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# EFFECT OF LOCALLY USED FUNGICIDE MANCOZEB AND INSECTICIDE PROFENOFOS IN RICE FIELDS OF MEGHALAYA ON THE SOIL MICROFLORA

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## ABSTRACT

Major loss in crop yield and quality are due to biotic stresses known to be a major threat to agriculture. During the past few decades, pesticides have been used frenziedly though, it is not a good measure of sustainable practice. Microalgae easily become the first, in the list of nontarget organisms that are affected by pesticides. The present study provides the first information about the effect of commonly used fungicide mancozeb and insecticide profenofos on growth, pigment, lipid peroxidation, superoxide dismutase, total peroxide, proline and protein content of two important algae *Anabaena* sp. and *Scenedesmus* sp. common inhabitants of the paddy fields. Rising concentrations of pesticide accelerated the formation of reactive oxygen species. An increase of 3-4 fold in MDA level at 60ppm of mancozeb and 40 ppm of profenofos reported just after the first day of treatment. Further, a dose dependent increase noticed with the duration of exposure to pesticides. An enhanced superoxide dismutase activity along with peroxide and proline content was also revealed.

**KEYWORDS: Cyanobacteria, Algae, Pesticides, Chlorophyll, Superoxide dismutase, Proline**

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# INTRODUCTION

The total arable land is decreasing and attempts to obtain more crop products from the unit area of land available is necessary to overcome the hunger problem due to the rapidly growing world population[1]. Today, more than 10,000 chemicals are used for industrial and agricultural purposes. In the past few decades, with the advancement in technology, chemical products are mostly used to accomplish success in improving agricultural production, human health and quality of life[2]. However, the pollution caused by both production process of these chemicals as well as chemicals themselves, offered many new problems. Particularly, chemical pesticides and fertilisers applied to increase the yield of the agricultural products and to protect them from pests are done in an uncontrolled way. Contamination of water bodies appears to be the unavoidable consequence of agricultural activities, mainly related to the use of plant protection products[3]. Several recent studies show that pesticide residues frequently occur in surface water in agricultural areas[4, 5, 6]. Pesticide use in India for crop protection and public health maintenance goes to a total of 85,000 tons per year. Of these, 77.8% are insecticides, and the rest are fungicides, herbicides, rodenticides, fumigants and miscellaneous pesticides. Of this, rice crop, occupies about 24% of the crop area, which accounts for 17.2% of the pesticides used[7]. Studies in experimental ecosystems help in understanding the direct and indirect processes involved in chemical stress of ecosystems, thus a prior prediction of effects on the ecosystem level can be made. Therefore, the levels of environment pollution can be screened with the presence of cyanobacteria the primary producers in fresh water ecosystems. These blue-green algae are a diverse group of gram-negative photosynthetic photoautotrophs. Their life processes require only water, carbon dioxide, inorganic substances and light. Many cyanobacteria contribute greatly to the nitrogen economy of aquatic and terrestrial habitats through their ability to fix atmospheric nitrogen. Studies on the interaction of cyanobacteria with agrochemicals have been widely conducted, but there have been an increased attention paid to pesticides, particularly insecticides and fungicides. Pesticides may induce many cellular disorders and overall growth performance in cyanobacteria. Depending on the type and concentration of pesticides and composition of growth media, pesticides has been found to exert stimulatory, inhibitory or no effect on the growth and nitrogen fixing ability by cyanobacteria[8]. Insecticides, which constitute 83% of the total pesticides produce, have been studied quite extensively with respect to their effect on cyanobacteria. Profenofos, a group of pesticides, is an organophosphate insecticide. The effect of insecticides on the population of cyanobacteria also depends on the pesticide/insecticide concentration. Toxicity is affected not only by type of pesticide, but also by the taxonomic groups and species. The inhibition of growth is due to an alteration in synthesis of nucleic acids, amino acids and proteins[9] as well as due to impairment in photosynthetic activity[10] of the cyanobacteria. Fungicides have potentially serious consequences on the overall productivity of soil, by interfering with the activity of cyanobacteria[11].The mancozeb fungicide is very toxic to aquatic organisms including cyanobacteria. However, they have developed defensive mechanisms to several stress factors. Proline accumulation has been reported to be an important index for stress tolerance capacity in plants and even in cyanobacteria. Organophosphorous (chlorfenvinphos) insecticides demonstrated by Mohapatra and Schiewer[12] with that toxicant-membrane interaction is responsible for changes in fluorescence behaviour and pigment content of *Synechocystis* PCC 6803. Growth and the macromolecular contents of *Anabaena cylindrica* and *Oscillatoria tenue* were adversely affected by diazinon at a concentration of 9ppm and 10ppm[13]. *Scenedesmus acutus* when exposed to different concentrations of diazinon in liquid cultures reduced algal growth by 5 to 96% of the growth population as compared to the control group[14]. Tebuconazole (fungicide) treatments at various treated concentrations

caused inhibitory effects on photosynthetic pigments like chlorophyll, carotenoids and phycobiliproteins decreased with increasing pesticide treatments in *Anabaena fertilissima, Aulosira fertilissima* and *Westiellopsis prolifica*[15]. Mancozeb was found to be toxic at a range of concentration from 40.0 to 72.2 ppm in four tested cyanobacteria *Nostoc ellipsosporum, Scytonema simplex, Tolypothrix tenuis, and Westiellopsis prolifica*[16].Though much work done on abiotic stress induced effect of growth, photosynthetic pigments content and nitrogen metabolism but pesticide induced effect on growth, ROS generation, antioxidants and lipid peroxidation in soil microbes are yet to be investigated. The purpose of the present study was to examine the exposure effects of insecticide (profenofos) and fungicide (mancozeb) on the growth, pigment, total peroxide, lipid peroxidation, key antioxidant enzyme like superoxide dismutase (SOD), proline and protein content of algae *Anabaena sp.* and *Scenedesmus sp.* in order to assess their application effects on soil microflora.

