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Comparison of the Toxicity of Chromium III and Chromium VI to Cyanobacteria

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Over the last decade the increasing concern over environmental pollution has led to extensive research into the toxic and non-toxic effects of environmental pollutants. Heavy metals are frequently cited as of particular concern and numerous studies have now been published describing potential methods for bioremediation and reclamation using micro-organisms. Chromium is a heavy metal of particular importance. It is extensively used in industry and high levels of chromium contamination of both terrestrial and aquatic (freshwater and marine) habitats occur. In general, Cr VI is considered to be of greatest concern due to studies in mammalian systems that show it to be carcinogenic and toxic (Rowbotham et al 2000). Reports suggest this action is likely to be due to uptake of Cr VI, via the sulphate anion channel, and then reduction of Cr VI to Cr III, which can then bind to DNA, leading eventually to DNA damage and mutation (Standeven and Wetterhahn 1989). In contrast, Cr III is considered relatively non-toxic in mammals due, in part, to the fact there is little or no cellular uptake of this chromium species. Studies on chromium bioremediation have therefore frequently concentrated on organisms capable of reducing Cr VI to Cr III which, up to now, has been considered relatively non-toxic in both eukaryotes and prokaryotes alike.

In this investigation the effects of chromium speciation on toxicity to cyanobacteria (*Synechococcus* PCC 7942 and *Nostoc* PCC 7120) were examined. Cyanobacteria are known to be relatively tolerant to heavy metals and are frequently cited as possible organisms for use in bioremediation of metal pollutants (Fiore and Trevors 1994) However, cyanobacteria are important primary producers in their own right; they produce high levels of oxygen, are capable of fixing atmospheric nitrogen in many cases and form an important part of the picoplankton. It is therefore important to consider what effects chromium may have on cyanobacteria in the environment. At present few studies have looked at Cr VI effects and little information currently exists on Cr III toxicity. This study partially addresses that shortfall.

MATERIALS AND METHODS

Two laboratory strains were chosen for this study: the unicellular strain

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Synechococcus PCC 7942 and the filamentous, nitrogen fixing strain *Nostoc* PCC 7120. Both were grown in Bg11 media (Rippka et al. 1979) using 100ml of culture in 250ml flasks. Conditions used were continuous light, 5% CO_2, 34°C with shaking at 120rpm. At various time points (see results) after the addition of chromium to the cultures, samples were removed from the flasks and analysed for growth by monitoring the absorbance at 730nm. This was performed for both low density and high density cultures. The low-density cultures were obtained by adding 1ml of culture (A_{730} 0.4) to 100ml media, followed immediately by addition of chromium as either $CrCl_3$ (Cr III) or Na_2CrO_4 (Cr VI). High-density cultures were set up as above then allowed to grow to mid-log phase (A_{730} 0.4) before addition of chromium.

In order to determine any differences in acute and chronic effects and whether toxicity leads to cell death or growth inhibition, recovery studies were performed. A 10ml volume was removed from each treatment after 2, 24 and 48 hours' exposure to chromium. The samples were centrifuged at 2000x g for 8 minutes and the supernatant discarded. The cells were washed with BG11, re-centrifuged as above then resuspended in fresh, chromium free media. Growth was analysed as above.

As well as absorbance, the pH of the media was monitored at the start of the experiments and through out growth. When large changes in the initial pH were seen, control flasks were set up with media altered to this pH and growth monitored in order to ensure effects were due to the chromium treatment rather than general pH effects.

RESULTS AND DISCUSSION

Low-density cultures should be better models for the likely effects of environmental contamination on cyanobacteria found under most environmental conditions (i.e. no eutrophication or bloom forming)(Whitton and Potts 2000). In general, chronic effects are unlikely to occur frequently in most freshwater habitats but may take place in more terrestrial habitats or in water bodies where little movement or mixing occurs. However, under low density conditions, even chronic treatment with Na_2CrO_4 (Cr VI) failed to produce much effect on growth for both *Synechococcus* PCC 7942 and *Nostoc* PCC 7120 until levels of 1mM or above were reached (figure 1). At 1mM for *Nostoc* PCC 7120 and 2mM for *Synechococcus* PCC 7942 total cessation of growth occurred. Levels just lower than these exhibited either a longer lag phase or a slight reduction in the growth rate but little overall effect on the cyanobacteria for the criteria tested was observed.

