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Bioremediation of Aquaculture Water using Nitrifying Bacteria-Microalga Consortium with Special Reference to Ammoniacal Nitrogen

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KEYWORDS

Bioremediation; Aquaculture; Nitrifying bacteria; *Chlorella vulgaris;* Zebra fish; Immobilized nitrifying bacteria-microalga consortium.

A B S T R A C T

The present study was aimed at reducing the ammonia content in aquaculture systems using nitrifying bacteria (*Nitrosomonas* sp. and *Nitrospira* sp.) and a microalga (*Chlorella vulgaris*) by immobilization technique. Immobilization was performed in alginate beads. One set of beads was prepared by immobilizing nitrifying bacteria and another by immobilizing nitrifying bacteria-microalga consortium. Three tanks were set up and Zebra fish were added to each tank .Tank 1 and 2 served as test to which beads immobilized with bacteria were added. Tank 3 served as control. Similar set up was followed for beads immobilized with bacteria and alga. The beads were placed at the bottom to form an even bed. The set up was allowed to run for 4 days. pH, alkalinity, total hardness, chlorine, TDS, ammonia, nitrite, nitrate and total phosphorous were analysed daily for 4 days. Independent sample t-test was used to interpret the data statistically. The results suggested that there was a significant reduction in the parameters, ammonia and nitrite, between test and control. There was no significant difference between the values of parameters of experiments 1 and 2, i.e., test with immobilized bacteria and test with immobilized bacteria and alga. It was found that the trial carried out using immobilized bacteria with algae reduced the parameters such as hardness, alkalinity and TDS compared to trial carried out with beads immobilized with only bacteria. This technique can be used in various aquaculture systems to reduce the problem of increasing ammonia content in the waters thereby reducing the death of fish due to ammonia toxicity.

Introduction

Most modern aquaculture practices require high-density cultivation for successful commercial operations.

High-density aquaculture will lead to the exposure of animals to elevated concentrations of nitrogenous wastes,

particularly ammonia and nitrite. High concentrations of ammonia will lead to decreased survival rate, inhibition of growth, and a variety of physiological dysfunctions in the aquatic animals. Nitrite toxicity will lead to nitrite toxicosis which is characterized by reduced survival and growth rates, methemoglobinemia, and other physiological dysfunctions. Both ammonia and nitrite act as stressors and they stimulate the release of corticosteroid hormones into circulation in fishes. Elevated concentrations of circulating corticosteroids in aquatic animals result in impaired immune function and therefore, decreased disease resistance. To limit losses and remain competitive, aquaculturists must recognize the specific detrimental effects as well as the probable immunosuppressive effects of ammonia and nitrite and management of the same (Tomasso, 1994).

Ammonia accumulation is the direct result of fish osmoregulation (directly tied to fish respiration) as well as due to action of heterotrophic bacteria. This could be fish food, a dead fish, a dead plant, and of course, fish excrement (Robert *et al.*, 1997). Ammonia is converted to nitrite (NO_2) and nitrate (NO_3) by nitrifying bacteria, which are then used by plants. Therefore, nitrate and ammonia remain the most common forms of nitrogen in aquatic systems. It is also one of the most important pollutants because it is relatively common but can be toxic, causing lower reproduction and growth, or death. The neutral, unionized form (NH_3) is highly toxic to fish and other aquatic life.

A by-product of protein metabolism, ammonia is primarily excreted across the gill membranes, with only a small amount excreted in the urine (Ruth *et al.*, 1990). The decay of uneaten feed and organic matter generates small amounts of ammonia, but in most aquaculture systems, fish themselves are the primary source of the ammonia release. The more feed a fish consumes, the more ammonia it will produce, although even a starved fish will produce some amounts of ammonia. Dangerous short-term levels of toxic unionized ammonia which are capable of killing fish over a few days start at about 0.6 mg/L. Chronic exposure to toxic unionized ammonia levels as low as 0.06 mg/L can cause gill and kidney damage, reduction in growth, possible brain malfunctioning, and reduction in the oxygen-carrying capacity of the fish circulatory system (Robert *et al.*, 1997). Other symptoms also include: purple, red or bleeding gills, clamping of fish, may appear darker in colour, red streaking can be seen on the fins or body, fish may gasp for air at the surface of the tank water, torn & jagged fins, and may appear weak and lay at the bottom of the tank