## MATERIALS AND METHODS

### *Organisms and growth conditions*

The test organisms used for the present study are *Anabaena* sp. and *Scenedesmus* sp. obtained from Botany Department, Banaras Hindu University, Varanasi and subcultured in BG-11 medium[17] (pH 7.5) in the culture room at 25±2°C with a photoperiod of 14:10 h. Exponentially grown cells were used for initial toxicity test.

### *Pesticides and chemicals*

For evaluating the toxicity of two pesticides, Saaf (Mancozeb 63% WT) is a dithiocarbamate fungicide and Anaconda 404 (Profenofos 40% EC) is an organophosphate insecticide manufactured by United Phosphorus Ltd, Gujarat and AIMCO Pesticides Ltd, Mumbai respectively. Other chemicals used for the study was obtained from Himedia labs, Mumbai.

### *Work Plan*

Various concentrations of mancozeb for *Anabaena sp*. (5, 15, 30, 45 & 60ppm) and *Scenedesmus sp.* (5, 10, 20, 30 & 40ppm) were prepared from the stock solution (10,000ppm) by diluting it with the sterilized distilled water. Similarly, for profenofos various concentrations used for *Anabaena sp.* (25, 50, 75, & 100ppm) and *Scenedesmus sp.* (30, 45, 60, 75 & 90ppm) were prepared from the stock solution (40,000ppm). Subsequently, the exponentially growing cultures were then inoculated to estimate effective concentration. EC 50 of each pesticide was determined as expressed in terms of concentration of pesticides, which reduces growth by 50% as compared to the control. On that basis, the dose treatment of each pesticide for each test organism was selected as shown in the Table 1. This pattern will be followed in all the figures. Photosynthetic pigments, oxidative stress and enzymatic response were carried out in triplicate at an interval of 1$^{st}$, 4$^{th}$ and 7$^{th}$ day. For each particular day, the treated as well as untreated (control) cultures were assayed for chlorophyll, carotenoids, lipid peroxidation, total peroxide, protein and proline content along with SOD enzyme.

### *Photosynthetic pigments*

The pigment content was estimated by extracting them in 80% acetone. The cell pellet were kept overnight in 3ml of 80% acetone at 4ºC. The next day the sample was centrifuged and the absorbance of supernatant taken for chlorophyll content at 650nm and 665nm and calculated[18] and at 450nm for carotenoid content and calculated[19].

### *Lipid peroxidation*

Oxidative damage of lipid was measured in terms of the total content of 2-thiobarbituric acid reactive substances (TBARS) and expressed as equivalent of malondialdehyde (MDA) with minor modifications[20]. These reactive substances were extracted in 3ml of 0.1% (w/v) trichloroacetic acid (TCA) at 4ºC following centrifugation at 13,000g for 2 mins. An aliquot of 0.5 ml from the supernatant was added to 1.5 ml TBARS (0.5% in 20% TCA). Samples were incubated at 90°C for 20 min and then in ice bath for 5 mins to stop the reaction. This sample was centrifuged at

1,000g for 5 mins, absorbance of the supernatant was measured at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated at its extinction coefficient (155 $mM^{-1}$ $cm^{-1}$).

### *Peroxide*

The total peroxide was measured[21]. The cell pellets collected from 20 ml culture were suspended in fresh BG-11 medium and crushed in liquid nitrogen. After that 5% (w/v) TCA (trichloroacetic acid) was added and the resulting suspension was centrifuged. 1.6 ml of the resulting supernatant was mixed with 0.4 ml 50% (w/v) TCA, 0.4 ml 10 mM ferrous ammonium sulfate, and 0.2 ml 2.5 M potassium thiocyanate (final concentration of TCA was 20%). This was then centrifuged and the absorbance of the supernatant was measured at 480 nm. A standard curve prepared using Sigma grade $H_2O_2$ was used for measuring the concentration of the peroxide.

### *Crude enzyme extract*

Pellets collected from exponentially grown cultures were suspended in cell lysis buffer (pH 7) and crushed in liquid nitrogen. The cell lysis buffer contained 1 mM EDTA and 1% (w/v) polyvinyl pyrrolidone (PVP). The sample was then centrifuged at 15,000g for 30 min at 4°C and the resulting supernatant was used for the assay.

### *Superoxide dismutase*

The SOD activity inhibits the photochemical reduction of nitroblue tetrazolium (NBT) at 560nm. The monitoring of this inhibition is used to assay SOD activity. The reaction mixture was prepared by taking 50 µL enzyme extract and adding 1 mL NBT (50 µM), 500 µL methionine (13 mM), 1mL riboflavin (1.3 µM), 950 µL (50 mM) phosphate buffer and 500µL EDTA (75 mM). The reaction mixture was then kept under light for 20 mins. Two blanks were prepared with the reaction mixture without the enzyme, one was kept in light and the other in dark. The one kept in light was taken as reference for calculating absorbance for one unit of SOD and the one kept in dark was used as blank. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction monitored at 560nm. The SOD activity was determined[22] and expressed as SOD IU min-1 mg-1 protein.

