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Evaluation of nanobubble technology in aquaculture

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Contents

Tables

Figures

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Abbreviations

BASO	Basophils
BW	Body weight
CFU	Colony forming unit
CP	Crude protein
DO	Dissolved oxygen
DPI	Department of Primary Industries
EOS	Eosinophils
FCR	Food conversion ratio
FW	Freshwater
GE	Gross energy
HCT	Haematocrit
HGB	Haemoglobin
LDO	Luminescent dissolved oxygen
LYMPH	Lymphocytes
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MJ	Megajoule of energy
MONO	Monocytes
MPV	Mean platelet volume
NBT	Nano Bubble Technologies Pty Ltd
NEUT	Neutrophils
NFE	Nitrogen free extract
NSW	New South Wales, Australia
NTA	Nanoparticle Tracking Analysis
PLT	Platelet count
RAS	Recirculation aquaculture system
RBC	Red blood cell
RDW	Red blood cell distribution width
SAE	Standard aeration efficiency
SGR	Specific Growth Rate
SOTE	Standard oxygen transfer efficiency
SOTR	Standard oxygen transfer rate
SW	Saltwater
TAN	Total ammonia nitrogen
TDS	Total dissolved solids
WBC	White blood cells

Executive Summary

Nanobubble (NB) technology, i.e. the production of ultrafine bubbles with diameters <1 µm, is an emerging field which has the potential to greatly improve oxygenation efficiencies in aquaculture production. Before this technology can be considered for adoption by the aquaculture industry, a thorough assessment of the health and growth effects on fish stock held in NB oxygenated water is required.

Three trials were conducted to compare NB technology with conventional oxygenation setup (ceramic diffusers and air stones, oxygen gas and air) in a recirculating aquaculture system (RAS) under a number of different aquaculture scenarios testing i) stocking density, ii) salinity, and iii) temperature.

Trial 1 investigated Yellowtail Kingfish, *Seriola lalandi*, (initial body weight (IBW) = 52g) stocked at 10 fish $tank^{-1}$ or 50 fish $tank^{-1}$. Trial 2 investigated Barramundi, *Lates calcarifer*, (IBW = 103g) grown in either freshwater (bore water) or saltwater (32‰), with 15 fish stocked per tank. Trial 3 investigated Barramundi (IBW = 360g) grown at 20 or 30°C, with 10 fish stocked per tank. All experiment tanks (n=3) were 500 L cylindroconical aquaculture polytanks integrated within an individual RAS for each treatment.

Assessments were made on growth performance, feed intake, and feed conversion efficiencies (all trials), carcass chemical proximate composition and histology (Trials 1 and 2; samples of gill tissue, eye, heart and fins), and haematology (Trial 3).

The results demonstrated excellent growth rates and no mortalities in any of the trials. Fish grown in NB injected water performed similarly to those grown in conventionally oxygenated systems. Histological assessment found no significant findings. There were also no significant differences when considering carcass proximate analyses between the control and treatment groups. Examination of haematology also revealed no significant differences between the control and treatments groups. From the results it is clear that when fish are cultured in conditions providing appropriate water quality and feed, application of NB oxygenated water does not promote improved growth, health or feed efficiency when compared to a control group.

Wastewater remediation in the RAS's was also assessed while conducting these trials. Nitrification rates, as evidenced by total ammonia nitrogen (TAN) measurements, were similar between the control and treatment groups (All Trials), as too were bacterial colony forming units (CFU's) cultured from the effluent water of foam fractionators from each RAS (Trial 3).

Oxygen transfer efficiency rates were established at various O_2 gas flow rates (0.1 – 1.5 L O_2 min^{-1}) and using different capacity pumps (0.53 and 1.5 kW) in 500 L aquaculture tanks. Standard Oxygen Transfer Rates (SOTR) ranged from 0.07 to 0.44 kg O_2 h^{-1}, Standard Oxygen Transfer Efficiencies (SOTE) ranged from 17.4 to 54.2%, and Standard Aeration Efficiencies (SAE) ranged from 0.04 to 0.65 kg O_2 kWh^{-1}. Clear trade-offs were found when considering operating costs vs oxygen transfer efficiencies; with costs estimated to range from \$6.38 to \$15.73 kg O_2^{-1} depending on O_2 gas flow rate and pump capacity. Preliminary tests in a 5000 L aquaculture tank using a 1.5 kW pump and O_2 flow at 0.5 L min^{-1} found that the operating cost to transfer 1 kg of O_2 using the NB injector was \$5.35 compared to a standard aquaculture ceramic diffuser at \$19.26, a 260% difference. SOTE (51.7%) in the 5000 L tank was much greater than in the 500 L tank (33.4%) at an equivalent O_2 flow (0.5 L min^{-1}) and pump capacity (1.5 kW), likely an artefact of the differences in tank geometry and dwell time.

Taken overall, observations from the trials conducted in this project provide encouraging results towards the use of NB technology in aquaculture as an alternative oxygenation system. Growth, feed conversion efficiencies, tissue histology and haematology were comparable to those of the control groups implying that decisions on the implementation of this technology will therefore be predominantly based on establishment and operating costs, and the suitability of this technology to particular aquaculture system configurations (tanks, ponds, raceways etc).

Keywords

Nanobubble technology, fine bubble oxygenation, dissolved oxygen, oxygen saturation, Barramundi, Yellowtail Kingfish, health, aquaculture

Introduction

Dissolved oxygen (DO) is the most critically limiting factor in intensive aquaculture systems. A number of aeration technologies are commercially available and routinely used in aquaculture such as air blowers and oxygen generators in recirculating aquaculture systems (RAS's), and propeller or paddle wheel aerators in ponds. Oxygen gas injection systems can provide oxygen gas supply through low pressure diffusers or high-pressure oxygenation cones. These technologies have been in use for many years and provide their own specific benefits and drawbacks in commercial aquaculture operations with respect to oxygenation efficiency, reliability, purchase price, operating costs, maintenance schedule and run life. Oxygen transfer rates (gas-liquid phase) and energy requirements are two important factors influencing operating costs. Standard air or oxygen gas diffusers are extremely inefficient as most of the gas bubbled through the water column escapes to the atmosphere before dissolving. Standard oxygen transfer efficiencies (SOTE) are estimated at between 2 and 6% per ft (0.3 m) submergence for coarse and fine bubble diffusers, respectively, at standard conditions of 0 ppt salinity and 20 °C (Krause et al., 2010). Airstones and diffusers are relatively inefficient in aquaculture as the shallow water of culture systems does not provide sufficient dwell time for the transfer of oxygen. In contrast, oxygen cones are extremely efficient in the gas-liquid transfer of oxygen yet pumping water under pressure can be costly (Lekang, 2020).

Advances in the efficiency of gas–liquid phase processes have seen the emergence of nanobubble (NB) technologies, i.e. the production of ultrafine bubbles with diameters <1 µm. Because of their small size NBs are typically neutrally buoyant resulting in extended dwell time in the water column (Azevedo et al., 2016; Favvas et al., 2021). Microbubbles are considered to have a diameter >10 µm and macrobubbles >100 µm (Temesgen et al., 2017). NB technologies now have a demonstrated application across a broad variety of industries including wastewater treatment (Temesgen et al., 2017), biomedical engineering (Dehariya et al., 2023), gas and oil industry (Bui et al., 2022), agriculture (Marcelino et al., 2023), and the food industry (Zhang et al., 2022). Aquaculture production is rapidly expanding globally. New and innovative technologies are required to facilitate this expansion and make aquaculture industries more sustainable and profitable.

NB technology is an emerging field which has the potential to greatly improve oxygenation efficiencies in aquaculture production, and coupled with monitoring technologies (e.g. Pissoat and Jerry, 2018) could see a transformation in oxygen delivery systems. Recent studies have also focused on treatments utilising NB coupled with ozone to reduce pathogenic loads in aquaculture systems that have demonstrated encouraging results (e.g. Jhunkeaw et al., 2021; Le et al., 2021; Nghia et al., 2021). There are several different types of NB systems which have potential application in aquaculture settings including NB generators (e.g. Mahasri et al., 2018), diffusers (e.g. Suriasni et al., 2023), venturi systems (e.g. Khan et al., 2020), and likely to a lesser extent NB generated using ultrasonic and electrolysis systems (Foudas et al., 2022; Senthilkumar et al., 2018).

In any intensive aquaculture system oxygen supplementation is necessary to prevent hypoxic conditions. Some studies focusing on NB technology have suggested that an increase in oxygen concentration of haemoglobin and hemolymph via NB oxygenated water promotes better growth in fish and shrimp (Ebina et al., 2013); however, oxygen hyper-saturation can cause gas bubble disease (Bohl, 1997; Espmark et al., 2010; Machova et al., 2017). Gunanti et al. (2019) theorised that a decrease in immune specific cells (white blood cells) of fish reared in NB oxygenated compared to conventional oxygenation systems can occur due to a lowering of pathogen concentrations in the NB water, although clear description of the NB system and control group weren't provided in that study. Improvements in growth rates have also been observed in fish and shrimp reared in NB oxygenated water compared to control groups (Galang et al., 2019; Mahasri and Harifa, 2019; Mahasri et al., 2018); however, few studies have been rigorously conducted with respect to control groups maintained with adequate levels of DO, have presented sufficient information on experiment design and water quality data or have measured the size of the gas bubbles that are being reported as "nanobubbles". The literature available on the application of NB technology in aquaculture and its potential to support the growth and health of cultured aquatic animals is still very limited. Before this technology can be considered for broader adoption, a more thorough investigation needs to be undertaken

to assess the health effects on fish stock held in NB oxygenated water and the suitability for application of this technology to the aquaculture industry.

The primary aim of this project was to test the application of NB technology in a RAS under a number of different culture scenarios which can impact DO demand i.e. considering different stocking densities, salinities, and temperatures. The health, growth, and feed conversion efficiencies of two finfish species were assessed: i) Yellowtail Kingfish, *Seriola lalandi*, are a high energy and oxygen demand species and therefore a good candidate with which test NB application at different stocking densities, and ii) Barramundi, *Lates calcarifer*, are euryhaline and eurythermal and a suitable species to culture in a wide range of salinities and temperatures. While conducting these trials, general assessments of wastewater remediation *in situ* of the operating RAS's were also undertaken to determine if NB oxygenated water affected remediation efficiencies. Further, gas transfer efficiencies were determined, and a preliminary cost benefit analysis conducted based on operating costs.

Objectives

1. Assessing the health, growth and feed conversion efficiencies of fish exposed to nanobubble oxygenated water and cultured in a recirculating aquaculture system at different temperatures, salinities and stocking densities
2. Determine the effect of nanobubble oxygenation on wastewater treatment efficiencies in a recirculating aquaculture system
3. Provide a preliminary cost benefit analysis on the implementation of nanobubble oxygenation to an aquaculture system based on gas transfer efficiencies and operating costs

Aquaculture trials

Methods

Animal Ethics

All experiment procedures involving live fish were performed under the NSW DPI Fisheries Animal Care & Ethics (ACEC) Research Authority 'ACEC-0479 Aquaculture Technology'. Care, husbandry and termination of fish were carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015)

Experiment systems

The experiment tanks used in this project were 500 L cylindroconical Polymaster aquaculture tanks. Tank dimensions were approximately 790mm depth and 920mm diameter with a central sump/drain at the base of a sloping (6.6°) floor. Each tank was fitted with netting to prevent the escape of fish.

Experiment tanks (n=3) from each treatment were integrated within an independent RAS. Each RAS consisted of an automated drum filter (Hydrotech HDF501 series), reverse cycle heater/chiller unit, PS300 protein skimmers (MAT), P-series UV lamps (UV Guard), and 750 L biological filter (MBBR C3 cell media). The influent water was pumped to the experiment tanks by a 2.1kW pool-pump connected to a distribution manifold. Influent flow rate in each tank was set at approximately 20 L min^{-1} and checked regularly throughout the trial.

Water quality parameters were measured regularly during the trial using dedicated water quality meters (Horiba). Total ammonia nitrogen (TAN; NH_4^+/NH_3) was measured using a rapid test kit. Lighting was controlled via programmable LED overhead lights at 12L:12D, ramping up steadily from 0600h – 0830h and then ramping down steadily from 1530h – 1800h. Peak light intensity (0830h – 1530h) was held at approximately 200 lux. Saltwater (SW) was obtained from Tilligerry Creek NSW which is located adjacent to PSFI and batch chlorinated prior to use in RAS's. Freshwater (FW) was pumped from a bore located on site at PSFI.

Fish stock and handling

Yellowtail Kingfish used in this project were progeny of wild caught broodstock held at PSFI. Prior to the experiment juveniles were reared at low densities in a RAS comprising 10 kL tanks at an ambient temperature of approximately 20–22 °C and fed once daily with a commercial marine fish feed (Table 1).

Barramundi were obtained from Tailor Made Fish Farm (Bob's Farm, Port Stephens, NSW) where they are cultured in water sourced from a FW bore. They were length graded prior to being transported to PSFI. On arrival at PSFI fish were transferred to a 5 kL holding tank. The fish were gradually acclimated from FW to estuarine SW over 5 days as required and fed daily with a commercial marine fish feed. As barramundi are a domestically translocated species effluent water was collected during all holding and experiment phases, batch chlorinated and then neutralised *in situ* according to NSW DPI bio-security procedures before releasing to site settlement ponds.

Fish were fasted 24 h prior to stocking each experiment, sedated with Aqui-S ™, individually weighed and measured (fork length) before being distributed to experiment tanks.

Fish were hand fed once daily at ~9am with a high protein commercial floating marine fish diet appropriate for marine carnivores. Feeding was conducted to apparent satiation over approximately one hour and determined when pellets began to accumulate uneaten on the surface water. Remaining uneaten pellets were netted from tanks, counted, and feed intake adjusted using a correction factor based on average

pellet weight (n=200) determined for each diet and daily feed intake. Test feeds were stored frozen (-20°C) between daily feeds. Compositional analyses of the diets are presented in **Table 1**.

At the conclusion of each trial all fish were again individually weighed and measured before being euthanized with an overdose of Aqui-s anaesthetic.

Table 1. Proximate compositional analyses (dry matter basis) of diets used in this project. Diets were a commercial marine fish floating feed. NFE = Nitrogen free extract (carbohydrate equivalent).

