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Determination of Effective Microbial Community for Biofloc Shrimp Culture System

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Abstract
This experiment was conducted for 50 days in four 100 L tanks with 15 cm of bottom area filled with soil. and completely randomized with three replicates for each one of four treatments. *Penaeus monodon* seeds were stocked at 30/m². All tanks were filled with chlorinated sea water and each tank received following microbes, Tank A – with only diatom,
Tank B – with only Nitrogen cycle bacteria, Tank C -- with only Sulphur cycle bacteria, Tank D -- with Diatom, bacteria involved in both nitrogen and sulphur cycles. The results, with a particular group of microbes were not effective, but when diatom, nitrogen and sulphur cycle bacteria work together in a culture environment, these can control water quality very effectively and gives best result. The tank D showed highest 7.5 gm ABW, 90% survival, 18 ml biofloc formation and lowest FCR 1:1.5. The results were statistically significant ($p<0.05$).

Keywords: Biofloc, BFT, shrimp, culture system

1. Introduction
The biofloc system consist non-living matter, and a variety of many microorganisms, principally algae and bacteria. The presence of biofloc has been reported to confer many beneficial effects on shrimp culture, the degree to which these effects are realized depends greatly on the community structure of the floc, which evolves over the course of culture and which is affected by the culture conditions (Boyd 1989; Burford 1997) [5, 9].
Diatoms are considered to be a beneficial algal group (Boyd 1989; Burford 1997) [5, 9] because they are a source of food and nutrients for most aquatic animals. And also, it has a major role in maintaining water quality through the process of photosynthesis (Hargreaves 2006) [16]. The importance of these microalgae in maintaining water quality and nutrition of the shrimp is already well known in conventional systems (Patil *et al.,* 2007) [30]. But, further studies need to be developed in order to assess its role in bio-floc culture systems.
The microbial community present in minimal-exchange systems not only provides supplemental nutrition, but also maintains water quality and controls waste accumulation (Bratvold and Browdy 1998, Avnimelech 2006, Hargreaves 2006) [7, 2, 16]. Bacteria are important microbial assemblage in biofloc systems to remove toxic ammonia in nitrogen cycle. So, the role of these bacteria on biofloc system has also to be studied. The bottom soil of old ponds tends to accumulate high organic load and turns in to black soil, which has high hydrogen sulfide. Sulfide toxicity is known to cause mortality due to hypoxia (Bagarinao and Lantin-Olaguer 1999) [4]. Sulphur oxidicing bacteria plays major role in removing toxic H_2S. So the role of sulphur oxidicing bacteria on biofloc system should also be studied.
For development and application of this biofloc technique, monitoring and managing the biofloc microbial community structure is very much needed. So, this study was aimed to determine the effect and role of diatom, bacteria involved in nitrogen and sulphur cycle on the water quality and growth of *Penaeus monodon*.

Materials and Method
Experimental design
The experimental design was for 50 days and completely randomized with three replicates for each one of four treatments. Each experimental unit consisted of a synthetic tank (100 L) with 15 cm of bottom area, filled with soil taken from ten year old pond bottom. The tank equipped with a circular hose with four porous stones set at the bottom to keep intense aeration.

Seeds were stocked at 30/m² in four different tanks as follows,
Tank A – Chlorinated water with only diatom,
Tank B – Chlorinated water with bacteria involved in only Nitrogen cycle,
Tank C -- Chlorinated water with bacteria involved in only Sulphur cycle,
Tank D -- Chlorinated water with Diatom, bacteria involved in both nitrogen and sulphur cycles,

Seed collection
Healthy *Penaeus monodon* seeds were collected from a commercial shrimp hatchery Lotus at Marakkanam.

Diatom collection
Chetoceros sp. was collected from Lotus shrimp hatchery at Marakkanam for the whole experiment.

Probiotic collection
Probiotic which contains *Nitrosomonas, Nitrosococcus, and Bacillus* sp. for nitrogen cycle called "Protect™" and *Thiobacillus* sp*., Desulfovibrio* sp., for sulphur cycle called "Thionil™" were collected from Poseidon BioTech, Chennai, India.