### *Proline*

Proline content was estimated by the method[23] in which the pellet obtained was crushed with 500µL of sulphosalicylic acid and 9.5ml more added later on. The crushed samples were then centrifuged at 9000g for 15 mins. The reaction mixture containing 2ml of the enzyme, 2ml ninhydrin and 2ml glacial acetic acid was kept in water bath (80ºC) for 60 mins. The reaction was then terminated in ice. 4ml toluene was then added to the reaction mixture and mixed vigorously with stirrer or vortex for 5 mins. The O.D of the coloured complex in toluene was then taken at 520nm.

### *Protein*

Algal sample suspensions were centrifuged at 4°C, 15,000*g* for 20 mins. Supernatant were thrown and the pellets re-extracted with 1.0mL 0.1N NaOH with 0.5% *β*-mercaptoethanol (v/v).The mixture of NaOH and pellets were kept at room temperature for 1 h with occasional manual shaking and then centrifuged at 21 °C, 15,000 *g* for 20 min. The supernatant were collected and pellets were discarded. The total cell protein was estimated by the method[24].The reaction volume contains 2ml of Bradford reagent (1X),180 µl of extraction buffer and 20 µl protein was added and incubated for 10 mins. The absorbance was read at 595 nm.

## RESULTS

### *Growth behaviour*

The growth pattern of *Anabaena sp*. subjected to varying concentrations (5-60ppm) of mancozeb was studied till 15 days. The $EC_{50}$ was determined to be 30ppm (Fig. 1A). Likewise, in case of *Scenedesmus sp.* (Fig. 1B) when subjected to varying concentrations (5-40ppm) of mancozeb, the $EC_{50}$ was resolved to be 20ppm. Also, the growth behaviour of *Anabaena sp*. shown here subjected to varying concentrations (25-100ppm) of profenofos was studied till 15

days. The $EC_{50}$ determined on the basis of growth pattern as 50ppm (Fig.2A). However, for *Scenedesmus sp.* (Fig. 2B) when subjected to varying concentrations (30-90ppm), the $EC_{50}$ was determined to be 60ppm.

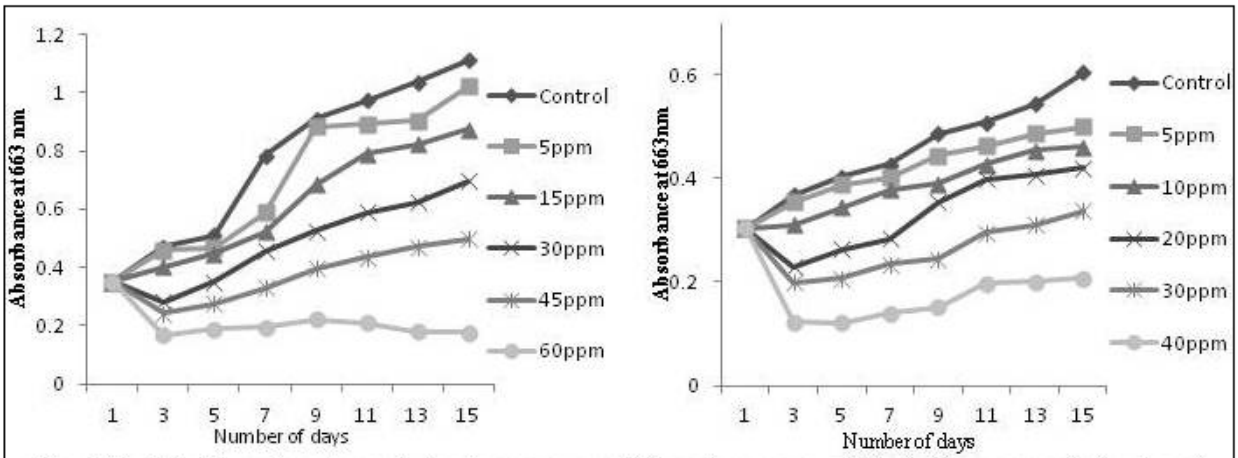


Fig. 1 (A&B): Growth pattern of *Anabaena sp.* and *Scenedesmus sp.* subjected to mancozeb treatment at different concentrations (5, 15, 30, 45 & 60ppm) & (5, 10, 20, 30 & 40ppm) respectively in comparison to control.

