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Chromate (VI) uptake by and interactions with cyanobacteria

Geoffrey W. Garnham and Martin Green

Company Research Laboratory, British Nuclear Fuels, Springfields Works, Salwick, Preston PR4 0XJ, UK

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SUMMARY

The short-term accumulation of chromate by the cyanobacteria *Anabaena variabilis* and *Synechococcus* PCC 6301 has been described as consisting of a rapid and relatively low level of biosorption of chromate to the cell walls; no energy-dependent uptake was detected. This biosorption was dependent on chromate concentration and could be described by a Freundlich adsorption isotherm for both cyanobacterial species studied. Decreasing the external pH increased the chromate accumulation by both species. Over a longer time period with growth it was shown that *A. variabilis* was capable of reducing chromate (VI) to chromium (III) and then accumulating the chromium (III). *Synechococcus* PCC 6301 showed no further interaction with chromate concentrations over the same time period after the initial biosorption.

INTRODUCTION

Chromate compounds have no known biological function and are highly toxic to biological systems. Many however, have industrial applications from which the waste is often the cause of environmental pollution [3]. The accumulation of highly toxic and mobile Cr (VI) or its reduction to stable and relatively less toxic Cr (III) by microorganisms are likely to be useful processes in the remediation of contaminated soils and waters [12]. A wide range of bacteria, both aerobic and anaerobic, remove Cr (VI) from solution by reducing it to Cr (III) [11]. A fraction of the reduced Cr (VI) may also then be taken up by the cells [16]. Cr (VI) reduction in aerobic conditions is not a resistance mechanism in bacteria but is a side activity for enzymes that have other as yet unidentified substrates [10]. To our knowledge, no one has yet described the accumulation of Cr (VI) by cyanobacteria in detail, or if they are capable of reducing Cr (VI). Cyanobacteria have been described as being able to reduce metals such as Au (III) [11] and to accumulate significant amounts of heavy metal ions both by biosorption and active processes [6]. Cyanobacteria have also been described as producing complex organic ligands which may bind metals [13].

This paper investigates the accumulation of chromate ions and the capability to reduce chromate to chromium of two common cyanobacterial species, one unicellular *Synechococcus* PCC 6301 and one filamentous with heterocysts and the capability to fix nitrogen (*Anabaena variabilis*).

Correspondence to: G.W. Garnham, Company Research Laboratory, British Nuclear Fuels, Springfields Works, Salwick, Preston PR4 0XJ, UK.

MATERIALS AND METHODS

Organisms, media and culture conditions

Axenic cultures of *Synechococcus* PCC 6301 and *Anabaena variabilis* were grown at 23 °C in 100 ml BG11 medium and in 100 ml BG11 medium without a nitrate source respectively. BG11 medium comprised 1.5 g $NaNO_3$; 0.04 g K_2HPO_3; 0.0075 g $MgSO_4 \cdot 7H_2O$; 0.036 g $CaCl_2 \cdot 2H_2O$; 0.006 g citric acid; 0.006 g ferric ammonium citrate; 0.001 g Na_2EDTA; 0.02 g Na_2CO_3; and 1 ml trace metal mix A5 (2.89 g H_3BO_3; 1.81 g $MnCl_2 \cdot 4H_2O$; 0.222 g $ZnSO_4 \cdot 7H_2O$; 0.0039 g $Na_2MoO_4 \cdot 2H_2O$; 0.079 g $CuSO_4 \cdot 5H_2O$; 0.049 g $Co(NO_3)_2 \cdot 6H_2O$ in 1 L distilled water) [1]. The medium was adjusted to pH 8 with tetramethylammonium hydroxide and autoclaved (120 °C, 15 min) before being inoculated to a density of 4×10^6 cells ml^{-1} *Synechococcus* PCC 6301 or with 2 ml of *Anabaena variabilis* culture of $OD_{680\,nm}$ of ~10 since this is a filamentous organism. Cultures were incubated in 250-ml conical flasks with rotary incubation at 150 cycles min^{-1}, at 23 °C and with a photon fluence irradiance rate on the surface of the flask of 12 μmol photon m^{-2} s^{-1} provided by white fluorescent tubes.