At high density *Nostoc* PCC 7120 grew for 24 hours after the addition of levels of Na_2CrO_4 (1.75mM and above) that eventually inhibited growth. High density cultures of this organism therefore show a two-fold increase in Na_2CrO_4 concentrations required to induce a toxic effect when compared to low density cultures. In contrast, *Synechococcus* PCC 7942 did not exhibit a toxic effect for

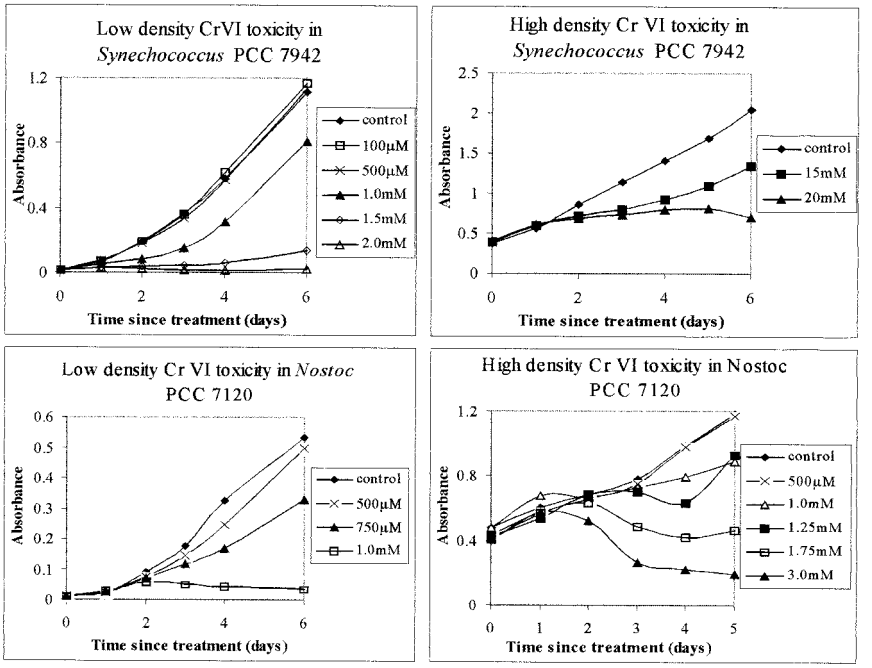

Figure 1. Effect of Cr VI on cyanobacterial growth for both *Synechococcus* PCC 7942 and *Nostoc* PCC 7120. Low density cultures were obtained by adding 1ml of culture (A_{730} 0.4) to 100ml media, followed immediately by addition of chromium as Na_2CrO_4 (Cr VI). High-density cultures were set up as above then allowed to grow to mid-log phase (A_{730} 0.4) before addition of chromium.

Na_2CrO_4 under high density conditions until a concentration greater than 15mM was reached, a ten-fold increase in concentration compared to low density cultures. *Synechococcus* PCC 7942 also exhibited a longer continuation of growth after treatment was initiated with cellular concentrations increasing for approximately 4 days before cell numbers declined. What causes the increased resistance of *Synechococcus* PCC 7942 to Na_2CrO_4 is unclear but may be due to differences in the cell wall between the two cyanobacteria tested (Fiore and Trevors 1994, unpublished data). The general decrease in toxicity in dense cultures was, however, expected as this has previously been noted for other metals. This was usually attributed to lower amounts available per cell (Singh and Yadava 1985) and the reduced total surface area available due to aggregation of cells (Schecher and Driscoll 1985) although aggregation was not noted in this case.

It is generally considered that Cr III is relatively non toxic compared to Cr VI (eg Bopp and Ehrlich 1988) but in this study chromium III proved highly toxic to both *Synechococcus* PCC 7942 and *Nostoc* PCC 7120 (Figure 2). Both cyanobacteria studied were susceptible to levels of Cr III (as $CrCl_3$) at least 10 fold lower than

