Report

on the

Effect of Oxygen Nano Bubble treatment on the welfare indices of fish and hydrological parameters on the rearing water

Contracted for

NICO Nanobubble India Co.

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Objective 1: Effect of Oxygen Nano Bubble treatment on the welfare indices of fish

Methodology

The study was carried out with two different groups: an air treatment group (control group) and a NB-O2 treatment group. Each group consisted of three replicates with 30 fish per tank. All fish tanks were equipped with normal aeration during the experimental period. For the NB-O2 treatment groups, a single 10-min treatment with NB-O2 was performed twice daily for up to 3 months. Water temperature, DO and pH were measured using a multiparameter (YSI Pro Plus 3 Inc., USA). These parameters were measured in both the control and NB-O2 treatment groups at initial point (i.e., 0 min) and during 10 min exposure NB-O2.

Nile tilapia fingerlings (mean weight 6.98±0.04 g) were obtained from RGCA hatchery, Kochi. Fish were acclimated for 2 weeks and fed commercial tilapia diet (28 % protein) twice daily at 2 % of body weight. After the acclimation period, a random selection of ten fish were carefully examined for signs of parasitic or bacterial infection before the experiment began to ensure that they were healthy. During the experiment, fifty percent of rearing water was changed in all tanks every 2–3 days. The use of fish in this study was in accordance with the guidelines of institutional animal ethics committee of KUFOS

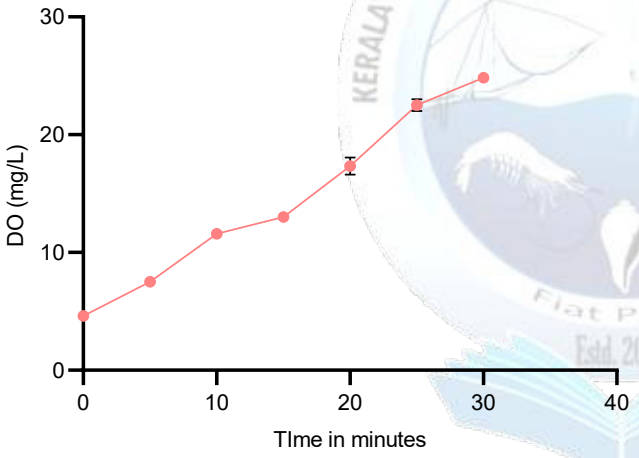
To investigate the growth and health parameters of Nile tilapia associated with long-term NB-O2 exposure, fish were exposed to NB-O2 twice daily for 10 min for 3 months. Weight and length were measured on 10 experimental fish weekly and averaged for a month. One fish from each group were sacrificed for the blood collection. After the procedures, the remaining fish were immediately returned to their living tanks for further analysis.

Haematology assays were performed with whole blood on an automated haematology analyser (BC-30 Vet, Mindray). Serum metabolites and enzyme values were measured using Dri-Chem Nx500V Veterinary Dri Chemistry Analyser. Serum oxidative stress markers were determined using commercially available kits (Origin, India) and the serum cortisol was measured using ELISA (Cusabio, USA).