**Figure 1**

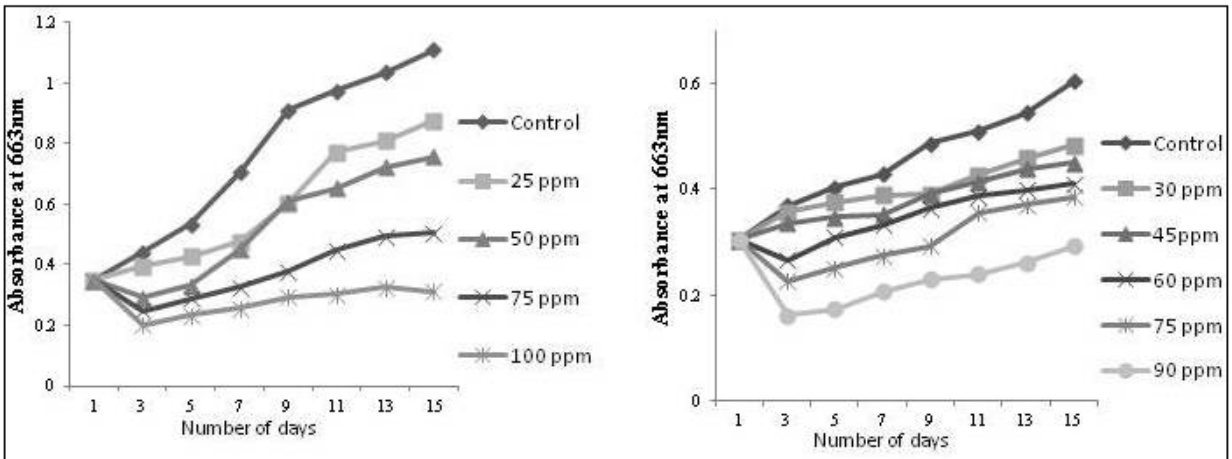


Fig. 2(A) & (B): Growth O.D of *Anabaena sp.* & *Scenedesmus sp.* subjected to profenofos treatment at different concentrations (25, 50, 75 & 100ppm) & (30, 45, 60, 75 & 90ppm) respectively in comparison with the control.

**Figure 2**

### *Lipid peroxidation*

The extent of oxidative damage measured in terms of MDA content in *Anabaena* sp. and *Scenedesmus* sp. subjected to different concentrations of mancozeb and profenofos separately attest an increase in their MDA level. The similar trends were also noticed with respect to duration of treatment in both the organisms under above mentioned pesticides (for details see the Fig 3A and Table1)

**Table 1**

| S. No. | Pesticide name | Organism selected | $EC_{50}$ values determined | Treatments decided based upon $LC_{50}$ |
|---|---|---|---|---|
| 1 | Mancozeb | *Anabaena sp.* **(AC)** | 30ppm | 15ppm (AM1)<br>30ppm (AM2)<br>60ppm (AM3) |
| | | *Scenedesmus sp.*(**SC**) | 20ppm | 10ppm (SM1)<br>20ppm (SM2)<br>40ppm (SM3) |
| 2 | Profenofos | *Anabaena sp.* | 50ppm | 25ppm (AP1)<br>50ppm (AP2)<br>100ppm(AP3) |
| | | *Scenedesmus sp.* | 60ppm | 30ppm (SP1)<br>60ppm (SP2)<br>90ppm(SP3) |

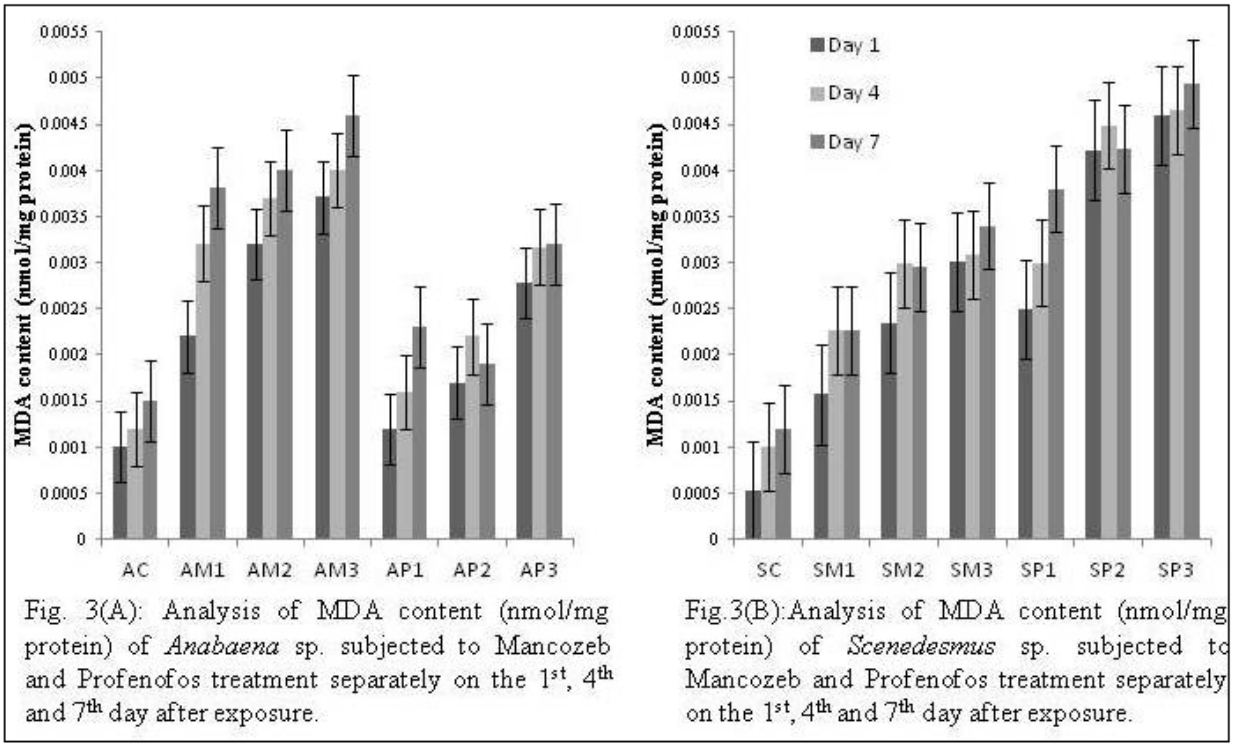


Fig. 3(A): Analysis of MDA content (nmol/mg protein) of *Anabaena* sp. subjected to Mancozeb and Profenofos treatment separately on the 1st, 4th and 7th day after exposure.