Trial	Pellet Size (mm)	Dry Matter (%)	Protein (%)	Fat (%)	NFE (%)	Ash (%)	Energy (MJ/kg)
1	3	92.7	55.1	9.7	22.3	12.9	23.8
	6	95.1	50.8	12.6	29.4	7.2	26.4
2	6	93.9	53.2	12.1	27.0	7.7	21.35
3	8	90.5	50.4	17.4	24.9	7.3	28.76

DO application and monitoring

Consistent and similar dissolved oxygen (DO levels) across all experiment tanks for each experiment was achieved through close and frequent observation of DO readings and frequent manual adjustment of oxygen gas (control tanks) or NB water flow rates (NB tanks) as required to maintain saturation levels at ~120%. DO in each experiment tank was monitored in real time using PyroScience FireSting dissolved oxygen meters.

Control tanks were aerated using compressed air pumped through 100mm cylindrical sintered airstones, and also supplied with industrial grade oxygen to ensure dissolved oxygen concentration in tanks remained within the target saturation range at all times. Flow of oxygen to each tank was regulated via flowmeters feeding 200mm ceramic diffusers placed within each tank. Air and ceramic oxygen gas diffusers were also placed in the NB experiment tanks, however gas was not supplied during the trials and were used to ensure that the tank environments were standardised across both control and treatment groups with respect to physical objects present in the tanks. While not needed, these also provided a rapid emergency backup supply of oxygen should the NB system fail.

For Trial 1, a single NB injector hyper-saturated (oxygen gas) saturation tank (200 L) supplied the experiment tanks (both stocking densities) with NB oxygenated water via 25 mm PVC and 25 mm poly-pipe manifolds from a single RAS (Figure 1). NB experiment tanks received two water influent sources: i) hyper-saturated oxygenated NB water from saturation tanks and ii) water supplied directly from the RAS sump. Water from the NB saturation tank also recirculated back through the sump, providing stable temperature control, and directly supplied other RAS components including the biofilter and foam fractionator. This configuration facilitated a continuous supply of NB oxygenated water throughout the RAS. Water flow rates were balanced across influent lines to experiment tanks to maintain similar total flow rates (~20 L min^{-1}) to the Control group while also maintaining target DO levels. As Trials 2 and 3 also required abiotic manipulation (temperature or salinity), two NB injector systems were set up for each abiotic treatment within a trial.

NB injectors were supplied by Nano Bubble Technologies Pty Ltd (NBT), South Granville, Sydney NSW. NBT injectors are a membrane style device and utilise NBT patented technology with gas passing through a membrane facilitating shearing of ultrafine NB's of gas into the water column. Trial 1 utilised a single cell injector, Trials 2 and 3 utilised a triple cell injector.

Oxygen gas flow rates to injectors were adjusted from <0.1 L min^{-1} (start of trial) to ~0.25 L min^{-1} (end of trial) during Trial 1 to maintain adequate DO in experiment tanks as biomass and feeding rates increased. The DO saturation in the saturation tank over the course of the trial was ~220%. Gas flow rates in Trials 2 and 3 were set at ~0.1 L min^{-1} maintaining saturation at ~300% in the saturation tanks. The difference in gas

flow rates between experiments was due to i) higher stocking density and higher energy demand species in Trial 1, ii) both NB density treatments in Trial 1 were supplied from a single saturation tank (i.e. supplying six experiment tanks compared to three tanks each in Trials 2 and 3), and iii) the use of more efficient triple cell NB injectors in the Trials 2 and 3.

Figure 1. Configuration of NB injected saturation tank. Arrows indicate direction of water flow. A) 200 L saturation tank, B) NB injector, C) RAS and experiment tanks supply pump of NB saturated water, D) water top-up recirculation line from RAS.

Nanobubble assessment

NB size has been previously demonstrated using NBT injectors similar to the type used in the current study (Kazeem et al., 2022a; Kazeem et al., 2022b). A preliminary assessment of NB particle size and concentration was also undertaken of water samples from the systems used in the aquaculture trials. Nanoparticle analyses was conducted at the Flow Cytometry Facility at the University of NSW with a NanoSight NS300 using Nanoparticle Tracking Analysis (NTA) particle characterization technology (Ke et al., 2019; Zhu et al., 2016) using the freeze-thaw method where nanoparticles are measured before freezing and nanobubbles estimated by difference after the elimination of bulk nanobubbles following the freeze–thaw process (Nirmalkar et al., 2018, 2019; Yasui et al., 2023). These data are presented in Appendix 1.

Biometric indices

The following biometric indices were calculated based on the mean data of all fish in each replicate tank.

- Initial weight (g fish^{-1}) = initial weight at stocking
- Final weight (g fish^{-1}) = final weight at harvest

- Initial length (mm $fish^{-1}$) = total length at stocking
- Final length (mm $fish^{-1}$) = total length at harvest
- Condition factor (*k*) = 10^5 x (final weight / total $length^3$)
- Feed intake (g $fish^{-1}$) = total dry matter feed intake
- Biomass gain (g) = final biomass per tank – initial biomass per tank
- Specific growth rate (SGR % $fish^{-1}$ day^{-1}) = [ln(final weight) – ln(initial weight)]/days x 100
- Feed conversion ratio (FCR) = total dry matter feed intake / weight gain

Carcass composition

Carcass composition of a subsampled number of fish from Trial 1 (Yellowtail Kingfish; n=5) and Trial 2 (Barramundi; n=5) were analysed at the conclusion of each trial. Proximate (dry matter, protein, fat, ash) and gross energy whole carcass composition was determined. Sample processing for whole carcass composition involved placing weighed subsampled fish into double bagged autoclave bags and then autoclaving for 99 min at 121°C to facilitate homogenisation. After cooling to room temperature any changes in weight were accounted for and the moisture value adjusted accordingly. The autoclaved samples were then transferred to a Robot-Coupe Blixer® 5 food processor and homogenised. A subsample (approximately 2 g) of the homogenate was used to determine whole carcass dry matter composition. A further subsample (approximately 20 g) of the wet homogenate was then stored frozen pending further chemical analyses.

Protein was calculated from total nitrogen based on N x 6.25 using the Dumas method. Dry matter was calculated gravimetrically after oven drying at 105°C. Ash was calculated gravimetrically after incineration at 550°C for 2 h. Fat was measured gravimetrically after hexane soxtec extraction. Gross energy was determined by bomb calorimetry. Carbohydrate (Nitrogen Free Extract; NFE) was calculated by difference as: 100 – (protein + fat + ash). Chemical analyses were conducted by NSW DPI EnviroAg Testing Services, Wagga Wagga, NSW. Gross energy analysis was subcontracted to ALS Global, Wetherill Park NSW.

Histology

Histology was conducted on sampled fish at the conclusion of Trials 1 and 2. Tissue samples (n=3) of fins, eyes, gills and heart collected for histological analyses were sampled after fish were euthanised with an overdose of Aqui-S. Fish approximating average size were sampled haphazardly from each replicate tank. Tissues were fixed in 10% buffered formalin. Histological slide preparation and examination was conducted by EMAI Veterinary Pathology Service, Menangle NSW.

Haematology

Blood sampling was conducted on Barramundi at the conclusion of Trial 3. Fish were anaesthetised with 30ppm Aqui-S and one fish of approximate average size was haphazardly selected from each replicate tank and transferred to a trough-shaped flow-through chamber for blood collection. The flow through chamber was 900x400x210mm and water was continuously recirculated from a 200 L reservoir tank via a small submersible pump at ~8 L min^{-1} containing Aqui-S anaesthetic and oxygenated at ~120% saturation. Fish were held in an inverted position to sample from the caudal vein and orientated into the water flow to permit continual ventilation with oxygenated water (Figure 2). DO was monitored continuously in the flow through chamber and adjusted as necessary. 500µl of blood was extracted from the caudal vein using a 19G needle and transferred immediately to EDTA tubes.

Haematology was performed by NSW Health Pathology at the John Hunter Hospital, NSW. Blood was examined for white blood cell count (WBC), red blood cell count (RBC) haematocrit (HCT), mean corpuscular volume (MCV), red blood cell distribution width (RDW), haemoglobin (HGB), mean corpuscular haemoglobin (MCH) mean corpuscular haemoglobin concentration (MCHC), neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EOS), basophils (BASO), platelet count (PLT), mean platelet volume (MPV).

Figure 2. Flow-through blood sampling chamber. Sedated fish were orientated upside-down and head-first into the water flow direction to ensure continual and adequate oxygenation. DO probe (top-left corner of chamber) continuously monitored DO and temperature.

Heterotrophic bacteria

Colony forming units (CFU's) were determined at the conclusion of Trial 3. Water (~15ml) was sampled from i) the outlet of each fractionator unit and ii) a sample of tank water within each RAS (i.e. control 20°C, NB 20°C, control 30°C, NB 30°C) and a count of viable heterotrophic bacteria performed. Appropriate dilution factors to perform viable CFU counts were based on the results of a pilot trial where appropriate fractionator wastewater dilution was determined to be to 10^3/mL and the tank water was undiluted (10^0/ml). Heterotrophic bacteria were cultured on Marine Agar (BD Difco™ 2216) plates in triplicate, after dilutions in 34 g/L sterile sodium chloride solution where relevant and incubated for 10 days at 25°C before final CFU counts were undertaken.

Data Analyses

The effect of oxygenation system (Control vs NB) and aquaculture treatment (Stocking Density, or Salinity, or Temperature) on the performance of fish was analysed using two-way ANOVA considering oxygenation system and aquaculture treatment as orthogonal fixed factors. The level of probability for which ANOVA was considered significant was α=0.05. Differences among significant treatment means were determined using Tukey's HSD post-hoc test, or Fishers LSD when Tukey's returned non-significant results. Data were statistically analysed using NCSS 2022 v.22.0.3. Comparison of nitrification rates expressed as TAN were analysed using repeated measures ANOVA.

Experiment Design

Each trial was conducted as a two-factor orthogonal design where:

Factor 1 = Oxygenation system; NB oxygenation vs Standard oxygenation (i.e. a mixture of compressed air and oxygen gas), hereafter termed "control" regime.

Each of the three trials examined a different second factor: stocking density, salinity or temperature

Replication for each trial was n=3 x 500 L cylindroconical tanks per treatment (i.e. 12 tanks in total).

Trial 1 - Stocking Density

Factor 2: Stocking density; High stocking density (50 fish $tank^{-1}$) vs Low stocking density (10 fish $tank^{-1}$).

Fish: Juvenile Yellowtail Kingfish *Seriola lalandi* (initial body weight ~52 g $fish^{-1}$)

Trial duration: 42 days

Variables assessed:

- Biometric indices
- Carcass composition
- Histology

Average water quality values throughout Trial 1 are presented in Table 2. Average DO concentrations are presented in Table 3.

Table 2. Trial 1 (Stocking Density; Yellowtail Kingfish) average RAS water quality (±SD) in both NB and standard oxygen regimes over 42 days.

System		pH	Temperature (°C)	Salinity (‰)	TAN (mg L^{-1})
Nanobubble	**Avg**	7.75	22.30	28.82	1.18
	SD	0.24	0.72	2.29	0.54
Control	**Avg**	7.86	22.56	28.86	1.39
	SD	0.19	0.38	2.20	0.83

Table 3. Trial 1 (Stocking Density; Yellowtail Kingfish). Average DO saturation (%) and concentration (mg/L) of individual experiment tanks over 42 days.

Tank#	System	Density	DO (% sat)	DO (mg L^{-1})
1	Nanobubble	High	116.0	8.51
2	Nanobubble	Low	115.1	8.45
3	Nanobubble	High	116.3	8.54
4	Nanobubble	Low	114.6	8.41
5	Nanobubble	Low	115.0	8.45
6	Nanobubble	High	116.0	8.52
7	Control	Low	113.5	8.33
8	Control	High	115.2	8.46
9	Control	High	113.6	8.34
10	Control	Low	114.0	8.37
11	Control	Low	115.8	8.50
12	Control	High	116.1	8.52

Trial 2 - Salinity

Factor 2: Salinity; SW (32.0 ppt) vs FW (bore water; 0.36 ppt).

Fish: Barramundi *Lates calcarifer* (initial weight 103 g fish^{-1}), 15 fish tank^{-1}

Trial duration: 42 days

Variables assessed:

- Biometric indices
- Carcass composition
- Histology

Average water quality values throughout Trial 2 are presented in **Table 4**. Average DO concentrations are presented in Table 5.

Table 4. Trial 2 (Salinity; Barramundi). Average RAS water quality (±SD) in both NB and standard oxygen regimes over 42 days. TAN results annotated with the same superscript letter are not significantly different (p>.05).

System	Salinity		Temperature (°C)	pH	Salinity (‰)	TAN (mg L^{-1})
Control	FW	Avg	28.22	7.45	0.36	0.97^{a}
		SD	0.45	0.23	0.06	0.63
	SW	Avg	28.26	8.63	32.00	0.68^{b}
		SD	0.20	0.09	2.18	0.76
Nanobubble	FW	Avg	28.21	7.48	0.35	1.16^{a}
		SD	0.19	0.25	0.01	0.65
	SW	Avg	28.57	8.49	31.93	0.40^{b}
		SD	0.29	0.15	2.16	0.54

Table 5. Trial 2 (Salinity; Barramundi). Average DO saturation (%) and concentration (mg/L) of individual experiment tanks over 42 days.

Experiment Tank	System	Salinity	DO (% sat)	DO (mg L^{-1})
1	Control	FW	120.95	9.41
2	Control	FW	123.01	9.57
3	Control	FW	122.62	9.54
4	Control	SW	122.55	7.99
5	Control	SW	123.77	8.07
6	Control	SW	121.93	7.95
7	Nanobubble	FW	120.82	9.40
8	Nanobubble	FW	124.42	9.68
9	Nanobubble	FW	124.16	9.66
10	Nanobubble	SW	122.65	7.96
11	Nanobubble	SW	122.34	7.94
12	Nanobubble	SW	125.27	8.13

Trial 3 - Temperature

Factor 2: Temperature; 30°C vs 20°C.

