3. MATERIALS AND METHODS

3.1. Chemicals

The test chemicals (PNP, PNSP and PAP), used in the present study (Figure 3) were purchased from Sigma Chemical Company, St Louis, U.S.A. Aqueous stock solution of PNP was prepared in sterilized distilled water. Aliquots from the stock were directly added to the sterilized culture medium. In view of their lower water solubility, PNSP and PAP were dissolved in analytical grade acetone, and aliquots of these stock solutions were added in desired concentrations to sterilized Erlenmeyer flasks. After evaporating the carrier solvent at room temperature, portions of steam-sterilized medium were added to the flask, and the residues were equilibrated for a day to obtain aqueous solutions of the test chemicals.

3.2. Culture growth and maintenance

Axenic cultures of two species of unicellular green algae, Chlorella vulgaris (Beijerinck), and Scenedesmus bijugatus (Turpin), and two cyanobacteria, Nostoc muscorum (Ag) B & F, and Nostoc linckia (Roth) B & F, originally isolated from a rice soil by following MPN method were used in the present study. The
Figure 3. Phenolic compounds used in the study
identity of these cultures was confirmed by the kind courtesy of Prof. V. N. Raja Rao, Department of Botany, University of Madras. The cultures were deposited in the Culture Collection Centre, University of Madras. The isolates of microalgae were cultivated in Bold’s basal medium, while those of cyanobacteria were propagated in Allen’s medium the compositions of which are given below.

**Bold’s basal medium**

Six stock solutions, 400 ml in volume, were prepared, each containing one of the following salts in the amounts listed.

\[
\begin{align*}
\text{NaNO}_3 & \quad 10 \text{ g} \\
\text{CaCl}_2 & \quad 1 \text{ g} \\
\text{MgSO}_4 & \quad 3 \text{ g} \\
\text{K}_2\text{HPO}_4 & \quad 3 \text{ g} \\
\text{KH}_2\text{PO}_4 & \quad 7 \text{ g} \\
\text{NaCl} & \quad 1 \text{ g}
\end{align*}
\]

To 940 ml distilled water were added ten millilitres of each stock solution and one millilitre each of the stock solutions containing trace elements prepared as follows.

1. 50 g EDTA and 31 g KOH dissolved in one litre distilled water
2. 4.98 g FeSO\textsubscript{4} dissolved in one litre of acidified distilled water (1.0 ml H\textsubscript{2}SO\textsubscript{4} added to 999 ml distilled water)
3. 11.42 g H\textsubscript{3}BO\textsubscript{4} dissolved in one litre distilled water
4. The following, in amounts indicated, dissolved in one litre distilled water.
Allen's and Arnon medium

Seven stock solutions, in 100 ml in volume, were prepared, each containing one of following salts in the concentrations listed below.

- **MgSO₄** 0.50 g
- **CaCl₂** 1.11 g
- **K₂HPO₄** 0.20 g
- **Ferric citrate** 0.06 g
- **Na₂SiO₃** 0.88 g
- **Na₂CO₃** 0.40 g
- **Citric acid** 0.06 g

Five millilitres each of the above stock solutions and 1.0 ml from the stock solutions of trace elements with the following composition (in 500 ml) were added to 964 ml distilled water.

- **H₃BO₄** 1.430 g
- **ZnSO₄** 0.110 g
- **CuSO₄** 0.039 g
- **MnCl₂** 0.950 g
- **MoO₃** 0.080 g

The culture flasks were shaken reciprocally at 120 rpm at 25°C under continuous illumination at a PPFD of 65 µE m⁻² s⁻¹ provided by a batch of fluorescent tubes.
Axenic cultures of the microalgae and cyanobacteria were maintained on slopes prepared with agarised medium for subsequent use. The stocks as well as experimental cultures were incubated at room temperature (28 ± 4°C) in a growth chamber. The cultures were regularly checked for any contamination by inoculating on to nutrient agar medium. In all studies, 14-day-old cultures were used as sources of inoculum, unless and otherwise mentioned.

3.3. Determination of effective concentrations of the toxicants

Aliquots from the stock solutions of PNP, PNSP and PAP were added to 50 ml portions of Bold's basal medium (in case of microalgae) or Allen's N-free medium (in case of cyanobacteria) to provide a desired concentration of a toxicant ranging from 1 to 100 μg/ml. Exponentially-growing cultures of C. vulgaris, S. bijugatus, N. linckia and N. muscorum were used as inocula. Inoculated media without a toxicant served as controls. All the treatments and controls were incubated under fluorescent tubes at a PPFD of 65 μE m⁻² s⁻¹ and a temperature of 28 ± 4°C. The culture flasks were shaken twice a day for aeration. At the exponential phase (14 days after incubation), growth yield of the cultures, in terms of optical density at 650 nm in a Spectronic 20D spectrophotometer (Bausch and Lomb, U.S.A.), was measured. For each concentration, percentage inhibition values relative to growth in controls were calculated. Data were subjected to linear regression analysis with the help of a standard package, and the effective concentrations such as EC25, EC50 and EC90 were calculated.