Shrimp culture system and performance
The experiment was started when the total suspended solids (TSS) in the medium for development of the bio-flocs reached a concentration of 250 mg L^{-1}, value within the recommended standards for bio-floc systems (Samocha *et al.,* 2007) [35]. The shrimps were fed with commercial diet (40% CP) at a ratio of 5% of the biomass four times a day (6.00 am, 12.00 noon, 6.00 pm, and 11.00 pm). Organic fertilization was performed daily for Tank A for diatom development. Other tanks received jaggery daily. The feed was adjusted daily according to the feed consumption.

Shrimp performance
Weekly sampling to estimate survival rate (%), Food conversion ratio (FCR), average final body weight (ABW), weight gain (g/ week) were used to assess dietary effects on shrimp performance. At the end of the 60- days experiment, the same performance indicators were estimated in addition to final biomass (kg/ pond) per treatment basis.

Water quality analysis
During the experimental period, water quality in the culture systems was monitored daily for dissolved oxygen (mg/L), salinity (ppt), pH and temperature (°C) in all tanks at morning 9.00 am using a DO meter, refractometer, pH pen, and standard digital Thermometer respectively (Attago™, Japan). The concentrations of ammonia of water in the tanks were measured according to the procedures of Parsons *et al.,* (1984) [29]. Hydrogen sulfide content was measured as described by Pachmayr (1960) [28].

Volume of biofloc
Bio floc formation was measured by the floc settlement in Imhoff cone through pouring one liter of tank water (Avnimelech 2009) [3]. Content of Biofloc particle were examined through microscope (Fig 1, 2, 3, 4, 5)

Statistical analysis
Two-way analysis of variance was used to detect differences between treatments.

Results
Shrimp performance
ABW and survival of P. monodon were minimum (4 g & 70% respectively) in Tank A with diatom and maximum (7.5g and 90%) in Tank D with diatom, Nitrogen and Sulphur cycle bacteria (Table 1). Tank B with nitrogen cycle bacteria, and tank C with sulphur cycle bacteria exhibited only intermediary values: 5.8 g and 80% for ABW and survival respectively for tank B and 5.8 g and 80% for tank C. Values are highly significant ($p< 0.05$) for ABW (Table 1, Fig 6) and Survival (Table 1, Fig 7). Food Convertion Ratio (FCR) (Fig 8) ranged from 1.5 in tank D and 2.0 in tank A with intermediate values of 1.7 and 1.8 in tank B and C respectively. Similarly the volume of biofloc was the maximum (18 ml) in tank D and minimum (5 ml) in tank A and with intermediate values of 12 and 10 respectively in tank B and C. The values are statistically highly significant between treatments.

Table 1: Growth performance parameters

Parameters	Tank A (with diatom)	Tank B (with nitrogen cycle bacteria)	Tank C (with sulphur cycle bacteria)	Tank D (with diatom, nitrogen and sulphur cycle bacteria)
Stocking Density(m²)	30	30	30	30
Days of Culture	50	50	50	50
Average Body Weight (g)	4.0	5.8	5.0	7.5
Feed Convert –ion Ratio	2.0	1.7	1.8	1.5
Survival%	70	80	80	90
Volume of biofloc (ml)	5	12	10	18

Water quality analysis
There was no significant difference ($p>0.05$) between treatment in terms of water quality parameters such as temperature, salinity, dissolved oxygen and pH. However, the levels of ammonia and H_2S were significant (Table 2, 6). Dissolved oxygen (DO) was high (6.5 mg/l) in tank A with diatom, all other tanks showed no significant DO levels.
Hydrogen Sulphide (Fig. 9) was high (0.4 mg/l) in tank A with diatom and low 0.01 mg/l in tank C with sulphur cycle bacteria. Ammonia (Fig. 10) was observed high (1.05 mg/l) in Tank A with diatom and low (0.31 mg/l) in Tank B with nitrogen cycle bacteria.

Table 2: Water quality parameters

Parameters	Tank A (with diatom)	Tank B (with nitrogen cycle bacteria)	Tank C (with sulphur cycle bacteria)	Tank D (with diatom, nitrogen and sulphur cycle bacteria)
Temperature (°C)	26.5	26.3	26.6	26.5
Salinity (‰)	30.5	32.5	32	32.8
Dissolved oxygen (mg /L)	6.5	4.6	4.8	5.5
pH	8.3	7.5	7.6	7.9
Ammonia (mg /L)	1.05	0.31	0.9	0.35
H2S (mg /L)	0.4	0.3	0.01	0.02