Fish: Barramundi (initial body weight 360 g fish^{-1}), 10 fish tank^{-1}

Trial duration: 45 days

Variables assessed:

- Biometric indices
- Haematology
- CFU's

Average water quality values throughout Trial 5 are presented in **Table 6**. Average DO concentrations are presented in **Table 7**.

Table 6. Trial 3 (Temperature; Barramundi). Average RAS water quality (±SD) in both NB and standard oxygen regimes over 45 days.

System		Temperature (°C)	pH	Salinity (‰)	TAN (mg L^{-1})
Control	Avg	20.42	8.02	33.36	0.22
	SD	0.03	0.08	1.88	0.22
	Avg	30.12	8.00	33.50	0.20
	SD	0.06	0.05	1.97	0.29
Nanobubble	Avg	20.43	8.01	33.40	0.20
	SD	0.04	0.08	1.89	0.18
	Avg	30.25	8.03	33.49	0.22
	SD	0.01	0.06	2.00	0.35

Table 7. Trial 3 (Temperature; Barramundi). Average DO saturation (%) and concentration (mg/L) of individual experiment tanks over 45 days.

Experiment Tank	System	Temperature (°C)	DO (% sat)	DO (mg L^{-1})
1	Control	20	120.79	8.95
2	Control	20	119.99	8.89
3	Control	20	120.66	8.94
4	Control	30	119.85	7.53
5	Control	30	119.07	7.48
6	Control	30	120.88	7.59
7	Nanobubble	20	119.76	8.87
8	Nanobubble	20	119.97	8.89
9	Nanobubble	20	120.89	8.96
10	Nanobubble	30	121.80	7.62
11	Nanobubble	30	121.50	7.61
12	Nanobubble	30	121.67	7.62

Results

Trial 1 – Stocking Density

There was 100% survival of all fish in all tanks after six weeks. Fish grew rapidly increasing in body weight by >350%. Statistical analyses (two-way ANOVA) indicated no differences between oxygen systems for all parameters tested nor any density interactions (Table 1). Significant differences were found only between stocking densities when considering individual final weight and FCR with Yellowtail Kingfish stocked at low densities performing relatively better than those stocked at high densities after 42 days (Table 8). The relative decrease in feed intake after three weeks indicates Yellowtail Kingfish stocked at the highest density had exceeded an optimal biomass (Figure 3). Fish harvested at the conclusion of the trial were in very good condition with clear eyes, fins and skin and no obvious signs of pathology (Figure 4).

Table 8. Trial 1 (Stocking Density; Yellowtail Kingfish). Average performance data of Yellowtail Kingfish exposed to either NB or standard (Control) oxygenation at high or low stocking densities after 42 days. Treatments annotated with the same superscript letter are not significantly different (p>.05). Density treatment groups: High = High stocking density (50 x 52 g fish tank-1, Low = Low stocking density (10 x 52 g fish tank-1. Two-way ANOVA level of significance represented as P>0.05 (NS), P <0.05 (*), P <0.01 (**), P < 0.001(***).

	Control		**Nanobubble**			**ANOVA**		
Parameter	**Low**	**High**	**Low**	**High**	**Pooled SEM**	**A: O_2 System**	**B: Density**	**AxB**
Initial Weight (g fish^{-1})	52.6	52.2	52.5	52.7	0.19	NS	NS	NS
Initial Density (kg m^{-3})	1.05^{a}	5.22^{b}	1.05^{a}	5.27^{b}	0.01	NS	***	NS
Initial K	1.21	1.23	1.20	1.21	0.01	NS	NS	NS
Survival (%)	100	100	100	100	-	-	-	-
Final Body Weight (g fish^{-1})	254.4^{b}	239.6^{a}	247.2^{a,b}	238.8^{a}	2.45	NS	**	NS
Final Density (kg m^{-3})	5.09^{a}	23.96^{b}	4.94^{a}	23.88^{b}	0.11	NS	***	NS
Final K	1.43	1.39	1.40	1.39	0.02	NS	NS	NS
Gain (g fish^{-1})	201.8^{a}	187.3^{b}	194.8^{a,b}	186.1^{a}	2.54	NS	*	NS
Gain (% fish^{-1})	383.4^{b}	358.7^{a,b}	371.3^{a,b}	353.0^{a}	0.06	NS	**	NS
SGR (% day^{-1})	3.75^{b}	3.63^{a,b}	3.69^{a,b}	3.60^{a}	0.03	NS	**	NS
Feed intake (g DM fish^{-1})	229.9	224.8	224.6	221.3	3.07	NS	NS	NS
FCR (DM basis)	1.14^{a}	1.20^{b}	1.15^{a,b}	1.19^{a,b}	0.02	NS	*	NS

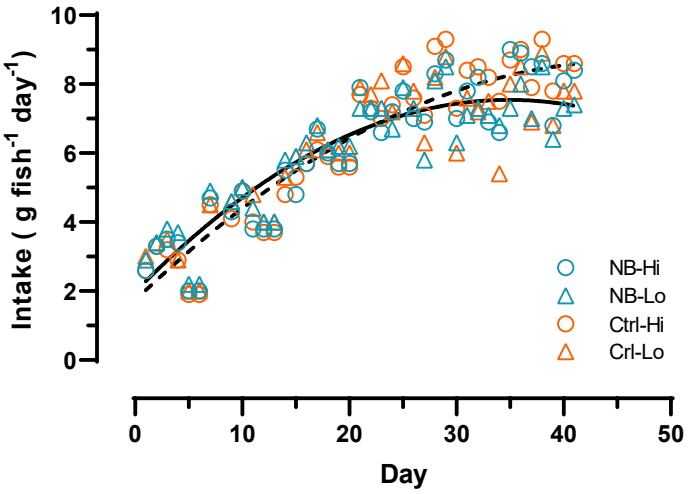


Figure 3. Trial 1 (Stocking Density; Yellowtail Kingfish). Daily feed intake of Yellowtail Kingfish exposed to either NB (blue) or control (orange) oxygenation regimes at high (circles; 50 fish tank-1) or low (triangles; 10 tank-1) stocking densities. Regression lines were pooled across oxygen regimes; solid line = high stocking density, dashed line = low stocking density.

Carcass composition

There were statistically significant differences between the control density groups when considering protein composition; however, proportionally this difference was relatively small at 1.3% (66.7% vs 68%) (**Table 9**). There were system differences when considering NFE and GE composition with 0.9% between NFE composition and 2.1 MJ kg^{-1} difference for GE composition.

Table 9. Trial 1 (Stocking Density; Yellowtail Kingfish). Average proximate whole carcass composition of Yellowtail Kingfish grown for 42 days at 22°C. Treatments annotated with the same superscript letter are not significantly different (p>.05). Density treatment groups: High = High stocking density (50 x 52 g fish tank-1, Low = Low stocking density (10 x 52 g fish tank-1. Two-way ANOVA level of significance represented as P>0.05 (NS), P <0.05 (*), P <0.01 (**).

	Control		Nanobubble			ANOVA		
Composition	**Low**	**High**	**Low**	**High**	**Pooled SEM**	**A:O_2 System**	**B: Density**	**AXB**
Protein	68.0^{b}	66.7^{a}	$67.3^{a,b}$	$67.1^{a,b}$	0.26	NS	*	NS
Fat	19.3	20.8	20.9	20.5	0.39	NS	NS	NS
Ash	10.4	10.7	10.7	11.1	0.17	NS	NS	NS
NFE	2.3^{b}	$1.8^{a,b}$	1.1^{a}	1.2^{a}	0.18	**	NS	NS
GE	$23.8^{a,b}$	24.0^{b}	$22.9^{a,b}$	20.6^{a}	0.56	*	NS	NS

Histology

No significant findings were reported on any of the sampled Yellowtail Kingfish tissue (gill, heart, fin and eye) for either of the oxygenation treatments (Control vs NB) or density treatments (low vs high).

Figure 4. Typical example of Yellowtail Kingfish (~250g) exposed to NB oxygenated water for 42 days. Note clear eyes, fins and skin and no visual indication of any obvious pathology.

TAN

There were no statistical differences (p=0.2) found between aeration systems when considering TAN nitrification. Mean TAN (mg L^{-1} day^{-1} ±SD) across both systems was 1.28±0.70.

Trial 2 – Salinity

There was 100% survival of all fish in all tanks after six weeks. Fish grew rapidly increasing their body weight by >210%, with growth of Barramundi in all treatment groups better than that of published data for this species (Figure 5). Statistical analyses (two-way ANOVA) indicated no differences between oxygen systems for all parameters tested; however, there was a significant interaction when considering FCR (Table 10). Significant differences were found between salinities when considering individual growth metrics, but not feed intake (Table 10) with Barramundi grown in SW performing relatively better than those grown in FW after 42 days (Figure 5, Table 10). FW was bore water pumped directly at PSFI, consequently the clarity of the bore water was low in comparison to SW (Figure 6).

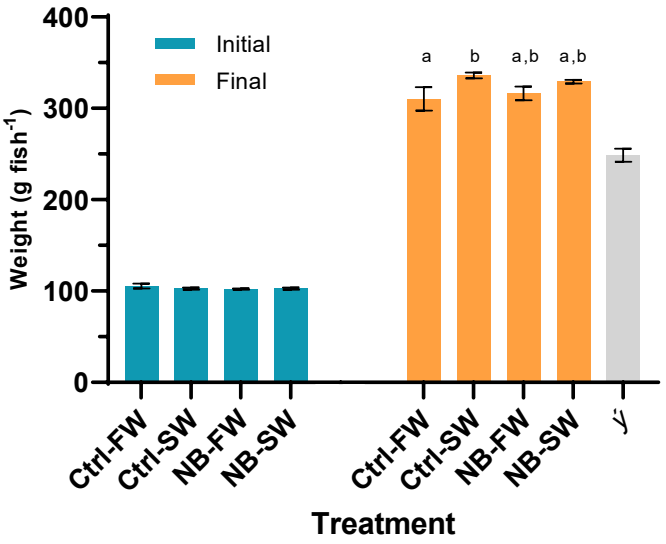


Figure 5. Trial 2 (Salinity; Barramundi). Average weight of Barramundi (g fish^{-1}; ±SE) after 6 weeks. Treatments annotated with the same superscript letter are not significantly different (p>.05). Ctrl = Control, NB = Nanobubble, FW = Freshwater, SW = Saltwater. Predicted (ý) body weight (±5%) also shown for comparison: Barramundi growth (g fish^{-1} day^{-1}) = (2.2495-0.3275T+0.0150T2-0.0002T3)*(BW)-0.0095T+0.72 where T = temperature and BW = body weight (g) (adapted from Glencross and Bermudes, 2012).

Figure 6. Barramundi in saltwater NB tank (L) and freshwater (bore water) control tank (R).

Table 10. Trial 2 (Salinity; Barramundi). Average growth and feed intake metrics of Barramundi grown for 42 days at 28°C. Treatments annotated with the same superscript letter are not significantly different (p>.05). Salinity treatment groups: FW = freshwater, SW = saltwater. Two-way ANOVA level of significance represented as P>0.05 (NS), P <0.05 (*), P <0.01 (**).

	Control		Nanobubble			ANOVA		
Parameter	**FW**	**SW**	**FW**	**SW**	**Pooled SEM**	**A: O_2 System**	**B: Salinity**	**AXB**
Initial Weight (g fish^{-1})	105.4	102.8	102.2	102.7	1.38	NS	NS	NS
Initial Biomass (kg tank^{-1})	1.581	1.542	1.533	1.540	0.02	NS	NS	NS
Initial K	1.2	1.2	1.2	1.21	0.01	NS	NS	NS
Survival (%)	100	100	100	100	-	NS	NS	NS
Final Weight (g fish^{-1})	310.2^{a}	336.0^{b}	316.2^{a,b}	329.0^{a,b}	6.5	NS	*	NS
Final Biomass (kg tank^{-1})	4.652^{a}	4.885^{b}	4.688^{a,b}	4.743^{a,b}	0.10	NS	*	NS
Final K	1.46	1.46	1.49	1.45	0.01	NS	NS	NS
Gain (g fish^{-1})	204.8^{a}	233.3^{b}	214^{a,b}	226.4^{a,b}	5.5	NS	*	NS
Gain (% fish^{-1})	194.3^{a}	209.9^{b}	194.8^{a,b}	209.3^{b}	4.8	NS	**	NS
SGR (% day^{-1})	2.57^{a}	2.69^{b}	2.57^{a,b}	2.69^{a,b}	0.04	NS	**	NS
Feed intake (g DM fish^{-1})	188.4	199	190.4	203.1	5.2	NS	NS	NS
FCR (DM basis)	0.92^{b}	0.85^{a}	0.89^{a,b}	0.90^{a,b}	0.01	NS	*	*