3.4. Toxicity of PNP, PNSP and PAP towards cellular constituents

Aliquots (25 ml) of Allen's medium and Bold's basal medium taken in 25- x 200-mm culture tubes were supplemented with technical grade PNP and its
metabolites, PNSP and PAP, at levels of EC25 and EC50, determined by linear regression analysis as described elsewhere (3.3). The medium was sterilized by autoclaving, and inoculated with exponentially-growing cultures of *C. vulgaris*, *S. bijugatus*, *N. muscorum* and *N. linckia*. The culture tubes were incubated under fluorescent tubes at a PPFD of 65 $\mu$E m$^{-2}$ s$^{-1}$ and a temperature of 28 ± 4°C and were shaken thrice a day for aeration.

The phototrophic growth response of the filamentous cyanobacteria and microalgae under the influence of the selected toxicants was determined by recording optical density at 650 nm of the culture suspensions in a Spectronic 20D. Toxicity studies involved the criteria such as chlorophyll a, phycocyanins, carotenoids, DNA and RNA (Bhaskar *et al.*, 1994).

**3.4.1. Chlorophyll a and carotenoids**

The influence of PNP and its reduction metabolites, PNSP and PAP, at EC values of 25 and 50 on isolates of cyanobacteria and microalgae was also assessed by determining the concentration of acetone-soluble chlorophyll a as outlined by Holden (1976). Samples from treated and untreated controls, withdrawn at desired intervals, were centrifuged and the supernatant was discarded. The pellet was weighed and suspended in 5 ml of 80% acetone overnight in a refrigerator at 10°C and the contents were centrifuged. The supernatant was transferred to a test tube. The absorbance of extracted acetone-soluble pigment was measured in a Spectronic 20D (Bausch and Lomb). The content of chlorophyll a was quantitated using the extinction coefficient of 82.04 at 663 nm.
The amount of carotenoids were also estimated by recording the absorbance of the above extract in acetone at 450 nm (Jenson, 1978) and using the formula:

\[
\text{carotenoids / mg protein} = \frac{D \times V \times f \times 10}{2500}
\]

where \( D \) = Absorbance at 450 nm in a 1.0 cm cell
\( V \) = Volume of the original extract in ml
\( f \) = Dilution factor, and
\( 2500 \) = Average extinction coefficient of the pigments.

### 3.4.2. Phycocyanin

Five millilitres of a test culture treated with PNP or its reduction metabolites were withdrawn after 14 days of incubation. The cells were collected by centrifugation at 5400 \( \times \) g for 10 min at room temperature. The pellets were washed with 4 ml of saline solution and the supernatant was taken for phycocyanin estimation. Optical density of the samples was measured at 625 nm with saline solution as a blank. The phycocyanin content of water-soluble extract upon repeated freezing and thawing was quantitated using the extinction coefficient of 7.5 at 625 nm (Brody and Brody, 1961).

### 3.4.3. Isolation of DNA and RNA

Five millilitres of a culture suspension were withdrawn after thorough shaking, and the pellet from the culture was collected by centrifugation at 5400 \( \times \) g for 10 min at room temperature. DNA and RNA contents in the cultures were estimated by the method of Webb and Levy (1958). The washed pellet was suspended in 0.9% saline, filtered through four layers of a cheese cloth, and the filtrate was used for further isolation. This extract was treated with equal volumes of 10% trichloroacetic acid (TCA). The contents were shaken well, centrifuged and the supernatant discarded. The residue was washed twice with 10% TCA followed...
by ethanol wash twice. The residue was then extracted twice with ether to remove the phospholipids. Further, 2.5 ml of 10% TCA was added, mixed and centrifuged. The supernatant was separated and residue was treated with 5 ml of 5% TCA again and kept at 100°C for 15-20 min. The contents were cooled, centrifuged and the supernatant was separated. The final residue was again treated with 2.5 ml of 5% TCA, heated as above and the supernatant was separated. All the fractions were pooled and 5 ml aliquots of clear extracts were used for DNA and RNA determinations.

3.4.3.1. DNA

To 1.0 ml of extract containing DNA, 2 ml of freshly prepared Dische's reagent (1.5 g of crystalline diphenylamine was added in 100 ml of glacial acetic acid and 1.5 ml of conc. H₂SO₄ was added, and at the time of use 0.1 ml of acetaldehyde was added for each 20 ml of the reagent) was added, and the total volume in each tube was made up to 5 ml with glass distilled water. The initial turbidity formed disappeared gradually. The tubes were covered with glass marbles and heated in a boiling water bath for 10-15 min. The contents were then cooled under tap water and the blue colour developed was measured at 565 nm in a Spectronic 20D. Blanks contained only water and the Dische’s reagent.

3.4.3.2. RNA

To 1.0 ml of extract solution containing RNA, 3 ml of ferric chloride was added and the total volume was made up to 5.5 ml with glass distilled water prior to the addition of orcinol reagent (250 mg of FeCl₃ was dissolved in 250 ml HCl. 600 mg of orcinol was dissolved separately in 10 ml ethanol to obtain 6% (w/v) orcinol solution, and finally 8.75 ml orcinol solution was added to 241.25 ml FeCl₃ in HCl). Aliquots (0.3 ml) of freshly prepared orcinol reagent was added and all
the tubes were kept in a boiling water bath for 20 min. The green colour
developed was read at 675 nm in a spectrophotometer.