Short term chromate uptake

Cultures in the exponential phase of growth (approximately 20-day incubation) were harvested by centrifugation (15 min, 1000 *g*). The supernatant fluid was removed and the cells were then washed once with 10 mM {[(2-hydroxymethyl)ethyl]-amino}-1-propanesulphonic acid (TAPS) buffer adjusted to pH 8.0 with solid tetramethylammonium hydroxide and resuspended to cell densities $\sim 5 \times 10^8$ cells ml^{-1} *Synechococcus* PCC 6301 or to an $OD_{680\,nm}$ of ~10 *A. variabilis* (each equivalent to 4 mg dry wt. of cells ml^{-1}). For CrO_4^{2-} uptake experiments, 25-ml cell suspensions were incubated in 100-ml acid-washed plastic beakers which were shaken in the light (30 μmol photon m^{-2} s^{-1}) at 23 °C unless stated otherwise.

Aliquots from a 0.01 M potassium chromate solution were added to give chromate concentrations in the range 1–1000 μM. Three replicate samples (1 ml) were taken from the cell suspensions at time intervals after the addition of potassium chromate and centrifuged using an Eppendorf 5412 microcentrifuge (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) (30 s, 8000 × *g*). The supernatant fluid was removed and the chromate concentration measured spectrophotometrically using 1,5-diphenylcarbazide [15]. The amount of chromate taken up by cells was calculated from the reduction of chromate in the buffer, after taking into account any binding of chromate to plastic beakers (determined in control experiments without cells). Where desired, cells were pre-treated 30 min prior to harvesting with 200 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) or incubated in the dark or light for 24 h.

The effect of pH on the short term uptake of chromate was investigated. Uptake experiments were performed in the following buffers with a chromate concentration of 50 μM; 10 mM {[(2-hydroxymethyl)ethyl]-amino}-1-propane sulphonic acid (TAPS) buffer, pH 9.0 and 8.0; 10 mM 2-(*N*-cyclohexylamino)ethanesulphonic acid (HEPES) buffer, pH 7.0 and 8.0; 10 mM 1,4-piperazinediethanesulphonic acid (PIPES) buffer, pH 6.0 and 7.0; 4-morpholinoethanesulphonic acid (MES) buffer, pH 6.0; 10 mM acetate buffer, pH 4.0 and 5.0.

Long term uptake of chromate during growth of cyanobacteria

Cultures were grown as described previously but in the presence of 10 and 100 μM potassium chromate concentrations. Growth of the cultures was monitored by measuring the optical density at 680 nm. Samples were taken during growth as in the short term uptake experiments. The chromate concentration in the media during growth was also monitored spectrophotometrically using 1,5-diphenylcarbazide. As a control, the chromate concentration was also monitored in BG11 media without cells over the same time period. After 18 days growth, the chromium concentration in the media and cells was measured using atomic adsorption spectrophotometry as described by Avery et al. [2].

After 18 days growth of 100-ml *A. variabilis* culture in BG11 as described previously, cells were separated from the medium by centrifugation (10 min, 12 000 × *g*). One millilitre of a 10 mM potassium chromate solution was then added to 99 ml of the separated media and left at room temperature for 24 h after which the chromate and chromium concentrations in the media were measured as described previously.

Chemicals and reagents

All chemicals used were of analytical grade. Inhibitors, buffers and tetramethylammonium hydroxide were supplied by Sigma (Poole, Dorset, UK).

RESULTS

Short term uptake of chromate

Short term uptake of chromate by both *Synechococcus* PCC 6301 and *A. variabilis* consisted of a single phase which was independent of light or the presence of CCCP, indicating an energy-independent process (Fig. 1). This phase was complete within 5 min with no further significant uptake over a further 4 h incubation. *Synechococcus* PCC 6301 accumulated approximately 10 nmoles more chromate per g dry weight of cells than *A. variabilis*.

