7 mg/L) by d3 (5.48 mg/L) with no change thereafter. Nitrate/nitrite concentration in the 100-Efl. treatment was significantly lower than initial (36.2 mg/L) by d3 (6.33 mg/L) with no change thereafter.

Initial ammonia concentrations in the culture media differed according to treatment type (Fig. 12). The control, f/2, 50-Efl. and 100-Efl. treatments had initial ammonia concentrations of

1.51, 1.94, 1.18 and 1.96 mg/L, respectively, which included residual ammonia with the microalgae inocula. There was no significant difference in initial ammonia concentrations among treatments. Ammonia concentration in the control treatment was significantly lower than initial (1.51 mg/L) by d9 (0.00 mg/L) with no change thereafter. Ammonia concentration in the f/2 treatment was significantly lower than initial (1.94 mg/L) by d9 (0.00 mg/L) with no change thereafter. Ammonia concentration in the 50-Efl. treatment was significantly lower than initial (1.18 mg/L) by d9 (0.00 mg/L) with no change thereafter. Ammonia concentration in the 100-Efl. treatment was significantly higher than initial (1.96 mg/L) by d1 (3.15 mg/L), but was significantly lower than initial (1.96 mg/L) by d5 (0.93 mg/L) with further reduction by d9 (0.00 mg/L).

Initial total phosphorous concentrations in the culture media differed according to treatment type (Fig. 13). The control, f/2, 50-Efl. and 100-Efl. treatments had initial total phosphorous concentrations of 6.57, 8.49, 18.5 and 36.4 mg/L, respectively, which included residual total phosphorous from the microalgae inocula. Initial concentration of total phosphorous was significantly higher in the 100-Efl. than in the control, f/2 and 50-Efl. treatments and higher in the 50-Efl compared to the control and f/2 treatments. Total phosphorous concentrations showed large and significant reductions by d3 in the control, f/2 and 100-Efl. treatments with little or no change thereafter. Total phosphorous concentration in the control treatment was significantly lower than initial (6.57 mg/l) by d3 (1.62 mg/L) with no change thereafter. Total phosphorous concentration in the f/2 treatment was significantly lower than initial (8.48 mg/L) by d3 (3.88 mg/L) with further significant reduction by d7 (2.62 mg/L). There was no significant change in total phosphorous concentration in the 50-Efl. treatment

throughout the culture trial. Total phosphorous concentration in the 100-Efl. treatment was significantly lower than initial (36.4 mg/L) by d3 (19.86 mg/L) with no change thereafter.

Initial total nitrogen concentrations in the culture media differed according to treatment type (Fig. 14). The control, f/2, 50-Efl. and 100-Efl. treatments had initial total nitrogen concentrations of 63.6, 99.2, 78.5 and 113.4 mg/L, respectively, and included residual total nitrogen with the microalgae inocula. Initial concentration of total nitrogen was significantly higher in the 100-Efl. treatment than in the control and 50-Efl. treatments and higher in the f/2 treatment compared to the control treatment. Total nitrogen concentration in the control treatment was significantly lower than initial (63.6 mg/L) by d1 (47.9 mg/L) with further significant reduction by d3 (28.6 mg/L) and no change thereafter. Total nitrogen concentration in the f/2 treatment was significantly lower than initial (99.2 mg/L) by d1 (87.6 mg/L) with further significant reduction by d3 (75.5 mg/L) and no change thereafter. There was no significant change in total nitrogen concentrations in the 50-Efl. and 100-Efl. treatments throughout the culture trial.

Laboratory-scale effluent-based *I. galbana* culture (Trial 2)

In trial 2 initial *I. galbana* cell densities were approximately 8×10^5 cells/mL in all four treatments (control, f, 50-Efl., 100-Efl.) (Fig. 15). Cell density in the control treatment was significantly ($P < 0.05$) lower than initial by d3 (4.6×10^5 cells/mL), but increased to initial levels by d7. Cell density in the f treatment was significantly higher than initial by d5 (2.8×10^6 cells/mL) and continued to increase by d7 (4.49×10^6 cells/mL). Cell density in the 50-Efl. treatment was significantly higher than initial by d3 (3.38×10^6 cells/mL) with no change

thereafter. Cell density in the 100-Efl. treatment was significantly higher than initial by d3 (5.18×10^6 cells/mL) and continued to increase through d7 (7.27×10^6 cells/mL). On d7 cell densities in the f, 50-Efl. and 100-Efl treatments were significantly higher than the control treatment (Fig. 16).