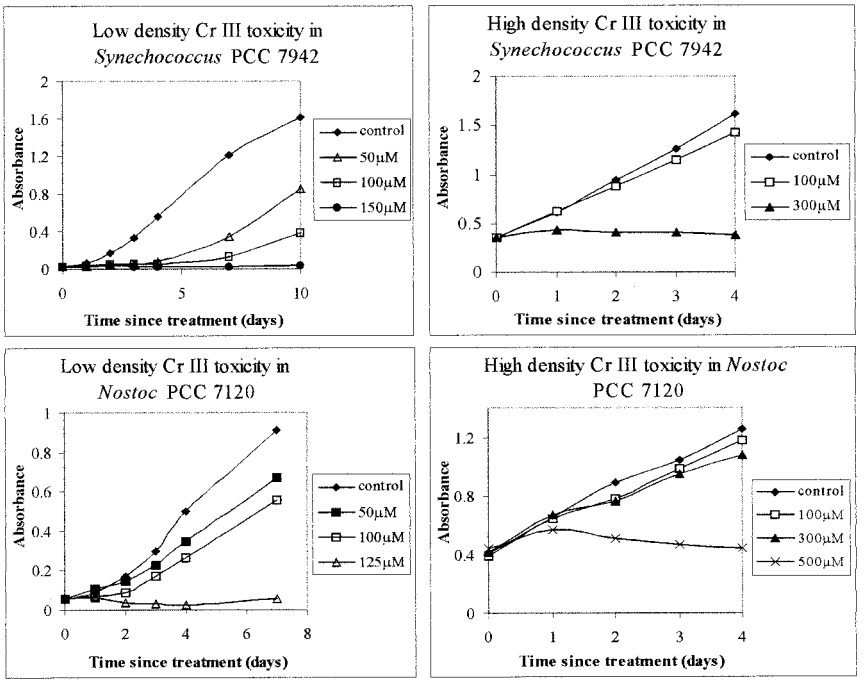

Figure 2. Effect of Cr III on cyanobacterial growth for both *Synechococcus* PCC 7942 and *Nostoc* PCC 7120. Low density cultures were obtained by adding 1ml of culture (A_{730} 0.4) to 100ml media, followed immediately by addition of chromium as $CrCl_3$ (Cr III). High-density cultures were set up as above then allowed to grow to mid-log phase (A_{730} 0.4) before addition of chromium.

those required for a similar response with Cr VI, under similar experimental conditions. For both low and high density cultures, concentrations above 100μM lead to a cessation in growth of *Synechococcus* PCC 7942, whilst for *Nostoc* PCC 7120 100μM Cr III inhibited growth in low density cultures with levels above 300μM required for this effect in high density cultures. However, for *Synechococcus* PCC 7942, a concentration of 150μM did not appear to lead immediately to cell death but initially inhibited cell growth as evidenced by the ability of treated cells to recover once chromium III is removed (Figure 3). This figure shows that 150μM $CrCl_3$ treatment and removal after 2 hours induced an apparent longer lag phase in the growth of *Synechococcus* PCC 7942 but the growth rate obtained following this period was similar to the control. Similar results are obtained for *Nostoc* PCC 7120 (data not shown). Following longer treatments the concentration of chromium at which no recovery was noted dropped and remained at levels comparable to the original toxicity data. The main mechanism for Cr III toxicity appears to be rapid occurring within 24 hours at the most with some effects noted within 2 hours. The apparent increased lag phase on recovery, as noted above, could be attributable to either time required for repair of

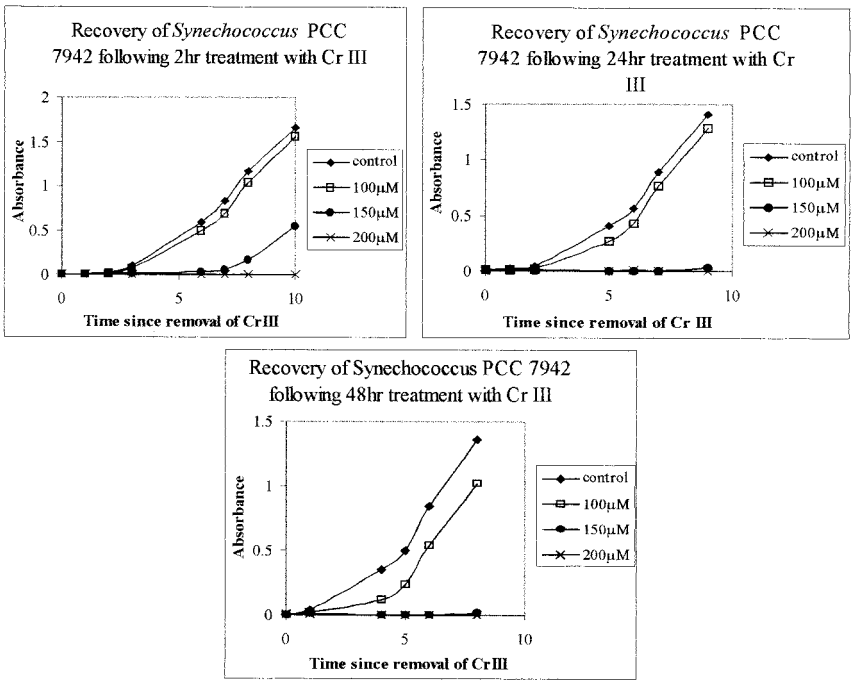

Figure 3. Recovery of *Synechococcus* PCC 7942 following treatment with Cr III. Low density cultures were treated as before then samples were removed and placed in chromium free media and growth monitored by absorbance at 730nm.