3.5. Test chemicals versus carbon metabolism

3.5.1. Total carbohydrates

Total carbohydrates were estimated as total anthrone positive substances in
TCA extracts. The TCA homogenates were centrifuged and the supernatants
were used directly for estimating total carbohydrates employing anthrone
reagent according to the method of Carrol et al. (1956). To aliquots of the
samples, 4 ml of anthrone reagent (200 mg of anthrone was dissolved in 100 ml
of cold 95% H₂SO₄) was added, mixed well, and the tubes were placed in a
boiling water bath for 10 min with a marble on top to prevent evaporation. The
contents were cooled and the extinction was read at 620 nm against a reagent
blank. Total anthrone positive substances referred here include free sugars in
addition to glycogen, and expressed as μg glucose/g cells.

3.5.1.1. Carbohydrate fractions

Cell pellet was extracted with 80% ethanol according to the method of Highkin
and Frankel (1962). The alcoholic extract was evaporated to 10 ml on a water
bath and cooled to room temperature. It was centrifuged and the supernatant
(alcoholic extract) was used for the estimation of reducing sugars.

3.5.1.2. Reducing sugars

The reducing sugars were estimated by the method of Nelson (1944) as
modified by Somogyi (1952). To 1.0 ml alcoholic extract taken into test tubes,
1.0 ml of a freshly prepared mixture of 25 parts of reagent 'A' (25 g anhydrous
sodium carbonate, 25 g sodium potassium tartrate, and 200 g of anhydrous sodium sulphate were dissolved in 800 ml distilled water, diluted to 1 litre, and the reagent was stored at room temperature), and 1 part of reagent ‘B’ (15 g of CuSO₄ 7H₂O was dissolved in 100 ml distilled water with a drop of conc. H₂SO₄ per 100 ml) were added, and thoroughly mixed. The test tubes were incubated in a boiling water bath for 20 min and cooled under running tap water. One millilitre of arsenomolybdate reagent (25 g of ammonium molybdate was dissolved in 450 ml of distilled water and 21 ml of conc. H₂SO₄ was added and mixed, and 3 g Na₂HSO₄ 7H₂O was dissolved in 25 ml distilled water. Both solutions were mixed and placed in an incubator at 37°C for 48 h) was added, and the coloured complex was diluted to 10 ml after 15 min. The absorbance of the solution was measured in a Spectronic 20D at 500 nm. Reducing sugar content was estimated using a standard curve prepared with glucose.

3.5.1.3. Non-reducing sugars

From the alcoholic extract, the non-reducing sugars were estimated by following the method of Scott (1960). Alcoholic extract (10 ml) was hydrolyzed with 10 ml of 3 NHCl for 30 min at 100°C in a water bath. The contents were cooled and neutralized with 30% NaOH. The total soluble sugars in the hydrolyzed extract were estimated by the Nelson method as described earlier.

3.5.1.4. Starch

The sediment left behind after the alcoholic extraction of the original material was taken for starch estimation according to the method of McCready et al. (1960). Starch was solubilized with 52% perchloric acid for 30 min, filtered and was made up to 5.0 ml. To this, 1.0 ml of perchloric acid extract, and 4.0 ml of freshly prepared anthrone reagent (200 mg anthrone was dissolved in 100 ml of cold
95% H₂SO₄) were added in cold. The contents were heated for 7.5 min at 100°C in a water bath, cooled rapidly to room temperature, and the colour intensity was measured at 630 nm against a reagent blank.

3.5.1.5. Adenosine triphosphate

Adenosine triphosphate (ATP) was estimated according to the method of Lamprecht and Trautschold (1958). The reaction mixture consisted of 2.27 ml of 50 mM triethanol amine buffer (pH 7.5), 0.10 ml of 10 mM NADP, 0.20 ml of 0.1 M MgCl₂, 0.10 ml glucose-6-phosphate dehydrogenase (3.3 µg/ml), 0.30 ml of 0.5 M glucose, 0.20 ml of hexokinase (13.3 µg/ml) and 0.5 ml enzyme extract. The reaction was started by the addition of glucose and hexokinase suspensions. ATP was measured in terms of NADPH formed/mg protein at 340 nm.

3.5.2. Enzymes related to carbon metabolism

3.5.2.1. Aldolase (E.C. 4.1.2.13)

Aldolase was assayed according to the method described by Stribling and Perham (1973). Cell culture (5.0 ml) was homogenized in ice-cold 0.1 M Tris-HCl (pH 7.5). The homogenate was centrifuged at 10800 x g for 15 min and the supernatant was used as an enzyme source.

The reaction mixture in a volume of 0.5 ml contained 0.056 M hydrazine hydrochloride (neutralized to pH 7.5 with solid Tris), 0.05 M fructose 1,6-diphosphate (neutralized with NaOH and finally in 0.05 M Tris buffer, pH 7.5), distilled water, and enzyme extract. After incubation at 37°C for 20 min, test tubes were kept at room temperature for 10 min and 1 ml of 2,4-dinitrophenyl hydrazine (1 mg/ml in 2 M HCl) was added, and kept at 37°C for 10 min. Finally, 7 ml of 3% NaOH was added and the colour intensity was
read at 540 nm against a reagent blank in a Spectronic 20D. The aldolase activity was expressed as μg pyruvate formed per mg protein.

