# GROWTH OF CYANOPHAGE N-1 UNDER THE INFLUENCE OF HEAVY METAL IONS

T.A. SARMA*, SUKHRISH P. KAUR

Department of Botany, Punjabi University, Patiala - 147 002, India

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**Summary. –** The growth of cyanophage N-1 in the cyanobacterium *Nostoc muscorum* under the influence of heavy metal ions, namely $Co^{2+}$, $Cr^{6+}$, $Cu^{2+}$, $Mn^{2+}$ and $Ni^{2+}$ has been studied. One-step growth experiments revealed that heavy metal ions extended the latent period by 1–2 hrs with a concomitant decrease in the phage burst size. The latter was reduced in the order $Cu^{2+}$/$Mn^{2+}$, $Ni^{2+}$, $Co^{2+}$ and $Cr^{6+}$. The treatment of the phage-infected bacteria with heavy metal ions did not induce mutations affecting either the phage plaque morphology or burst size. The final phage titer after such a treatments was lowest with $Co^{2+}$, $Cu^{2+}$ and $Cr^{6+}$. The inhibition of the phage growth under the influence of heavy metal ions is discussed in context with the interaction of cyanophage N-1 with the photosynthetic reactions in the host bacteria.

**Key words:** cyanophage N-1; heavy metal ions; growth; Nostoc muscorum; photosynthesis

## Introduction

Large quantities of heavy metals are generally discharged in the form of industrial effluents into aquatic bodies thus posing a great threat to aquatic organisms. Effluents from electroplating, dyeing and other industrial processes contain mainly chromium, copper and nickel. These together with other heavy metals affect the growth of algae including cyanobacteria and cyanophages. Use of algae in monitoring heavy metal toxicity is greatly increasing due to their ubiquity in aquatic ecosystems where they influence and are influenced by most aquatic processes. A number of species of algae belonging to *Chlorophyta* and *Cyanobacteria* have been employed to study the heavy metal toxicity. Knowledge of heavy metal toxicity for eukaryotic algae and cyanobacteria has been repeatedly reviewed (Rai *et al.,* 1981; Stokes, 1983; Whitton, 1984; Singh, 1993; Singh *et al.,* 1994; Fiore and Trevors, 1994).

$Cu^{2+}$ has been shown to be mutagenic for *Escherichia coli* B (Weed and Longfellow, 1954; Hirsch, 1961), *E. coli* K-12 (Clowes and Rowley, 1955) and yeast (Lindegren *et al.,* 1958). On the other hand, $Cu^{2+}$ has been shown to be anti-mutagenic for the cyanobacterium *Anacystis nidulans* (Sarma, 1978). Further, the mutagenicity of $Mn^{2+}$ for *E. coli* (Demerec and Hanson, 1951; Roberts and Aldous, 1951; Durham and Wyss, 1957), bacteriophage $T_4$ (Orgel and Orgel, 1965) and the cyanobacterium *A. nidulans* (Sarma, 1977) has been well documented.

However, the effects of heavy metal ions on the growth of cyanophages and the probability of induction of mutations in cyanophages by heavy metals have not been investigated. Therefore, this study was focused on the growth of cyanophage N-1 under the influence of heavy metal ions, such as $Co^{2+}$, $Cr^{6+}$, $Cu^{2+}$, $Mn^{2+}$ and $Ni^{2+}$.

## Materials and Methods

*Bacterium and phage.* The cyanobacterium *Nostoc muscorum* ISU (ATCC 27893) and cyanophage N-1 were used (Sarma and Kaur, 1993). The host organism was propagated in the Chu-10 broth (Safferman and Morris, 1964) modified with micronutrient solution $A_6$ of Allen and Arnon (1955), in which calcium chloride

*E-mail: tas_11@rediffmail.com; fax: +91175-2283891.