There are several ways to reduce ammonia concentration, such as to reduce feeding rate, increase the aeration, and addition of ion-exchange materials and lime. But most approaches are considered unsuitable for large ponds used in commercial aquaculture. However, removal of ammonia using microbes is a widely-used approach. Nitrifying bacteria are classified as obligate chemolithotrophs (use inorganic salts as energy source) belonging to family *Nitrobacteriaceae.* They use ammonia and nitrites for energy and fix inorganic carbon dioxide to fulfil their carbon requirements. These are non-motile and colonize on the surface for optimum growth. *Nitrosomonas* sp. and *Nitrospira* sp. are gram negative rod-shaped bacteria and cannot multiply or convert ammonia/nitrite in the absence of oxygen. They are very efficient that a single cell can convert ammonia at a rate that would require up to 1 million heterotrophs to accomplish. Most of their energy is used

to fix carbon dioxide and the remaining for growth and reproduction and therefore, they have slow growth rate (doubles every 15-20 h). Similarly, microalgae have the ability to use inorganic nitrogen and phosphorus for their growth. Moreover, they have the capacity to remove heavy metals, as well as some toxic organic compounds from water (Rao *et al*., 2011a,b; Abdel *et al.*, 2012).

Immobilization is a general term describing a wide variety of the cell or particle attachment or entrapment. Precisely, cell immobilization has been defined as the physical confinement of viable microbial cells in a defined region in such a way as to limit their free migration (Suzana *et al.*, 2013). Currently, various types of immobilization methods find wide applications not only in the field of biotechnology, but also in pharmaceutical, environmental, food and biosensor industries. Entrapment is an irreversible method, where immobilized cells are entrapped in a support matrix or inside fibres. This technique creates a protective barrier around the immobilized microbes, ensuring their prolonged viability. The commonly used matrices are agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatine, epoxy resin, photo cross-linkable resins, polyacrylamide, polyester, polystyrene and polyurethane. The technique of immobilizing whole cells was used in the present study to entrap nitrifying bacteria (*Nitrospora* sp. and *Nitrosomonas* sp.) and a microalga (*Chlorella vulgaris*) in alginate beads.

Zebra fish were chosen in the present study as they are more resistant to fluctuations. The zebra fish (*Danio rerio*) is a tropical freshwater fish belonging to the family Cyprinidae of the order Cypriniformes. Native to Himalayan region, it is a popular aquarium fish, frequently sold under the trade name Zebra danio. The Zebra fish is also a widely used vertebrate model organism in scientific research, and was the first vertebrate to be cloned. It is particularly notable for its regenerative abilities, and has been modified by researchers to produce several transgenic strains (White *et al.*, 2008).

Aquatic systems find it difficult to maintain their fish with all the external and internal problems. It is therefore necessary to come up with solutions to minimize death of fish due to high ammonia content and in turn to prevent commercial loss not only in aquariums but also in fish breeding industries**.**

Of all the water quality parameters which affect fish, ammonia is the most important (Ruth *et al*., 1990). As nitrifying bacteria have been reported to have the ability to reduce ammonia by converting it to nitrate and algae improves the overall parameters of water, it was used to study their effect in fish tank. In addition, immobilized nitrifying bacteria and microalga consortium was used to study the reduction of parameters, especially ammonia.

Materials and Methods

Procurement/ culturing/ maintenance of microorganisms

Two nitrifying bacteria, *Nitrosomonas* sp. and *Nitrospira* sp., were obtained commercially as lyophilized cultures and revived using inorganic salt medium (Modified Raggios Medium).

The microalgal culture, *Chlorella vulgaris*, was obtained from the algal culture collection facility of the University of Madras, and was subcultured/maintained in Bold Basal Medium (BBM) in an

illuminated culture rack at 24°C with 12/12 hours light/dark cycle.

Set up of aquaculture tanks

Three fish tanks were bought and set up. An aerating motor was fixed to ensure proper aeration. Each tank contained 8 L of water. Ten numbers of zebra fish were added to each tank and the fish were fed twice a day. The fish was introduced into the tanks 2 days prior to addition of immobilized beads.