3.5.2.2. Sucrose phosphate synthase (E.C. 2.4.1.14)

Sucrose phosphate synthase activity was determined by the method of Huber et al. (1989). Enzyme extract was incubated for 15 min at 25°C with 50 mM NaOH, 10 mM fructose-6-phosphate, 10 mM uridine phosphate glucose, 40 mM glucose-6-phosphate, 15 mM MgCl₂ and 2.5 mM dithiothreitol.

The reaction was stopped by adding 0.5 ml of 0.5 M KOH and untreated fructose-6-phosphate was destroyed by placing the tubes in a boiling water bath for 10 min. After cooling, 1 ml of 0.14% (w/v) anthrone in 13.8 M H₂SO₄ was added, and the absorbance was read at 620 nm.

3.5.2.3. Fructose 1,6-diphosphatase (E.C. 3.1.3.11)

Fructose biphosphatase was assayed according to the method described by Kelly et al. (1982). The reaction mixture (1.0 ml) contained 0.1 ml of 1 M Tris buffer (pH 7.5), 0.05 ml of 0.1 M MgCl₂, 0.1 ml of 3 mM NADP, 0.02 units of glucose-6-phosphate dehydrogenase and enzyme extract. After incubation at 25°C for 3-5 min, 0.05 ml fructose biphosphate (1.2 mM) was added, and the enzyme activity was calculated from the recorded change in absorbance at 340 nm.

3.5.2.4. Amylase (E.C. 3.2.1.1)

Amylase activity was measured according to the method of Sridhar and Ou (1972). Cell material was extracted from 5.0 ml culture suspensions into 10 ml of cold 1 M acetic acid-sodium acetate buffer (pH 6) by macerating in a mortar and pestle. The homogenate was filtered through a muslin cloth and the filtrate was
transferred into centrifuge tubes and centrifuged at 5,400 x g in a refrigerated high-speed centrifuge for 20 min at 0°C. Supernatant (5.0 ml) was taken into a Erlenmeyer flask, and 10 ml of 1 M acetic acid-sodium acetate buffer (pH 5) and 5.0 ml of 1% starch were added. The contents were mixed and incubated at 37°C for 24 hours.

After incubation, 1.0 ml of the reaction mixture was used to estimate the reducing sugars released by the Nelson’s method as described earlier. Amylase activity was expressed in //g of reducing sugars released per ml.

3.5.2.5. Starch phosphorylase (E.C. 2.4.1.1)

The activity of this enzyme was studied according to the method developed by Kumar (1983). Cell pellet from 5 ml culture was taken and homogenized with 0.01 M Tris-HCl buffer (pH 7.5) containing 0.02 M 2-mercaptoethanol and 0.1% Triton X-100. The contents were filtered through a muslin cloth and the extract was made up to 25 ml with the same buffer. This was used as the enzyme source. The enzyme extract (3.0 ml) was taken and incubated at 30°C for 1 h in 0.1 M Tris-Maleate buffer (pH 7.5), 0.5 ml of 5% soluble starch, and 0.5 ml of potassium dihydrogen phosphate.

After incubation, 5.0 ml of 10% TCA was added to precipitate the proteins and to stop the enzyme activity. The assay mixture was centrifuged at 2160 x g for 10 min under cold conditions. The sediment was discarded and the supernatant volume was noted and pH was adjusted to 7.0, and double the volume of 0.2 M MgCl₂ was added to the above supernatant. To this, sufficient volume of 5 N NaOH was added to bring to 0.1 N NaOH in the above solution and incubated for 5 min at room temperature. The above extract was
centrifuged and the precipitated magnesium phosphate was completely removed. Supernatant was collected and the volume was made up to 25.0 ml. Equal volume of 2 N H$_2$SO$_4$ was added and kept at 100°C for 10 min. Inorganic phosphate present was estimated according to the procedure of Fiske and Subba Row (1925).

Five millilitres of the above solution was pipetted into a 10 ml graduated stoppered cylinder. To this, 1.0 ml of 2.5% molybdate reagent (2.5 g ammonium molybdate was dissolved in 100 ml of 5 N H$_2$SO$_4$) was added and mixed. Then 0.4 ml of 0.25% ANSA (15 g ammonium molybdate was dissolved in water and 0.25 g 1-amino-2-naphthol-4-sulphonic acid) powder was added and the volume was made up to 90 ml with vigorous stirring. 9.0 g crystalline sodium sulphite was slowly added until the solution was clear, and the solution was made up to 100 ml) was added, mixed, and the volume was made up to the mark with distilled water. After allowing for 5 min, the absorbance was read at 680 nm in a Spectronic 20D. The inorganic phosphate present was calculated from a standard curve prepared from potassium dihydrogen orthophosphate.