This short term accumulation by both species was dependent on concentration and followed a Freundlich adsorption isotherm [5] (Fig. 2). Scatchard plots (not shown) of the same data were bi-phasic; the binding constants (K_{ads}) and maximal binding capacities (R_{Tads}) [17] calculated for *Synechococcus* PCC 6301 and *A. variabilis* were (0.020 and 0.021 nmol^{-1}) and (60 and 55 nmol (g dry wt)$^{-1}$) respectively.

As the pH was reduced from 9 to 7, a slight increase in the short term accumulation of chromate was observed for both species, but as the pH was further reduced to pH 4, a much greater increase in short term accumulation occurred, from 0.13 to 4.70 μmol chromate (g dry wt.)$^{-1}$ by *A. variabilis* and from 0.14 to 2.8 μmol chromate (g dry wt.)$^{-1}$ by *Synechococcus* PCC 6301 (Fig. 3).

Long term accumulation of chromate during growth of cyanobacteria

After the initial short term uptake as described previously in *A. variabilis*, further removal of chromate from both 100 and 10 μM concentrations in the medium by this species was observed (Fig. 4). No further removal of chromate was observed with *Synechococcus* PCC 6301 (Fig. 5). During an

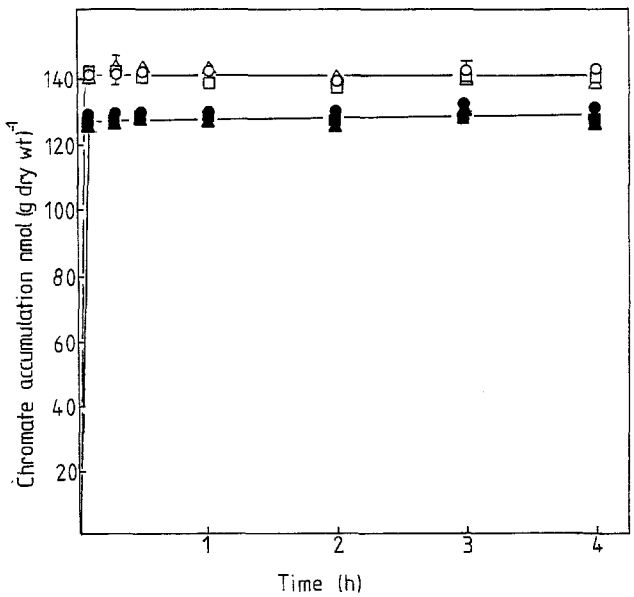

Fig. 1. Short-term uptake of chromate at 23 °C by cultures of *Synechococcus* PCC 6301 at a cell density of 5×10^8 cells ml^{-1} (open symbols) and *Anabaena variabilis* at an $OD_{680\,nm}$ ~10 (closed symbols) in {[(2-hydroxymethyl)ethyl]-amino}-1-propanesulphonic acid (TAPS) buffer, pH 8.0, containing 50 μM potassium chromate: (○,●) light incubation; (□,■) dark incubation; (△,▲) cells treated prior to incubation in the light with 200 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) for 30 min. Each point is a mean of three replicates; bars indicate standard error of mean and when not shown were smaller than the dimensions of the symbols.