Initial phosphate concentrations in the culture media differed according to treatment type (Fig. 17). The control, f, 50-Efl. and 100-Efl. treatments had initial concentrations of 0.43, 28.4, 2.96 and 4.62 mg/L, respectively, which included residual phosphate with the microalgae inocula. The initial concentration of phosphate was significantly higher in the f treatment compared to the control, 50-Efl., and 100-Efl., treatments and higher in the 100-Efl. and 50-Efl. compared to the control treatment. Phosphate concentration showed large and significant reduction in all treatments except the control. Phosphate concentration in the control treatment showed no significant change throughout the trial. Phosphate concentration in the f treatment was significantly lower than initial (28.4 mg/L) by d1 (18.0 mg/L) and continued to decrease to d7 (5.62 mg/L). Phosphate concentration in the 50-Efl. treatment was significantly lower than initial (2.96 mg/L) by d1 (1.96 mg/L) and continued to decrease to d7 (0.51 mg/L). Phosphate concentration in the 100-Efl. treatment was significantly lower than initial (4.62 mg/L) by d1 (4.00 mg/L) and continued to decrease to d7 (2.19 mg/L).

Initial nitrate/nitrite concentrations in the culture media differed according to treatment type (Fig. 18). The control, f, 50-Efl. and 100-Efl. treatments had initial nitrate/nitrite concentrations of 7.47, 475, 29.9 and 43.3 mg/L, respectively, which included residual nitrate/nitrite with the microalgae inocula. The initial concentration of nitrate/nitrite in the f treatment was significantly higher than the control, 50-Efl. and 100-Efl. treatments and higher in the 100-Efl. compared to the 50-Efl. and control treatments. Nitrate/nitrite concentration in the

control and f treatments showed no significant change throughout the trial. Nitrate/nitrite concentration in the 50-Efl. treatment was significantly lower than initial (29.9 mg/L) by d1 (19.6 mg/L) and continued to decrease to d7 (11.5 mg/L). Nitrate/nitrite concentration in the 100-Efl. treatment was significantly lower than initial (43.3 mg/L) by d3 (20.83 mg/L) with no change thereafter.

Initial ammonia concentrations in the culture media differed according to treatment type (Fig. 19). The control, f, 50-Efl. and 100-Efl. treatments had initial ammonia concentrations of 1.04, 2.06, 3.47 and 4.76 mg/L, respectively, which included residual ammonia with the microalgae inocula. Ammonia concentrations in the control treatment were higher than initial (1.04 mg/L) by d3 (5.31 mg/L) with no change thereafter. Ammonia concentrations in the f treatment were higher than initial (2.06 mg/L) by d3 (4.74 mg/L) but decreased by d7 (2.84 mg/L). Ammonia concentration in the 50-Efl. treatment was significantly lower than initial (3.47 mg/L) on d1 (1.33 mg/L) but returned to initial levels by d3 (4.02 mg/L) with another reduction by d5 (1.48 mg/L), with no change thereafter. Ammonia concentration in the 100-Efl. was significantly lower than initial (4.76 mg/L) by d1 (3.30 mg/L) and continued to decline to d7 (2.49 mg/L).

Initial total phosphorous concentrations in the culture media differed according to treatment type (Fig. 20). The control, f, 50-Efl. and 100-Efl. treatments had initial total phosphorous concentrations of 3.13, 37.5, 6.68 and 12.0 mg/L, respectively, which included residual total phosphorous with the microalgae inocula. Initial concentration of total phosphorous was significantly higher in the f treatment than the control, 50-Efl. and 100-Efl. treatments and higher in the 100-Efl. treatment compared to the 50-Efl. and control treatments and higher in the 50-Efl. treatment compared to the control treatment. Total phosphorous

concentration in the control treatment showed no significant change throughout the culture trial. Total phosphorous concentration in the f treatment was significantly lower than initial (37.5 mg/L) by d1 (27.0 mg/L) and continued to decline to d3 (15.1 mg/L), with no change thereafter. Total phosphorous concentration in the 50-Efl. and 100-Efl. treatment showed no significant change throughout the culture trial.

