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Role of Different N/P Ratios of some Manures and Phosphate Fertilizer on Bacterial Enzyme and Algal Growth as Eutrophication Control Strategy

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Abstract

The growth responses of green algae and blue green algae were estimated in small simulated eutrophic systems receiving either high N/P ratios maintained with some commonly used manures like Badam Oil Cake (BOC), Mustard Oil Cake (MOC), mixture of BOC and MOC (MIX) or high P/N ratios created by Single Super Phosphate (SSP). Prior to the main experiment a pilot study was carried out with different manures and fertilizers with algal rich pond water to select the suitable N/P ratios of the manures for the main experiment. Based on the results of the primary productivity of the pilot studies, treatments with varying N/P ratios of 60:1(BOC), 50:1(MOC) and 40:1(MIX) were set up in the main experiment along with N/P ratios of 1:20 and 1:40 with SSP. In both the experiments, pond water rich in mixed algal population was added to each jar. The bacterial enzyme activity in the MOC treatment played an important role as a driving factor ensuring the role of microbial heterotrophic pathway in controlling harmful algal bloom. The role of N/P ratio in shifting the food chain from blue green algae to benign algal food chain, *i.e.* green algae is evident from a better positive correlation between the N/P ratio with green algal growth rather than the *Microcystis* sp. bloom. So, the effects of N/P ratios of Badam Oil Cake (60:1), Mustard Oil Cake (50:1) and Mix (Badam Oil Cake + Mustard Oil cake) showed favourable conditions for the growth of green algae thus controlling the major eutrophication syndrome.

Keywords: Eutrophication; N/P ratio; Green Algae; Blue-green Algae; Organic Manures; Fertilizer.

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INTRODUCTION

Eutrophication as a result of pollution from point and non-point sources, possesses serious threat to environment and human being (Harper, 1992; Carpenter et al., 1998). This is specially due to nutrient enrichment of phosphorus (P) and nitrogen (N) in water bodies, more specifically P and N in freshwater and N in oceanic water (Le *et al.,* 2010; Zhou *et al.,* 2022). Tropical eutrophic waters often

experience cyanophycae or myxophyceae blooms, known as Harmful Algal Bloom (HAB) which is the accumulation or aggregations of planktonic blue-green algae at the surface of lakes and reservoirs (Chislock *et al.*, 2013). Because of warmer temperature, they multiply fast and form scum in the surface of numerous stagnant lakes, wetlands and ponds. Cyanophyta algal bloom causes depletion of water quality and serious health hazards of aquatic animals, humans and livestock (Hwang et al., 2020; Wang *et al.*, 2021; Wang *et al.*, 2022).

The growth of blue-green algae has been recorded year-round in some tropical lakes in Uganda (Lake George) and Ethiopia (Lake Aranguadi and Lake Kilotes), although in temperate locations, the production of bloom is seasonal, occurring in the summer when temperatures are high (Ganf 1974). It is common knowledge that cyanophytes spend the winter in the sediments at the bottom of bodies of water, either as dormant spores or as the source of new growth in the spring (Ho *et al.*, 2024). Similarly, for *Microcystis* sp., a successful overwintering is essential because the sediment offers an inoculum for population expansion in the spring (Verspagen *et al.*, 2005). Microcystis colonies were found to double in number after being resuspended from the top 80-100 mm of mud in Lake George (Uganda) by open-water algal blooms with a high concentration of thriving species. (Piehler *et al.*, 2009). Under this backdrop, it is most important to have a lake management plan for controlling eutrophication (Schindler, 1974; Moal *et al.*, 2021).

The release of organic matter in large amount from the decomposed bloom of cyanobacteria can be transformed into inorganic nutrients by bacteria (Wand *et al.*, 2021) which can be utilised by *Microcystis* sp. This decomposition of organic bloom can influence the composition of bacterial community (Shi *et al.*, 2017). The present study envisages to examine the primacy of nutrient manipulation on the growth criteria of Cyanophyceae using particularly organic nutrient manure. The extensive review of literature reveals that N/P regulation have a tremendous regulation on the growth of blue green algae (Plinski and Jozwiak, 1999). One of the big problems of the eutrophication is the wrong direction of food energy as a huge algal biomass or primary producers are not being used by the next trophic level consumers (Liu *et al.*, 2019) and therefore they are not directly linked with grazing food chain. One of the challenge is to direct this wrong pathway of food chain into right direction. In other words, it is possible to shift the food chain from blue green algae to benign algal food chain, *i.e.* green algae.

Therefore, the present study was undertaken to explore the possibility of shifting the food chain by nutrient manipulation with the view of understanding the shifting of the cyanobacterial algal bloom to a benign green algal growth. Due to lack of research in this discipline the experiment was undertaken. The novelty of the present study was to examine the role of organic manure as a nutrient manipulating agent to favour the growth of green algae.