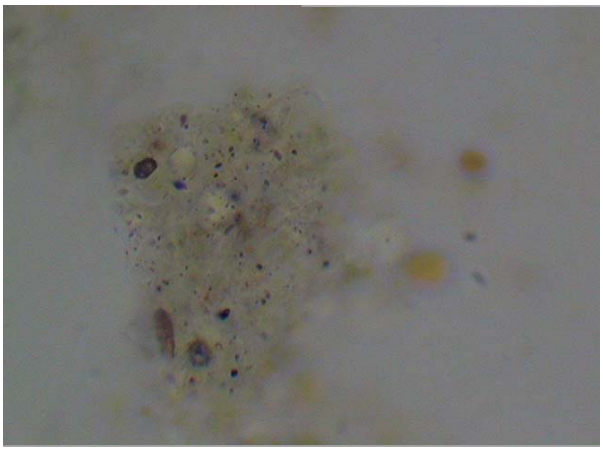

Fig 1: Biofloc particle (100x)

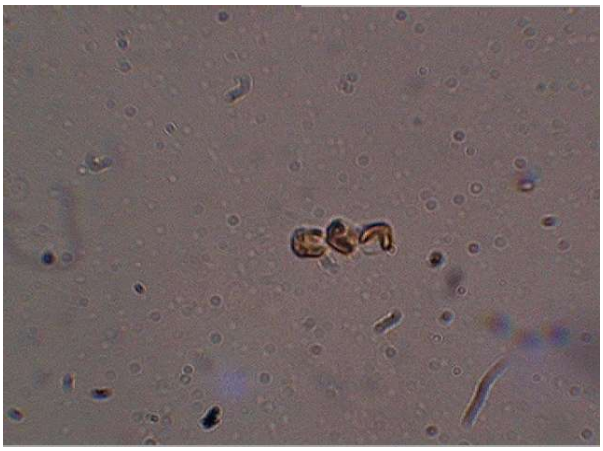

Fig 4: Biofloc particle with accumulated *Chetoceros* (100x)

Fig 2: *Chetoceros* sp. used as diatom in experiment (100x)

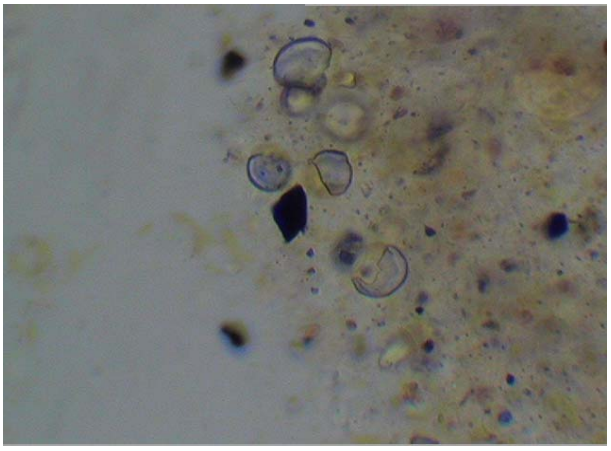

Fig 5: Biofloc particle with organic matter and shells (100x)

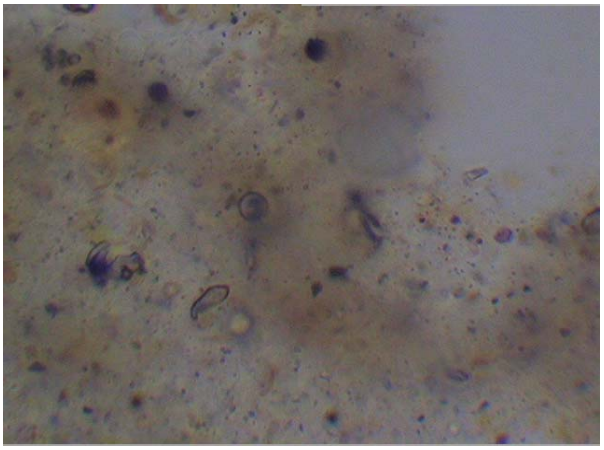

Fig 3: Biofloc particle with detritus and plankton (100x)