Standardization of beads

Sodium alginate beads were standardized by preparing various concentrations of sodium alginate slurry and calcium chloride solution. The beads were allowed to stand overnight to check its stability in water (Fig. 1).

Immobilization

The immobilizate was prepared by dropping the bacteria-sodium alginate slurry into calcium chloride using a dropper. The beads which formed (3 mm diameters) were left in the calcium chloride solution for about 3 h, and then washed with water and used for the experiments. Twenty grams of alginate was dissolved in 500 ml of water to which 0.25 g of bacteria was added (Fig. 2). For algal immobilization similar protocol was followed with the addition of 100 ml of algal sample to the slurry.

Layering of immobilized beads

Three tanks were set up. Tanks 1 and 2 served as test to which beads immobilized with bacteria were added. Tank 3 served as control. The beads were added to the bottom of the tank and were spread evenly to form a uniform bed of beads (Fig 3). The same procedure was followed for beads immobilized with bacteria and microalga. The set up with beads was allowed to run for 4 days.

Parameter testing

pH, alkalinity, hardness, chlorine, TDS, ammonia, nitrite, nitrate and total phosphorous were analysed on a daily basis for 4 days. The above parameters were analysed following the methods of APHA (2000). All analyses were carried out in duplicate.

Statistical analysis

Independent sample t-test was used to analyse and compare the data for significant difference in the mean values of chemical parameters between test and control.

Results and Discussion

Microscopic examination

The bacterial culture was identified as Gram negative bacilli. There was no appearance of contaminants. During subculture and maintenance, the microalgal culture was confirmed as *Chlorella vulgaris* following the monograph of Philipose (1967). Both cultures were used in immobilization technique.

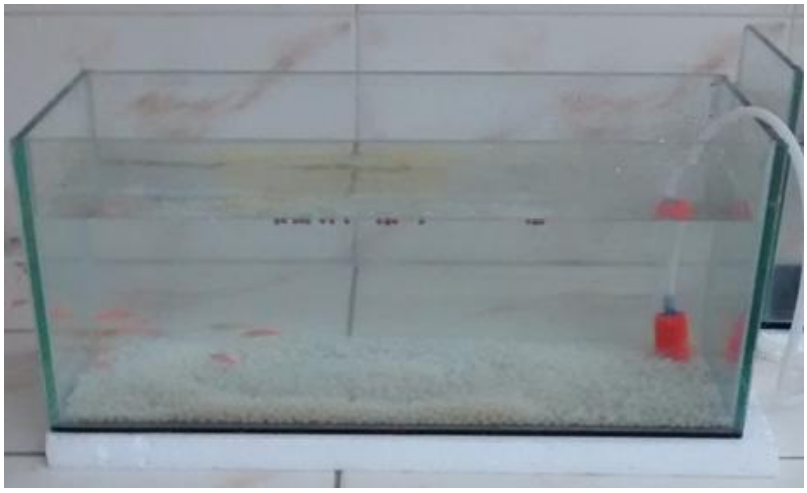
Standardization of beads

The slurry which contained 2 g of sodium alginate in 50 ml of distilled water where the beads were dropped into $CaCl_2$ solution (50 ml of 4% $CaCl_2$) and was used for bead preparation. The beads were stable with uniform size and did not disintegrate after overnight immersion in distilled water. The organisms from the beads were again cultured in their respective media to confirm its viability inside the substrate.

Fig.1 Standardization of beads

Fig.2 Immobilization of bacteria in alginate beads

Fig.3 Test tank with beads



Chemical parameters

Statistical analysis of the data-independent sample t-test

Independent sample t-test was used to analyse and compare the data for significant difference in the mean values of chemical parameters.

It was found that ammonia, nitrite and nitrate showed significant difference between test and control values for both experiments i.e. with bacteria and with bacteria-alga consortium because ($p<0.5$) at 5 percent level of significance we reject H0, null hypothesis. There was also significant difference in alkalinity, hardness and TDS in experiment 1 (bacteria).

There was no significant difference in phosphate for experiment 1 and no significant difference in alkalinity, hardness, TDS and phosphate in experiment 2 (bacteria with alga).