Carcass Composition

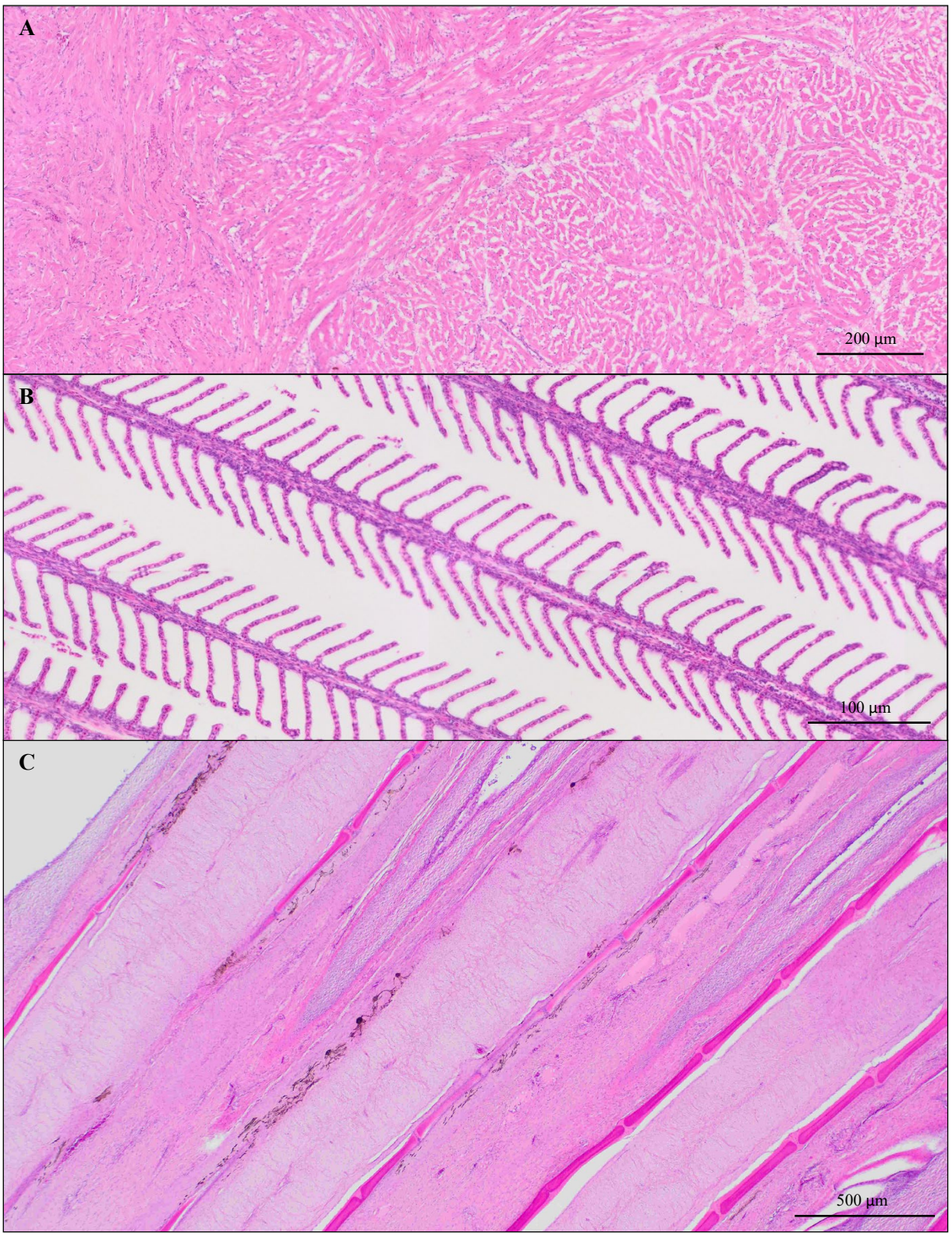
There were no statistically significant differences between the control density groups when considering protein composition for any of the variables tested (Table 11). Average proximate composition of Barramundi was protein = 53.3%, fat = 33.8, ash = 15.1%, and Gros energy = 25.0 MJ kg^{-1}. The small negative values for NFE are likely an artefact of calculating NFE by difference, i.e. NFE = 100 – (protein + fat + ash).

Table 11. Trial 2 (Salinity; Barramundi). Average proximate whole carcass composition of Barramundi grown for 42 days at 28°C. Treatments annotated with the same superscript letter are not significantly different (p>.05). Density treatment groups: FW= Freshwater, SW = Saltwater. Two-way ANOVA level of significance represented as P>0.05 (NS), P <0.05 (*), P <0.01 (**).

System	**Control**		**Nanobubble**			**ANOVA**		
Salinity	**FW**	**SW**	**FW**	**SW**	**Pooled SEM**	**A:O2 System**	**B: Salinity**	**AXB**
Protein	52.9	54.5	54.8	53.3	1.04	NS	NS	NS
Fat	33.1	33.2	30.8	33.8	0.86	NS	NS	NS
Ash	12.7	14.2	13.6	15.1	1.16	NS	NS	NS
NFE	1.4	-1.9	0.7	-2.2	1.41	NS	NS	NS
GE	25.2	25.0	24.8	25.1	0.35	NS	NS	NS

Histology

No significant abnormalities were identified in any of the tissues examined (gill, heart, fin and eye) for either of the oxygenation treatments (Control vs NB) or salinity treatments (FW vs SW). Histological images of barramundi grown in NB water are presented in (Figure 7).



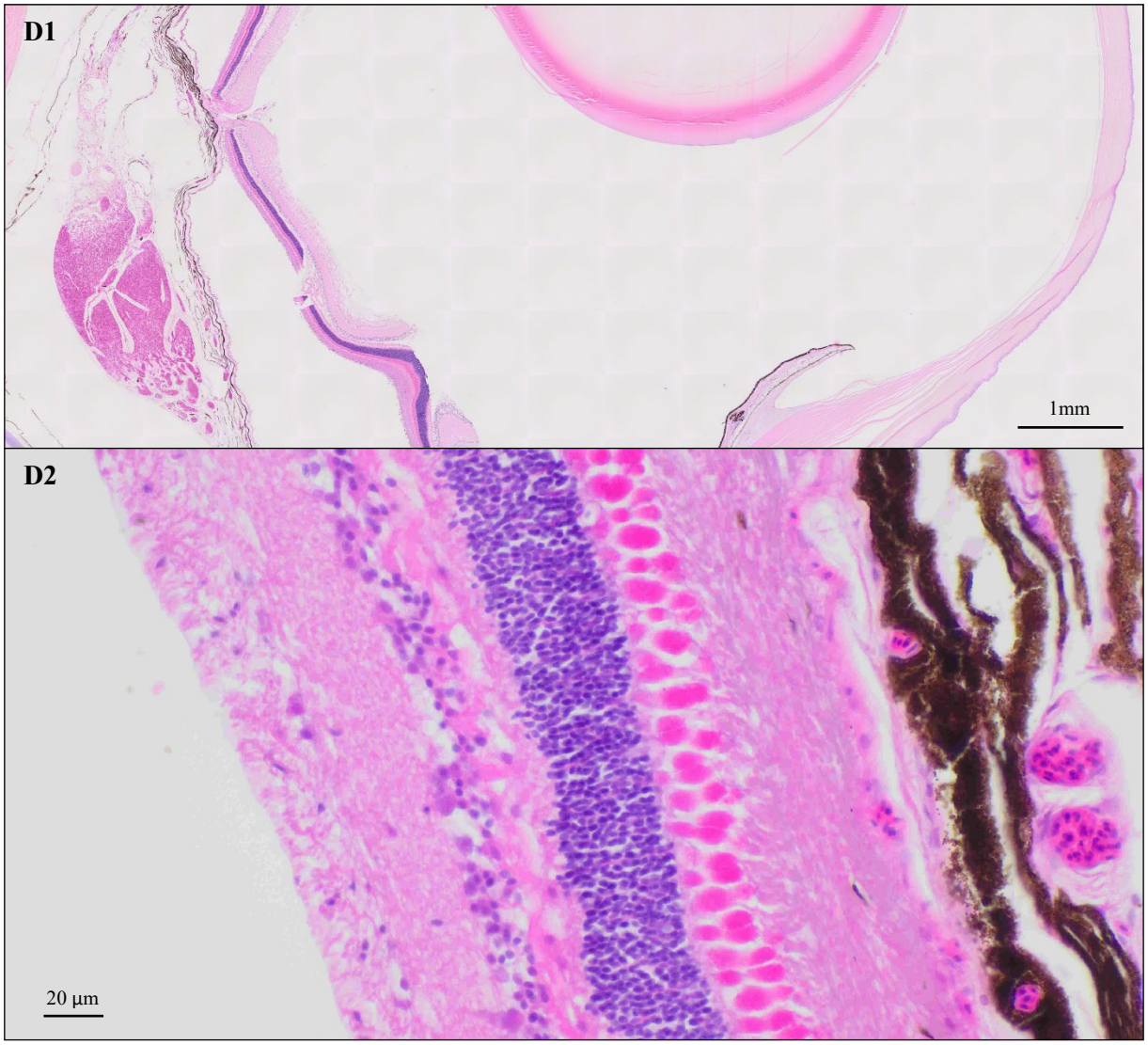


Figure 7. Trial 2 (Salinity; Barramundi). Histology of barramundi grown in water oxygenated using nanobubble injectors. A) Heart (myocardium), B) Gill (lamellae), C) Fin, D1) Eye, D2) Eye (retina).

TAN

Significant differences were found between salinity treatment ($p<0.000001$) but not between aeration system ($p=0.36$), and there was no significant interaction ($p=0.08$) when considering TAN nitrification among treatments (Table 4). Mean (mg L^{-1} day^{-1} ±SD) TAN in FW (1.09±0.66) was significantly higher compared to SW (0.54±0.67).

Trial 3 – Temperature

There was 100% survival of all fish in all tanks after 45 days. Cool water fish increased in body weight on average by 34.7% and warm water fish by 92.4%. Growth of Barramundi in all treatment groups were better than that of published data for this species (Figure 8). Statistical analyses indicated no significant differences between oxygen systems nor interactions with temperature for all parameters tested. Significant differences were found between temperature treatments when considering all individual growth metrics (Table 12) with Barramundi grown at 30°C typically performing significantly better than those grown at 20°C after 45 days. The condition (*K*) of Barramundi grown at 20°C was significantly greater by, on average, 6.9% than those grown at 30°C (Table 12).

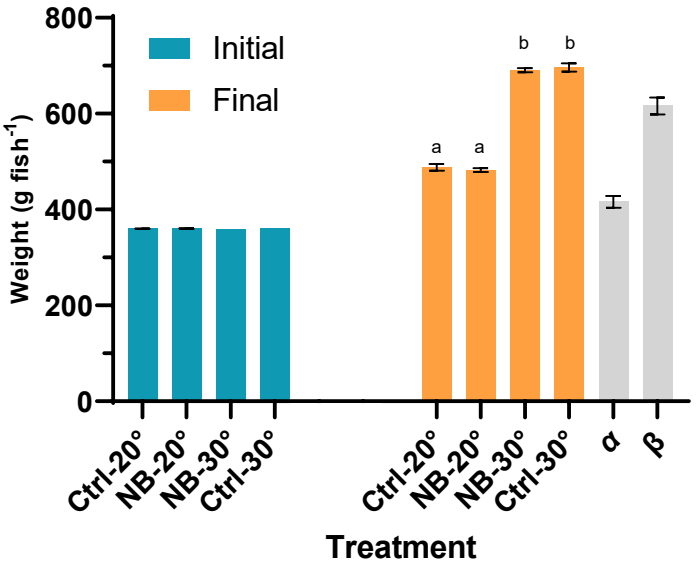


Figure 8. Trial 3 (Temperature; Barramundi). Average weight of Barramundi (g fish^{-1}; ±SE) after 45 days. Treatments annotated with the same superscript letter are not significantly different (p>.05). Ctrl = Control, NB = Nanobubble at 20 or 30°C. Predicted body weight (±5%) at 20°C (α) and 30°C (β) also shown for comparison: Barramundi growth (g fish^{-1} day^{-1}) = (2.2495-0.3275T+0.0150T2-0.0002T3)*(BW)-0.0095T+0.72 where T = temperature and BW = body weight (g) (adapted from Glencross and Bermudes, 2012).

Table 12. Trial 3 (Temperature; Barramundi). Average growth and feed intake metrics of Barramundi (n=3) grown for 45 days in saltwater. Treatments annotated with the same superscript letter within rows are not significantly different (p>.05). Temperature treatment groups: 20°C and 30°C. Two-way ANOVA level of significance represented as P>0.05 (NS), P <0.05 (*), P <0.01 (**), P <0.001 (***).

	Control		Nanobubble			ANOVA		
Parameter	**20°C**	**30°C**	**20°C**	**30°C**	**Pooled SEM**	**A: O_2 System**	**B: Temperature**	**AXB**
Initial Weight (g $fish^{-1}$)	360.1	359.7	360.5	361.0	0.32	NS	NS	NS
Initial Biomass (kg $tank^{-1}$)	3.601	3.597	3.605	3.610	0.003	NS	NS	NS
Initial K	1.21	1.21	1.22	1.21	0.01	NS	NS	NS
Survival (%)	100	100	100	100	-	-	-	-
Final Weight (g $fish^{-1}$)	488.2^{a}	690.4^{b}	482.2^{a}	696.1^{b}	6.05	NS	***	NS
Final Biomass (kg $tank^{-1}$)	4.882^{a}	6.904^{b}	4.822^{a}	6.961^{b}	0.06	NS	***	NS
Final K	1.52^{a}	1.41^{b}	1.51^{a}	1.41^{b}	0.02	NS	**	NS
Gain (g $fish^{-1}$)	128.0^{a}	330.6^{b}	121.7^{a}	335.0^{b}	5.88	NS	***	NS
Gain (% $fish^{-1}$)	35.6^{a}	91.9^{b}	33.8^{a}	92.8^{b}	1.62	NS	***	NS
SGR (% day^{-1})	0.68^{a}	1.45^{b}	0.65^{a}	1.46^{b}	0.02	NS	***	NS
Feed intake (g DM $fish^{-1}$)	175.5^{a}	370.0^{b}	175.1^{a}	373.0^{b}	5.83	NS	***	NS
FCR (DM basis)	1.37^{a}	1.12^{b}	1.44^{a}	1.11^{b}	0.02	NS	***	NS

Haematology

There was no significant effect of oxygenation system nor its interaction with temperature on any of the blood parameters assessed (Table 13). Temperature had a significant effect on RBC, HB and RDW with a general trend for a positive correlation with RBC and HB counts, and a negative correlation with RDW (Table 13).

Table 13. Trial 3 (Temperature; Barramundi). Haematological blood parameters of barramundi (n=3) grown in saltwater and exposed to NB or standard oxygenation regimes at 20 or 30°C after 45 days. Treatments annotated with the same superscript letter within columns are not significantly different (p>.05). Two-way ANOVA level of significance represented as P>0.05 (NS), P <0.05 (*), P <0.01 (**). Post-hoc multiple comparison tests on RBC and Hb was conducted with Fishers LSD as Tukey's returned non-significant results.