**Abbreviations:** Chl = chlorophyll; MOI = multiplicity of infection; p.i. = post infection; PS = photosystem

**Table 1. Photosynthetic oxygen evolution and growth of phage N-1 under the influence of heavy metal ions**

| Heavy metal ions | Concentration (µmol/l) | Inhibition by % | | |
|---|---|---|---|---|
| | | | Phage burst size[b] | |
| | | Oxygen evolution[a] | Bacteria grown on heavy metal ions | Phage-infected bacteria treated with heavy metal ions during latent phase' |
| $Co^{2+}$ | 5 | – | 42.31 | – |
| | 10 | – | 50.00 | – |
| | 20 | – | 53.85 | – |
| | 50 | – | – | 99.99 |
| | 100 | 46.57 | – | 99.97 |
| | 150 | 56.11 | – | – |
| $Cr^{6+}$ | 20 | – | 25.00 | – |
| | 50 | – | 50.00 | – |
| | 100 | – | 99.90 | – |
| | 150 | 32.83 | – | 76.48 |
| | 250 | 86.54 | – | 94.12 |
| $Cu^{2+}$ | 2.5 | – | 92.31 | – |
| | 5.0 | – | 99.90 | 81.50 |
| | 7.5 | – | 100.00 | – |
| | 10.0 | 47.5 | 100.00 | 86.25 |
| $Mn^{2+}$ | 150 | – | 53.85 | – |
| | 200 | – | 92.31 | – |
| | 1000 | 71.24 | 98.47 | 60.00 |
| | 2000 | – | – | 60.00 |
| $Ni^{2+}$ | 1.0 | – | 61.54 | – |
| | 2.5 | – | 65.39 | – |
| | 5.0 | – | 80.00 | 34.05 |
| | 10.0 | – | – | 36.18 |
| | 20.0 | – | – | – |
| | 40.0 | 32.57 | – | – |
| | 60.0 | 58.0 | – | – |

[a]115.23 nmoles of oxygen evolved in metal ions-free control was taken for 100%.
[b]The burst size of 254 PFU/cell in metal ions-free control was taken for 100%.
(–) = not tested.

was replaced with calcium nitrate in the same molar concentration. The propagation proceeded under illumination with day light fluorescent tubes (2000 Lux) at the 14:10 light/dark regime. The preparation, purification and maintenance of lysates of the phage have been described (Sarma and Kaur, 1993). Unless otherwise specified, the growth and treatments of both the bacterium and phage proceeded at 28±1°C.

*Heavy metal ions.* Stock solutions of cobalt chloride ($Co^{2+}$), potassium chromate ($Cr^{6+}$), copper sulphate ($Cu^{2+}$), manganese chloride ($Mn^{2+}$), and nickel chloride ($Ni^{2+}$) were prepared in distilled water sterilized through Millipore 0.45 µm membrane filters.

*Photosynthetic oxygen evolution.* Suspensions of *N. muscorum* treated with heavy metals for 24 hrs (Table 1) were used for the assay of oxygen evolution. Measurement of oxygen evolved in light (3.6 k Lux) at 28±1°C was done by using an oxygen electrode (Oximeter-ox1-191; Toshniwal Process Instruments Private Limited, New Delhi) fitted to a reaction vessel of 10 ml capacity. Heavy metal ions-treated suspensions were added to the reaction vessel at a cell density of 4–5 µg Chl/ml (Chl = chlorophyll). The amount of $O_2$ evolved was expressed in nmoles of $O_2$ evolved/hr/µg Chl. The Chl content was determined according to Holm (1964).

*Phage titration.* Diluted phage lysates (0.1 ml) were mixed with host cultures (3 ml), incubated for 60 mins to allow adsorption and plated by the double agar layer technique (Adams, 1959). The number of plaques was counted after three days of incubation and the phage titer was expressed in PFU/ml.

*One-step growth experiments* were performed in a standard way (Adams, 1959). An exponentially growing culture of *N. muscorum* was blended to obtain a homogeneous single cell suspension of approximately 1 x $10^6$ cells/ml. The phage (1 ml) was left to adsorb on the bacteria (9 ml) at multiplicity of infection (MOI) of 1 PFU/cell for 90 mins. Under these conditions a final titer of approximately 1 x $10^6$ PFU/ml was obtained. The infected bacteria were washed twice with fresh medium by centrifugation to remove unadsorbed phage and resuspended in original volume of medium. The bacteria were diluted 1000-fold in the Chu-10 broth supplemented with heavy metals in various concentrations and incubated for up to 9 hrs. Aliquots (1 ml) were taken at 1-hr intervals for phage titration. The linear increase of phage titer was taken as a means of determining the latent and rise periods. Unless otherwise stated all treatments of the bacteria, phage or phage-infected bacteria proceeded at 28±1°C.