Comparison of parameters between test and control for bacteria is shown in figures 4 to 12 and table 1. Figures 13 to 20 and table 2 show the comparison of parameters between test and control for bacteria-microalga consortium.

The present study was aimed to search for an alternate solution to the problem of ammonia toxicity in fish tanks, a serious problem faced in aquaculture systems. Breakpoint chlorination, air stripping, ion exchange and biological methods have been used for ammonia reduction. Jeffery (1984) used water hyacinth water treatment system to reduce ammonia. Shin *et al.* (1998) worked on the removal of ammonia by precipitating it using magnesium salts. Endong *et al.* (2008) isolated *Scendesmus* sp. and entrapped in calcium alginate as algal sheets to remove nitrogen and phosphate from secondary effluent of a bioreactor.

The focus of this study was to use the biological method where the organisms would uptake ammonia during biological growth. Therefore, nitrifying bacteria were tested for their ammonia removal efficiency in fish tanks.

A similar procedure was carried out by Shan *et al*. (2001) where the nitrifying bacteria were immobilized in clay pellets and their efficiency was tested in prawn aquaculture ponds. Christopher *et al.* (2008) used nitrifying bacteria to remove ammonia from anaerobic sludge digesters. Both experiment showed positive results.

Fig.4

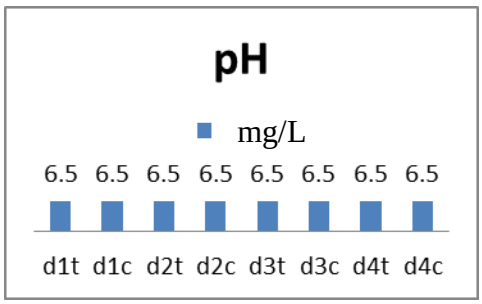


Fig.5

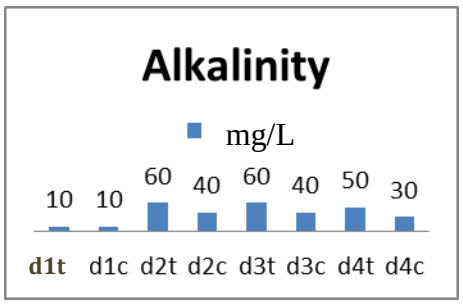


Fig.6

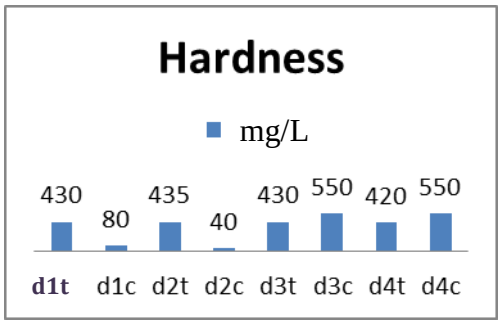


Fig.7

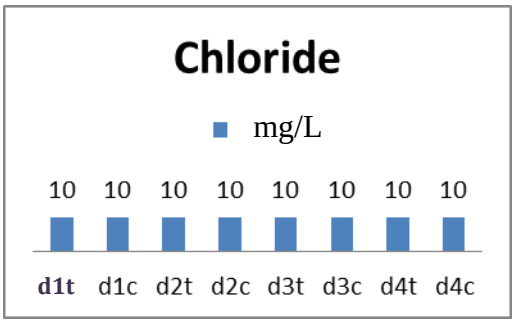


Fig.8

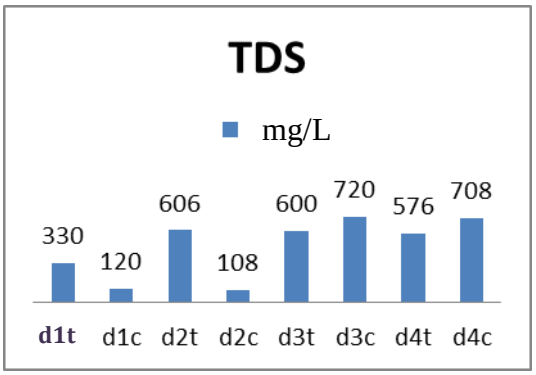


Fig.9

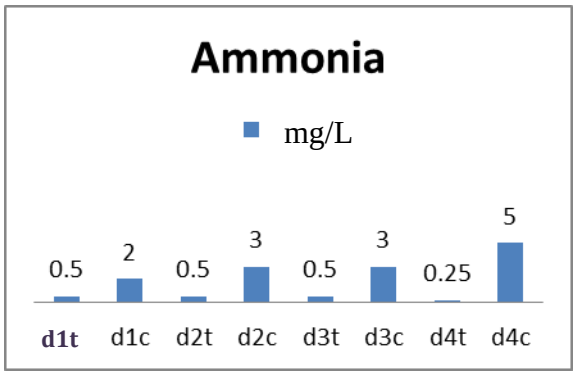


Fig.10

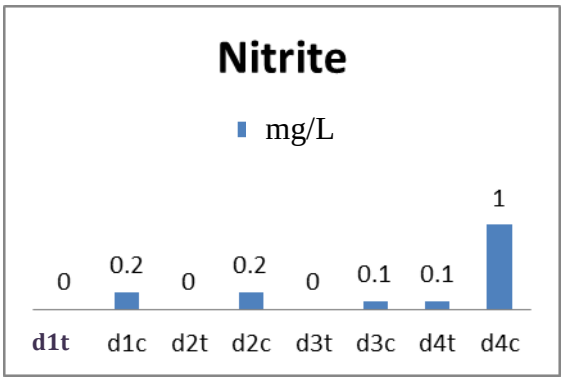


Fig.11

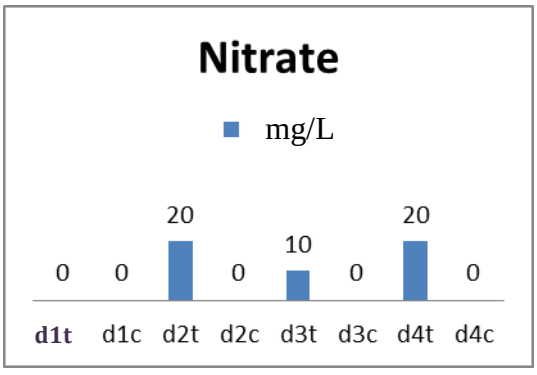
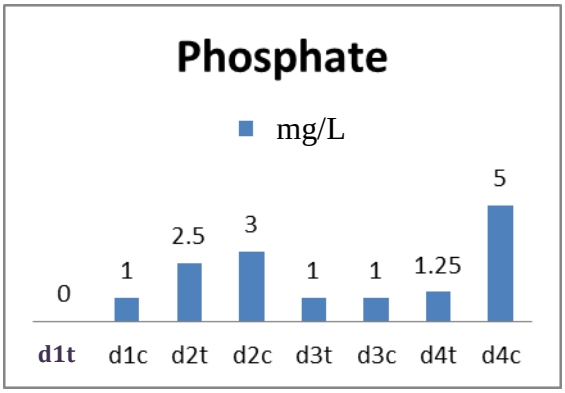


Fig.12



Figures 4 to 12 show the physico-chemical parameters for test and control after treatment with nitrifying bacteria.

(Day 1 test-d1t, day 1 control- d1c, day 2 test- d2t, day 2 control- d2c, day 3 test- d3t, day 3 control- d3c, day 4 test- d4t, day 4 control- d4c)