Fig.3(B):Analysis of MDA content (nmol/mg protein) of *Scenedesmus* sp. subjected to Mancozeb and Profenofos treatment separately on the 1st, 4th and 7th day after exposure.

**Figure 3**

***Peroxide content***

The peroxide content was enhanced under all treated cultures of mancozeb and profenofos as compared to the control from 1st to 7th day. The increase in peroxide content in *Anabaena* sp. *Scenedesmus* sp. (Fig. 4A&B) found highest at 60ppm of mancozeb (AM3) with respect to control. A significant increase has been observed at 90ppm of profenofos (SP3) treated culture on the 1st, 4th and 7th day of exposure.

***Superoxide Dismutase***

A pronounced increase in SOD activity observed in all treated cultures of *Anabaena sp*. and *Scenedesmus* sp. after treatment with mancozeb and profenofos for 1st, 4th and 7th day. In Fig. 5(A), the SOD activity was more pronounced in the AM3 treated culture right from the 1st day. On 7th day an increase by 89% in the AM3 against 86% of AP3 noticed compared to the control. However, in case of *Scenedesmus* sp. the SOD activity was more pronounced in SP3 (93% at 90ppm) than that of SM3 (89% at 40ppm) compared to control (Fig.5B).

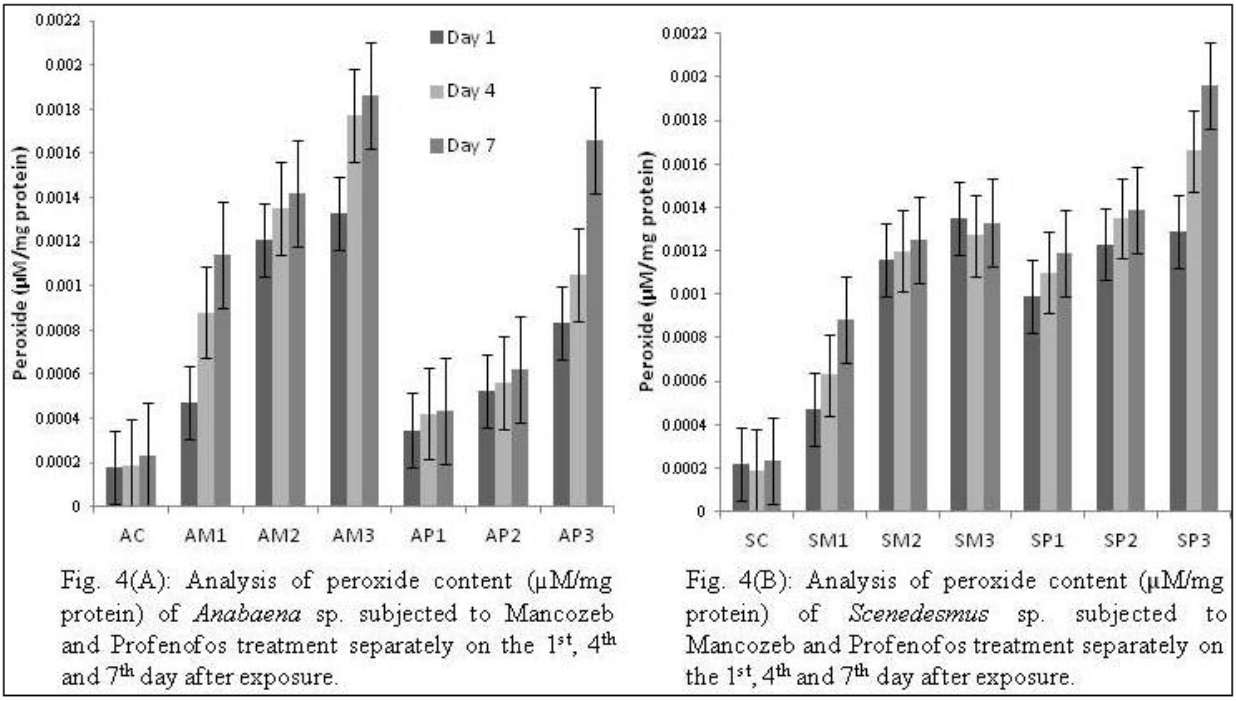


Fig. 4(A): Analysis of peroxide content (µM/mg protein) of *Anabaena* sp. subjected to Mancozeb and Profenofos treatment separately on the 1st, 4th and 7th day after exposure.

Fig. 4(B): Analysis of peroxide content (µM/mg protein) of *Scenedesmus* sp. subjected to Mancozeb and Profenofos treatment separately on the 1st, 4th and 7th day after exposure.