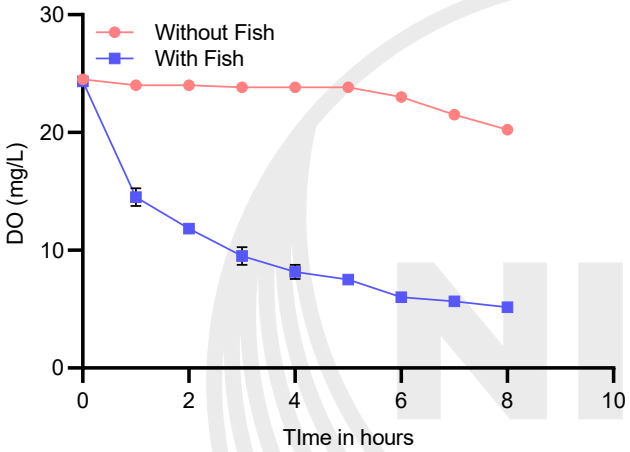
Results

Initial NB-O2 validation

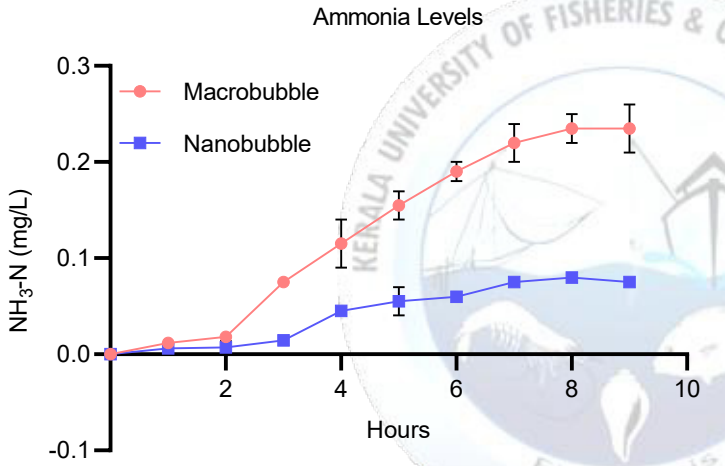
1. Time to increase DO in tanks



2. Stability of DO levels in nanobubble oxygenated tanks with and without fishes



3 Ammonia production in tanks



From the initial validation experiments, oxygenation of tanks for 10 minutes was found to be sufficient to raise DO level to 15 ppm. Stability of NB-O2 in the tanks maintained a level of 6 ppm with an initial oxygenation to 20 ppm for a time frame of 8 hours. The free ammonia generation in the tanks over the period in the fish rearing tanks were below the optimal for 8 hours and maintained the stability. Thus the NB-O2 operation was standardized at 8 hr interval for 10 minutes in each tanks.

Initial health validation of the experimental animals

Sl. No	Parameters	Observed value
1.	**Total weight**	6.98±0.04 grams
2.	**Haematology parameters**	
	Hb (g/dL)	9.30 ± 0.50
	RBC ($\times 10^6/mm^3$)	1.16 ± 0.08
	Hct (%)	31.36 1.45
	MCV(fL)	270.06 ± 1.70
	MCH (pg)	80.03 ± 1.74
	MCHC (g/dL)	29.65 ± 0.60
	TLC (x $10^3/mm^3$)	63.70 ± 1.72
	TLØ (x $10^3/mm^3$)	49.32 ± 0.97
	NØ (x $10^3/mm^3$)	11.10 ± 1.21
	MØ (x $10^3/mm^3$)	3.2 ± 1.78
	TØ (x $10^3/mm^3$)	42.29 ± 1.92
3.	**Serum metabolites**	
	Glucose ($mmolL^{-1}$)	2.12 ± 0.12
	T. protein (gdL^{-1})	3.17 ± 0.10
	Albumin (gdL^{-1})	1.36 ± 0.09
	Globulin (gdL^{-1})	1.80 ± 0.10
	T. cholesterol ($mmolL^{-1}$)	4.99 ± 0.70
	Triglycerides($mmolL^{-1}$)	2.65 ± 0.27
	BUN($mmolL^{-1}$)	1.63 ± 0.52
	Creatinine($\mu molL^{-1}$)	30.14 ± 11.45
	T. Bilirubin($\mu molL^{-1}$)	4.33 ± 0.60
	Uric acid($\mu molL^{-1}$)	29.92 ± 6.63
4.	**Serum Enzyme**	
	ALT (µkatL)	0.17 ± 0.06
	AST (µkatL)	6.24± 2.26
	ALP (µkatL)	1.42 ± 0.57
	CK (µkatL)	23.42± 4.17
5.	**Serum Oxidative stress markers**	
	AChE ($\mu M\ min^{-1}\ gm\ protein^{-1}$)	12.18±0.21
	SOD (U/mg protein)	3.51±0.01
	CAT (U/mg protein)	5.10±0.21
	GPx (U/mg protein)	2.21±0.01
	MDA (n mole/g)	41.35±0.15
	GST (U/mg protein)	8.1±0.05
	GSH (U/mg protein)	7.3±0.03
	Cortisol (mg/mL)	11.04±0.11

Hb = hemoglobin; RBC = red blood cell; Hct = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; TLC = total leukocyte count; TLØ = total lymphocytes; NØ = neutrophils; MØ = monocytes; TØ = thrombocytes; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; CK = creatine kinase; AChE = Acetry cholinesterase; SOD = superoxide dismutase; Cat = catalase; GPx = glutathione peroxidase; MDA = malonaldehyde; GST = glutathione S transferase; GSH = glutathione reductase