MATERIALS AND METHODS

The present study was conducted in two phases:-

(a) Pilot study

(b) Main experimental trial

(a) Pilot Study

Experimental set-up: Prior to start of the main experiment a pilot study was undertaken to examine the responses of different organic manures and inorganic fertilizers to create an eutrophic condition. 1 litre of pond water was taken in each jar of 3.5 litres capacity in replicate which was treated with following 13 treatments (Table 1).

Table 1: Types and doses of treatments applied in the pilot study

Treatment	Type of manure/fertilizer	Quantity (gm/l)
T-1	Solid Urea	0.20
T-2	Mustard Oil Cake (MOC)	2
T-3	Neem Oil Cake	2
T-4	Badam or Peanut Oil Cake (PNOC/BOC)	2
T-5	Liquid Urea	2 (in ml/l)
T-6	Single Super Phosphate (SSP)	1
T-7	Mix-1:	BOC: 1
	BOC,MOC,SSP and Charcoal	MOC: 1; SSP: 0.5; Charcoal: 1

Table Cont...

T-8	Mix-2: BOC,MOC,SSP and Glucose	BOC: 1; MOC: 1; SSP: 0.5; Glucose: 1
T-9	Mix-3: BOC, MOC, Urea and Charcoal	BOC: 1; MOC: 1; Urea: 1; Charcoal: 1
T-10	Charcoal	1
T-11	Glucose	1
T-12	Tea leaves and Methi leaves	Not measured and given arbitrarily
T-13	Reference or Control	Nothing given

The whole set-up of the thirteen jars were kept under the sun till evening for the next nine days so that the sunlight can penetrate inside the water in order to create algal bloom which is an important indicator of eutrophication. The treatment water was kept stirring in every two days interval so that the nutrients do not settle down at the bottom. Different water quality parameters along with gross primary productivity (GPP), net primary productivity (NPP) and community respiration (CR) during the pilot study were measured following the standard protocol (APHA, 2021) on day 1 before treatment, and then on day 4, day 10. After day-10 the pilot study was terminated.

(b) Main Study

Experimental design: The main study was conducted in eighteen 3.5l plastic jars allotted to 5 treatments and one control in triplicate. The jars were filled with pond water containing dominant blue green algae. Green algae and diatoms were present to a lesser extent. The N/P ratios of the manures were selected based upon the result of primary productivity of phytoplankton in different treatments in the pilot study. The doses of different manure for N/P ratio selection was primarily based on approximate composition of manure. Since the manure is chemically composite in nature, the residual phosphorus content of the selected manure was not considered in the selection of N/P ratio of treatment. Finally following N/P ratios with higher and lower values of both nitrogen and phosphorus were selected and allotted to the five treatments

i) BOC- 60:1,

ii) MOC- 50:1,

iii) MIX of BOC and MOC - 40:1,

In case of SSP fertilizer the ratio was

i) SSP-1:20

ii) SSP-1:40

iii) a set of control.

Each treatment as well as control had 3 replicates. The manures were added during the beginning of the experiment, while second installment was applied on 17th day of the trial. All the jars were exposed to sunlight during the day and transferred to laboratory keeping under artificial light after sunset. The experiment was continued for 30 days.

Water Quality Analysis

The standard protocols of APHA (2021) were followed to examine the different physico-chemical parameters of water such as dissolved oxygen (DO), pH, conductivity, redox potential, total alkalinity, chemical oxygen demand (COD), dissolved organic-C, orthophosphate (PO_4-P), ammonium-nitrogen (NH_4-N), nitrite-nitrogen (NO_2-N) and nitrate-nitrogen (NO_3-N). Sampling was done at 5-10 days interval.

Plankton Analysis

Subsamples of plankton were collected from each jar on day 4, day 8, day 13, day 24, day 29 and analyzed for both qualitatively and quantitatively (APHA, 2021). The planktons were categorized on the dominance of Chlorophyceae and Cyanophyceae and remaining few species were expressed as others.

Isolation of bacteria

All the routine procedures were followed (sterilization of glassware, media preparation, inoculation of sample and incubation) for culture of heterotrophic bacteria (HB) and phosphate solubilizing bacteria (PSB). Screening for microbes was done in specific medium in order to isolate the tested groups of microbes.

Quantitative assay of bacterial enzyme

The bacterial strains were individually introduced into nutrient broth within Erlenmeyer flasks and incubated for 24 hours. This is followed by centrifugation of the cultures at 10000 rpm for 15 minutes. The resulting pellets were washed twice with 0.85% saline solution and resuspended to achieve a final optical density of 0.100 at 600 nm.

The resuspended supernatants were utilized for the assessment of extracellular phosphatase activity. Acid and alkaline phosphatase activities were assessed by combining two milliliters of reaction buffer (citric acid/sodium-citrate buffer, pH 5.0 for acid phosphatase evaluation and glycine-NaOH buffer, pH 10.0 for alkaline phosphatase evaluation) with 500 µl of p-nitrophenyl phosphate solution (0.115M pNPP in diethanolamine), followed by the addition of 2 ml of supernatant. The reaction mixtures were then incubated at 37°C for 90 minutes and halted by the addition of 500µl 0.5 M calcium chloride and 2 ml of 3M NaOH. One unit of activity was defined as the quantity of enzyme capable of hydrolyzing 1 µM PNPP per minute. Distilled water was employed in place of supernatants for the preparation of blanks. The hydrolysis of p-nitrophenyl phosphate was quantified by measuring the concentration of p-nitrophenol using a spectrophotometer at a wavelength of 410 nm. The concentration of p-nitrophenol was determined by comparison with a standard curve. The quantity of enzyme (mg) necessary to release 1µmole of p-nitrophenol per minute signifies one unit of enzyme activity.