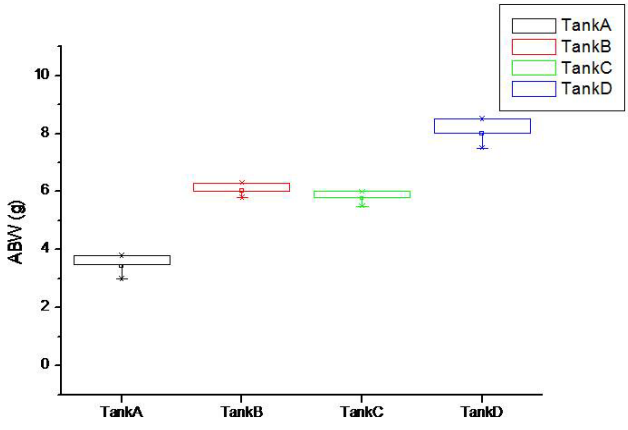

Fig 6: Average Body Weight (ABW) of *P. monodon* under different treatments

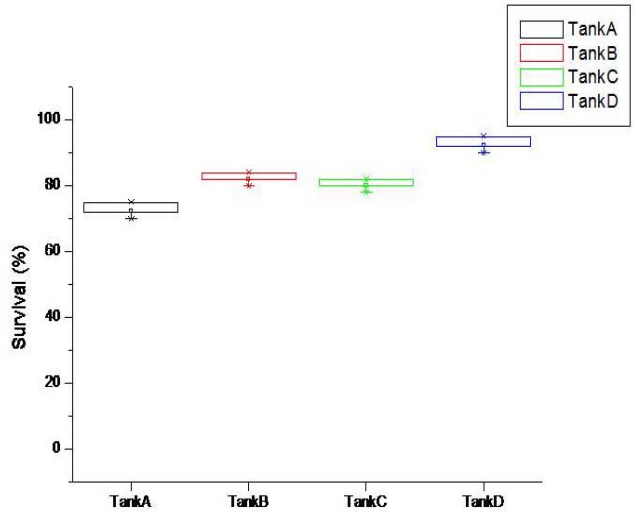

Fig 7: Percentage of survival of *P. monodon* under different treatments

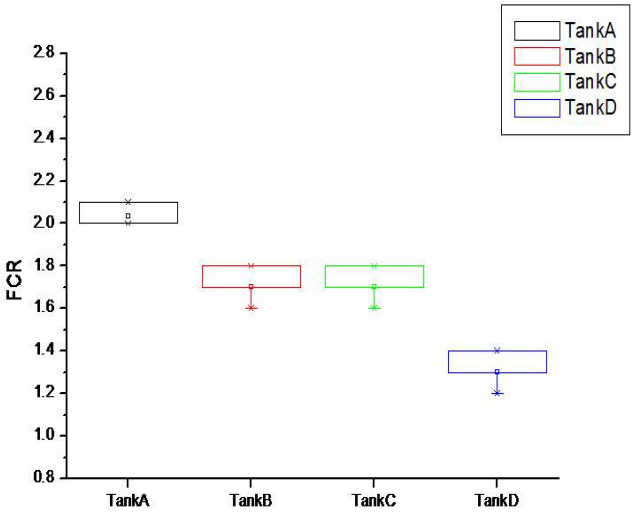

Fig 8: Food conversion ratio in *P. monodon* under different treatments

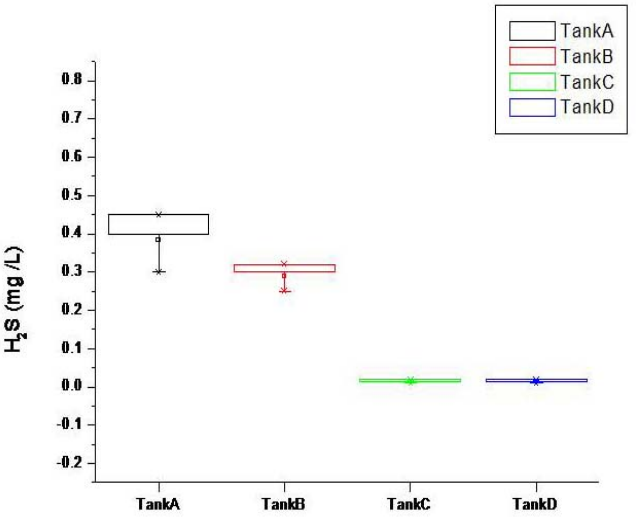

Fig 9: Concentration of hydrogen sulphide in Tank A- diatom, Tank B- nitrogen cycle bacteria, Tank C- sulphur cycle bacteria, Tank D- diatom, nitrogen and sulphur cycle bacteria