*The phage grown on bacteria treated with heavy metals.* The bacteria grown in various concentrations of heavy metals for 20 days were concentrated, washed with sterile double-distilled water and resuspended in fresh medium. The phage was added at MOI of 1 PFU/cell and allowed to adsorb to the bacteria for 90 mins. The phage-infected bacteria were resuspended in the same concentrations of heavy metals as those used for the growth of the bacteria and incubated for 72 hrs to ensure a complete lysis. The lysates were centrifuged at 5,000 x g for 15 mins in the cold to remove cell debris. The supernatants were taken for phage titration.

*The phage-infected bacteria treated with heavy metals during latent phase of phage growth.* The bacteria mixed with the phage at the MOI of 1 PFU/cell were allowed to undergo adsorption for 90 mins. The phage-infected bacteria were treated with various concentrations of heavy metals during latent phase of the phage growth for 3 hrs. Then the infected bacteria were pelleted, resuspended, lysed and titrated in standard way. In counting plaques the possible appearance of minute plaque and rapid lysis mutants was considered. Suspected variant plaques were isolated, propagated and plated to determine the nature of variation.

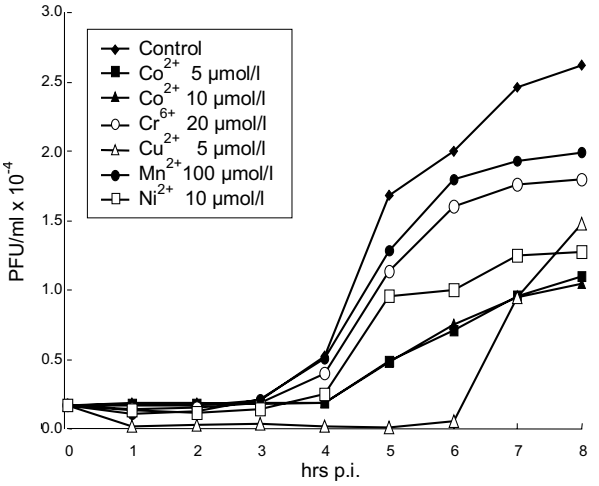


**Fig. 1**

**Growth of cyanophage N-1 under the influence of heavy metal ions**

One-step growth experiment.

## Results

*Effects of heavy metal ions on photosynthetic oxygen evolution in the bacteria*

The sublethal concentrations of the heavy metal ions under study for *N. muscorum* were determined to be 10, 100, 5, 1500 and 15 µmol/l for $Co^{2+}$, $Cr^{6+}$, $Cu^{2+}$, $Mn^{2+}$ and $Ni^{2+}$, respectively. The treatment of the bacterium with these ions resulted in inhibition of oxygen evolution (Table 1). The highest inhibition of oxygen evolution by 86.54 % was observed with $Cr^{6+}$ at 250 µmol/l. Regarding the toxicity for photosynthetic evolution of oxygen the five heavy metal ions can be ordered as follows: $Cr^{6+} > Mn^{2+} > Ni^{2+} > Cu^{2+} > Co^{2+}$.

*Effects of heavy metal ions on the phage growth in the one-step growth experiment*

To determine inhibitory effects of heavy metal ions on the phage growth the one-step growth experiment was employed (Fig. 1, Table 2). Under normal conditions, in the absence of heavy metal ions the phage titer was constant during the first 3 hrs post infection (p.i.). At 4 hrs p.i., the titer began to rise and kept rising during the next 5 hrs. An average burst size of 254 PFU/cell was noted. $Co^{2+}$ in concentrations 1.0 and 2.5 µmol/l did not alter both the latent and rise phase of the phage growth, but the burst size was reduced by 29% and 33.4%, respectively.

However, 5.0 and 10 µmol/l $Co^{2+}$ extended the latent and rise phase by 1 and 3–4 hrs, respectively and inhibited the burst size by 40% and 77%, respectively.

In the presence of 2.5 and 5 µmol/l $Cr^{6+}$ the latent phase was extended by 1 hr, while the rise phase and burst size were unchanged. In 10 and 20 µmol/l $Cr^{6+}$ both the latent and rise phase were extended by 1 hr, while the burst size was inhibited by 64.6% and 76.4%, respectively.