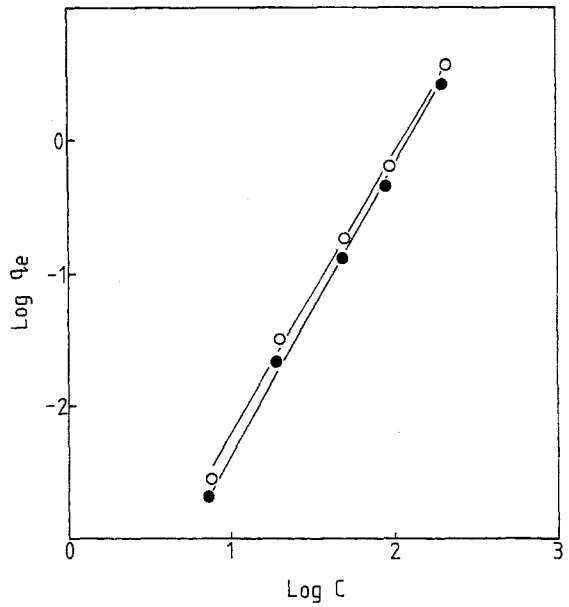

Fig. 2. Freundlich plot of chromate biosorption by *Synechococcus* PCC 6301 (●) and *A. variabilis* (○) in 10 mM TAPS buffer, pH 8.0, at 23 °C. Each point is a mean of three replicates with a standard error not greater than 0.01; q_e = the quantity of chromate adsorbed/mass of biomass at a fixed temperature (μmol (g dry weight of cells)$^{-1}$); C = the concentration of chromate remaining in solution at equilibrum (μM).

18-day period *A. variabilis* removed approximately 2 nmoles of chromate from a total of 10 nmoles in the medium and 0.6 nmoles from a total of 1 nmole in the medium. A 100-μM concentration had no effect on the growth of *A. variabilis* but the same concentration of chromate inhibited the growth of *Synechococcus* PCC 6301. In the absence of cyanobacterial cells no reduction in the chromate concentration in BG11 media was observed over an 18-day period. Analysis of the medium and cells from the two cultures after 18 days growth revealed the presence of chromium (III) associated with *A. variabilis* and in the growth media used for its growth. No significant amount of chromium was detected in the growth medium or with *Synechococcus* PCC 6301 cells (Table 1). Growth medium alone after 18 days growth of *A. variabilis* had no effect when added to a 100-μM chromate concentration.