3.5.2.6. Glucose-6-phosphate dehydrogenase (E.C. 1.1.49)

Glucose-6-phosphate dehydrogenase activity was measured according to the method of Bergmeyer and Bernt (1965). The reaction mixture contained 20 $\mu$mol glucose-6-phosphate dehydrogenase, 100 $\mu$mol triethanolamine buffer (pH 7.4), 4.0 $\mu$moles of 2-(4-iodophenyl) 3-(4-nitrophenyl) 5-phenyltetrazlium chloride (INT), 10.0 $\mu$mol MgCl$_2$ and 0.3 $\mu$mol NADP. The reaction was initiated by the addition of 0.5 ml enzyme extract. The reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 5 ml of glacial acetic acid. The
formazone formed was extracted into 5 ml of toluene overnight at 0°C and the colour intensity was read at 495 nm against reagent blank.

3.5.2.7. Isocitrate dehydrogenase (E.C. 1.1.1.42)

Isocitrate dehydrogenase was assayed according to the method outlined by Bergmeyer and Bernt (1965). The reaction mixture in a final volume of 3 ml contained 1.0 ml of 0.4 M phosphate buffer, 0.5 ml of 4 mM INT, 0.5 ml 10 μM MgCl₂, 0.25 ml 10.0 μM ADP, 0.25 ml of 12 μM NADP and 0.25 ml of 0.32 mM isocitrate and 0.5 ml enzyme extract. The reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 5 ml glacial acetic acid. The formazone formed was extracted into 5 ml toluene overnight at 0°C and the colour intensity was read at 495 nm against a reagent blank.

3.5.2.8. Succinate dehydrogenase (E.C. 1.3.99.1)

Succinate dehydrogenase activity was measured according to the method of Nachlas et al. (1966). The incubation mixture consisted of 0.2 ml of 0.4 M phosphate buffer (pH 7.4), 0.2 ml of 0.35 mM sodium succinate, 1 ml of 4 mM INT, 0.1 ml of 5 mM phenozene methosulphate and 0.5 ml enzyme extract. The mixture was incubated at 37°C for 30 min, and the reaction was terminated by the addition of 5 ml glacial acetic acid. The formazone formed was extracted into 5 ml toluene overnight at 0°C and the colour intensity was read at 495 nm against a reagent blank.

3.5.2.9. Malate dehydrogenase (E.C. 1.1.1.37)

Malate dehydrogenase was assayed according to the procedure of Bergmeyer and Bernt (1965). The incubation mixture consisted of 0.5 ml of 0.4 M phosphate buffer, 0.5 ml of 4 mM INT, 0.05 ml of 14 μM NAD, 0.5 ml of 0.35 mM sodium
malate and 0.5 ml extract. The contents were incubated at 37°C for 30 min. and the reaction was terminated by the addition of 5 ml glacial acetic acid. The formazone formed was extracted into 5 ml toluene overnight at 0°C and the colour intensity was read at 495 nm against a reagent blank.

3.5.2.10. Acid phosphatase activity (E.C. 3.1.3.2)

Acid phosphatase was assayed according to the method of Rao et al. (1990). Enzyme extract was prepared with 0.1 N citrate buffer (pH 5.6). Two millilitres of 15 mM p-nitrophenyl phosphate were taken into test tubes and 0.5 ml enzyme extract was added, and the tubes were kept in a water bath. After incubation at 37°C for 30 min, the reaction was stopped by adding 9.5 ml of 0.01 N NaOH. The absorbance was measured at 405 nm using 0.01 N NaOH as a blank. Acid phosphatase activity was calculated using a standard curve prepared from PNP, and the activity was expressed in μmoles of PNP released/mg protein.

3.5.2.11. Alkaline phosphatase (E.C. 3.1.3.1)

Alkaline phosphatase activity was determined according to the method of Rao et al. (1990). Enzyme extract was prepared with glycine buffer (pH 9.8). Two millilitres of 15 mM p-nitrophenyl phosphate and 0.5 ml enzyme extract were added, and incubated in a water bath at 37°C for 30 min. About 9.5 ml of 0.1 N NaOH was added to terminate the reaction, and the absorbance was measured at 405 nm in a spectrophotometer using 0.1 N NaOH as a blank. Standard curve was prepared with PNP, and the alkaline phosphatase activity was expressed in μmoles of PNP released/mg protein.
3.6. Test chemicals versus nitrogen metabolism

3.6.1. Total proteins

The total protein content was determined with Folin Ciocalteu reagent according to the method of Lowry et al. (1951). Five millilitres of the test culture solution were withdrawn to collect the pellet by centrifugation at 5400 \( \times g \) for 10 min at room temperature. The pellet was washed with 5 ml saline. The washed pellets were suspended in 5 ml of 10% TCA and allowed to stay at 20\(^{\circ}\)C overnight, and centrifuged at 10,800 \( \times g \) for 15 min. The pellet was suspended in 2 ml 0.1 N NaOH and thoroughly mixed. About 0.95 ml of 1 N NaOH was added to 1 ml of test solution. Then 1 ml of protein solution and 5 ml alkaline copper sulphate solution were added, and allowed to stand for 10 min at room temperature. Diluted Folin-Ciocalteu reagent (0.5 ml) was added rapidly, thoroughly mixed, and allowed to stand for 30 min for complete colour development. The optical density was measured at 750 nm in a spectrophotometer against a reagent blank. Bovine serum albumin was used as a standard.