System	Temp.	WBC (10^9 L^{-1})	RBC (10^{12} L^{-1})	HB (g L^{-1})	HCT (L L^{-1})	MCV (fl)	MCH (pg)	MCHC (g L^{-1})	RDW (%)	PLT (10^9 L^{-1})	MPV (fl)	NEUT (10^9 L^{-1})	LYMPH (10^9 L^{-1})	MONO (10^9 L^{-1})	EOS (10^9 L^{-1})	BASO (10^9 L^{-1})
Control	20°C	70.6	$3.3^{a,b}$	86.3^{a}	0.37	110.3	26.0	236.0	$25.6^{a,b}$	2.3	10.4	2.1	63.2	0.00)	5.3	0.0
	30°C	150.8	4.6^{c}	117.7^{b}	0.47	104.0	26.0	249.3	12.7a	12.0	7.4	5.7	103.8	0.43	40.8	0.0
NB	20°C	105.4	3.0^{a}	81.3^{a}	0.45	148.3	26.7	185.3	33.2^{b}	16.7	7.3	3.6	99.0	0.50	2.3	0.0
	30°C	89.2	$4.4^{b,c}$	$92.7^{a,b}$	0.50	114.0	21.7	202.3	$19.0^{a,b}$	11.7	9.3	5.4	63.9	1.63	18.2	0.0
	Pooled SEM	20.2	0.3	7.7	0.06	8.7	1.2	21.8	2.7	5.2	1.0	2.6	20.7	0.57	12.6	0.0
ANOVA	A: System	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-
	B: Temp	NS	**	**	NS	NS	NS	NS	**	NS	NS	NS	NS	NS	NS	-
	AXB	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-

TAN

There were no statistical differences found between aeration systems (p=1.0) or temperature (p=1.0), nor was there a significant interaction (p=0.56) when considering TAN nitrification. Mean TAN (mg L^{-1} day^{-1} ±SD) across all systems and treatment groups was 0.21±0.26.

Heterotrophic bacterial counts (CFU's)

There was no significant effect of oxygenation system on CFU's sampled from either the foam fractionator waste outlets or directly from the experiment tank water (Table 14). The 30°C treatment had significantly higher CFU's than the 20°C treatment and there was no interaction effect between O2 system and temperature (Table 14).

Table 14. Trial 3 (Temperature; Barramundi). Average concentration of heterotrophic bacteria (CFU/ml) from experiment tank water and foam fractionator outlet at two oxygenation methods (Control or NB) and two temperatures (20°C or 30°C) (n=3). Treatments annotated with the same superscript letter within rows are not significantly different (p>.05). Two-way ANOVA level of significance represented as P>0.05 (NS), P <0.05 (*), P <0.01 (**)., P <0.001 (***).

	Control		**Nanobubble**		**ANOVA**			
Sample	**20°C**	**30°C**	**20°C**	**30°C**	**A:O_2 System**	**B: Temp**	**AxB**	**Pooled SEM**
Tank (x10^0)	14.7	6.7	25.3	18.7	NS	NS	NS	7.2
Fractionator (x10^3)	11.7^a	19.7^{a,b}	8.7^a	40.7^b	NS	**	NS	4.2

Oxygen transfer efficiency and operating costs

Methods

Oxygen transfer rates were determined as per protocols outlined in ASCE (2022) and Boyd (1998). The oxygen transfer efficiencies of the triple tube NB injectors were assessed in 500 L (nominally) aquaculture tanks used in the above Trials 1-3. Oxygen transfer rates were measured at seven different O_2 gas flow rates: 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 or 1.5 L min^{-1}, and using two different capacity centrifugal pumps; a 0.53 kW Davey XF 171S or a 1.5 kW Lowara Com350/15 with each pump plumbed to a single 500 L tank (Figure 9). The measured water flowrates in the 500 L test tank setups were 147.2(±0.6) and 277.5(±3.0) L min^{-1} (±SD; n=3) for the 0.53 and 1.5 kW pumps, respectively. O_2 gas regulator pressure was 50 psi. The oxygen transfer tests were conducted in clean freshwater that was held at approximately 20°C using Schego Titanium Heaters (3x 300w). Deoxygenation was achieved by supplying nitrogen gas to the NB injectors until the DO was <1 mg L^{-1}. Once the start DO level was reached O_2 gas was then applied to the NB injectors and re-saturation rates recorded. This deoxygenation/re-saturation cycle was repeated for each gas flow treatment in triplicate. Total dissolved solids were always <120 mg L^{-1} and well below the 2000 mg L^{-1} TDS limits of a valid oxygen transfer test (ASCE, 2022), the same water was used for each measurement cycle within each pump/tank setup. DO measurements were taken every 10 sec using Firesting oxygen meters with two LDO sensors in each tank until saturation was reached. As the application of flow via pumping created water movement and turbulence within the tanks, a control deoxygenation/re-saturation cycle, i.e. without the supply of O_2 gas, was also conducted in duplicate to quantify the contribution of atmospheric oxygen to re-saturation when running the tests. This was determined to be very low at 0.043(±0.005) and 0.063(±0.006) mg O_2 L^{-1} min^{-1} for the 0.53 and 1.5 kW pumps respectively. Nonetheless, SOTR values were adjusted to account for the contribution of ambient atmospheric oxygenation.

A second test was conducted using a larger 5,000 L Polymaster aquaculture tank assessing the NB injector supplied with O_2 gas and operated with the 1.5 kW pump described above. The 5,000 L tanks had a central sump/drain at the base of a sloping (4.8°) floor with an approximate depth of 900 mm and a diameter of approximately 2700mm. Measurements were also taken using a standard ceramic stone diffuser (approximately 400 mm length and 50 mm Ø) which was also supplied with O_2 gas, and a standard sintered airstone (190 mm length and 40mm Ø) supplied with compressed air. All gas to each aeration/oxygenation apparatus was delivered at a single flow rate of 0.5 L min^{-1}. Measurements for the NB injector were repeated three times. The ceramic diffuser and airstone measurements were not replicated as these were done to mainly provide a general comparison of the oxygenation rates and efficiencies compared to the NB injector in the same tank system, and SOTR for these types of diffusers are already established (Boyd and Ahmad, 1987; Lawson, 1995; Tharp, 2020).

Figure 9. 500 L tank setup with NB injector and O_2 gas line attached, and 0.53 kW pump.

Oxygen transfer efficiency calculations

The DO deficit was determined as the difference between the saturated DO and the measured oxygen level (Boyd, 1998). The slope of ln (deficit) vs. time (h^{-1}) provided the oxygen transfer coefficient (K_La_T) at water temperature *T*, calculated as:

$$K_La_T = \frac{\ln[(C_S^* - C_1)/\ C_S^* - C_2]}{t_2 - t_1}$$

Where C_S* is the DO solubility (mg L^{-1}) at ambient barometric pressure and temperature, C_1 is the DO concentration (mg L^{-1}) at time t_1, and C_2 is the DO concentration at time t_2.

K_La_t can be standardised to 20°C (K_La_{20}) as (ASCE, 2022):

$$K_La_{20} = K_La_t\Theta^{(20-T)}$$

where Θ is the Arrhenius temperature correction coefficient (1.024).

The Standard Oxygen Transfer Rate (SOTR) was calculated as:

$$SOTR\ (kg\ O_2\ h^{-1}) = K_La_{20}\ C_{s20}\ V$$

where C_{s20} is the dissolved oxygen concentration (mg L^{-1}) at saturation at 20°C and standard pressure (760 mm Hg) and *V* is the volume of water in the tank (m^3).

The Standard Oxygen Transfer Efficiency (SOTE) was calculated as:

SOTE (%) = $\frac{SOTR}{W_{O2}}$ x 100

where W_{O2} is the mass flow rate of O_2 in the gas flow stream at 20°C (g L^{-1}). This was calculated for both types of O_2 gas (i.e. 99.5% purity industrial O_2 gas or 21% O_2 in air).

The Standard Aeration Efficiency (SAE) was calculated as:

SAE = SOTR / power input (kW)

where the power input was the total delivered power which was measured at 0.529 and 1.725 kW for the Davey XF 171S and the Lowara Com350 pumps, respectively.

A cost estimate to transfer 1 kg O_2 was then calculated using a price of \$0.507 kWh^{-1} which was based on the average default market offer (DMO) price for businesses from July 2023 according to the Australian Energy Regulator (AER https://www.aer.gov.au/). The DMO is the maximum retail price that can be charged and is used here for illustrative purposes, retail prices will vary depending on the electricity provider. O_2 gas price was estimated at \$1.92 m^{-3} (Air Liquide Australia), which is equivalent to \$1.44 kg O_2^{-1} at 20°C. Equipment establishment and/or rental costs were not considered in this analysis.

Data analyses

Statistical comparison (e.g. two-way ANOVA) of data sets for oxygen transfer efficiencies were not carried out as, by design, the large range in O_2 gas flow rates and difference in pump capacities would obviously illicit strongly significant interactions where the magnitude of the effect of O_2 gas flow rates on DO transfer efficiencies is dependent on the pump velocity. The main aim of this section was to establish values for O_2 gas transfer efficiencies. Non-linear regression analyses were conducted to establish the relationship between O_2 gas flow rates, SOTR, SAE, SOTE and pumping cost.

Results

500 L system

500 L tank system metrics and O_2 transfer efficiencies are presented in Table 15. Oxygenation rates from initial concentrations of <1.0 mg DO L^{-1} to saturation were linear ($p<0001$; $r^2>0.99$) for all O_2 gas flow rates and pump velocities. Re-saturation times ranged from approximately 12 minutes for the 0.53 kW pump at 0.1 L min^{-1} O_2 flow rate, to approximately 1.6 minutes for the 1.5 kW pump at 1.5 L min^{-1} O_2 flow.

SOTR values in the 500 L tanks ranged from 0.067 kg O_2 h^{-1} (at 0.1 L O_2 min^{-1} and 1.5 kW) to 0.440 kg O_2 h^{-1} (at 1.5 L O_2 min^{-1} and 1.5 kW) with the difference in magnitude of SOTR between the different pumps increasing with increasing O_2 gas flow (Figure 10, Table 15).

Modelling SOTR as a function of O_2 gas flow rates demonstrated a curvilinear response (Figure 10) where:

SOTR (kg O_2 h^{-1}) at 0.53kW = 0.2594(O_2 gas flow (L min^{-1}))$^{0.634}$ (r^2=0.985)

SOTR (kg O_2 h^{-1}) at 1.5kW = 0.3356(O_2 gas flow (L min^{-1}))$^{0.666}$ (r^2=0.983)

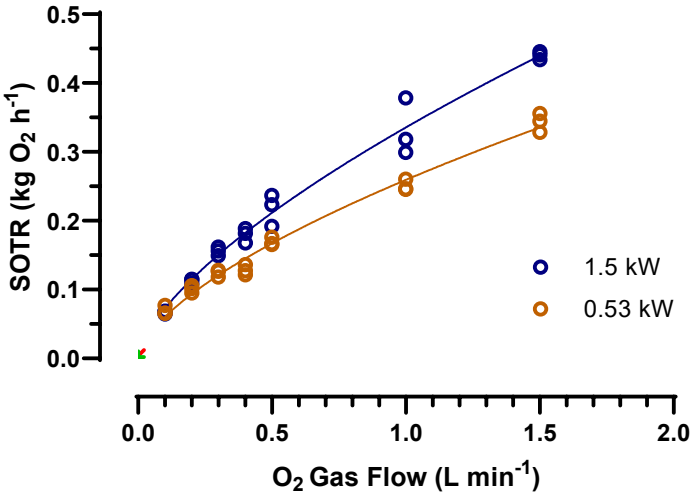


Figure 10. Relationship between adjusted (for ambient resaturation) SOTR and O2 gas flow relative to different pump capacities. 0.53 kW pump: SOTR = $0.2594x^{0.6343}$ (r^2=0.985), 1.5 kW pump: SOTR = $0.3356x^{0.666}$ (r^2=0.983). Ambient SOTR data points also shown for context; green "+" (0.0022 kg O_2 h^{-1}; 0.53 kW pump) and red "x" (0.0038 kg O_2 h^{-1}; 1.5 kW pump). Data are shown for SOTR established in 500 L aquaculture tanks.

SOTE values ranged from 17.4 - 54.2% with efficiencies between the different pumps very similar at the lowest gas flow rate of 0.1 L min^{-1}, although SOTE was generally higher for the 1.5 kW pump at all other O_2 flow rates (Figure 11, Table 15). There was an overall trend, regardless of the pump, for SOTE to decrease as O_2 gas flow rates increased (Figure 11, Table 15).

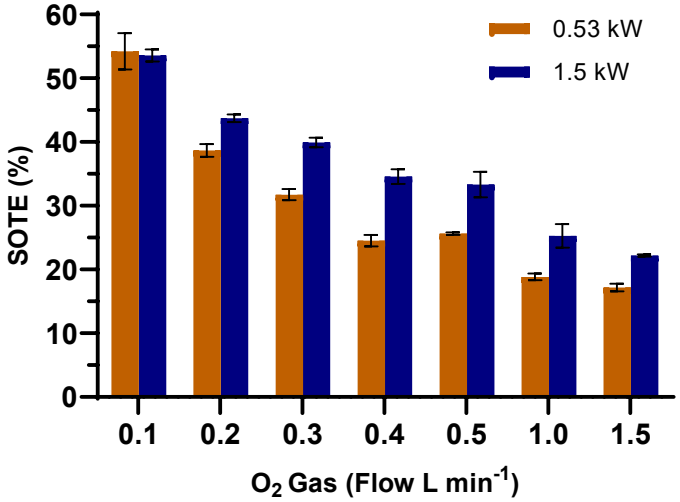


Figure 11. Standard oxygen transfer efficiencies (±SEM; n=3) for different capacity pumps and O2 flow rates

SAE values in the 500 L tanks ranged from 0.041 kg O_2 kW h^{-1} (at 0.1 L O_2 min^{-1} and 1.5 kW) to 0.652 kg O_2 kW h^{-1} (at 0.1 L O_2 min^{-1} and 0.53 kW) (Table 15).

As with increasing O_2 gas flow rate, SOTE deteriorated with increasing SAE (Figure 12). This relationship can be described as:

SOTE (%) at 0.53kW = $10.22(\text{SAE (kg } O_2 \text{ kW } h^{-1}))^{-0.8044}$ (r^2=0.866)

SOTE (%) at 1.5kW = $12.74(\text{SAE (kg } O_2 \text{ kW } h^{-1}))^{-0.4547}$ (r^2=0.917)

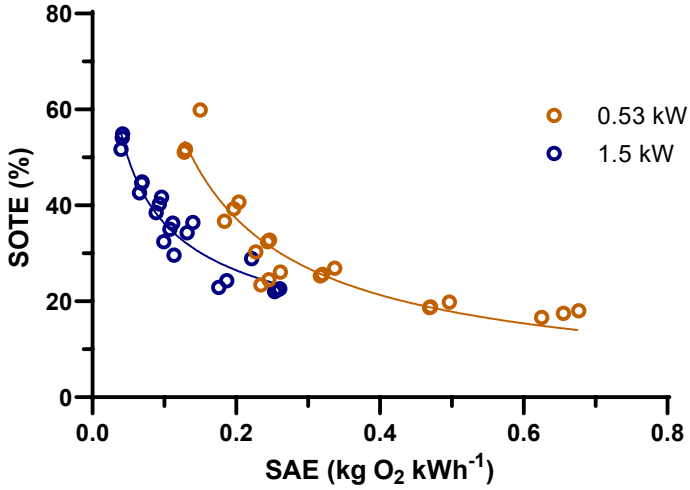


Figure 12. Relationship between SOTE and SAE.