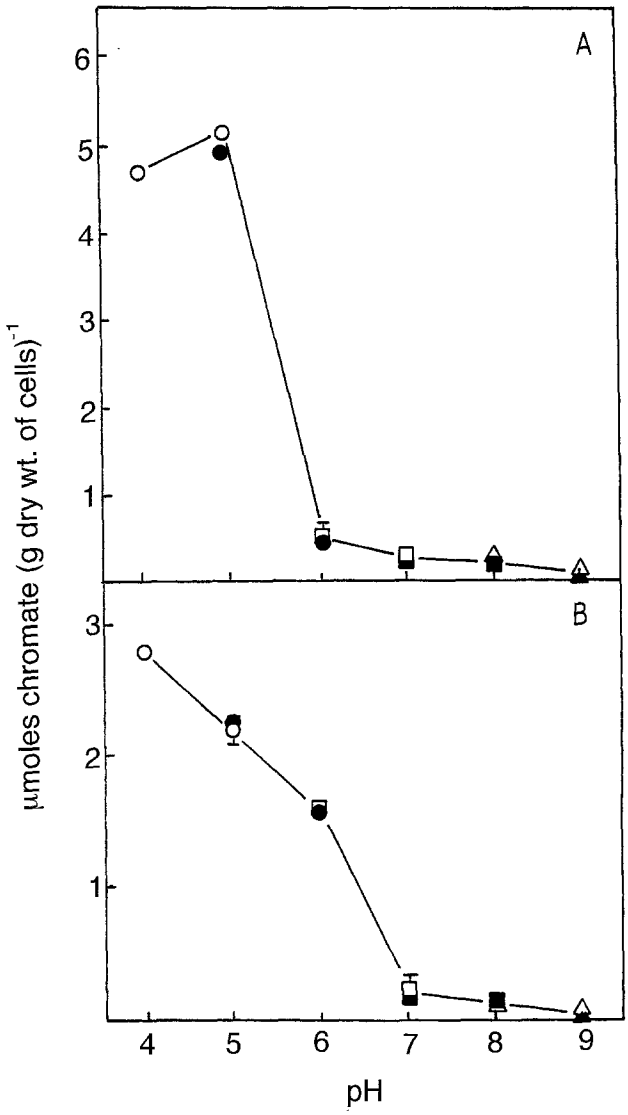

Fig. 3. Effect of pH on short-term uptake of chromate by (A) *A. variabilis* ($OD_{680\,nm}$ ~10) and (B) *Synechococcus* PCC 6301 (5×10^8 cells ml^{-1}) from 50 μM chromate in the following buffer solutions at 23 °C: (○) 10 mM acetate buffer, (●) 10 mM MES, (□) 10 mM PIPES, (■) 10 mM HEPES, (△) 10 mM TAPS, (▲) 10 mM CHES (see materials and methods for buffers). Each point is a mean of three replicates; bars indicate standard errors and when not shown, were smaller than the dimensions of the symbols.

DISCUSSION

The short term uptake of chromate (CrO_4^{2-}) by the cyanobacterial species studied was due to a single phase of metabolism-independent 'biosorption' with no apparent intracellular uptake. To our knowledge, no other study has described chromate accumulation by cyanobacteria, although there are studies which describe accumulation of other metals by cyanobacteria [4]. The amounts of cations accumulated are usually many times greater than the amounts of chromate accumulated in this study. The cell walls of cyanobacteria have a similar structure to those of Gram-negative bacteria, where the major component is murein containing diaminopimelic acid, muramic acid and *N*-acetyl glucosamine with a sheath composed of a matrix of pectic acids and mucopolysaccharide [9]. Cyanobacterial cell walls have a negative charge thus attracting cations and repelling species such as CrO_4^{2-}, which explains the low levels accumulated by biosorption. Examinations of the biosorption of anionic species such as MoO_4^{2-}, SeO_4^{2-}, $AuCl_4^-$ and TcO_4^- by cyanobacteria [8,9] described such biosorption as being weak with the species easily desorbed, and due to electrostatic attraction between anions and the cationic groups already associated with or possibly bound to the cell wall. The difference in the amount of chromate accumulated by the two cyanobacterial species is possibly due to variations in cell wall structure. The difference

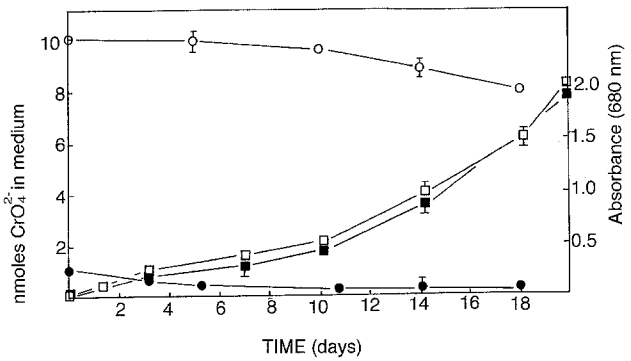

Fig. 4. Removal of chromate from BG11 media during growth of *A. variabilis*. (□, ■) growth measured by adsorbance at 680 nm; (○, ●) nmoles of chromate in media. Open symbols indicate a 100-μM concentration of chromate in media and closed symbols a 10-μM concentration of chromate in BG11 medium. Each point is a mean of three replicates; bars indicate standard error of mean and when not shown, were smaller than the dimensions of the symbols.

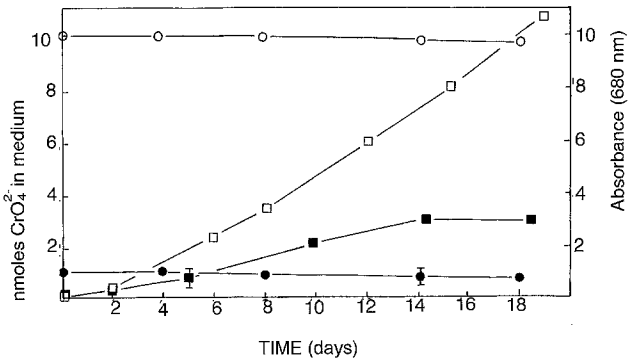

Fig. 5. Removal of chromate from BG11 media during growth of *Synechococcus* PCC 6301. (□, ■) growth measured by adsorbance at 680 nm; (○, ●) nmoles of chromate in media. Open symbols indicate a 100-μM concentration and closed symbols a 10-μM concentration of chromate in BG11 medium. Each point is a mean of three replicates; bars indicate standard error of mean and when not shown, were smaller than the dimensions of the symbols.

however, could also be due to the size of cells and the cell morphologies of the two species since this can affect the surface area available for biosorption. In this study, accumulation was only expressed on a dry weight basis.