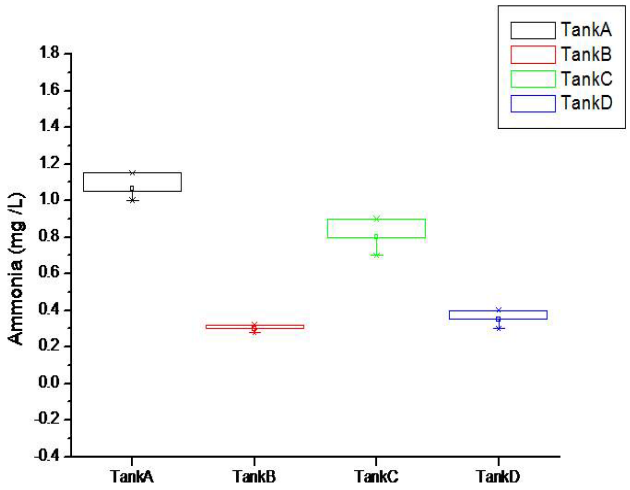

Fig 10: Concentration of ammonia in different treatments

Discussion

Temperature is one of the most important factors that control the growth of marine shrimp, because it directly affects metabolism, oxygen consumption, growth, molting and survival. In general, a sudden change of temperature affects the shrimp immune system. The optimum range of temperature for the black tiger shrimp is between 26 to 30 °C (Ramanathan *et al.,* 2005) [33]. The temperature in the present study was maintained at 26.5. Temperature range of 28 to 33 °C supports normal growth (MPEDA, 1992) [27] as observed in the present study.

The highest DO concentration 6.5 mg/l (super saturation) in tank A was the result of intense photosynthetic activity of the diatoms. The lowest DO concentration in other tanks was probably due to a higher respiration rate of the heterotrophic microbial community. However, the DO concentration was kept within the optimum range for *P. monodon* growth in all treatments. Similar results have been reported by Burford *et al.*, (2003) [10]. The high pH 8.3 observed in the tank A was also due to the photosynthesis process conducted by the microalgae, with the removal of CO_2 from the medium. The pH variation was low in all the other tanks throughout the experiment and remained close to neutrality. Even with significant differences between treatments the pH remained in a range considered adequate for the proper performance of penaeid shrimp (Cohen *et al.,* 2005) [12].

Antony and Philip (2006) [1] state that bacteriological nitrification is the most practical method for the removal of ammonium from closed aquaculture systems. Among bacteria involved in nitrogen cycling the ammonium oxidizers are generally high in aerated conditions. Ammonium oxidizers are usually sensitive to sudden change in temperature, reduction in available nutrients and H2S (Joye and Hollibaugh 1995) [20]. This might be a reason for the increased ammonia 1.05 mg/L in tank A because it contains high H2S level (0.4 mg/L). They also require an optimum temperature between 20 and 30° C (Foch and Verstate 1977) [14]. Most importantly, nitrification rates are dependent on ambient dissolved oxygen concentrations. Gunderson and Mountain (1973) [15] have found that nitrification rate decreases if dissolved oxygen concentration is less than 0.3 mg L^{-1}. In addition, the optimal pH for nitrification is 7–7.5 (Wong-Chong and Loehr 1975) [38]. Cheng and Liu (2001) [11] have shown that aeration creates favorable conditions for nitrifying bacteria at dissolved oxygen concentrations of 4–6 mg L^{-1}.

Total ammonia concentration was significantly high in Tank A, probably due to the inoculation of diatoms and the input of nitrogen compound residues used in the preparation of the culture media of microalgae (Jensen *et al.,* 2004) [19], and also algae tend to bloom in response to nutrient additions. Often, blooms are followed by an algal crash in which toxic ammonia is released back into the system (Ray *et al.,* 2009) [34]. Tank C also showed high ammonia (0.9 mg/L) concentration which might due to absence of nitrifying bacteria. However, in all treatments the concentrations of ammonia remained below the safety levels recommended for juveniles of *L. vannamei* (Lin and Chen 2001; 2003) [11, 23, 24]. The low concentration of ammonia in Tank B and D was due to the microbial community that uses these sources of nitrogen to form their biomass (microbial protein). Moreover, there is a high nitrification rate in biofloc systems due to the nitrifying bacteria that ensure the rapid oxidation of ammonia and toxic nitrite to nitrate, relatively harmless to shrimp (Holl *et al.,* 2006; Boyd 2007) [17, 9]. Joye and Hollibaugh (1995) [20] have observed that nitrification rates either get reduced or inhibited when hydrogen sulphide concentration increases in the marine sediment; it would be main reason for high ammonia in tank A, because H_2S was also high in this tank.