While the adjusted SOTR values for each pump were similar when gas flow rates were 0.1 L O_2 min^{-1}, the pumping cost differed by 236%, ranging from \$3.87 kg O_2^{-1} for the 0.53 kW pump to \$13.03 kg O_2^{-1} for the 1.5 kW pump (Table 15). Generally, pumping costs (kWh) to transfer of 1 kg O_2 tended to decrease with increased O_2 gas flow (Table 15); however, when also factoring in the total O_2 gas used (i.e. SOTE) as part of the overall cost (pumping + O_2 gas) cost tended to decrease with increasing gas flow for the higher wattage pump, while cost tended to increase for the lower wattage pump (Figure 13) where the nexus (equivalent cost) occurred at a flow rate of 0.7766 L O_2 min^{-1}:

Total cost at 0.53kW = $8.465(O_2 \text{ gas flow (L } min^{-1}))^{0.1446}$ (r^2=0.767)

Total cost at 1.5kW = $7.618(O_2 \text{ gas flow (L } min^{-1}))^{-0.2724}$ (r^2=0.760)

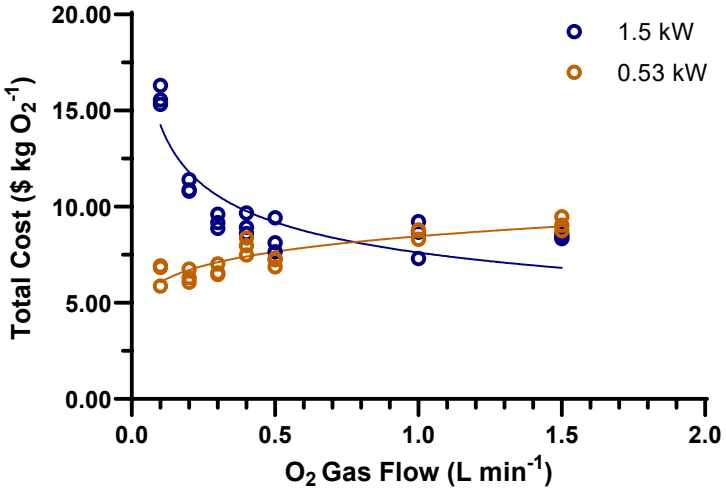


Figure 13. Total cost (pumping (kWh) + O_2 gas cost (transferred + lost) to transfer 1 kg DO into freshwater using two different wattage pumps. Nexus = 0.78 L min^{-1}. Data shown for values established in 500 L aquaculture tanks.

Table 15. Mean (n=3) NB injector system metrics in 500 L aquaculture tanks and O2 gas transfer rates. Price estimate based on \$0.507 kWh^{-1}

Pump Capacity (kW)	O_2 Flow Rate (L min^{-1})	Operating Temp. (°C)	Operating Atm. Press. (mbar)	Operating C_{sat}	K_La_T (h^{-1})	KLa_{20} (h^{-1})	SOTR (kg O_2 h^{-1})	SOTE (%)	SOTE (% m^{-1})	SAE (kg O_2 kW h^{-1})	Transfer Time (h kg O_2^{-1})	Pumping Cost[a] (kg O_2^{-1})	Total Cost[b] (kg O_2^{-1})
0.5	0.1	20.1	1027.3	9.20	0.306	18.289	0.070	54.2	77.5	0.132	14.45	\$3.87	\$6.55
0.5	0.2	20.4	1026.9	9.14	0.446	26.495	0.101	38.9	55.6	0.191	9.94	\$2.67	\$6.38
0.5	0.3	20.7	1026.9	9.09	0.546	32.220	0.124	31.9	45.5	0.235	8.05	\$2.16	\$6.69
0.5	0.4	20.6	1026.7	9.11	0.594	35.108	0.129	24.7	35.3	0.243	7.80	\$2.09	\$7.95
0.5	0.5	20.2	1022.8	9.15	0.732	43.751	0.170	26.0	37.1	0.321	5.90	\$1.58	\$7.14
0.5	1.0	20.3	1021.9	9.12	1.083	64.475	0.251	19.1	27.3	0.475	3.99	\$1.07	\$8.61
0.5	1.5	19.9	1020.7	9.08	1.442	86.650	0.343	17.4	24.8	0.648	2.92	\$0.78	\$9.09
1.5	0.1	21.2	1024.8	9.04	0.332	19.380	0.067	53.6	76.5	0.039	14.90	\$13.03	\$15.73
1.5	0.2	22.5	1024.5	8.85	0.532	30.102	0.113	44.0	62.9	0.065	8.87	\$7.75	\$11.03
1.5	0.3	22.3	1027.9	8.83	0.734	41.679	0.156	40.2	57.4	0.090	6.42	\$5.62	\$9.21
1.5	0.4	21.8	1024.3	8.93	0.871	50.052	0.179	34.6	49.4	0.104	5.59	\$4.89	\$9.07
1.5	0.5	21.6	1022.3	8.99	1.043	60.226	0.217	33.4	47.7	0.126	4.63	\$4.05	\$8.40
1.5	1.0	21.8	1023.6	8.86	1.524	87.533	0.332	25.4	36.2	0.192	3.04	\$2.66	\$8.40
1.5	1.5	22.7	1020.3	8.77	2.063	116.086	0.440	22.4	31.9	0.255	2.27	\$1.99	\$8.44
Pooled SEM		0.4	1.6	0.06	0.029	1.626	0.006	1.04	1.49	0.006	0.219	\$0.12	\$0.11

[a] Estimated at \$0.507 kWh^{-1}

[b] Total cost estimated as pumping cost + total O^2 gas used (at \$1.44 kg O_2^{-1}) to transfer 1 kg DO

5000 L system

The re-saturation response for both the NB injector and ceramic diffuser were strongly linear, while the response for the airstone was curvilinear (Figure 14). Times to re-saturation in the 5000 L tank were 26 min, 143 min, and 58.17 h for the NB injector, ceramic diffuser, and airstone respectively when gas flow rates were at 0.5 L min^{-1} (Figure 14).

SOTR (kg O2 h^{-1}) and SOTE values for the NB injector (n=3; mean±SEM) (O_2 gas), ceramic diffuser (O_2 gas), and airstone (compressed air) were 0.342±0.01 and 51.7±0.8 %, 0.050 and 7.49 %, and 0.004 and 2.67% respectively. SOTE standardised for depth was 61.9±1.0, 8.97 and 2.67% m^{-1} for the NB injector, ceramic diffuser, and airstone respectively.

SAE for the NB injector was 0.198±0.003 kg O_2 kW h^{-1} using the 1.5 kW pump. The total O_2 gas used to transfer 1 kg of DO was 1.936±0.03 and 13.354 kg for the NB injector and ceramic stone respectively when flow rates were at 0.5 L min^{-1}. The total cost (pumping costs kWh^{-1} + O_2 gas (SOTE)) for the NB injector to transfer 1 kg of DO was $5.35±0.09, while the cost to transfer 1 kg of DO via the ceramic stone (no pumping costs) was $19.26 in O_2 gas alone.

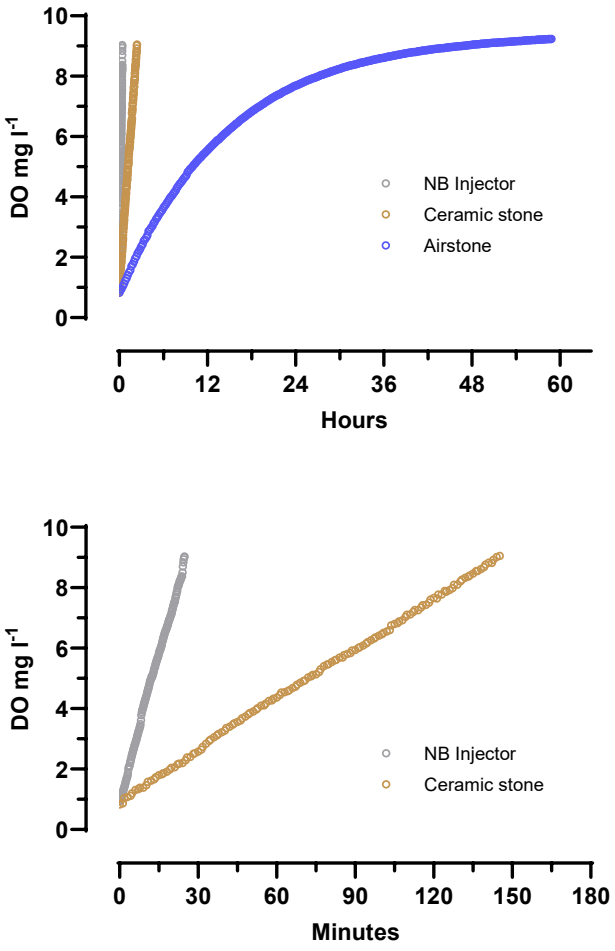


Figure 14. Relative DO re-saturation rates comparing the NB injector supplied with a 1.5kW Lowara pump at 277.5 L min^{-1}, a ceramic O2 diffuser stone (n=1), and an air stone (n=1) in a 5,000 L aquaculture tank. The NB injector and ceramic stone O2 supply pressure was 50 psi. Gas flow rate to all devices supplied at 0.5 L min^{-1}. Re-saturation times from <1.0 mg L^{-1} DO were: NB injector (O_2 gas) = 25 min, ceramic stone (O_2 gas) = 143 min, airstone (compressed air) = 58.17 h.

Discussion

Over the three trials conducted during this project using 660 fish (360 Yellowtail Kingfish, 300 Barramundi) there were no mortalities, and all fish grew as well if not better than expected based on published data for Yellowtail Kingfish and Barramundi (Booth et al., 2010; Glencross and Bermudes, 2012; Pirozzi et al., 2019). Fish reared in NB oxygenated water did not appear to be affected, positively or negatively, by NB exposure when considering growth, feed intake and feeding efficiencies, histology, haematology (Barramundi), and carcass compositional data demonstrating the efficacy and safety of using a NB injection system in aquaculture. The absence of any pathology associated with gas bubble disease implies that the total gas pressures, while not measured, were below levels that would cause gas-bubble trauma. The assessment of nitrification rates (total ammonia production (TAN); NH_3/NH_4^+) and CFU counts of fractionator waste were similar between oxygenation systems indicating no significant influence on waste metabolite remediation in operating RAS's supplied with oxygenated NB injected water compared to control RAS's. In this context the benefit of applying NB technology to a RAS provides an alternative, and efficient, oxygen delivery system but has no demonstrable effect as a growth or health promoter in fish.

In a typical RAS the water is continually recirculating and there are many points of turbulence and aeration such as in biofilters, foam fractionators, pumps, water delivery lines and sumps. These highly dynamic microenvironments of constant and forceful water movement facilitate the degassing of hyper-saturated (i.e. >100% saturated) solutions and this was the case in the RAS's used in this project. While the experiment tanks were maintained slightly hyper-saturated at ~120%, oxygen saturation levels measured in the sumps and biofilters were always ~100% indicating a decrease in DO and potentially, the relative NB concentration. Oxygen and NB dynamics will likely be very different in the relatively highly turbid, and less turbulent pond environments which may present a culture system type that is even more suited to NB oxygenation and is an area worth further investigation.

The biotic and abiotic treatments used in this project were chosen to promote a wide-ranging demand for DO requirement in the RAS's. The NB injection system was found to be very stable and required virtually no further adjustment of gas and water flow within the hyper-saturation tanks after initial setup, and only minor daily flow adjustments to individual experiment tanks throughout the trial periods. It should be noted however that daily flow adjustments were done to maintain precise DO concentrations to facilitate rigorous statistical comparison among treatment groups. In a practical setting such fine scale adjustments would not be necessary and could realistically be close to, within reason, a set-and-forget arrangement, particularly when coupled with remote DO monitoring sensors.

The magnitude of the response to the different biotic and abiotic treatments implemented in this study by Yellowtail Kingfish and Barramundi were by far the main drivers for the differences seen between treatments within each experiment. The differences between oxygenation treatment when considering GE carcass composition in Yellowtail Kingfish (Trial 1 - Table 9), while statistically significant, were in absolute numerical terms quite small. As there were no other significant differences when considering growth and feed intake, these anomalies may simply be due to some remaining undigested feed in the gut influencing carcass composition. These results were not seen with Barramundi (Trial 2 - Table 11).

Supra optimal stocking density eventually had an adverse effect on Yellowtail Kingfish in Trial 1, indicated by a slight increase in FCR and inferior growth, but this response was not dependant on the oxygenation regime. There were differences in growth rates between Barramundi grown in different salinities in Trial 2. While there is an energetic cost associated with osmoregulation which may in part explain these observations, the differences may simply be an artefact of water clarity, where the FW (bore water) was quite turbid in comparison to SW (Figure 6) potentially influencing behaviour. For example, while there was no statistical difference in feed intake, Barramundi in FW consumed on average 189 g $fish^{-1}$ compared to 201 g $fish^{-1}$ in SW. This difference may explain the significant interaction seen with FCR with respect to System X Salinity. Barramundi grown at 30°C performed far better than those grown at 20°C in Trial 3; this was to be expected as 30°C is considered the thermal optima for Barramundi (Glencross and Bermudes, 2012). Similarly, some temperature (RBC, Hb, RDW) but no system dependent effects were observed when

considering haematology. Most haematological values of barramundi were generally within the range for clinically healthy farmed barramundi (Anderson et al., 1996; Chew and Gibson-Kueh, 2023). The exception were WBC in the 30°C Control and 20°C NB treatment groups. While these values seemed to be somewhat elevated compared to published values (Anderson et al., 1996; Chew and Gibson-Kueh, 2023), there were no obvious signs of pathology, and growth and feed intake were better than predicted for this species (Glencross and Bermudes, 2012).