The acid and alkaline phosphatase activity was calculated using the formula:

Enzyme activity (U/mL) = {OD_{410} nm x 1/0.0208 x V (mL) }/ ε x incubation time (min) x E (mL)

V = Total reaction volume (in milliliters) of assay

1/.0208 = Dilution factor of pNP from standard graph

ε = 18.5 millimolar extinction coefficient of p-Nitrophenol at 410 nm

E = Enzyme reaction volume (in milliliters) used

Statistical analysis

One-way analysis of variance (ANOVA) was done with the help of computer software SPSS (Version 7.5) at 1% and 5% level of significance to find the treatment differences on all days of observation. Standard Error values have been provided for each mean value. Correlation study was done in MSExcel to determine the relationship between the variable observed parameters.

RESULTS

(a) Water Quality

(i) Pilot Study

High primary productivity was recorded from the treatments of MIX-3, MIX-1, BOC, MOC. This formed the basis of the final selection (Table 2) of the manure in the main experiment of the study.

Table 2: The Primary Productivity Table of Pilot Study

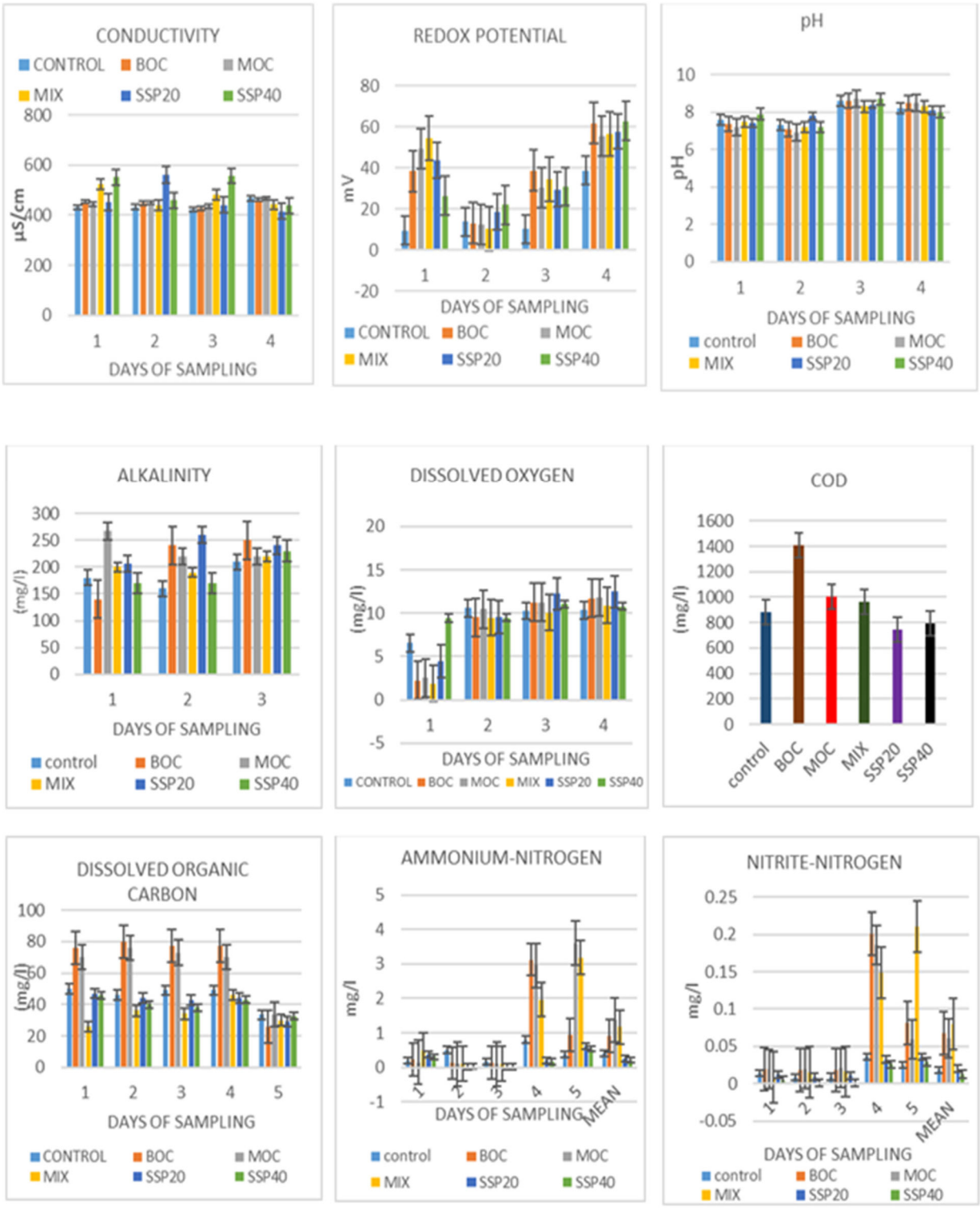
Treatment	NPP (mgC/l/hr)	GPP (mgC/l/hr)	CR (mgC/l/hr)
Solid Urea	0.2	0.389	0.189
Charcoal	0.589	1.369	0.78
Reference	1.2	2.2	1
Mustard Oil Cake	2.42	5.22	2.8
Badam Oil Cake	4.2	12	7.8
Mix-3	7.8	18.2	10.4
Mix-1	12.2	25	12.8