3.6.2. Total free amino acids

The extraction and estimation of amino acids was according to the method of More and Stein (1948). The cell pellet was digested in 80% ethanol and centrifuged. The supernatant was collected and made up to 5.0 ml and used for the estimation of total amino acids. To 1.0 ml sample, 1.0 ml ninhydrin reagent (800 mg of hydrated stannous chloride was dissolved 500 ml of 0.5 M citrate buffer (pH 5.0) and 20 g recrystallized ninhydrin was added to in 500 ml of methyl cellosolve (ethylene glycol monomethyl ether) and both the solutions were mixed) was added in test tube. Glass marbles were placed on each test tube to avoid evaporation. The contents of the tubes were kept in a boiling water
bath for 20 min. Five millilitres of diluent solution (equal volumes of \textit{n}\textsuperscript{-}propanol and distilled water were mixed) were added after incubation. Blank was maintained with distilled water. The tubes were cooled under running tap water and the contents were mixed thoroughly, and were diluted if necessary with the diluent solution. The absorbance of the colour was read at 570 nm in a Spectronic 20D against a reagent blank. The amount of amino acids was calculated using a standard curve prepared with glycine.

3.6.3. Enzymes related to nitrogen metabolism

3.6.3.1. Transaminases

Aspartate amino transferase (glutamate oxaloacetate transminase, GOT) and alanine amino transferase (glutamate pyruvate amino transferase, GPT) were extracted and estimated according to the procedure of Hedley and Stoddart (1971), and Reitman and Frankel (1957), respectively. Extraction in buffer (Tris 0.04 M, and EDTA 0.25 M, pH 7.5) was performed by centrifugation at 10,800 \textit{x} g at 0°C for 15 min and the supernatant was used as an enzyme source.

3.6.3.1.1. Alanine aminotransferase (E.C. 2.6.1.2)

The activity of the enzyme was determined colorimetrically. The reaction mixture consisted 1.0 ml of the substrate (0.2 M DL- alanine,) and 0.02 M ketoglutarate in 0.1 M Tris buffer (pH 6.4). After incubation at 37°C for 30 min, the ketoacid produced was determined according to the method of Reitman and Frankel (1957). The reaction was stopped by the addition of 1.0 ml of 0.01 M 2,4-dinitrophenyl hydrazine solution (ketone reagent). The reaction mixture was made up to 10.0 ml with 0.4 N NaOH and the optical density of the colour
developed was measured in a Spectronic 20D at 545 nm and the enzyme activity was expressed as amount of ketoacids per mg protein.

3.6.3.1.2. Aspartate aminotransferase (E.C. 2.6.1.1)

The activity of this enzyme was determined in a similar way to that of alanine aminotransferase. The reaction mixture of 1.0 ml consisted of 0.1 M L-aspartate, and 0.002 M oxaloacetate in 0.01 M Tris buffer (pH 7.4). Incubation time was extended to 1 hour. The enzyme activity was calculated.

3.6.3.2. Glutamate dehydrogenase (E.C. 1.4.1.3)

Glutamate dehydrogenase activity was measured according to the method of Lee and Lardy (1965). The incubation mixture consisted of 0.4 ml of 4 mM sodium glutamate, 0.5 ml of 0.2 M phosphate buffer (pH 7.4), 0.1 ml of 1 mM NAD, 0.5 ml of 4 mM INT and 0.5 ml enzyme extract. The mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 5 ml glacial acetic acid. The formazone formed was extracted at 0°C and the intensity was read at 495 nm against a reagent blank.

3.6.3.3. Protease

The protease enzyme was assayed according to the method of Davis and Smith (1995). Cell pellet was homogenated in chilled acetic acid sodium acetate buffer (pH 5.2) at 4°C. The homogenate was filtered and transferred into centrifuge tubes and spun at 3240 x g for 20 min at 4°C. The supernatant was removed and was used as an enzyme source.

Five millilitres of casein solution (since casein is sparingly soluble in water, 1.0 g was dissolved in a minimum quantity of 0.1 N NaOH and the volume
was made up to 100 ml with phosphate buffer pH 7.0) was taken into test tubes
and 2.5 ml of 0.1 M phosphate buffer (pH 7.0) and 2.5 ml enzyme extract were
added. The contents were mixed and incubated in a water bath at 30°C for 30
min. Boiled enzyme at zero time was used as control. After incubation, 1.0 ml
solution was taken from the mixture and 2.0 ml ninhydrin reagent was added and
the contents were heated to 100°C for 1 min in a boiling water bath. About 2.5
ml diluent solution was added to the mixture, and blanks were maintained with
1.0 ml of distilled water. The contents were cooled and the absorbance was
measured at 570 nm in a Spectronic 20D. The amount of amino acids released
were calculated using a standard curve prepared from aspartic acid. Protease
activity was expressed as μg amino acids formed per mg protein.

3.6.3.4. Glutamine synthetase (E.C. 6.3.1.2)

Glutamine synthetase (L-glutamate : ADP ammonia ligase) was assayed
essentially as per the method of O'Neal and Joy (1973). All operations were
performed at 2-4°C. Cell pellet was ground in a prechilled mortar with a pestle
using 5 ml of 0.05 M Tris-HCl buffer (pH 7.9) containing 10 μM mercaptoethanol.
and the extract was centrifuged at 3240 x g for 15 min. The supernatant thus
obtained was used as an enzyme source.