**Figure 4**

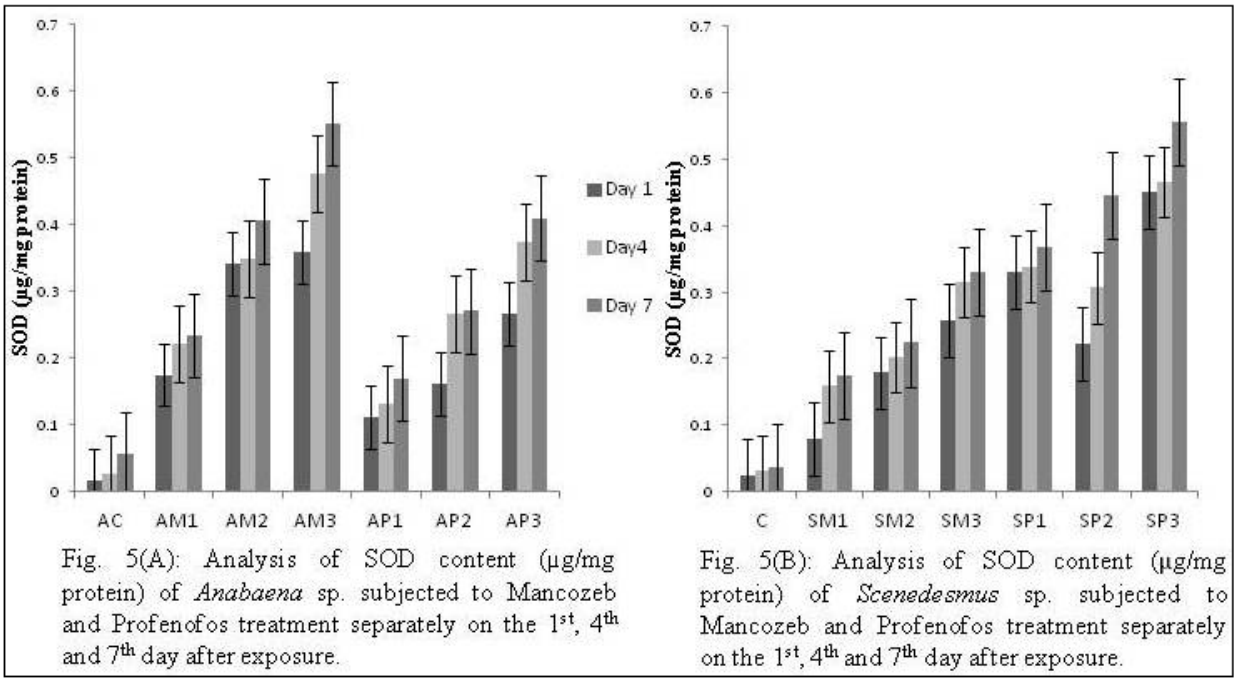


Fig. 5(A): Analysis of SOD content (µg/mg protein) of *Anabaena* sp. subjected to Mancozeb and Profenofos treatment separately on the 1st, 4th and 7th day after exposure.

Fig. 5(B): Analysis of SOD content (µg/mg protein) of *Scenedesmus* sp. subjected to Mancozeb and Profenofos treatment separately on the 1st, 4th and 7th day after exposure.

**Figure 5**

***Proline Content***

The proline accumulation enhanced with increase in concentration of mancozeb and profenofos on the treated cultures of *Anabaena* sp. and *Scenedesmus* sp. (Fig.6A& B). There has been a pronounced increase in AM3 compared to AP3 treated culture on the 7th day of exposure.

***Protein content***

A declining trend in the concentration of proteins observed in all the treated cultures of *Anabaena* sp. and *Scenedesmus* sp. compared to the control. In *Anabaena* sp., the maximum decline was found 83.8% in AM3 (60ppm) treated culture on 7th day of exposure. However, in case of *Scenedesmus* sp. it was found to be 82% in SP3 (90ppm) (Fig.7A & B).

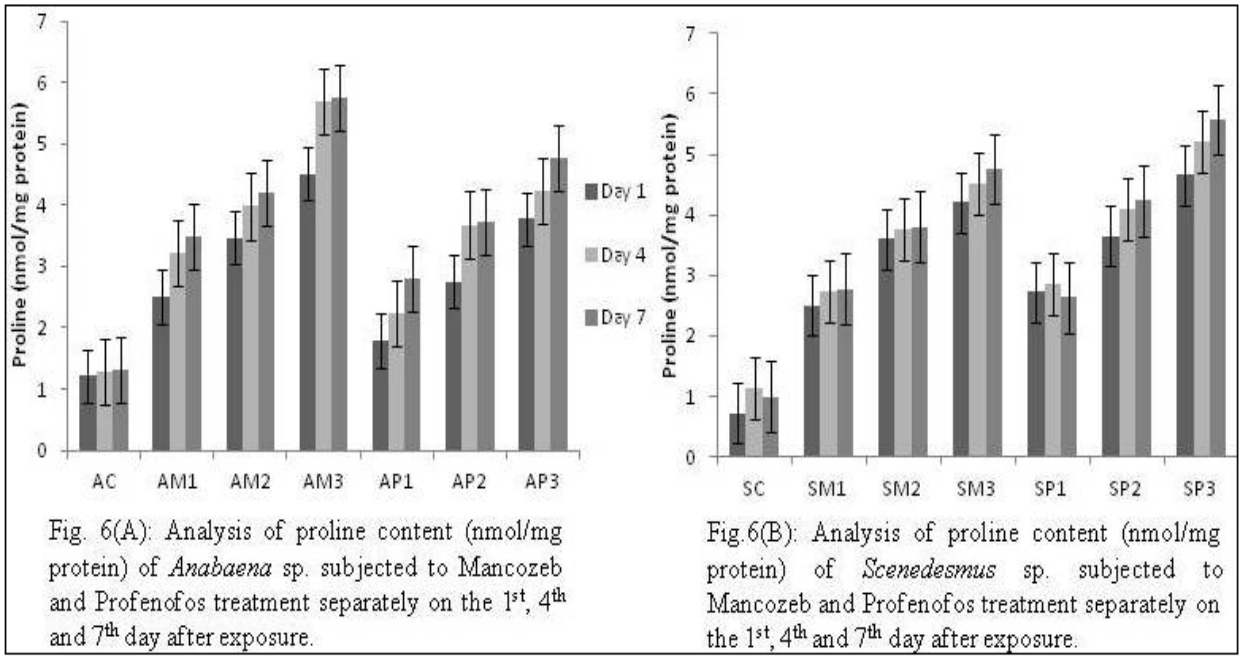


Fig. 6(A): Analysis of proline content (nmol/mg protein) of *Anabaena* sp. subjected to Mancozeb and Profenofos treatment separately on the 1st, 4th and 7th day after exposure.