Table 1. pH of media containing chromium

Cr III concentration	control	50μM	100μM	125μM	150μM	300mM
pH day 0	7.65	7.27	7.0	6.84	6.7	6.14
Final pH	9.5	9.53	9.41	9.42	9.47	9.51
Cr VI concentration	control	0.05mM	0.1mM	0.5mM	1mM	2mM
pH day 0	7.65	7.56	7.58	7.75	7.75	7.75

cellular damage, or immediate growth by a small fraction of cells that weren't killed by the treatment. Interestingly, whilst a slight increase in dose is required for toxicity at high densities this is not as marked as with Cr VI above.

A factor that can alter both the growth of an organism and its ability to bind or adsorb metal ions is pH. Consequently the pH of the media was monitored during these experiments. As shown in table 1, addition of Cr III caused some changes in the initial pH of the media. These changes observed did not continue throughout growth and quickly both controls and treated media obtained the same pH suggesting pH was not a factor in the growth inhibition of the cyanobacteria. This

was confirmed by growth curves for the two cyanobacteria under the various pH obtained during which the cyanobacteria grew at a similar rate to the control (Data not shown). Cr VI had little or no effect on the pH of the media.

Previous work indicates that most bacteria are sensitive to Cr VI at levels much lower than used here. At levels of 50 and 100mg/L (1 and 2mM respectively), only 0.1% of isolatable microorganisms from environments regularly suffering acute contamination with similar levels of Cr VI were capable of growth (Baldi et al. 1990; Luli et al 1983).). These levels are comparable to those at which the cyanobacteria tested survive and the levels at which *Synechococcus* PCC 7942 will continue growing under high density conditions is over 15 fold higher than this. Why these cyanobacteria should show this resistance to chromium is unclear. Levels of total chromium in freshwater usually fall within 1-10μg/L and it is only in areas of high industrial contamination that levels at millimolar concentrations and above are regularly seen (Palmer and Wittbrodt 1991). The mechanism for this resistance also requires further analysis but there are a number of possibilities including plasmid borne resistance, extracellular binding or precipitation, impermeability and exclusion, internal detoxification and metal transformations (Fiore and Trevors 1994). So far two genes specifically involved in metal resistance have been studied in *Synechococcus* PCC 7942. These are *smt* A, which codes a metallothionein protein involved in zinc homeostasis and metabolism (Turner and Robinson 1995) and *srp*C , a plasmid borne gene regulated by availability of sulphur (Nicholson and Laudenbach 1995) However, no genes have been isolated which are specifically involved in chromium resistance. Whatever mechanism exists this study confirms that some cyanobacteria are relatively resistant to chromium VI compared to most other bacteria and that it may be possible to use cyanobacteria in bioremediation of metal contaminated sites. Laboratory strains as used here however, would probably only be useful in a bioreactor system as has been previously proposed. (Khattar et al 1999) It must also be considered that *Synechococcus* PCC 6301, a closely related strain to *Synechococcus* PCC 7942, has been shown to exhibit inhibition of growth at 100 μM chromate under conditions differing only in the temperature used (23°C instead of 34°C) from this present study (Garnham and Green 1994). Therefore care must be taken when considering either the strain to be used, or the conditions under which to use it.

Whilst the pH had no direct effect on the growth of the organisms used, the pH of the media may have a bearing on the low toxicity of Cr VI and the apparent increased toxicity of Cr III. Earlier work has shown that the short-term uptake of chromate is pH dependent such that at low pH Cr VI is readily and strongly bound whilst above pH 5 little binding occurs (Garnham and Green 1995; Greene and Darnell 1990). In contrast, Cr III exhibits the opposite behaviour, binding more strongly as pH increases. In the environment the pH may have a broad range dependent on numerous factors. The results given here may only be true within systems where the pH remains above 5 and, under conditions causing a lowering of pH, Cr VI may well lead to toxicity.

These results show that it can not be assumed Cr VI is the toxic form of chromium with Cr III being relatively non-toxic. In earlier studies it was assumed that one reason for increased resistance of some cyanobacteria to Cr VI was due to reduction to Cr III (Garnham and Green 1995; Azeez and Banerjee 1988), but these result do not agree with that assumption. For the two cyanobacteria tested here the toxicity is the opposite to that expected, and, whilst other factors may influence the relative toxicity in the environment, this illustrates that care must be taken in considering bioremediation methods and re-emphasises the importance of speciation in metal toxicity.

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