$Cu^{2+}$ in the concentrations of 1 and 2.5 µmol/l did not affect the latent phase but inhibited the burst size by 16% and 18%, respectively. In 5 µmol/l $Cu^{2+}$ both the latent and rise phase

**Table 2. Inhibition of phage N-1 growth under the influence of heavy metal ions during one-step growth experiment**

| Heavy metal ions | Concentration (mmol/l) | Phage burst size inhibition by %[a] |
|---|---|---|
| $Co^{2+}$ | 1.0 | 29.0 |
| | 2.5 | 33.4 |
| | 5.0 | 40.0 |
| | 10.0 | 77.0 |
| $Cr^{6+}$ | 2.5 | 0 |
| | 5.0 | 0 |
| | 10.0 | 64.6 |
| | 20.0 | 76.4 |
| $Cu^{2+}$ | 1.0 | 16.0 |
| | 2.5 | 18.0 |
| | 5.0 | 52.8 |
| | 10.0 | 100.0 |
| $Mn^{2+}$ | 10.0 | 0 |
| | 20.0 | 0 |
| | 50.0 | 84.0 |
| | 100.0 | 84.0 |
| $Ni^{2+}$ | 1.0 | 50.3 |
| | 2.5 | 65.0 |
| | 5.0 | 76.8 |
| | 10.0 | 84.3 |

[a]The burst size of 254 PFU/cell in metal ions-free control was taken for 100%.

were extended by 2 hrs, while the burst size was inhibited by 52.8%. No phage growth took place in 10 µmol/l $Cu^{2+}$.

$Mn^{2+}$ in the concentrations of 10 and 20 µmol/l did not affect either the latent phase or burst size. However, 50 and 100 µmol/l $Mn^{2+}$ inhibited the burst size by 84%, while the latent period was unaffected and the rise phase was reduced by 1 hr.

$Ni^{2+}$ in the concentrations of 1.0 and 2.5 µmol/l did not affect the latent phase but inhibited the burst size by 50.3% and 65%, respectively. In 5.0 and 10 µmol/l $Ni^{2+}$, the latent and rise phase were extended by 2 hrs and 3 hrs, respectively. The burst size was inhibited by 76.8% and 84.3%, respectively.

*Phage growth in the bacteria grown on heavy metal ions*

The experiments on the phage growth in the bacteria grown on heavy metals revealed that the burst size was inhibited as the concentration of the metal ions increased (Table 1). Thus, in the bacteria grown on 5.0, 10 and 20 µmol/l $Co^{2+}$, the burst size was reduced by 42.31%, 50.00% and 53.85%, respectively. The bacteria grown on 20 µmol/l $Cr^{6+}$ yielded only 75% of phage particles (inhibition by 25%) and there was continuous decrease in the phage titer as the metal concentration increased. The burst size in 2.5 and 5 µmol/l $Cu^{2+}$ was reduced by 92.31% and 99.99%, respectively and in 7.5 and 10 µmol/l $Cu^{2+}$ the phage growth was totally inhibited; 150 and 200 µmol/l $Mn^{2+}$ inhibited the burst size by 53.85% and 92.31%, respectively.

The burst size was inhibited by 1.0, 2.0 and 5.0 µmol/l $Ni^{2+}$ by 61.54%, 65.39% and 80%, respectively.

*Phage growth in the bacteria treated with heavy metal ions during latent phase*

The treatment of the phage-infected bacteria with heavy metal ions during latent phase revealed that the phage growth in 50 and 100 µmol/l $Co^{2+}$ was completely inhibited (Table 1). Likewise, 150 and 250 µmol/l $Cr^{6+}$ led to an inhibition by 76.48% and 94.12%, respectively. In 5.0 and 10 µmol/l $Cu^{2+}$, the phage growth was inhibited by 81.5 and 86.25%, respectively. $Mn^{2+}$ in the concentrations of 1000 and 2000 µmol/l reduced the burst size by 60%. A weakest inhibition of the phage growth, namely by 34.05% and 36.18% was obtained with 5.0 and 10 µmol/l $Ni^{2+}$.