Fig.6(B): Analysis of proline content (nmol/mg protein) of *Scenedesmus* sp. subjected to Mancozeb and Profenofos treatment separately on the 1st, 4th and 7th day after exposure.

**Figure 6**

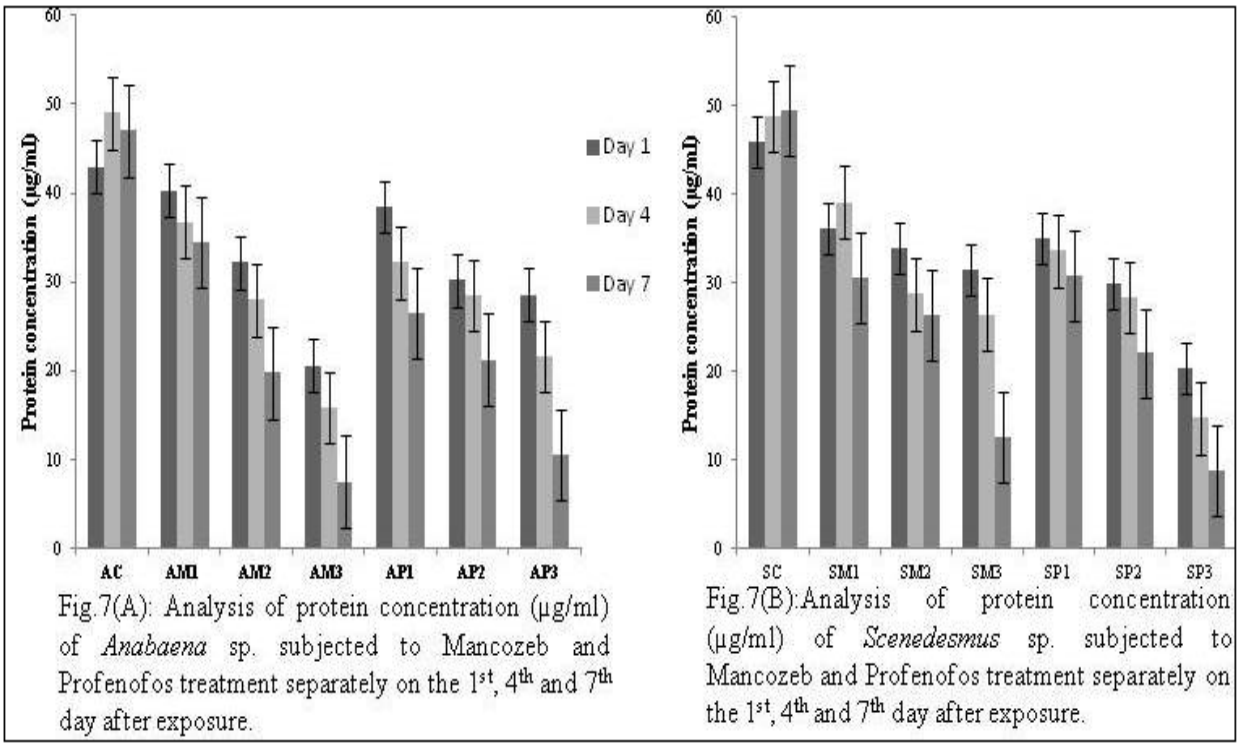


Fig.7(A): Analysis of protein concentration (µg/ml) of *Anabaena* sp. subjected to Mancozeb and Profenofos treatment separately on the 1st, 4th and 7th day after exposure.

Fig.7(B):Analysis of protein concentration (µg/ml) of *Scenedesmus* sp. subjected to Mancozeb and Profenofos treatment separately on the 1st, 4th and 7th day after exposure.