Initial total nitrogen concentrations in the culture media differed according to treatment type (Fig. 21). The control, f, 50-Efl. and 100-Efl. treatments had initial total nitrogen concentrations of 46.2, 513, 62.3 and 99.6 mg/L, respectively, which included residual total nitrogen with the microalgae inocula. Initial concentration of total nitrogen was significantly higher in the f treatment compared to the control, 50-Efl. and 100-Efl. treatments, higher in the 100-Efl. treatment compared to the 50-Efl. and control treatments and higher in the 50-Efl. compared to the control treatment. Total nitrogen concentration in the control treatment showed no significant change throughout the culture trial. Total nitrogen concentration in the f treatment was significantly lower than initial (513 mg/L) by d5 (488 mg/L), with no change thereafter. There was no significant change in total nitrogen concentrations in the 50-Efl. and 100-Efl. treatments throughout the culture trial.

Laboratory-scale effluent-based wild plankton culture

Initial wild plankton cell densities averaged 0.65×10^6 cells/mL in the four treatments (control f/2, 50-Efl., 100-Efl.) (Fig. 22). Cell density in the control treatment was significantly ($P < 0.05$) higher than initial by d3 (1.02×10^6 cells/mL), with no change thereafter. Cell density in the f/2 was significantly higher than initial by d5 (8.17×10^6 cells/mL). Cell density in the 50-

Efl. treatment was significantly higher than initial by d3 (23.67×10^6 cells/mL), with no change thereafter. Cell density in the 100-Efl. treatment was significantly higher than initial by d3 (24.67×10^6 cells/mL) with further increase through d5 (38.0×10^6 cells/mL). On d5 cell density in the 100-Efl. treatment was significantly higher than the control, f/2 and 50-Efl.treatments, and higher in the 50-Efl. compared to the control and f/2 treatments (Fig. 23).

Initial phosphate concentrations in the culture media differed according to treatment type (Fig. 24). The phosphate concentration in the control, f/2, 50-Efl. and 100-Efl. treatments were 0.0, 2.56, 0.0 and 2.24 mg/L, respectively, and included residual phosphate from the microalgae inocula. The initial concentration of phosphate was significantly higher in the f/2 and 100-Efl. treatments compared to the 50-Efl. and control treatments. Phosphate concentration was undetectable in the control treatment throughout the culture trial. Phosphate concentration in the f/2 treatment was significantly lower than initial (2.56 mg/L) by d3 (0.014 mg/L), with no change thereafter. Phosphate concentration in the 50-Efl. and 100-Efl. treatments showed no significant change throughout the culture trial.

Nitrate/nitrite concentrations were undetectable in the control, 50-Efl. and 100-Efl. treatments throughout the culture trial. Nitrate/nitrite concentration in the f/2 treatment ranged from 194.2 to 165.9 mg/L, with no significant change throughout the trial (Fig. 25).

Initial ammonia concentrations in the culture media differed according to treatment type (Fig. 26). The control, f/2, 50-Efl. and 100-Efl. treatments had initial ammonia concentrations of 1.50, 1.83, 4.22 and 8.13 mg/L, respectively, which included residual ammonia with the microalgae inocula. The initial concentration of ammonia was significantly higher in the 100-Efl. treatment compared to the control, f/2 and 50-Efl. treatments. Ammonia concentrations in the control and f/2 treatments showed no significant change throughout the culture trial.

Ammonia concentration in the 50-Efl. was significantly lower than initial (4.22 mg/L) by d1 (0.79 mg/L) with no change thereafter. Ammonia concentration in the 100-Efl. treatment was significantly lower than initial (8.13 mg/L) by d1 (2.08 mg/L), with no change thereafter.

Initial total phosphorous concentrations in the culture media differed according to treatment type (Fig. 27). The control, f/2, 50-Efl. and 100-Efl. treatments had initial total phosphorous concentrations of 1.16, 7.74, 14.6 and 26.3 mg/L, respectively, which included residual total phosphorous with the microalgae inoculum. Initial concentration of total phosphorous was significantly higher in the 100-Efl. treatment than the control, f/2 and 50-Efl. treatments, higher in the 50-Efl. treatment compared to the control and f/2 treatments and higher in the f/2 treatment compared to the control treatment. Total phosphorous concentration in the control treatment did not change significantly throughout the culture trial. Total phosphorous concentration in the f/2 treatment was significantly lower than initial (7.74 mg/L) by d3 (5.16 mg/L) and continued to decline by d5 (3.92 mg/L). Total phosphorous concentration in the 50-Efl. treatment was significantly lower than initial (14.6 mg/L) by d5 (8.37 mg/L). Total phosphorous concentration in the 100-Efl. treatment was significantly lower than initial (26.3 mg/L) by d5 (19.6 mg/L).