The standard reaction mixture in 1 ml consisted of 0.05 M Tris- HCl
buffer (pH 7.9), 150 μM MgSO4, 20 μM α-ketoglutarate, 80 μM hydroxyl amine, 6
μM ATP and 0.5 ml enzyme extract! The reaction was started after an initial 2
minutes incubation at 35°C by the addition of the enzyme preparation. After 30
minutes incubation at 35°C, the reaction was stopped with the addition of 1 ml
solution containing 0.37 M FeCl3, 0.67 M HCl and 0.20 M TCA. The reaction
mixture was centrifuged at 3240 x g for 10 min to remove precipitated proteins.
The colour of the ferric hydroxymate complex was measured at 540 nm in a Bausch and Lomb Spectronic 20D. Glutamine synthetase activity was expressed in terms of units of glutamine hydroxymate formed/mg protein.

3.6.3.5. Glutamate synthase  (E.C. 6.3.1.3)

The extraction and estimation of glutamine (amide) 2-oxoglutarate aminotransferase (GOGAT) was done according to the method of Rachin and Nicholas (1985). All the operations were performed at 2-4°C. Cell pellet was homogenated in Tris-HCl buffer (50 mM, pH 7.5). the extract was centrifuged at 10800 x g for 20 min, and the supernatant was used as crude enzyme extract.

Glutamate synthase was assayed at 30°C by measuring the initial rate of oxidation of NADPH in a 1 cm quartz cuvette at 340 nm in a UV-Vis spectrophotometer. The reaction mixture, in 3 ml final volume, contained 6 mM glutamine, 3 mM ketoglutarate, 0.1 mM NADPH, and 100 mM sodium phosphate buffer (pH 8.0). The reaction was started by adding ketoglutarate. The enzyme activity was expressed as \(\mu\)mol of NADPH oxidised/mg of protein.

3.6.3.6. Nitrate reductase  (E.C. 1.6.6.1)

The method used for assay of nitrate reductase was that of Lewis et al. (1981) with slight modifications. Cell pellet was ground in 8 ml of ice-cold extraction medium with little acid-washed sand. The extraction medium contained 0.1 M phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM DTT and 2.5% casein. Casein was dissolved by stirring 2.5 g in 100 ml of the medium at 50°C for 30 minutes. The extract was first centrifuged at 10800 x g at 4°C and the supernatant was used for the assay immediately (3-5 min) after centrifugation. Nitrate reductase was assayed at 37°C in a reaction mixture containing 0.1 ml of 1 M potassium
phosphate buffer (pH 7.5), 0.2 ml of 0.1 M KNO₃, 0.1 ml of 1 mg/ml NADH, 0.5 ml extract, 0.1 ml of 1% Triton X-100 and 1.3 ml distilled water. The assay was done in duplicates along with a control having no NADH. The reaction was allowed to proceed for 15 min at 37°C and was stopped by adding 0.1 ml zinc acetate and 1.9 ml of 80% alcohol. The assay medium was then aged for 10 min after which NO₂ formed was measured by diazotization method (Snell and Snell, 1971).

**3.6.3.7. Heterocyst frequency**

To determine the heterocyst frequency, cultures were initially grown in KNO₃ since no heterocysts were observed in this medium. Heterocyst differentiation was initiated by transferring the washed cyanobacterial suspension to the basal medium (N-free medium). The number of heterocysts was recorded microscopically following incubation at desired intervals and the frequency was determined (average of at least 10 random samples). The number of heterocysts has been expressed in terms of percentages.

**3.7. Siderophore production by *Nostoc* spp.**

Aliquots (25 ml) of Allen’s medium lacking iron, taken in 25- x 200-mm culture tubes, were sterilized by autoclaving and then inoculated with exponentially-growing cultures of *N. muscorum* and *N. linckia*. The culture tubes were maintained at a PPFD of 65 μE m⁻² s⁻¹ and a temperature of 28 ± 4°C. and were shaken thrice a day for aeration. Triplicate samples were withdrawn after 5, 10, 15, 20, 22, 25 and 27 days of incubation for siderophore assay.

Siderophore was assayed according to the method of Clark *et al.* (1987). Siderophore was not found in cell-free extract. In order to determine the cell-associated siderophore, cells were collected from 25 ml culture by
centrifugation. The resulting cell pellet was suspended in 5 ml of distilled water and the pH was adjusted to 1.5 with 0.1 M HNO$_3$. Siderophore was extracted into 10 ml of chloroform phenol (1:1 v/v). Diethyl ether (40 ml) was then added and the siderophore was back extracted into 4 ml of distilled water. Residual phenol was removed by extraction with 8 ml of ether followed by bubbling with nitrogen. The water layer was adjusted to pH 7.0 with 0.1 M NaOH, lyophilized and suspended in 1.5 ml of 5 mM FeCl$_3$ in 0.14 M HClO$_4$. Particulate material was removed by millipore filtration, and the resulting ferric schizokinen solution was quantified by measuring OD at 490 (ε 2600 m$^{-1}$ cm$^{-1}$).