Fig.13

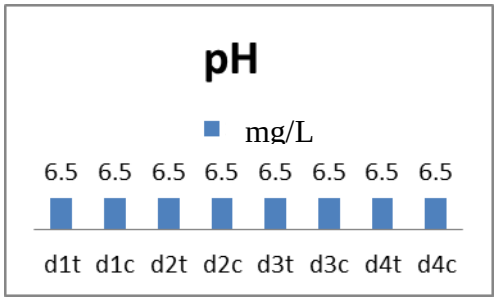


Fig.14

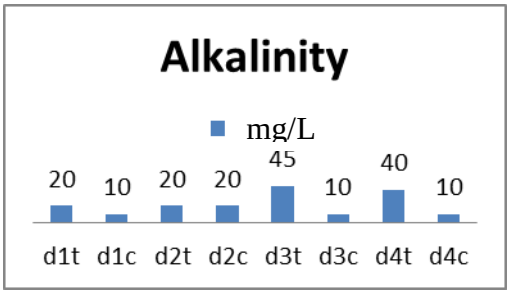


Fig.15

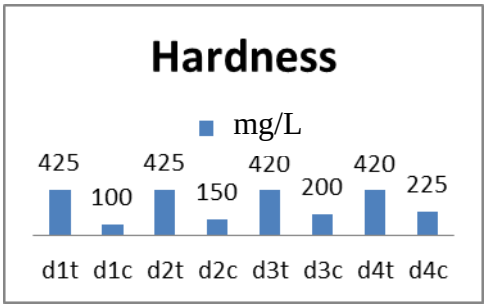


Fig16

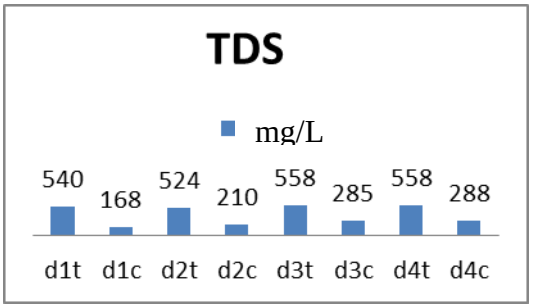


Fig.17

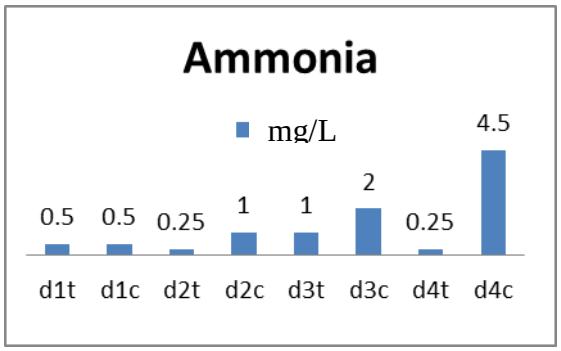


Fig.18

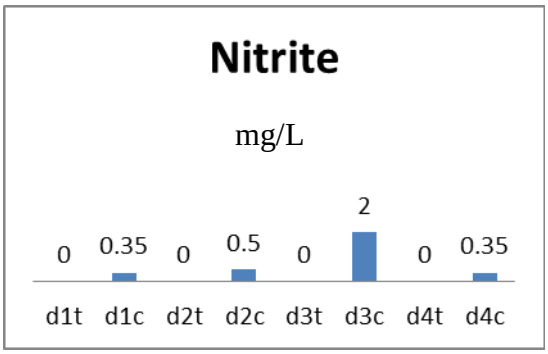


Fig.19

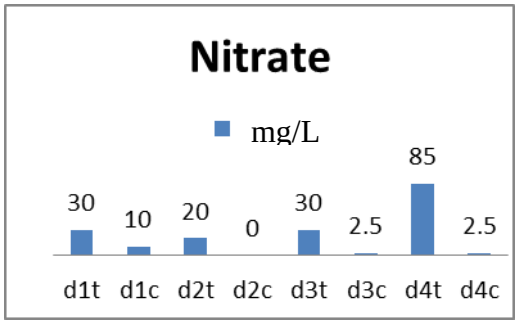
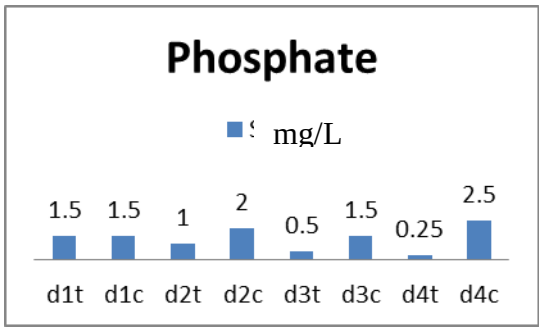


Fig.20



Figures 13 to 20 show the physico-chemical parameters for test and control after treatment with nitrifying bacteria-microalga consortium.