Initial total nitrogen concentrations in the culture media differed according to treatment type. The control, f/2, 50-Efl. and 100-Efl. treatments had initial total nitrogen concentrations of 15.6, 125, 37.4 and 64.3 mg/L, respectively, which include residual total nitrogen with the microalgae inoculum. Total nitrogen concentration showed no significant change throughout the culture trial for any treatment (Fig. 28).

Pilot scale effluent based *I. galbana* culture

I. galbana cell densities appeared to increase from an initial 0.82×10^6 cells/mL to 2.97×10^6 cells/mL by d13, however, no significant change in density was detected (Fig. 29).

Dissolved nutrient concentrations generally declined throughout the culture trial. Phosphate concentration was significantly lower than initial (4.27 mg/L) by d10 (3.28 mg/L), with no change thereafter (Fig. 30). Nitrate/nitrite concentration was significantly lower than initial (12.0 mg/L) by d4 (4.3 mg/L) and continued to decline to d13 (1.77 mg/L). Ammonia concentration showed no significant change throughout the culture trial (Fig. 30).

Total phosphorous and total nitrogen averaged 11.9 and 145 mg/L, respectively, and showed no significant change throughout the culture trial (Fig. 31).

Pilot scale effluent based wild plankton culture

Initial wild plankton cell densities averaged 4.67×10^6 cells/mL and generally increased throughout the trial. Cell densities were significantly higher by d8 (12.3×10^6 cells/mL) when compared to d1 (3.23×10^6 cells/mL) and d2 (3.98×10^6 cells/mL) (Fig. 32).

Phosphate concentration was significantly lower than initial (13.8 mg/L) by d1 (5.84 mg/L), with no change thereafter (Fig. 33). Nitrate/nitrite concentration was significantly higher than initial (4.21 mg/L) by d8 (13.1 mg/L). Ammonia concentration was significantly lower than initial (8.58 mg/L) by d4 (5.50 mg/L) and continued to decrease to d8 (2.47 mg/L) (Fig. 33).

Total phosphorous showed no significant change throughout the culture trial (Fig. 34). Total nitrogen concentration was significantly lower than initial (154 mg/L) by d2 (98.0 mg/L) with no significant change thereafter (Fig. 34).

DISCUSSION

RAS waste characterization

The effluent volume over the 12 month study period averaged 1,357L and ranged from 9 to 11% of the system volume exchanged during each 24 h period which represented a typical exchange rate for an intensive recirculating aquaculture system (Masser 1999). This low exchange rate resulted in a highly concentrated effluent that included settleable and suspended solids averaging 1403 mg/L (range = 1076 to 1750 mg/L), which correspond to an average percentage of 39% (range = 30 to 52%) feed fed to the experimental animals per day. These values are consistent with published values of solids in the effluent of RAS (20 to 40% of feed fed) (Timmons et al. 2001).

The environmental parameters were measured in the RAS sump after a 24 h quiescent period that promoted vigorous bacterial activity that consumed oxygen (12 month mean = 0.06 mg/L). The mean pH for the 12-month study period was low (6.62) probably due to increased CO₂ via microbial degradation of organic matter in the collection sump promoting the formation of carbonic acid. The water temperature in the RAS sump varied seasonally with a high of 28.5⁰ C during summer and a low of 18.0⁰ C during winter; however no apparent influence on the examined parameters due to varying temperature was observed.

Previous studies characterizing the effluent of marine recirculating systems have focused on evaluating the efficiency of a particular filter component or different combinations of components (Twarowska 1997, Singh 1999, Easter 2000), and the effluent was sampled as it

entered and exited these components. This study examined the effluent from a marine recirculating system from a mass balance approach and required collecting all of the effluent produced by all of the filtration components during a period of 24 h in a reservoir that was not part of the recirculating stream. In the first 4 months of the study period nitrate/nitrite concentration was higher (4 month mean = 39.6 mg/L) than ammonia (4 month mean = 4.88 mg/L) (Fig.5). This result was expected due to the nitrifying environment of the biological filter which was well-conditioned before the study, however, this relationship between nitrate/nitrite and ammonia concentrations reversed in the last eight months of the study when ammonia concentration (8 month mean = 3.80 mg/L) was higher than the nitrate/nitrite concentration (8 month mean = 1.32 mg/L) (Fig.5). It appears that the increased exchange rate in the intervals between sampling events flushed the nitrate/nitrite from the system.