(ii) Main experiment

Conductivity of water ranged from 422.3µS/cm- 523.6 µS/cm in different treatments. One-way analysis of variance carried out on each day which showed a clear-cut differences among the treatments (ANOVA $F_{5,12} \geq 4.873$, $P<0.05$). The highest values differed on different dates of observation. pH of the water ranged from 7.1-8.6. There was no significant difference ($P>0.05$) between the treatments. Redox Potential of water ranges from 9.5 mV- 62.9mV in different treatments (ANOVA $F_{5,12} \geq 109.4$, $P<0.05$). The ranking in values in each treatment was not same on all days of observation and increased considerable towards the end of the experiment (Fig. 1).

Significant treatment differences were observed for PO_4-P ($F_{5,12} \geq 79.54$; $P<0.05$) with the concentrations in BOC, MIX and MOC treatments remained lower than that in the SSP treatments. The concentration of phosphate decreased by 34.22% at the end of the experiment from the 1st treatment. The values peaked in SSP20, SSP40 and MIX after 2nd treatment. The concentration of ammonium-nitrogen ranged from 0.014mg/l to 3.598 mg/l in different treatments employed. The values were consistently higher in MIX and MOC and lower in SSP20 and SSP40 ($F_{5,12} \geq 59.154$; $P<0.05$). After 2nd treatment the values of ammonium-nitrogen peaked. Nitrate nitrogen was entirely absent during the entire period of experiment. Although initially in the pond water it was present at concentration 0.351 mg/l. DO increased sharply on the 4th day of observation showing prominent treatment differences (*ANOVA $F_{5,12} \geq 14.15$, $P<0.05$)* with high values ranging from 14-18 mg/l in MIX, BOC and MOC treatments (Fig. 1).

All the treatments were contributed mainly by chemical oxidation of organic substances rather than biological oxidation since BOD was either negligible or undetected from the samples. The average value of COD was high in BOC followed by MOC and MIX (ANOVA: $F_{5,12} \geq 38.76$: $P<0.05$). Organic carbon of water ranged from 34.4mg/l – 67.2mg/l in different treatments with clear cut differences of treatments on all the days examined ($P<0.05$). The value decreased considerable at the end of the experiment. Alkalinity of water ranged from 140mg/l – 267mg/l in different treatments ($F_{5,12} \geq 5.17$: $P<0.05$) (Fig. 1).



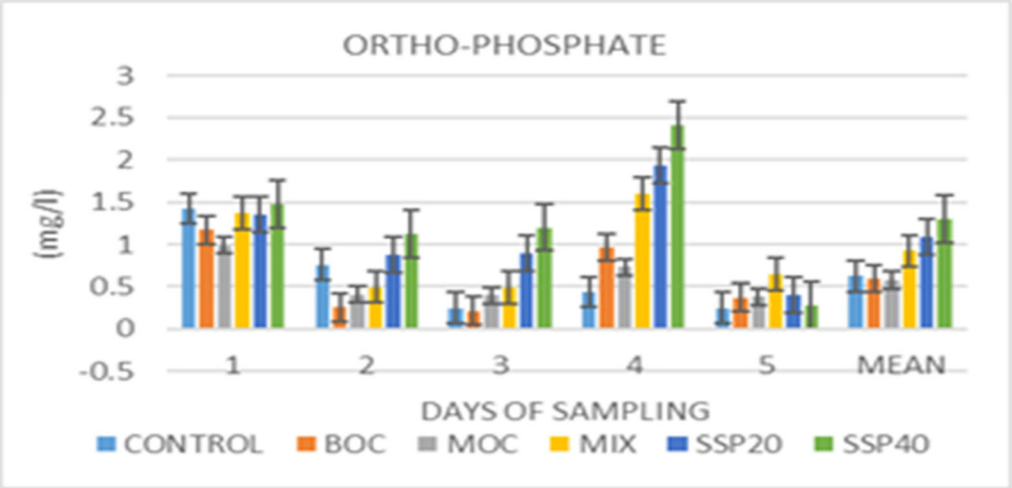


Fig. 1: Results of the different water quality parameters (mean ±SE values) observed on different days of sampling.