Promising results are obtained by Kuhn *et al.,* (2008) [22] in an experiment where the effluent from a commercial farm of tilapia receives biological treatment and the bio-floc produced is supplied as food supplement for the cultivation of marine shrimp. Best water quality with low concentrations of ammonia (0.05 mg/ L) results in higher survival and growth of shrimp. The safe levels of ammonia for shrimp culture has been reported to be <1mg/L (MPEDA, 1992) [27]. It was confirmed in the present study by low survival (70%) at Tank A and high survival (90) at tank D, because these two tanks showed highest (1.02 mg/ l) and lowest (0.04 mg/ l) ammonia concentrations respectively.

The greatest weight gain and more efficient food conversion in Tank D confirmed that diatom, nitrifying and sulphur oxidizing bacteria participated significantly to the better performance of the shrimp because they consumed less food (79% of provided commercial diet) than those reared in treatment Tank A, B, and C (87% of provided commercial diet). The food conversion rate is an extremely important index in the aquaculture activity, since the cost of food generally represents up to 60% of the total cost of the production (Wasielesky *et al.,* 2006) [37]. A strict technical control is necessary so that the feed supplied to the cultured organism is efficiently converted into biomass. Shrimp reared in Tank D had Food conversion ratio of 1.5 and its weight gain was significantly higher (17%) while consuming less commercial diet when compared to the tank A, B and C treatments. *Chaetoceros* sp., is a microalgae rich in essential nutrients for the larval stage of the shrimp and they are commonly used in hatcheries (Brown *et al.,* 1997) [8]. Analyses of the proximal composition show that *Chaetoceros* sp., has protein and total lipid contents of 43.11 and 21.48%, respectively (Jaime-Ceballos *et al.,* 2006) [18]. Diatoms are easily digestible by shrimp due to its low fiber content (Moss 2000) [26]. Ju *et al.,* (2009) [21] have evaluated different biochemical components of the diatom *Thalassiosira weissflogii* which may enhance shrimp growth, by adding whole diatom, carotenoid, and residue fractions to a formulated diet.

The sources of sulphide in the pond soils are the organic matter (shrimp food and excreta) and seawater (Prabnarong *et al.,* 1994) [32]. After the shrimp raising activities stop, no further sulphide is added to the pond soils. Moreover, it is generally known that Sulphur in soils is taken up by *Thiobacillus* sp. and released as SO_2 under aerobic conditions during the summer season, and utilized by *Desulfovibrio* sp. and released as H_2S under anaerobic conditions during the rainy season (Tisdale *et al.,* 1985) [36]. The presence of sulfate reducers and sulfide oxidizers is suggestive of efficient sulfur cycling in an environment (Madrid *et al.,* 2001) [25]. The sulfide oxidizers and sulfate reducers were relatively less in their abundance than the heterotrophs and nitrifiers in the pond (Devaraja *et al.,* 2002) [13]. So, it is necessary to add Sulphur reducing bacteria to the culture system. Paulraj *et al.,* (1998) [31] suggest <0.03 mg l^{-1} H_2S levels for shrimp culture. It was observed in the present study that in Tank C and D sulfide was efficiently oxidized by sulphur cycle bacterial community.

Conclusion

The diatom is nutritive but it cannot involve in the process of denitrification and sulphur oxidation, it can only produce oxygen, Nitrifiers can involve only in the nitrogen cycle, thus it can eliminate toxic ammonia but it cannot oxidize sulphur and produce oxygen, Sulphur oxidizers can involve only in sulphur cycle, thus it can eliminate toxic sulphur but it cannot eliminate ammonia and produce oxygen. Biofloc culture system has good microbial environment through floc formation, but the result with a particular group of microbes is not properly ratified, but when diatom, nitrogen and sulphur cycle bacteria work together in a culture environment these can control water quality very effectively. Thus it can be used as an effective microbial community for the biofloc systems.

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