Laboratory-scale effluent-based microalgae culture (Trial 1)

In the first laboratory culture trial involving *I. galbana*, microalgae growth in the four treatments (control, f/2, 50-Efl. and 100-Efl.) followed predictable trends with increasing algal biomass over time in the treatments containing nutrients (Fig. 8) as previously reported for macroalgae (Neori, et al. 2005; Schuenhoff, et al. 2002) and microalgae growth (Dunstan, 1971) using effluent from animal rearing systems as a nutrient source. In the control treatment, cellular density decreased over time as expected for nutrient free media. The growth curves in the f/2 and 50-Efl. treatments followed typical microalgae growth curves with an initial adaptive phase of slow or no growth, a period of rapid growth and an eventual plateau. In contrast, in the 100-Efl. treatment, cell density continued to increase through d15 with no evidence of a plateau. The

100-Efl. treatment produced the highest cell densities among the four treatments, while the 50-Efl. treatment produced higher cell densities than the f/2 treatment (Fig. 9).

A reduction of dissolved nutrients (PO_4 , NH_3 , NO_3/NO_2) that was associated with *I. galbana* cell growth, similar to what was reported in an earlier study (Dunstan 1971). In the control treatment, all the nutrients were introduced when the culture units were inoculated with microalgae (*I. galbana*). The observation of declining cell density in the control (Fig. 8) while nutrient concentrations declined (Figs. 10, 11 and 12) was likely due to cell death exceeding cell growth from d0 to d5.

In the f/2 treatment, the observed plateau in cell density occurred on d9 (Fig. 8) and was associated with the elimination of phosphate and ammonia from the culture media (Figs. 10 and 12).

In the 50-Efl. treatment cell growth plateaued by d9 corresponding with the elimination of ammonia from the culture media. Phosphate and nitrate/nitrite concentrations were still appreciable in this treatment on d9 (Figs. 10 and 11) suggesting that ammonia was the limiting nutrient in this treatment.

In the 100-Efl. treatment, no plateau in the growth curve was observed (Fig. 5) even though ammonia was eliminated in this treatment by d9 (Fig.8). However, there was a moderate concentration of nitrate/nitrite in the culture media (Fig. 12) that likely supported continued cell growth in this treatment. In addition, the microbial conversion of organic nitrogen from breakdown of solids to inorganic nitrogen (ammonia) may have exceeded microalgae uptake. Ammonia appeared to have been the limiting nutrient in the 50-Efl. treatment and was eliminated before nitrate/nitrite in the 100-Efl. treatment, suggesting that *I. galbana* preferably utilized ammonia, then nitrate/nitrite as nutrient sources in effluent from a marine recirculating system.

As the *I. galbana* cultures grew they assimilated dissolved phosphorous and nitrogen from the culture media and converted these nutrients to biomass. Since nutrient concentrations in the culture media were determined without filtering out solids (i.e. microalgal cells plus effluent solids) it was expected that no net change in concentrations of total nitrogen and total phosphorous would be observed. An unexpected result was the reduction of total phosphorous in the control, f/2 and 100-Efl. treatments (Fig.13) and the reduction of total nitrogen in the control and f/2 treatments (Fig.14). I speculate that a reason for these reductions may be through the processes of phosphate precipitation and ammonia stripping. As microalgae cultures grow and become densely populated, the reduction of CO₂ in the media drives the pH up to very high levels. In order to reestablish pH equilibrium the hydrogen ions are stripped from NH₄ to form N₂ gas (Garcia 2000). As these treatment vessels are open, this nitrogen is released from the system. Also, at high pH values (i.e. 9), phosphate can form a precipitate with calcium carbonate (Olsen 2006). This precipitate may be unavailable for detection by the analytical procedure used to determine phosphate concentration i.e. persulfate digestion procedure.

In summary, an increase in cell densities in the three treatments containing nutrients (f/2, 50-Efl. and 100-Efl.) occurred with a concurrent reduction of dissolved PO₄, NH₃, and NO₃/NO₂ in the culture media (Figs 10, 11 and 12). This experiment demonstrated that effluent from a marine recirculating aquaculture system (even at 50% strength) was a more effective nutrient source than commercially prepared media (Guillard's f/2).

Laboratory-scale effluent-based microalgae culture (Trial 2)

A second laboratory culture trial utilizing the microalgae *I. galbana* was performed to confirm that cellular growth and nutrient reductions would occur in a manner similar to those observed in the first seven days of trial 1. In this trial Guillard's f strength nutrient concentration was used to determine if a higher level of nutrients in this treatment would produce higher cell densities than those observed in the f/2 treatment of trial 1.