(b) Plankton analysis

(i) Qualitative Analysis

The genera present in water of Blue green algae and Green algae (Table 3).

Table 3: Genera of Blue green algae and Green algae found in the water samples.

Genera of Blue Green Algae	Genera of Green Algae
Microcystis sp	Chlorella sp
Oscillatoria sp	Coelastrum sp
Merismopedia sp	Scenedesmus
Anabena sp	Dictyoshaerium sp
	Pediastrum sp
	Ankistrodesmus sp

(ii) Quantitative Analysis

Microcystis Colony Growth

Microcystis Colony ranged from 114 count/l – 3543 count/l in different treatments. One-way analysis of variance carried out on each day showed that the clear cut differences of treatments in all the days examined (ANOVA: $F_{5,12} \geq 1.93$: $P<0.05$). Among the treatments the counts remained quite high on most of the days of observation in BOC, MOC and MIX. Although, differences among treatments were significant on different days of observation, overall mean values for each treatment were not different from some of the treatments to other for example, the overall mean of MIX (913 Count/l) and SSP20 (914 Count/l) respectively (Fig. 2).

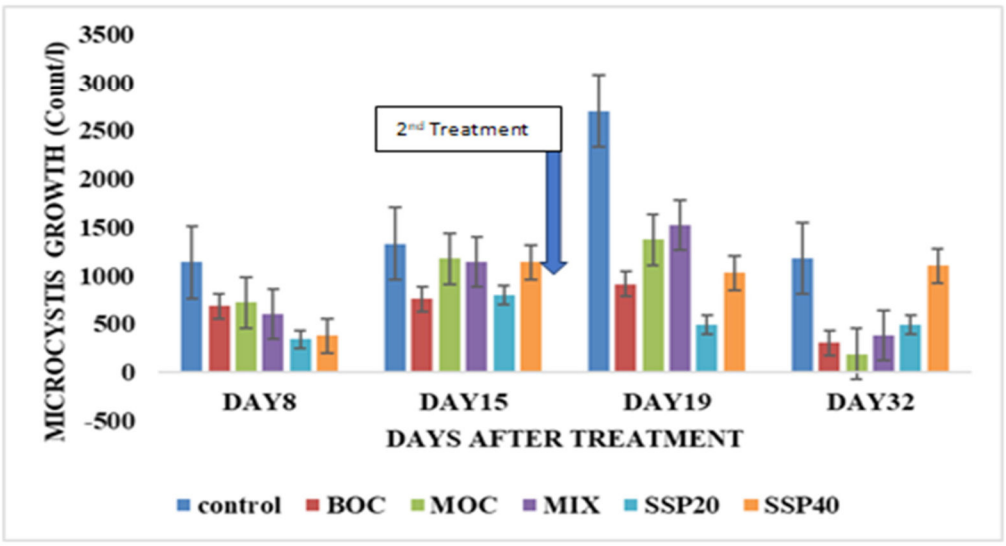


Fig. 2: Treatment wise growth of *Microcystis* sp. (Mean count ±SE) on different days after treatments

Green algae Colony Growth

Green algae ranged from 9240 count/l –69020 count/l in different treatments with significant differences among the treatments on all days of observation (ANOVA: $F_{5,12} \geq 11.12$: $P<0.05$). During the later half of the experiment the population of green algae in BOC and MOC increased sharply in comparison with that in other treatments (Fig. 3).

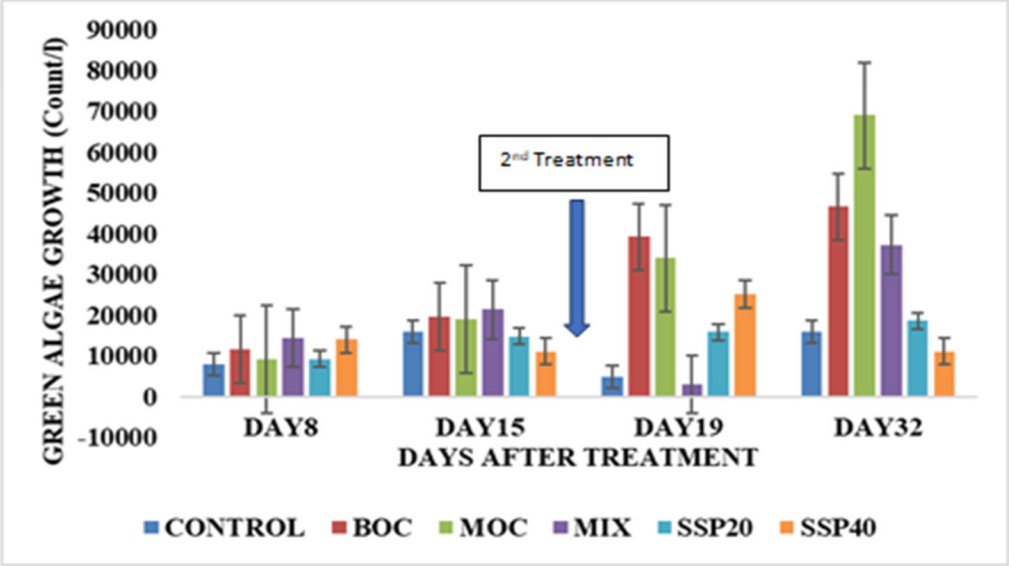


Fig. 3: Treatment wise growth of green algae (Mean count ±SE) in different days after treatments.