**Figure 7**

# DISCUSSION

The *Anabaena* sp. and *Scenedesmus* sp. showed an inhibitory growth response against the fungicide (mancozeb) and insecticide (profenofos) and it was found to be dose dependent. In the case of *Anabaena sp*., the treatment of profenofos has less inhibitory effect on its growth (Fig. 3A) when compared to the mancozeb that has been found more toxic than profenofos. It has been suggested that the monochrotophos, an organophosphorous insecticide were used by filamentous heterocystous cyanobacteria as a sole source of phosphorous in the absence of inorganic phosphate in the medium and as an additional source of phosphorous when inorganic phosphorus was available in the medium[25]. However, in case of *Scenedesmus* sp., with the profenofos treatment the growth (Fig. 3B) decreased significantly when compared to mancozeb. It has been found that mancozeb proved highly toxic when compared to profenofos adversely affects the morphology of *Anabaena* sp. especially at 60ppm dose (denoted as AM3) on the 7th day exposure, which shows broken single or double cell fragments of *Anabaena* sp. At dose of 90ppm (SP3) of profenofos insecticide, the cells has been found to contain very less chloroplasts in it, which have been found to be more toxic when compared to mancozeb (40ppm) on the $7^{th}$ day exposure. The degree of toxicity varied with the pesticides and species. The toxicity of pesticide to cyanobacteria is generally a function of the concentration of pesticide. Inter-species variation in sensitivity to the organophosphate pesticides may also be attributed to the chemical nature of pesticides. Number and types of esters present in organophosphates and their stereochemistry regulates pesticide potency, spectrum of activity, and toxicology[26, 27, 28]. The photosynthetic pigments also found to decrease significantly with respect to increasing mancozeb and profenofos concentrations as well as an increase in duration of exposure. The deleterious effect was more pronounced on chlorophyll compared to carotenoids. Such decrease in chlorophyll and carotenoid contents may be ascribed to the inhibition of pigment synthesis directly by pesticide or accelerated degradation of pigments due to increased ROS formation at the various sites of the photosynthetic electron transport chain during stress[29]. As most of the carotenoids synthesizing enzymes are membrane-bound, reduction of carotenoid biosynthesis might be due to the interaction of the insecticide with these enzymes. As carotenoids provide photoprotection to Chl *a*, pigment degradation may make the cyanobacterium more susceptible to photoinhibition under natural conditions[30]. Fungicides reported to cause deleterious effects on chlorophyll, carotenoids and phycobiliproteins of marine microalgal communities[31]. Under pesticide stress conditions, the stimulated generation of Reactive oxygen species (ROS) caused increased lipid peroxidation in cyanobacteria and thus increased MDA production. Pesticide induced oxidative stress accelerates lipid peroxidation, thereby affecting the structural integrity and permeability of cellular membranes[32]. During pesticide stress, the compound in illuminated cells turns away energized electrons from photosystem I during the light phase of photosynthesis, enhances the formation of superoxide radicals and hydrogen peroxide, two dangerous reactive oxygen species that damage the membrane structures of different cell compartments. As a consequence of this oxidative stress, toxic products of lipid peroxidation (thiobarbituric acid reactive substances) accumulate in the algal and cyanobacterial cells[33]. Here, it has been found that in case of *Anabaena sp*., the MDA content was more in mancozeb treated cells compared to profenofos. However, in case of *Scenedesmus sp*. the MDA content is more in profenofos treated sample than mancozeb. Recent evidence has shown that ROS, especially $H_2O_2$ and $O_2^-$ are involved in cellular signalling processes as secondary messengers to induce a number of genes and enzymes such as POD and SOD, which invoke active oxygen species in stressed organisms[34]. The increased level of $O_2^-$ and $H_2O_2$ triggered the activity of several antioxidant enzymes such as superoxide dismutase. It has been found here, with increase in concentration of mancozeb and

profenofos, the SOD activity also increases significantly. The Proline accumulation has been reported to be an important index for stress tolerance capacity in cyanobacteria, algae and plants. It functions as a stabilizer, a metal chelator, an inhibitor of lipid peroxidation and a singlet scavenger. Cytosol harbors proline accumulation and maintains cellular osmotic adjustment. Oxidative stress by free radicals is reduced by proline[35]. From the above study, proline accumulation has been found to increase with dose dependent or with an increase in concentration of the above particular pesticides. The protein content also exhibited decreasing trend with increasing duration and pesticide concentration. It has been emphasized that the decrease in protein content was observed in starved cyanobacterial cells of pesticide treated culture[36]. Thus, the present study revealed that the mancozeb fungicide and profenofos insecticide treatments adversely affected the photosynthetic pigments (Chl, Carotenoids) at high concentrations. Furthermore, with increasing doses of mancozeb and profenofos, they accelerated the formation of active oxygen species, i.e., $O_2$ and $H_2O_2$ in cells progressively, whereby an enhanced peroxide content and peroxidation of lipid and leakage of cell membrane were noticed. As a consequence of ROS generation in treated cells, the activity of SOD and the proline, which is a stress induced substance, was enhanced considerably. So, it can be concluded that mancozeb fungicide is more toxic compared to profenofos insecticide in case of *Anabaena sp*. and in case of *Scenedesmus sp.*, profenofos proves to be more toxic compared to mancozeb.

## CONCLUSION

The present paper addresses the preliminary effect of commonly used pesticide concentration on the soil inhabitants such as *Anabaena* and *Scenedesmus*. As in present state Meghalaya, rice is a staple food crop of this area so people, mainly depend on chemical pesticides for rice cultivation. These organisms not only make an important part of the food chain, but also play a vital role in ecosystem balance. They help in maintaining soil fertility and providing the required nutrition to the crops. As evident in this study, exposure to pesticides adversely affected the growth, pigment and protein synthesis in the microorganisms. A 3-4fold increase in SOD activity, peroxide and proline content noticed after the few days of treatment. Enhanced content of proline clearly shows the stressed status of the organisms. The uninhibited use of pesticides on the crops and soil has destructive consequences for agriculture and public health in the future. In view of that, these studies extended further can be helpful in risk assessment and measures can be adopted to use a standard minimal concentration in the fields which only affects the target organisms. Several superfluities can be proposed and prepared in the fields where once these pesticides are used, then at the same place they get degraded after a certain period of time. More emphasis should be given on the use of biopesticides for which education to farmers about them is very essential.

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***Conflict of Interest***

The author declares that there is no conflict of interests regarding the publication of this paper.

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