The control treatment cell densities decreased by d3 but then returned to original densities by d5 (Fig. 15). A possible explanation for this could be that the initial reduction in densities occurred as a result of cell death from d0 to d3 and then the cultures rebounded in the presence of nutrients introduced with the inoculum. In contrast, cell growth in the f, 50-Efl. and 100-Efl. treatments increased through d7 (Fig. 15). When the 100-Efl treatment produced the highest cell densities, while the f treatment produced higher cell densities than the 50-Efl. treatment (Fig. 16).

There was no reduction of dissolved nutrients (PO_4 , NH_3 , NO_3/NO_2) in the control treatment through d7 (Figs. 17, 18 and 19) and an increase in the concentration of ammonia in the control treatment by d3 (Fig. 19) that corresponded to a decrease in cell densities on this day (Fig. 15). This may be explained by cell lysis and liberation of nutrients into the media.

In the f treatment, phosphate concentrations decreased to d3 (Fig. 17) while nitrate/nitrite concentrations remained unchanged (Fig. 18). Ammonia concentration fluctuated through d3, but returned to initial levels by d7 (Fig. 19). This fluctuation was possibly due to interconversion of molecular forms of nitrogen while algal uptake of ammonia was taking place.

In the 50-Efl. treatment a clear reduction of phosphate and nitrate/nitrite concentrations was observed (Figs. 17 and 18) with increasing cell densities indicating significant uptake of these dissolved nutrients. Ammonia concentrations fluctuated through d5, but returned to initial

levels by d7 possibly due to interconversion of molecular forms of nitrogen while algal uptake of ammonia was taking place.

(Fig. 19). There was a clear reduction of dissolved nutrients (PO_4 , NO_3/NO_2 , NH_4) in the 100-Efl. treatment (Figs. 17, 18 and 19) with a concurrent increase in cellular density through d7 (Fig. 15).

In trial 2 total phosphorous and total nitrogen concentrations remained stable in the control, 50-Efl. and 100-Efl. treatments but decreased in the f treatment through d7 (Fig. 17). In the f treatment the majority of the total phosphorous and total nitrogen was originally in the dissolved form and would have been readily available to the processes of ammonia stripping and phosphate precipitation. In contrast, total phosphorous and total nitrogen in the control, 50-Efl. and 100-Efl. treatments originated primarily from the effluent solids and algal biomass and were less available to these processes.

Trial 2 verified that effluent from a marine recirculating system was an excellent nutrient source for the culture of *I. galbana* and that effluent of 100% strength produced higher cell densities than either the f or 50-Efl. media. Although the f treatment and 50-Efl. treatments had similar growth curves in trial 2, the f treatment showed more significant reduction of phosphate and total phosphorous (Figs. 17 and 20), however, the phosphate and total phosphorous concentrations in the f treatment were extremely high and primarily in the dissolved form and readily available for phosphate precipitation. The 50-Efl. treatment was more effective in reducing nitrate/nitrite concentrations than the f treatment (Fig. 18) with no concurrent reduction in total nitrogen (Fig. 21). These observations make it difficult to differentiate between these two treatments (f and 50-Efl.) in the second *I. galbana* trial. As these cultures (f,

50-Efl. and 100-Efl.) were terminated before a plateau was achieved it was impossible to determine a limiting nutrient.

A clear result of both *I. galbana* laboratory trials 1 & 2 was the significant reduction of dissolved phosphorous and nitrogen with a concurrent increase in cellular densities in the 100-Efl. treatment as compared to the other treatments. It is possible that the effective component in this treatment was the abundance of solids available for microbial conversion to dissolved nutrients thus continually providing nutrient resource throughout the trial.

Laboratory-scale effluent-based wild plankton culture

The wild plankton cultures were dominated by small (4-6 μm) green microalgae that exhibited very fast growth in previous laboratory studies. This experiment was terminated when the 50-Efl. treatment plateaued after following an initial adaptive phase followed by 2 days of rapid growth (Fig. 22) by d3. Cell densities in the control increased significantly by d3 (Fig. 22) while there was no change in cell densities in the f/2 treatment throughout the culture trial. Cell densities increased significantly in the 50-Efl. and 100-Efl. treatments (Fig. 22), with no evidence of a plateau in the 100-Efl. treatment. The 100-Efl. treatment produced the highest cell densities amongst the four treatments, while the 50-Efl. treatment produced higher cell densities than the f/2 treatment (Fig. 23) by d5.