3.7.1. Effect of iron on siderophore production

In another experiment, the culture medium was supplemented with iron in the form of FeCl$_3$ at desired concentrations ranging from 0.5 to 5.0 M. Aliquots of medium without iron served as controls. The medium in the culture tubes was inoculated with exponentially-growing cultures of *N. muscorum* and *N. linckia*. The cultures used as inocula were grown initially in iron-lacking medium. Fifteen days after inoculation, triplicate samples of each treatment were assayed for siderophore.

3.7.2. Characterization of siderophore

Siderophore produced by the two species of *Nostoc* were characterized by following the thin-layer chromatography and infra-red spectrophotometry.

Siderophore isolated from *Nostoc* spp. was spotted on 200 μM-thick silica gel-G plates. The plates were developed for a distance of 15 cm with butanol-water-acetic acid (3:2:1. v/v/v) and air-dried.
Characterization of siderophore was also done by infra-red spectrophotometry. Sample containing siderophore was mixed with nujal-mull and subjected to IR spectrophotometry (Mullis et al., 1971)

3.7.3. Effect of PNP, PNSP and PAP on siderophore production

Portions (25 ml) of Allen’s medium lacking iron were treated with technical grade PNP, PNSP and PAP at levels of EC25 and EC50. Untreated medium without iron served as control. After incubation for 15 days, triplicate samples of each treatment were withdrawn for assaying siderophore.

3.7.4. Influence of glucose, succinate and ATP on toxicity towards siderophore production

In a repeat experiment, iron-lacking culture medium with or without a toxicant was supplemented with glucose (0.5%), succinate (0.5%) or ATP (10 μM) to determine whether these sources could reverse the phenolic toxicity towards siderophore production. Aliquots of iron-lacking medium without the addition of phenol and with no source of carbon and/or energy served as controls. Exponentially-growing cultures were used as inocula. The extent of siderophore production was determined in triplicates of each treatment.

3.8. Biodegradation of PNP

3.8.1. Degradation of PNP with free cells of microalgae and cyanobacteria

The ability of C. vulgaris and S. bijugatus, N. muscorum and N. linckia was tested for PNP degradation following the procedure outlined by Barik et al. (1976). Aliquots (50 ml) of medium were taken in 100 ml Erlenmeyer flasks and PNP was supplemented at 180 μM and 360 μM concentration. The growth medium was inoculated with exponentially-growing cultures to 1.0 OD600 nm.
Triplicate flasks of each treatment together with unioculated controls were incubated at a PPFD 65 μE m$^{-2}$ s$^{-1}$ and a temperature of 28 ± 4°C and were shaken thrice a day for aeration. At desired intervals, 2 ml portions of the culture medium from triplicate flasks were withdrawn for quantitating PNP remaining and nitrite accumulated. Five millilitres of the culture medium was taken and 0.1 ml 1 N NaOH was added and centrifuged at 5,400 x g for 10 min. The supernatant was read at 410 nm for PNP quantitation.

Nitrite was estimated by diazotisation following the method of Barnes and Folkard (1951). One millilitre portions of the culture medium were withdrawn and centrifuged at 5,400 x g for 10 min, 0.5 ml of 0.1% sulfanilamide in 1 N HCl was added to 0.5 ml of the supernatant, shaken thoroughly, and 0.5 ml of 0.12% N-(1-naphthyl) ethylenediamine in distilled water was added. The colour was allowed to develop for 10 min, and the volume was made up to 10 ml with distilled water. Absorbance of the pink colour was read at 520 nm in a spectrophotometer.

3.8.2. Degradation of PNP by immobilized cultures of microalgae and cyanobacteria

Cells of both microalgae and cyanobacteria grown were aseptically harvested by centrifugation at 5400 x g for 20 min and suspended in 50 ml sterilized distilled water. Sodium alginate was added to another 50 ml sterilized water (5% w/v), gently stirred for 1 hour, boiled and cooled. Cell suspensions were then added to sodium alginate solution and stirred for 30 minutes to ensure proper mixing. All these steps were done under aseptic conditions in a laminar flow hood. The mixture of sodium alginate and cultures were run dropwise through a burette into 0.4 M CaCl$_2$ solution. The beads were washed twice with sterilized distilled water before use.
A single alginate bead was dissolved by immersing in 1.0 ml of 0.2 M sodium phosphate buffer (pH 6.9) for 30 min for obtaining count of the test cultures (Chibata and Toser, 1977). After dissolving the bead, dilutions were made further and triplicates of 0.1 ml suspension from suitable dilution was plated on agar and incubated at a PPFD of 65 μE m⁻² s⁻¹ and a temperature of 28 ± 4°C. Discrete colonies developed after incubation at 37°C were counted to determine the cell density entrapped in a bead.

About 150 beads of cultures were transferred to 100 ml Allen's or Bold's basal medium supplemented with 180 μM PNP contained in 250 ml Erlenmeyer flasks and incubated at 37°C. At desired intervals, aliquots of samples were withdrawn for determining nitrite formed besides quantitating PNP. The culture suspensions after the disappearance of PNP were used for solvent extraction and subsequent TLC analysis for detection of metabolites, if any, of PNP.