Nitrification rates were similar among aeration treatments. The current study did not set out to investigate the dedicated application of NB injection to foam fractionation or biofiltration in isolation; rather the primary aim was to investigate the health and performance of fish reared in NB water, and secondly, to then assess any potential effects on waste remediation in an operating RAS supplied with NB water. In this context, and in practical terms, there were no demonstrable differences between the NB and control systems in this study. The potential of NB technology has however been demonstrated in water treatment (e.g. Atkinson et al., 2019), and wastewater environmental remediation (e.g. Xiao et al., 2019) with both Atkinson et al. (2019) and Xiao et al. (2019) highlighting the potential to move away from chemical-based oxidants for water treatment. In aquaculture, the use of ozone NB has been the focus of much research recently (e.g. Farid et al., 2022; Jhunkeaw et al., 2021; Linh et al., 2022; Ng et al., 2023; Seridou and Kalogerakis, 2021) and can potentially provide an alternative, more efficient disinfection system where hygiene requirements are critical such as in hatcheries. The differences in fractionator waste with respect to CFU's between temperature treatments in Trial 3 were likely due to the greater faecal and metabolic waste output in the warm water group feeding more and growing more rapidly providing a rich nutrient source in warm water for bacterial growth.

It is difficult to establish why other published studies report better growth responses in NB cultured fish compared to a standard aeration or control group as there are often not enough details presented in their methodologies to establish what the culture conditions were with respect to water quality, experiment design, and treatment vs control groups. For example some studies don't standardise for dissolved oxygen concentration, provide information on the type of culture system used, how many fish and what size were used, information on control group details or *n* replication (e.g. Ebina et al., 2013) yet become widely cited and their conclusions republished without objective critique in review (Marcelino et al., 2023) and research articles (Mauladani et al., 2020). Without appropriate controls the differences observed between treatments are more likely due to differences in DO concentration or other system artefact rather than some special quality of NB to enhance the physiology of an animal. As NB technology is relatively new to the aquaculture field it is important that studies assessing the efficacy of these technologies are carefully designed. A review of the literature reveals many instances made of grand claims for improving growth and health of aquatic species, yet very little rigorous data to support such claims.

Oxygen transfer rates will vary widely depending on a suite of system variables such as application technology, gas type (air or oxygen), bubble size ("nanobubble" vs "microbubble" vs "fine bubble"), tank geometry, and abiotic factors etc. therefore any direct comparison across different studies should be approached cautiously. This caveat notwithstanding, the SOTR values determined in this study are comparable to those using similar types of approaches to evaluate novel oxygenation technologies; such as Park et al. (2022) who achieved a SOTR of 0.1278 kg h^{-1} and a SOTE of 45.3% using a vortex aerator supplied with O_2 gas (90% purity) at 1 L min^{-1}, and Ashley, et al. (2008) with a SOTR of 0.0479 kg h^{-1} and a SOTE of 61.1% using a laboratory-scale Speece cone supplied with O_2 gas (90-95% purity) at 1 L min^{-1}. Taukhid et al (2021) achieved SOTR values of up to 0.0207 kg h^{-1} and a SOTE of 49% using an aeration microbubble generator. It is not clear if these studies accounted for ambient atmospheric oxygenation to calculate SOTR; however, in the current study this was demonstrated to be negligible. Based on the SOTR values in the 500 L system, it is estimated that it would take in excess of 448 or 263 h to transfer 1 kg of atmospheric oxygen using the 0.53 or 1.5 kW pumps alone, respectively (assuming adequate volume to 100% air saturation solubility). Rates of ambient re-saturation via atmospheric oxygen have been shown to be negligible when conducting respirometry studies on fish (Gamble et al., 2014; Pirozzi and Booth, 2009a, 2009b). Similarly in the current study, ambient re-saturation was proportionally extremely small compared to the application of O_2 gas via the NB injectors despite water movement and turbulence in the tanks.

The results of the assessment of gas transfer efficiency rates and operating costs clearly demonstrate the trade-off between pumping capacity and O_2 gas flow rates when using the NB injectors resulting in a very large range of SOTR, SAE and SOTE values. Improving SAE can be achieved by simply increasing gas flow rates; however, oxygen transfer efficiencies will then deteriorate. In an aquaculture context, optimising systems for the cheapest running cost may not necessarily be the best strategy if DO is compromised for livestock, particularly for those that are held at high stocking densities. Optimising for the health of livestock should always be prioritised. While the NB injection system requires the addition of a pump, the overall running cost to transfer 1 kg O_2 in the 5000 L tank system was 72.2% cheaper compared to using a single ceramic diffuser. This considers the application of only one pump; however, there is still a significant cost saving (58.9%) even if applying a second pump, for e.g., a recirculation pump to saturate a holding tank of water, and a second transfer pump to supply stock tanks, as was the design used in Trials 1-3 of the current study. Conversely, a diffuser with a SOTE of approximately 27% would have an equivalent operating cost of a single pump operated NB injector in this scenario. It is important to note that these estimations are applicable for the systems used in the current study, and values will likely be different if different system configurations are considered.

The tests using the 5000 L tank system were not replicated for the ceramic and airstone diffusers however SOTE values established for the ceramic diffuser and airstone in this study are similar to those suggested by commercial manufacturers and the aquaculture literature for fine and coarse bubble diffusers (SSI, 2019; Tharp, 2020; Timmons and Ebeling, 2010; Xylem, 2022). Cost estimate comparisons can of course also be done simply by using these published values. The improved SOTR and SOTE, and therefore cheaper operating cost, for NB injection in the 5000 L tank compared to the 500 L tank at the same O_2 flow rate (0.5 L min^{-1}) and pump velocity is likely an artefact of the difference in tank geometry and greater dwell time in the larger tank. This is something to consider with respect to the efficient design implementation of NB injection in an aquaculture system.

Oxygenation using nano and fine bubble technologies have been demonstrated to be highly efficient; however, overall setup costs should be considered (i.e. additional pumps, NB injector costs, and ancillary equipment (plumbing, tanks etc)) when making decisions on implementing this technology into an aquaculture setup. As the NB injection system relies on one or more pumps, back-up systems must be in place should a pump fail.

Of the different types of NB systems (i.e. generators, diffusers, ultrasonic, electrolytic) the diffuser/membrane type system of the NBT injectors used in the current project are likely one of the most practical and simple to implement in an aquaculture system, with no moving parts or speciality pumps required, and operating at relatively low pressures, making them simple to operate and maintain. The injectors performed reliably and without any problems throughout all the trials conducted in this project providing constant rates of oxygenation in the saturation tanks used for the fish trials. Any high cost of implementing NB technology presents a significant barrier for the broadscale adoption in the aquaculture industry. The cost and maintenance of NB generators, compared to NB diffusers, for example may be expensive, making it economically unviable for small-scale farmers or developing regions. Therefore, the return on investment and cost-effectiveness of this technology needs to be carefully assessed.

From the nanoparticle size and concentration measurements (Appendix 1) it is clear that NB's in the treatment sample are on average smaller than the control sample, but both are of a similar concentration. This makes sense when considering gas dissolution capacity in water at equilibrium held under normal atmospheric pressure and temperature. These measurements are insightful as this demonstrates that NBs will form following processes of gas transfer, diffusion, and dissolution regardless of the oxygenation mechanism implemented; however, the relative size will vary. It should be noted that the nanoparticle tracking analysis methodology used to quantify NB's in the water samples only detects particle sizes from 1 – 600 nm, therefore the relative proportion of fine bubble sizes >600 nm in the systems used in this study are unknown.

Conclusion

Taken overall, observations from the experiments in this study provide encouraging results towards the use of NB technology in aquaculture. The clear benefit of NB technology lies in its efficient oxygenation capacity, its scalability, and potential application to a wide variety of aquaculture systems, and other industries. This study found that the growth, feed conversion efficiencies, histology and haematology in the NB treatment groups were comparable to the control groups confirming the viability and safety of a relatively new oxygenation technology for the application to aquaculture systems. The abiotic (salinity and temperature) and biotic (stocking density) variables tested in this study clearly had a greater impact on fish growth than the type of oxygenation system utilised. Targeted DO levels should dictate the oxygen delivery rates in culture systems which should be within optimal ranges required for the species being cultured. The operating costs of a NB injection system will vary considerably depending on set up and configuration, and while improved SOTR compared to other aeration devices may potentially provide a significant benefit to some operations; the establishment, maintenance, and ancillary equipment costs etc. relevant to specific operations will need to be considered to determine overall cost effectiveness.

While the focus of this study was on the use of O_2 gas with the NB injection system in RAS's, the assessment of air injection may also have merit, particularly for areas or sites where O_2 gas supply and/or operating costs may be restrictive. The application of NB technology to larger water bodies such as ponds is an area also worth investigation, as to is the application to waste water treatment such as dedicated NB fractionation.

Implications

This project has demonstrated the effectiveness of NB injectors in a recirculating aquaculture system, providing an alternative oxygen delivery technology to conventional oxygenation equipment without adversely affecting the growth and health of fish. Operating costs using the NB injection system imply a potential for cost effectiveness; however, this must be considered in context with overall establishment and maintenance costs to determine value for money for commercial operations.

Recommendations

The trials conducted in this report were focused predominantly on assessing health and performance of Yellowtail Kingfish and Barramundi grown in RAS's and provides some valuable insight into the utility of NB technology in an aquaculture setting. Investigation of dedicated NB application to wastewater remediation, pond aquaculture and fish transport are warranted. Further, as this technology is scalable, it may have potential for environmental applications such remediating certain blackwater events in natural water systems.

Extension and Adoption

Results and recommendations will be made publicly available through this report. Scientific publication/s and presentations will extend the results of this work to the national and international scientific communities.

References

ACEC, 2015. A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research, 4th Edition, NSW DPI Animal Care and Ethics Committee. p. 56.

Anderson, I., Schaumuller, L., Kramer, H., 1996. A preliminary study on the hematology of freshwater-reared seabass/barramundi, *Lates calcarifer*. Asian fisheries science 9, 101-108.

ASCE, 2022. Measurement of oxygen transfer in clean water. American Society of Civil Engineers.

Ashley, K.I., Mavinic, D.S., Hall, K.J., 2008. Oxygenation performance of a laboratory-scale Speece Cone hypolimnetic aerator: preliminary assessment. Canadian Journal of Civil Engineering 35(7), 663-675. https://doi.org/10.1139/l08-011

Atkinson, A.J., Apul, O.G., Schneider, O., Garcia-Segura, S., Westerhoff, P., 2019. Nanobubble technologies offer opportunities to improve water treatment. Accounts of chemical research 52(5), 1196-1205.

Azevedo, A., Etchepare, R., Calgaroto, S., Rubio, J., 2016. Aqueous dispersions of nanobubbles: Generation, properties and features. Minerals Engineering 94, 29-37.

Bohl, M., 1997. Gas bubble disease of fish. Tierarztliche Prax. 25(3), 284-288.

Booth, M.A., Allan, G.L., Pirozzi, I., 2010. Estimation of digestible protein and energy requirements of yellowtail kingfish *Seriola lalandi* using a factorial approach. Aquaculture 307, 247-259.

Boyd, C., Ahmad, T., 1987. Evaluation of aerators for channel catfish farming. Bulletin 584. Alabama Agricultural Experiment Station, Auurn University, Alabama, 52.

Boyd, C.E., 1998. Pond water aeration systems. Aquac. Eng. 18(1), 9-40. https://doi.org/https://doi.org/10.1016/S0144-8609(98)00019-3

Bui, T.T., Nguyen, C.D., Shim, N., Han, M., 2022. Total petroleum hydrocarbon removal from oil-sand by injecting charged nanobubbles. Environmental Nanotechnology, Monitoring & Management 17, 100653.

Chew, X.Z., Gibson-Kueh, S., 2023. The haematology of clinically healthy, farmed juvenile Asian seabass (*Lates calcarifer* Bloch)—reference intervals, and indicators of subclinical disease. J. Fish Dis. 46(10), 1109-1124. https://doi.org/https://doi.org/10.1111/jfd.13831

Dehariya, D., Eswar, K., Tarafdar, A., Balusamy, S., Rengan, A.K., 2023. Recent advances of nanobubble-based systems in cancer therapeutics: A Review. Biomedical Engineering Advances 5, 100080. https://doi.org/https://doi.org/10.1016/j.bea.2023.100080

Ebina, K., Shi, K., Hirao, M., Hashimoto, J., Kawato, Y., Kaneshiro, S., Morimoto, T., Koizumi, K., Yoshikawa, H., 2013. Oxygen and air nanobubble water solution promote the growth of plants, fishes, and mice. PLoS One 8(6), e65339.

Espmark, Å.M., Hjelde, K., Baeverfjord, G., 2010. Development of gas bubble disease in juvenile Atlantic salmon exposed to water supersaturated with oxygen. Aquaculture 306(1-4), 198-204. https://doi.org/10.1016/j.aquaculture.2010.05.001

Farid, M.U., Choi, P.J., Kharraz, J.A., Lao, J.-Y., St-Hilaire, S., Ruan, Y., Lam, P.K.S., An, A.K., 2022. Hybrid nanobubble-forward osmosis system for aquaculture wastewater treatment and reuse. Chem. Eng. J. 435, 135164.

Favvas, E.P., Kyzas, G.Z., Efthimiadou, E.K., Mitropoulos, A.C., 2021. Bulk nanobubbles, generation methods and potential applications. Current Opinion in Colloid & Interface Science 54, 101455.

Foudas, A., Kosheleva, R.I., Favvas, E.P., Kostoglou, M., Mitropoulos, A.C., Kyzas, G.Z., 2022. Fundamentals and applications of nanobubbles: A review. Chemical Engineering Research and Design.

Galang, D., Ashari, A., Sulmatiwi, L., Mahasri, G., Sari, L., 2019. The oxygen content and dissolved oxygen consumption level of white shrimp *Litopenaeus vannamei* in the nanobubble cultivation system, IOP Conference Series: Earth and Environmental Science. IOP Publishing, p. 012014.