*Effects of heavy metal ions on plaque morphology*

A change in plaque morphology was observed in phage progeny released after the treatment with 20 µmol/l $Ni^{2+}$. Minute plaques (1–2 mm in diameter) as compared to control ones (4–5 mm) were observed after 72 hrs. Such plaques, when isolated and plated, did not maintain the minute morphology, suggesting a transient feature. No rapid lysis mutants were detected.

## Discussion

One-step growth experiments performed in the presence of the heavy metal ions $Co^{2+}$, $Cr^{6+}$, $Cu^{2+}$, $Mn^{2+}$ and $Ni^{2+}$ revealed a delay in the latent period by 1–2 hrs and a decreased burst size, both effects depending on the ion concentration. At higher concentrations of $Cu^{2+}$ the inhibition of phage growth was complete while with other heavy metals it was partial only. Also the other two types of experiments confirmed the inhibitory effects of heavy metal ions on the phage burst size.

Heavy metal toxicity for photosynthetic electron transport (Clijster and Van Assche, 1985; Mohanty and Mohanty, 1988; Murthy and Mohanty, 1991) and carbon dioxide incorporation in cyanobacteria have been reported earlier (Raizada and Rai, 1985; Pandey *et al*., 1992). These studies have also suggested that the photosystem (PS)-II is more susceptible to heavy metal ion-induced damage than PS-I. At least in the case of $Cu^{2+}$ the overall sensitivity of the reactions was found to decrease in the order $^{14}CO_2$ incorporation>PS-II>ATP>PS-I in *Nostoc calcicola* (Pandey *et al*., 1992). The inhibition of $O_2$ evolution observed by us is in accord with this finding. In this context it should be noted that the PS-II inhibition did not affect the growth of the phages LPP-1 (Sherman and Haselkorn, 1971), SM-1 (Mackenzie and Haselkorn, 1972) and N-1 (Kashyap and Singh, 1989).

Although the dependence of multiplication of N-1 (Adolph and Haselkorn, 1972) as well as other cyanophages such as LPP-1 G (Padan *et al.,* 1970), SM-1 (Mackenzie and Haselkorn, 1972), AS-1 M (Sherman, 1976) and SM-2 (Benson and Martin, 1981) on photosynthesis is known, the energy for the phage biosynthesis has been suggested to originate from PS-I by cyclic photophosphorylation (Padan *et al.,* 1970; Wu *et al.,* 1968; Mackenzie and Haselkorn, 1972; Adolph and Haselkorn, 1972). This is in line with the observation of Gusev *et al*. (1980) who have suggested the PS-I reaction center of *Anabaena variabilis* to be most resistant to degeneration caused by the phage A-1L infection.

Singh *et al*. (1994) have reported that the inhibition of $^{14}CO_2$ fixation was accompanied by a rise in oxygen uptake 4 hrs after infection of *N. muscorum* with phage N-1, suggesting that oxidative phosphorylation is more crucial for the phage growth than photophosphorylation under photoautotrophic conditions. As enzymes of nitrogen metabolism such as nitrogenase, glutamine synthetase and glutamate-oxaloacetate transaminase were inhibited after the infection it has been suggested that the N-1 phage multiplication proceeds in the absence of nitrogen fixation and the metabolism of amino acids is altered in favor of phage multiplication.

As discussed above, the inhibition of $^{14}CO_2$ fixation impinged on the bacteria both by phage infection and heavy metal ions could act synergistically leading to a decreased

phage burst size. Moreover, the toxicity of heavy metals seems to be more intricate rather than simply causing inhibition of PS-II and/or $^{14}CO_2$ fixation. This has been exemplified by a damage caused by heavy metals to the cell membrane leading to a loss of $K^+$ and $Na^+$ from the cells of *N. muscorum* (Rai *et al*., 1990), reduced levels of $Ca^{2+}$ and $Mg^{2+}$ in the cells of *Anabaena* PCC 7119 (Bolanos *et al*., 1992), decreased nutrient uptake by the cells of *A. doliolum* (Mallick and Rai, 1994) and $PO_4^{3-}$ deficiency in the cells of *A. doliolum* (Mallick and Rai, 1990; Singh *et al*., 1994) and *N. muscorum*.

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