(Day 1 test-d1t, day 1 control- d1c, day 2 test- d2t, day 2 control- d2c, day 3 test- d3t, day 3 control- d3c, day 4 test- d4t, day 4 control- d4c)

Table.1 Comparison of parameters between test and control (for bacteria)

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
alkalinity	Equal variances assumed	21.600	.004	2.666	6	.037	18.75000	7.03414	1.53807	35.96193
	Equal variances not assumed			2.666	3.850	.058	18.75000	7.03414	-1.08431	38.58431
hardness	Equal variances assumed	16.197	.007	9.765	6	.000	251.25000	25.72896	188.29351	314.20649
	Equal variances not assumed			9.765	3.019	.002	251.25000	25.72896	169.65886	332.84114
chloride	Equal variances assumed	1.000	.356	-.655	6	.537	-1.25000	1.90941	-5.92215	3.42215
	Equal variances not assumed			-.655	5.880	.537	-1.25000	1.90941	-5.94536	3.44536
TDS	Equal variances assumed	15.118	.008	10.059	6	.000	307.25000	30.54607	232.50646	381.99354
	Equal variances not assumed			10.059	3.461	.001	307.25000	30.54607	216.97383	397.52617
ammonia	Equal variances assumed	4.975	.067	-3.599	6	.011	-.92500	.25699	-1.55382	-.29618
	Equal variances not assumed			-3.599	3.339	.031	-.92500	.25699	-1.69783	-.15217
nitrite	Equal variances assumed	.	.	-5.196	6	.002	-1.50000	.28868	-2.20636	-.79364
	Equal variances not assumed			-5.196	3.000	.014	-1.50000	.28868	-2.41869	-.58131
Nitrate	Equal variances assumed	5.844	.052	2.512	6	.046	37.50000	14.93039	.96664	74.03336
	Equal variances not assumed			2.512	3.129	.083	37.50000	14.93039	-8.92741	83.92741
phosphate	Equal variances assumed	1.000	.356	-1.022	6	.346	-.50000	.48947	-1.69770	.69770
	Equal variances not assumed			-1.022	4.716	.357	-.50000	.48947	-1.78135	.78135

Table 1 inference

H0: there is no significant difference in parameters due to bacteria.
Procedure**:** Student independent paired t-test was carried out taking control and test as the grouping variables. (Considering they don't make much influence)
Calculation**:** Test was conducted and output was determined.

Note: Alkalinity, Hardness, TDS, Ammonia, Nitrite, Nitrate have made a significant difference. As ($p<0.05$) at 5 percent level of significance we reject H0, null hypothesis. There is no significant difference in chloride and phosphorous as $p> 0.05$ at 5 percent level of significance we accept H0.

Table.2 Comparison of parameters between test and control (for bacteria and microalga)

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Alkanility	Equal variances assumed	1.000	.356	1.083	6	.320	15.00000	13.84437	-18.87596	48.87596
	Equal variances not assumed			1.083	4.883	.329	15.00000	13.84437	-20.84593	50.84593
Hardness	Equal variances assumed	825.000	.000	.873	6	.416	123.75000	141.72119	-223.02926	470.52926
	Equal variances not assumed			.873	3.003	.447	123.75000	141.72119	-327.01888	574.51888
TDS	Equal variances assumed	1140.833	.000	.958	6	.375	166.50000	173.88574	-258.98307	591.98307
	Equal variances not assumed			.958	3.045	.408	166.50000	173.88574	-382.30122	715.30122
Ammonia	Equal variances assumed	4.310	.083	-4.448	6	.004	-2.81250	.63225	-4.35956	-1.26544
	Equal variances not assumed			-4.448	3.059	.020	-2.81250	.63225	-4.80275	-.82225
Nitrite	Equal variances assumed	7.218	.036	-4.876	6	.003	-1.22500	.25125	-1.83978	-.61022
	Equal variances not assumed			-4.876	3.060	.016	-1.22500	.25125	-2.01578	-.43422
Nitrate	Equal variances assumed	13.500	.010	2.611	6	.040	12.50000	4.78714	.78630	24.21370
	Equal variances not assumed			2.611	3.000	.080	12.50000	4.78714	-2.73480	27.73480
phosphate	Equal variances assumed	2.267	.183	-1.317	6	.236	-1.43750	1.09152	-4.10834	1.23334
	Equal variances not assumed			-1.317	4.650	.249	-1.43750	1.09152	-4.30804	1.43304