(c) Bacterial Enzyme

(ii) Alkaline Phosphatase Enzyme Activity

Initially, from the initial pond water the alkaline phosphatase activity was shown by 3 PSB isolates showing the maximum value of 27.5077 U/ml and lowest value 20.1732 U/ml.

After 1st treatment, each PSB isolates from the treatments SSP20, SSP40, MOC and MIX, the highest value was shown by PSB isolate of treatment MOC (47.747 U/ml) and lowest value by PSB isolate of treatment SSP40 (8.103 U/ml). After 2nd treatment, each PSB isolates from the treatments SSP20, SSP40, MOC and MIX; the highest value was shown by PSB isolate of SSP20 (30.571 U/ml) and lowest value of 17.295U/l by MOC treatment isolate (Table 4)

Table 4: Maximum alkaline phosphatase enzyme activity of different bacterial isolates from the treatments.

Sl. No.	Treatment	Bacteria Group	No of Isolates	Maximum Enzyme Activity (U/Ml)	Day of Maximum Activity
1	Initial	PSB	1	20.544	2nd
2	Initial	PSB	2	20.173	3rd
3	Initial	PSB	3	27.508	4th
Treatment 1					
1	SSP40	PSB	1	8.103	3rd
2	MOC	PSB	1	47.747	3rd
3	MIX	PSB	1	13.024	2rd
4	SSP20	PSB	1	25.372	3rd
Treatment 2					
1	SSP40	PSB	1	20.173	2nd
2	MOC	PSB	1	17.295	3rd
3	MIx	PSB	1	24.908	2nd
4	SSP20	PSB	1	30.571	3rd

(iii) Acid Phosphatase enzyme activity

Initially, from the initial pond water the acid phosphatase activity was shown by 3 PSB isolates showing the maximum value of 66.594 U/ml and minimum value 49.047

After 1st treatment, each PSB isolates from the treatments SSP20, SSP40, MOC and MIX, the highest value was shown by PSB isolate of MIX (57.309 U/ml) and lowest value of 42.746 by SSP20 treatment isolate.

After 2nd treatment, each PSB isolates from the treatments SSP20, SSP40, MOC and MIX, the highest value was shown by PSB isolate of SSP40 (50.068 U/ml) and lowest value of 37.813 U/ml by SSP20 treatment isolate (Table 5).

Table 5: Maximum Acid phosphatase enzyme activity of different bacterial isolates from the treatments.

Sl. No.	Treatment	Bacteria Group	No of Isolates	Maximum Enzyme Activity (U/Ml)	Day of Maximum Activity
1	Initial	PSB	1	63.159	3rd
2	Initial	PSB	2	66.594	4th
3	Initial	PSB	3	49.047	3td
Treatment 1					
1	Ssp40	PSB	1	45.423	4th
2	Moc	PSB	1	48.675	3rd
3	Mix	PSB	1	57.309	3rd
4	Ssp20	PSB	1	42.176	3rd
Treatment 2					
1	Mix	PSB	1	37.813	
2	Moc	PSB	1	44.869	
3	Ssp20	PSB	1	20.173	
4	Ssp40	PSB	1	50.068	

DISCUSSION

Green algae vs Phosphatase enzyme activity

Both the acid and alkaline phosphatase enzyme do not clearly show any inducing correlation (r^2=0.0877; 0.0436) in the growth of the green algae but has selected a favourable growth of the green algae within the range of 1000-1500 which occurred in the treatments of BOC, MOC and MIX (Fig. 4)

Since the plankton growth is limited by the nutrient availability, the interaction between the grazing and heterotrophic pathways being influenced by microbial enzymatic degradation has finally limited the enzyme activity within an optimum organic matter availability resulting in favorable phosphorus concentration for green algae growth. This interaction is mostly based on close nutrient complementary relationship between algae and the bacterial population which might provide scientific support for the research of *Microcystis sp.* bloom control (Cao *et al.*, 2016)