Growth parameters

	NB-O2 (n=12)	MB (n=12)
Initial	6.98±0.04	6.98±0.04
Final BW	12.75±0.65*	8.74±0.41
% weight gain	82.72% (5.77g)	25.22% (1.76g)
Growth rate	0.064g/day	0.0196g/day
Specific Growth rate	0.67 %/day	0.25%/day

Health parameters

Data represents the three month averaged values of the health indices (n=12)

Sl. No	Parameters	Observed values	
		Control	NB group
1.	**Haematology parameters**		
	Hb (g/dL)	14.61 ± 0.29	15.83 ± 1.05
	RBC ($\times 10^6$/mm^3)	4.64 ± 0.14	4.87 ± 0.41
	TLC (x 10^3/mm^3)	70.25± 1.12	71.81 ± 0.54
	Hct (%)	32.45 ± 0.24	35.40 ± 1.04
2.	**Serum metabolites**		
	Glucose (mmolL^{-1})	2.64 ± 0.32	3.12 ± 1.02
	BUN(mmolL^{-1})	1.84 ± 0.14	1.32 ± 0.74
	Creatinine(µmolL^{-1})	31.55 ± 1.41	25.51 ± 0.40*
	T. Bilirubin(µmolL^{-1})	6.15 ± 0.24	4.05 ± 1.01
	Uric acid(µmolL^{-1})	32.61 ± 0.27	22.12 ± 0.29*
3.	**Serum Enzyme**		
	ALT (µkatL^{1})	1.55 ± 0.74	1.08 ± 0.42
	AST (µkatL^{1})	11.51± 0.64	10.81± 1.0
	ALP (µkatL^{1})	5.19 ± 0.51	4.52 ± 0.41
	CK (µkatL^{-1})	31.83± 0.44	27.71± 0.87
4.	**Serum Oxidative stress markers**		
	AChE (µM min^{-1} gm protein^{-1})	17.54±0.81	12.71±0.81*
	SOD (U/mg protein)	9.20±0.13	4.85±1.71*
	CAT (U/mg protein)	10.15±1.15	6.95±1.51*
	GPx (U/mg protein)	11.81±1.01	7.89±0.83*
	MDA (n mole/g)	43.54±0.55	39.41±1.55
	GST (U/mg protein)	12.98±1.05	8.05±0.07
	GSH (U/mg protein)	11.62±0.45	8.95±0.16
	Cortisol (mg/mL)	19.26±1.17	10.20±1.31*

The general health parameters have not varied with respect to the haematology and serum enzymes where, no significant differences were noted between the groups. Significant changes in the serum oxidative markers like AChE, SOD, CAT, and GPx were noted which might be attributed to the lower oxidative stress min the NB-O2 treated fishes. The results were concurrent with the levels of lower cortisol levels in the plasma of the NB-O2 treated group displaying lower level of stress.

In conclusion, the lower oxidative stress and metabolic stress in the fishes might have contributed to the increase in growth rate observed in the NB-O2 treated groups. However, the experimental trial needs to be further evaluated on pilot level to understand the on-farm performance of the NB-O2

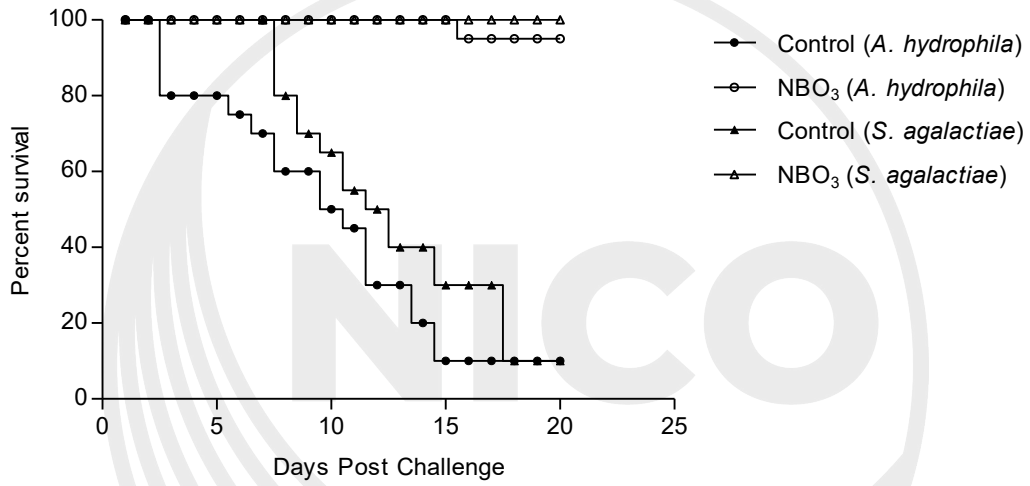
Objective 2: To study the effect of ozone Nano bubble on pathogenic bacterial load in water