Table 2: inference

H0: there is no significant difference in parameters due to alga and bacteria.
Procedure: Student independent paired t test taking control and test as the grouping variables. (Considering they don't make much influence)
Calculation: Test was conducted and output was determined

Note: Ammonia, Nitrite and Nitrate have made a significant difference. Since ($p<.05$) at 5 percent level of significance we reject H0, null hypothesis. Whereas in Alkalinity, TDS, Phosphorous, Hardness there is no significant difference since $p> 0.05$ at 5 percent level of significance we accept H0.

In this study, immobilization of nitrifying bacteria *Nitrosomonas* sp. and *Nitrospira* sp. was performed using sodium alginate beads. *Chlorella vulgaris* was also immobilized along with nitrifying bacteria to test the efficiency of the alga along with bacteria in improving the water quality. Immobilization technique was also used by Tam et al. (2000) where *Chlorella vulgaris* was entrapped in calcium alginate as algal beads and employed to remove nutrients (N and P) from simulated settled domestic wastewater.

Adsorption of microalgae on alginate gels was found to increase the algal uptake of N and phosphate. In 1985, Chevalier reported that hyperconcentrated microalgae cultures immobilized in kappa-carrageenan beads were able to efficiently remove nitrogen and phosphorus from urban wastewaters.

Immobilization of various bacteria has also been studied previously by many authors for the removal of ammonia from river water, sea water and municipal waste water, respectively, and was proved effective [David *et al.* (1982); Endong *et al.* (2008); Lianpeng *et al.* (2009)]. Therefore this procedure of immobilizing whole cells was used in the present study to entrap nitrifying bacteria (*Nitrospora* and *Nitrosomonas* sp.) and microalga (*Chlorella vulgaris*) in sodium alginate beads.

Zebra fish possesses several advantages over other animal models such as high fecundity, ease of maintenance, optical clearance of embryos, break of daylight triggers mating in zebra fish (many other fish only lay eggs in the dark), rapid embryonic development, and low maintenance cost (Avdesh *et al.*, 2012). They are also highly resistant to physical/chemical changes in water.

The results in this study were positive i.e. there was a significant reduction in ammonia and nitrite content in the test tanks of both experiments. There was not much difference in the reduction of the above parameters between experiments 1 and 2 (bacteria and bacteria-microalga respectively). In addition, other parameters such as alkalinity, hardness and TDS were reduced in experiment with algae. Therefore, it can be concluded that the parameters of water improved while testing it with the consortium. pH remained constant at around 6.5.

Problems encountered

Turbidity (due to overgrowth), alkalinity, hardness and TDS were high in test compared to control in experiment 1. One of the main problems was preparation of beads. As Pasteur pipette was used, it was time consuming and cannot be used in large scale production. This can be overcome by the following techniques.

- Rotating nozzle ring which sprays the gum solution–bacterium mixture into rotating vessels containing cross linking solution (Matulovic *et al.*, 1986). This apparatus is capable of producing beads of the requested size in large quantities per unit time.

- A dual fluid atomizer in which sodium alginate solution droplets are sheared off the tips of hypodermic needles into calcium chloride solutions to produce beads with an average diameter of 1 mm (Rehg *et al.*, 1986).

Future prospects

From the present study, it is concluded that ammonia and nitrite showed reduction in test compared to control in both experiments (with bacteria and alga). Test carried out with beads immobilized with bacteria and

microalga improved water parameters like hardness, alkalinity and TDS. Further research is warranted to minimize the hardness, turbidity and alkalinity when using beads immobilized with bacteria. This technique of immobilization could be used in fish breeding industries to minimize the loss faced due to fish death caused by ammonia toxicity. It can also be used in various other aquatic systems like prawn, shrimp and oyster aquacultures. Immobilization allows multiple uses of beads, and these beads are easy to retrieve due to their size. Usage of water could be decreased to a large extent as the toxic factors in water can be controlled.

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