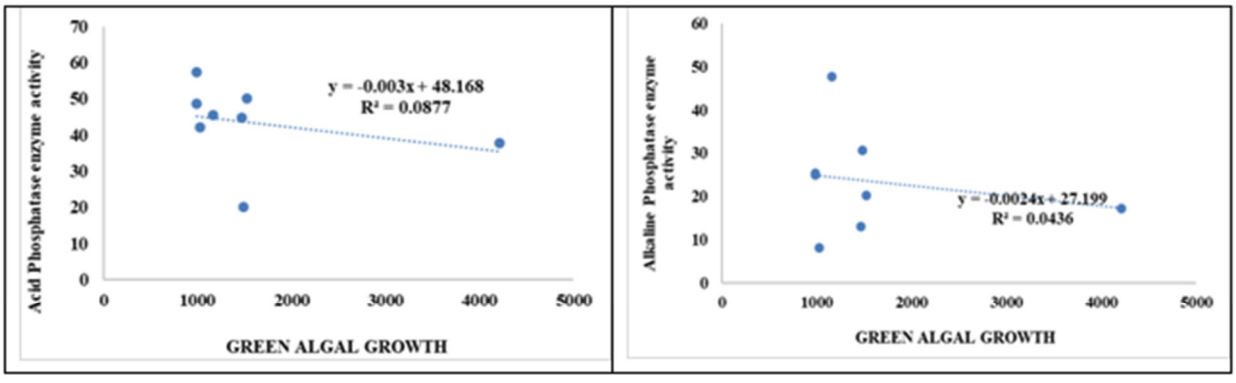


Fig. 4: Correlation between Green algae and Acid and alkaline phosphatase enzyme activity

Studies have found that during the decomposition of single bacterial bloom, large amount of deserved enzymatically hydrolysable phosphorus is released which can be hydrolysed by extracellular alkaline phosphatase producing bacteria can inhibit or promote bacterial growth as well as the alkaline phosphatase activity. PhoX gene in alkaline phosphatase can be used as biomarkers during Microcystis bloom decomposition (Shi *et al.*, 2017).

Therefore, the organic manipulated treatment has favored the maximum growth of green algae within a favourable phosphate range which is being influenced by the bacterial phosphate mineralization metabolic pathways mediated by phosphatase enzymes.

Green Algae vs Phosphate

In low phosphate concentration, green algae population is high. This indicated moderate nutrient concentration is suitable for maintaining the green algae population, this indicates good ecosystem health of the aquatic body showing poor eutrophication syndrome.

Therefore, Badam Oil Cake, Mustard Oil Cake treatments are suitable for favoring the growth of green algae and can be considered as a valuable organic nutrient manipulated eutrophication control strategies (Fig. 5).

Green algae vs N/P and P/N Ratio

The abundance of green algae in all the treatments was directly related to N/P or P/N ratio of water samples regardless of treatments. The relationship was more strongly related when nitrogen was more predominant compared to phosphorus The relation between N/P ratio and green algae has shown a better positive correlation (r^2= 0.7227) (Fig 6A) than the P/N ratio (r^2= 0.5127) (Fig. 6B). Thus N/P ratio has favored the green algae in treatments of BOC and MOC as well as the mixed combination. This implied that green algae had a preference for nitrogen rather than phosphorus in eutrophic conditions.