The algae used in this culture trial was harvested from local waters that may have had very low nutrient concentrations and had to be reared to appropriate densities to inoculate the treatment vessels at their initial densities (0.6×10^6 cells/mL) (Fig. 22). The media used to culture the wild plankton was effluent collected from the sump during the period that

nitrate/nitrite was eliminated by system exchange, but ammonia was still present. This may explain why no nitrate/nitrite concentrations were detected in the control, 50-Efl. and 100-Efl. treatments.

The initial presence of ammonia in the control treatment (Fig. 26) was introduced with the algae inocula. In the control treatment there was no significant reduction in any of the examined parameters.

Unexpectedly, in the f/2 treatment, no change in cellular density occurred during the culture trial (Fig. 22) and all nitrogen-based nutrients remained unchanged (Figs. 25, 26 and 28); however, there was a significant reduction in phosphate (Fig. 24) and total phosphorous (Fig. 27).

On the other hand, while nitrate/nitrite was absent from the 50-Efl. treatment, cell density increased with no change in phosphate concentration and an elimination of ammonia by d3 (Fig. 22). It appears that the elimination of ammonia coincided with a plateau in the growth curve in this treatment (Fig. 22) suggesting that ammonia was the limiting nutrient in this treatment.

Nitrate/nitrite concentration was undetectable in the 100-Efl. treatment, and ammonia was eliminated by d5, however, cell growth continued through d5 (Fig. 22), with no evidence of a plateau. As observed in the 50-Efl. treatment, there was no observed reduction in phosphate (Fig. 24); however, total phosphorous was significantly lower by d5 (Fig. 27). This treatment showed a continued increase in cellular densities even in the absence of nitrate/nitrite or ammonia. It may be concluded that the rate of microbial conversion of organic nitrogen to inorganic nitrogen, i.e. ammonia, exceeded uptake by microalgae. This experiment demonstrated that the effluent from a marine recirculating system (even at 50% strength) is a

more effective nutrient source for the growth of indigenous algae than commercially prepared f/2 media.

Pilot-scale effluent based *I. galbana* culture

As the laboratory trials confirmed that the 100% effluent from the UNCW marine RAS was most effective for the culture of *I. galbana* and indigenous plankton, this effluent strength was chosen for the pilot-scale cultures. At the pilot scale the volumes of effluent used prohibited any sterilization treatment so the culture vessels contained effluent with its associated population of microbes. My preliminary studies of *I. galbana* cultures in the bioreactors demonstrated that the maximum density obtainable in these vessels was approximately 3×10^6 cells/mL, therefore, the experiment was terminated when this density was reached on d13 (Fig. 29). The apparent increase in cell densities in the pilot-scale *I. galbana* culture was associated with a reduction of phosphate and nitrate/nitrite while ammonia remained unchanged (Fig. 30). As the bioreactors were untreated for microbes and well aerated, there was probably an active conversion of organic nitrogen to dissolved nitrogen, i.e. ammonia, at a rate that kept pace with algal uptake, therefore it is difficult to determine if one nitrogen-based nutrient was preferred over another. There was no change in total phosphorous or total nitrogen (Fig. 31) in this experiment as expected.

This experiment confirmed that effluent from a marine recirculating system at 100% strength is an excellent nutrient source for the outdoor production of *I. galbana* and that *I. galbana* effectively reduced the nutrient load associated with the effluent from a marine RAS.

Pilot-scale effluent-based wild plankton culture

As with the pilot-scale *I. galbana* culture, the effluent was untreated and 100% strength. The increase in cell densities in the pilot-scale wild plankton culture (Fig. 32) was associated with reduction of phosphate, ammonia and total nitrogen (Figs. 33 and 34), while nitrate/nitrite concentration increased through d7 (Fig. 33). A possible explanation for the increase in nitrate/nitrite concentrations may be the presence of nitrifying bacteria in the well aerated bioreactors. It would be useful to continue these studies to determine if a prolonged culture period would result in a trend of nutrient reduction, especially with respect to ammonia and total nitrogen.

This experiment verified that effluent from a marine recirculating system is an excellent nutrient source for wild plankton growth in outdoor bioreactors and that indigenous plankton is a suitable microalgae for the reduction of nutrients associated with effluent from a marine RAS for the production of southern flounder.