Gamble, S., Carton, A.G., Pirozzi, I., 2014. Open-top static respirometry is a reliable method to determine the routine metabolic rate of barramundi, *Lates calcarifer*. Mar. Freshw. Behav. Physiol., 1-10. https://doi.org/10.1080/10236244.2013.874119

Glencross, B.D., Bermudes, M., 2012. Adapting bioenergetic factorial modelling to understand the implications of heat stress on barramundi (*Lates calcarifer*) growth, feed utilisation and optimal protein and energy requirements - potential strategies for dealing with climate change? Aquac. Nutr. 18(4), 411-422. https://doi.org/10.1111/j.1365-2095.2011.00913.x

Gunanti, M., Wulansari, P., Kinzella, K., 2019. The erythrocyte and leucocyte profile of saline tilapia (*Oreochromis niloticus*) in a cultivation system with nanobubbles, IOP Conference Series: Earth and Environmental Science. IOP Publishing, p. 012089.

Jhunkeaw, C., Khongcharoen, N., Rungrueng, N., Sangpo, P., Panphut, W., Thapinta, A., Senapin, S., St-Hilaire, S., Dong, H.T., 2021. Ozone nanobubble treatment in freshwater effectively reduced pathogenic fish bacteria and is safe for Nile tilapia (*Oreochromis niloticus*). Aquaculture 534, 736286.

Kazeem, T., Muhammad, N., Stone, G., Rao, N.R.H., Prescott, S., Henderson, R.K., Leslie, G., 2022a. Temporal stability of nanobubbles generated at scale in water applications, Oz Water: Our Water Journey. Australia Water Association.

Kazeem, T., Rao, N.R.H., Prescott, S., Henderson, R., Leslie, G., 2022b. Elucidating the fate of nanobubbles in aqueous phase, Australian Colloid and Surface Science Student Conference. Australian Colloid and Interface Society.

Ke, S., Xiao, W., Quan, N., Dong, Y., Zhang, L., Hu, J., 2019. Formation and stability of bulk nanobubbles in different solutions. Langmuir 35(15), 5250-5256.

Khan, P., Zhu, W., Huang, F., Gao, W., Khan, N.A., 2020. Micro–nanobubble technology and water-related application. Water Supply 20(6), 2021-2035.

Krause, T.L., Reardon, R.D.J., al., e., 2010. Design of munnicipal wastewater treatment plants: WEF Manual of Practice No. 8. Chapter 12. Alexandria, Water Environ. Fed.

Lawson, T.B., 1995. Fundamentals of aquacultural engineering. Springer Science & Business Media.

Le, T.D., Linh, N.V., Sangpo, P., Senapin, S., St-Hilaire, S., Rodkhum, C., Dong, H.T., 2021. Ozone nanobubble treatments improve survivability of Nile tilapia (*Oreochromis niloticus*) challenged with a pathogenic multi-drug-resistant *Aeromonas hydrophila*. J. Fish Dis. 44(9), 1435-1447.

Lekang, O.-I., 2020. Aquaculture engineering. John Wiley & Sons.

Linh, N.V., Mai, T.T., Senapin, S., St-Hilaire, S., Rodkhum, C., Dong, H.T., 2022. Impacts of oxygen and ozone nanobubbles on bacteriophage in aquaculture system. Aquaculture 551, 737894.

Machova, J., Faina, R., Randak, T., Valentova, O., Steinbach, C., Kroupova, H.K., Svobodova, Z., 2017. Fish death caused by gas bubble disease: a case report. Veterinarni Medicina 62(4), 231-237. https://doi.org/10.17221/153/2016-vetmed

Mahasri, G., Harifa, A.I., 2019. Oxygen dissolved nanobubble technology improved to quality of pacific white shrimp cultivation. The Indian Veterinary Journal 96(05), 37-39.

Mahasri, G., Saskia, A., Apandi, P., Dewi, N., Usuman, N., 2018. Development of an aquaculture system using nanobubble technology for the optimation of dissolved oxygen in culture media for nile tilapia (*Oreochromis niloticus*), IOP Conference Series: Earth and Environmental Science. IOP Publishing, p. 012046.

Marcelino, K.R., Ling, L., Wongkiew, S., Nhan, H.T., Surendra, K., Shitanaka, T., Lu, H., Khanal, S.K., 2023. Nanobubble technology applications in environmental and agricultural systems: Opportunities and challenges. Crit. Rev. Environ. Sci. Technol. 53(14), 1378-1403.

Mauladani, S., Rahmawati, A.I., Absirin, M.F., Saputra, R.N., Pratama, A.F., Hidayatullah, A., Dwiarto, A., Syarif, A., Junaedi, H., Cahyadi, D., 2020. Economic feasibility study of *Litopenaeus vannamei* shrimp farming: nanobubble investment in increasing harvest productivity. Jurnal Akuakultur Indonesia 19(1), 30-38.

Ng, P.H., Huang, Q., Huang, L., Cheng, T.H., Man, K.Y., Cheng, K.P., Rita, P.M.A., Zhang, J., St-Hilaire, S., 2023. Assessment of ozone nanobubble technology to reduce freshwater algae. Aquac. Res. 2023.

Nghia, N.H., Van, P.T., Giang, P.T., Hanh, N.T., St-Hilaire, S., Domingos, J.A., 2021. Control of *Vibrio parahaemolyticus* (AHPND strain) and improvement of water quality using nanobubble technology. Aquac. Res. 52(6), 2727-2739.

Nirmalkar, N., Pacek, A., Barigou, M., 2018. On the existence and stability of bulk nanobubbles. Langmuir 34(37), 10964-10973.

Nirmalkar, N., Pacek, A., Barigou, M., 2019. Bulk nanobubbles from acoustically cavitated aqueous organic solvent mixtures. Langmuir 35(6), 2188-2195.

Park, S.H., Batchelor, B., Ghosh, A., 2022. Gas transfer model for a multistage vortex aerator: A novel oxygen transfer system for dissolved oxygen improvement. J. Environ. Manag. 319, 115704.

Pirozzi, I., Benito, M.R., Booth, M., 2019. Protein, amino acid and energy utilisation of juvenile Yellowtail Kingfish (*Seriola lalandi*): Quantifying abiotic influences. Aquaculture 513, 734439. https://doi.org/https://doi.org/10.1016/j.aquaculture.2019.734439

Pirozzi, I., Booth, M.A., 2009a. The effect of temperature and body weight on the routine metabolic rate and postprandial metabolic response in mulloway, *Argyrosomus japonicus*. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 154, 110-118.

Pirozzi, I., Booth, M.A., 2009b. The routine metabolic rate of mulloway (*Argyrosomus japonicus*: Sciaenidae) and yellowtail kingfish (*Seriola lalandi*: Carangidae) acclimated to six different temperatures. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 152(4), 586-592.

Pissoat, C., Jerry, D., 2018. Real time monitoring of water quality and mechanisation of pond management to boost productivity and increase profit in Barramundi (*Lates calcarifer*) farming. FRDC project no. 2017-170.

Senthilkumar, G., Rameshkumar, C., Nikhil, M., Kumar, J.N.R., 2018. An investigation of nanobubbles in aqueous solutions for various applications. Applied Nanoscience 8, 1557-1567.

Seridou, P., Kalogerakis, N., 2021. Disinfection applications of ozone micro-and nanobubbles. Environmental Science: Nano 8(12), 3493-3510.

SSI, 2019. Fine bubble vs. Coarse bubble diffusers. https://www.ssiaeration.com.

Suriasni, P.A., Faizal, F., Panatarani, C., Hermawan, W., Joni, I.M., 2023. A review of bubble aeration in biofilter to reduce total ammonia nitrogen of recirculating aquaculture system. Water 15(4), 808.

Taukhid, I., Trijuno, D.D., Karim, M.Y., Syah, R., Makmur, M., 2021. Effect of power pump and nozzle diameter microbubble generator to increase oxygen concentration in aquaculture.

Temesgen, T., Bui, T.T., Han, M., Kim, T.-i., Park, H., 2017. Micro and nanobubble technologies as a new horizon for water-treatment techniques: A review. Advances in Colloid and Interface Science 246, 40-51. https://doi.org/https://doi.org/10.1016/j.cis.2017.06.011

Tharp, P.E., 2020. Field performance of fine and ultra fine bubble diffusers. Technical Bulletin Environmental Dynamics International. Retrieved from : https://blog.wastewater.com/field-performance-of-fine-and-ultra-fine-bubble-diffusers.

Timmons, M.B., Ebeling, J.M., 2010. Recirculating aquaculture. Cayuga Aqua Ventures Ithaca, NY, USA.

Wang, L., Ali, J., Wang, Z., Oladoja, N., Cheng, R., Zhang, C., Mailhot, G., Pan, G., 2020. Oxygen nanobubbles enhanced photodegradation of oxytetracycline under visible light: Synergistic effect and mechanism. Chem. Eng. J. 388, 124227.

Xiao, Z., Aftab, T.B., Li, D., 2019. Applications of micro–nano bubble technology in environmental pollution control. Micro & Nano Letters 14(7), 782-787. https://doi.org/https://doi.org/10.1049/mnl.2018.5710

Xylem, 2022. Sanitaire® ceramic disc diffusers. https://www.xylem.com.

Yasui, K., Tuziuti, T., Kanematsu, W., 2023. Mechanism of the Decrease in Surface Tension by Bulk Nanobubbles (Ultrafine Bubbles). Langmuir 39(46), 16574-16583.

Zhang, Z.-H., Wang, S., Cheng, L., Ma, H., Gao, X., Brennan, C.S., Yan, J.-K., 2022. Micro-nano-bubble technology and its applications in food industry: A critical review. Food Rev. Int., 1-23.

Zhu, J., An, H., Alheshibri, M., Liu, L., Terpstra, P.M., Liu, G., Craig, V.S., 2016. Cleaning with bulk nanobubbles. Langmuir 32(43), 11203-11211.

Appendix 1

Nanobubble concentration and particle size distribution

A preliminary assessment of nanobubble size and concentration was conducted using sampled water from the 500 L aquaculture system described in Trials 2 and 3. An individual sample completely filling a submerged 70ml jar was sampled from both the control and nanobubble experiment tank and analysed using a NanoSight NS300 Nanoparticle Tracking Analysis (NTA) following standard protocols (Wang et al., 2020; Zhu et al., 2016). The particle size range detectable using the NanoSight was 1 – 600 nm. Analyses was conducted by the University of NSW. The results on nanobubble size and concentration are presented in Figure 15. The D50 size distribution for the control water sample was 252.9±28.6 nm, and the NB water sample was 171.3±20.3 nm.

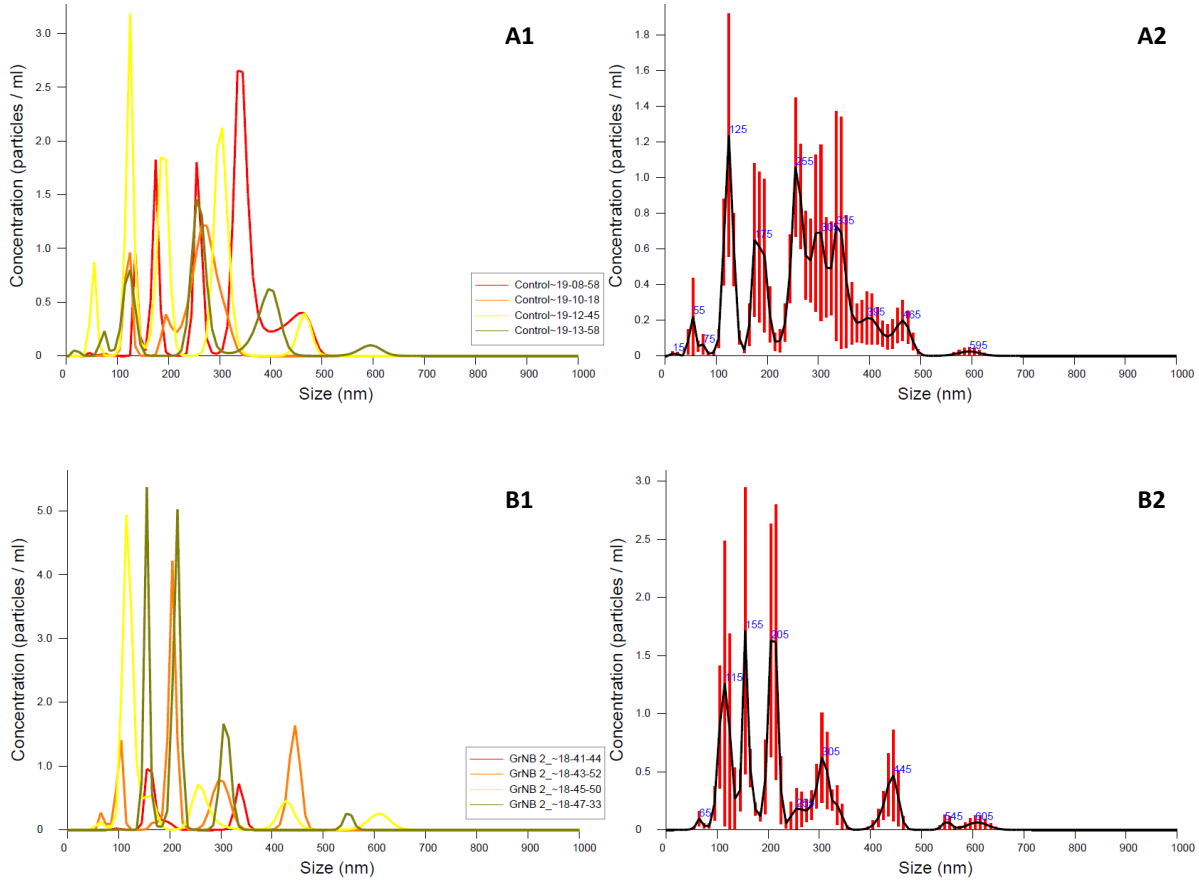


Figure 15. Nanobubble concentration and size in saltwater control (A) and nanobubble (B) treatments. (1) Finite track length adjustment (FTLA) Concentration/Size (n=4 runs), and (2) averaged FTLA Concentration/Size. Error bars indicate +/- 1 SEM.