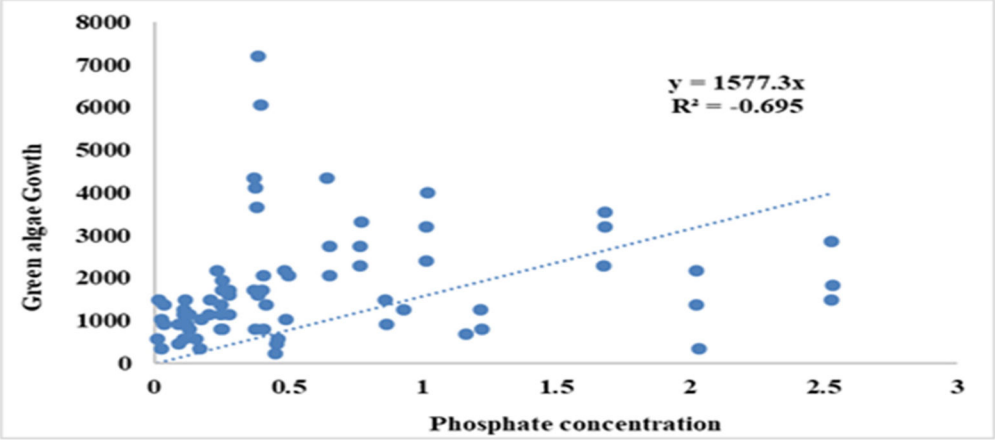


Fig. 5: Correlation between Green algae and phosphate

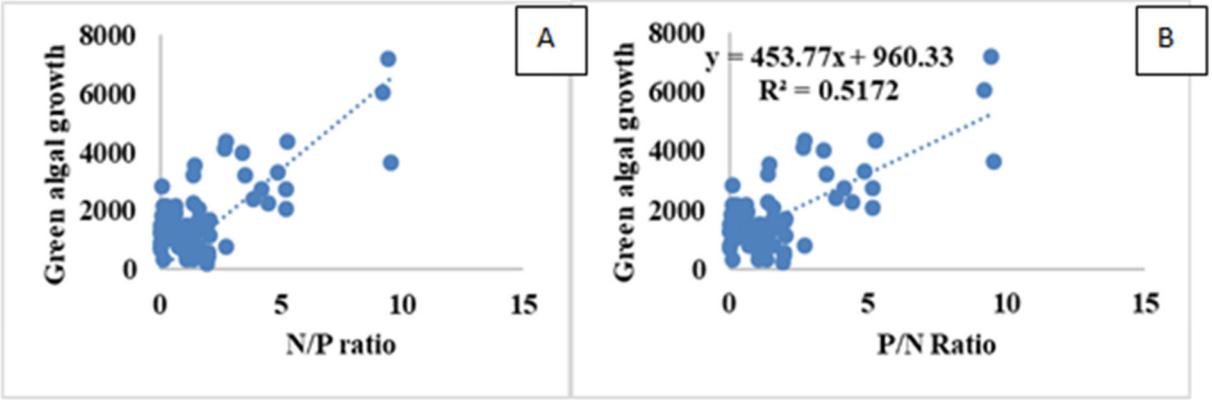


Fig. 6: Correlation between Green algae and (A) N/P ratio (B) P/N ratio

Microcystis vs N/P Ratio and P/N Ratio

The relationship between the Microcystis count and N/P (R=0.0533) (Fig. 7A) or P/N (r^2= 1.76) (Fig. 7B) of water revealed poor correlation. Since, addition of phosphorus or increased in concentration of phosphorus to nitrogen in water is likely to inhibit the growth of blue green algae. More nitrogen than phosphorus was inhibitory to Myxophyceae (Smith, 1983) as well as *Anabaena plantonic* (Wood *et al.*, 2010) at ratio more than N/P 29:1.

In present study, the blue green algae favored the low nitrogen and high phosphorus conditions according to its correlation of growth with ortho-phosphate. The growth of green algae, on the other hand was favored by more nitrogen than phosphorus concentration in water created as a result of nutrient manipulation by organic manure introduction.

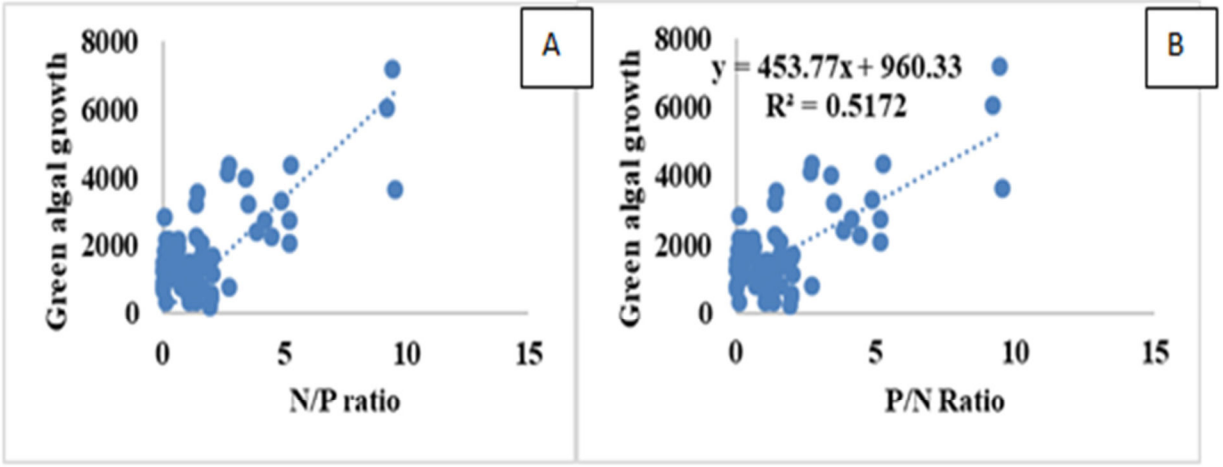


Fig. 7: Correlation between Microcystis growth and (A) N/P ratio (B) P/N ratio.

CONCLUSION

Bacterial enzymes did not play any specific influencing role in shifting the microcystis bloom to green algae population as a result of organic nutrient manipulation but due to interplay of different driving factors based on dynamic interactions between the grazing and heterotrophic pathways, the microbial enzymes have selected a favorable zone for optimum green algae as well as blue green algae growth. The bacterial phosphatase activity in the MOC treatment indicated a role in regulating N/P ratio by their metabolic adjustment for modifying the phosphorus interactive pathways that has driven the growth of Chlorophyceae, although the activity was maximum in SSP treatment. Thus the microbial heterotrophic pathway played a major driving factor in controlling harmful algal bloom.

The relation between N/P ratio and green algae has shown a better positive correlation than the Microcystis bloom. Thus N/P ratio has favored the green algae in treatments Badam Oil Cake, Mustard Oil Cake and the mixed combination of Badam Oil Cake and Mustard Oil Cake. So, the effects of N/P ratios of Badam Oil Cake (60:1), Mustard Oil Cake (50:1) and Mix (Badam Oil Cake + Mustard Oil cake showed favourable conditions for the growth of green algae. Therefore, self-designing of an eutrophic system can be enhanced by nutrient manipulation towards shifting the food chain in a right direction from harmful algal bloom to benign algal bloom.

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