Methodology

The experiment tested two rearing systems; one with nanobubble ozone supplementation and one without, which is marked as control. The nanobubble O3 supplementation was done at a rate of 10% water volume every 24 hours with 50% water change (40% normal UV treated water to 10% O3 NB enriched water) in the tank till the ORP value stabilizes at 650±150 mV (*ORP dropped to normal (~330 mV) after 15 min of every treatment time.). The water in control tanks were maintained with 50% water exchange with UV treated water with a stabilised ORP at 250±100 mV. Two separate challenge trials were conducted with *Aeromonas hydrophila* and *Streptococcus agalactiae*. The lethal dose concentrations of both bacteria were introduced into tanks and maintained the fishes for one hour in all tanks with normal aeration. Following infection, the fishes were maintained with normal aeration for 24 hours. Post 24 hours the water exchange regimen as explained before were introduced into experimental tanks.

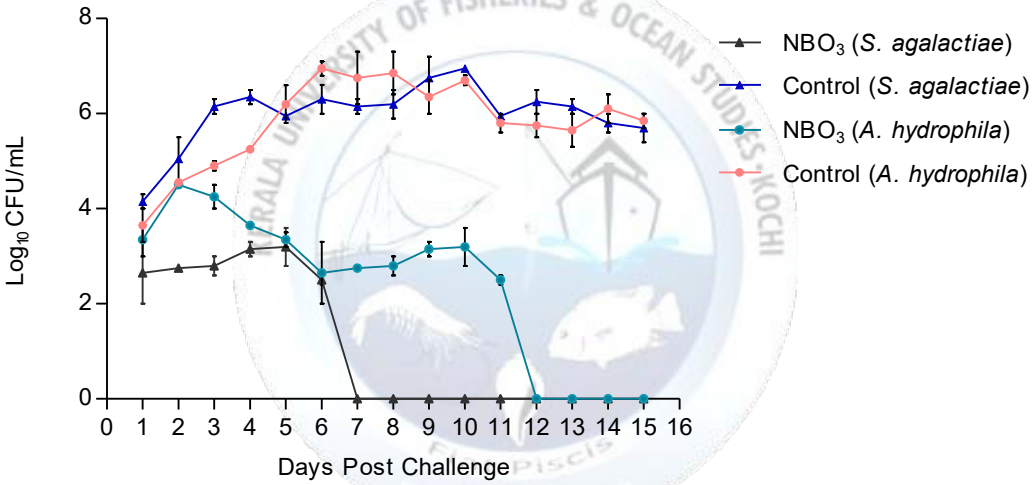
Results

Fig. Kaplan Meir survival curve analysis for the experimental challenge groups



The survival analysis following challenge have shown significant reduction in mortality in the NBO3 treated groups. The dosage for the NBO3 measured as ORP was adequate to reduce the bacterial load in the water thereby reducing the mortality in the infected fishes. *A. hydrophila* challenged and NBO3 treated group have displayed 95% survival compared to control ($P<0.05$), and no mortality was recorded in the *S. agalactiae* infected group.

Fig 2. Bacterial load in water post challenge



The bacterial load in water has displayed a significant reduction following the NBO3 treatment. The results are promising that the treatment standardised at ORP of 650±150 mV every 24 hours is sufficient to reduce the bacterial load in the rearing water without causing damage to the gills of the fishes. The ORP values above 800mV have shown significant adaptive change in the gills of the fishes.

The results are conclusive that the NBO3 have significant impact in reducing pathogenic bacterial load in the rearing water and have promoted the survival of the fishes from both gram positive and gram-negative bacterial infections.

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