CONCLUSION

These studies confirmed that microalgae, *I. galbana* and wild plankton, could be a valuable and efficient mitigator of wastes generated by marine recirculating aquaculture systems. They also confirm that the effluent from a marine RAS is an excellent nutrient source for the production of marine microalgae, especially at 100% strength. The suggestion that the presence

of organic material in the effluent facilitates a healthy culture is promising in that it could eliminate the expense of filtering prior to use. It is promising that the effluent from this RAS need not be sterilized prior to inoculation as this treatment process is expensive and complex and would destroy the microbes that are responsible for the breakdown of the solids into dissolved nutrients, and would further burden the financial responsibilities and resources of the fish farmer. It would be useful to continue studies at the bioreactor scale to determine if longer culture periods would result in continued reduction of nutrient concentrations despite a plateau in cellular densities. Although the cultures reach a maximum density there may be an equilibrium that exists between the microbial production of nutrients and CO₂ from organic metabolism and the production of O₂ from continued cellular respiration. In addition, instead of the batch culture methods utilized in this study it may be useful to pursue continuous culture techniques that would be more appropriate to integrate into a farm practice that produces this effluent continuously.

The integration of a microalgae bioreactor as the downstream treatment component of a recirculating stream presents many challenges despite its apparent advantages. One challenge would be to determine the scale of a production system that would be required to meaningfully reduce the nutrient concentrations in the effluent to a level suitable for discharge or recirculating back to the animal rearing system. For instance, by utilizing the data from the *I. galbana* bioreactor study we can propose the scale of a microalgae biofilter component required to reduce nutrient levels to an acceptable level for discharge. In this example we will assume the complete removal of nitrate/nitrite from the effluent of our experimental RAS on a continuous basis. In the pilot-scale bioreactor experiment it took 13d for *I. galbana* to remove 0.014 kg of nitrate/nitrite (7.7 mg/L x 1,800 L). Over that same time period the RAS produced 0.41 kg of

nitrate/nitrite ($17.4 \text{ mg/L} \times 1800 \text{ L} \times 13\text{d}$). This corresponds to a feed input of approximately 48 kg. By dividing the amount of nitrate/nitrite produced by the RAS over the 13 d cycle (0.41 kg) by the amount consumed in the bioreactor (0.014 kg) we determine the number of 2000-L bioreactors (29 or 52,000 L) it would require to reduce the nitrate/nitrite produced by the RAS in 13 days. This corresponds to a ratio of 1000 L of bioreactor volume per kg feed fed. A commercial-scale marine RAS (sixteen 8.2 m diameter tanks = $1 \times 10^6 \text{ L}$) for the production of southern flounder would produce at harvest 38,000 kg of fish and would require a feed input of 750 kg (2% of biomass) on the last day of feeding. This commercial-scale RAS would require a microalgae biofilter of $7.5 \times 10^5 \text{ L}$ or 12 – 8.2 m diameter bioreactors.

Microalgae culturing systems require land and water resources as do the fish rearing systems so it would be imperative to find a value for the particular species of microalgae that is produced. However, like recirculating aquaculture systems, high density microalgae production systems can significantly reduce the amount of natural resources (land and water) required to reduce inorganic effluent nutrient concentrations by designing high density culture systems in a controlled environment. This would also allow the production of specific monocultures that have high market values such as those used for the production of larval fish, oysters, protein or pharmaceuticals. Further studies on these intensive systems are required.

One possible economic advantage of utilizing microalgae to mitigate effluent wastes would be the elimination of the bacterial filtration unit (i.e. biofilter) of the recirculating system, a significant expense for the culturist and often unreliable component of filtration, thereby interrupting the process of nitrification but still providing dissolved nitrogen (NH_4/NH_3) to the microalgae biofilter. In addition, by taking advantage of the mechanical filtration component's (dual drain, microscreen and foam fractionator) waste streams, a culturist could select the solid

fraction in the effluent that would most benefit their effluent treatment design. A geotextile bag could be used to filter the solids separated by the particle trap and microscreen ($> 60 \mu\text{m}$) while the fines from the foam fractionator ($<30 \mu\text{m}$) could be retained and recombined with the dissolved nutrients to serve as a nutritive base for microalgae growth and as a food source for bivalves. Also, as demonstrated in these studies, the solids would benefit the microalgae cultures by providing a continual source of dissolved nutrients through microbial breakdown. The net effect of this process would be to eliminate dissolved nitrogen from the recirculating stream, as opposed to reducing its toxicity to a level tolerable by the fish species being cultured (i.e. nitrification), while producing valuable downstream products (microalgae and shellfish) and reducing the scale of the operation required to process all of the wastes.