The short term uptake of chromate (biosorption) was concentration-dependent and conformed to a Freundlich adsorption isotherm, as does the biosorption of other metals by cyanobacteria [4]. When data were plotted according to Scatchard [14], the relationship indicated a primary binding of chromate up to the R_{Tads} values given in results followed by weaker secondary binding. This may indicate that two mechanisms are involved in the chromate uptake.

The short term uptake of chromate by both species was pH-dependent, with increased uptake at decreased pH values [7], in contrast with decreased accumulation of many cationic metal species with decreased pH. This effect was also observed with cyanobacterial accumulation of pertechnetate [8]. It can be explained by increased binding of protons to

TABLE 1

Effect of cyanobacterial growth on the concentrations of chromium (III) and chromate (VI) in BG11 media and amount of chromium (III) and chromate (VI) associated with cells after growth

Experimental condition[a]	Cr species	Amount of Cr (nmol)	
		In media	In cells
Anabaena variabilis	chromate (VI)	0.56	—
	chromium (III)	4.10	4.20
Synechococcus PCC 6301	chromate (VI)	9.10	0.90
	chromium (III)	0.00	0.00
Cell-free BG11	chromate (VI)	10.00	—
	chromium (III)	0.00	0.00

[a] Samples of media and cells were taken from 18-day-old cultures. The data are means of three replicates each with standard errors no greater than (0.3).

binding sites on the cell wall, which in turn increases anionic binding. In addition, the isoelectric point for many cyanobacterial walls may lie in a low pH range (4–5), so that as the pH is decreased the overall net charge of the cell wall promotes easier access of anions to positively charged binding sites.

During the growth of *A. variabilis* approximately 50% of the chromate in the medium was removed, more than could be accounted for by biosorption to cells. Further analysis revealed that this was due to the reduction of chromate (CrO_4^{2-}) to chromium (Cr^{3+}) and uptake of the reduced chromate by the cells, probably through biosorption as described for other cyanobacteria and microorganisms [6,13]. Chromium (Cr^{3+}) concentrations were associated with both the media and *A. variabilis* cells. At this time the site of chromate reduction is unknown. Many microorganisms reduce chromate under aerobic conditions, but to our knowledge this is the first report of chromate reduction by a cyanobacterium. This type of chromate reduction is described as not being a resistance mechanism but as a side activity of enzymes that have other as yet unidentified natural substrates [11]. However, the reduction of chromate to less toxic chromium could confer increased resistance. This could have been a contributory factor to the ability of *A. variabilis* to tolerate 100 μM chromate while *Synechococcus* PCC 6301, which did not appear to be able to reduce chromate, showed inhibition of growth at this concentration. Since growth media with cells removed after 18 days growth of *A. variabilis* did not reduce chromate, the chromate reduction observed was probably not due to the production of extracellular products such as polysaccharides, but was mediated by a metabolic process. It is possible that the heterocysts of *A. variabilis* were the site of the chromate reduction. This would explain why no chromate reduction was observed with *Synechococcus* PCC 6301 which does not fix nitrogen or possess heterocysts. The only way in which *Synechococcus* PCC 6301 appeared to interact with chromate concentrations

was through biosorption. Further studies are needed to determine the mechanism of chromate reduction by *A. variabilis*.

It has been proposed that reduction of chromate by microorganisms could be a useful process for the remediation of contaminated waters and soils [12]. *A. variabilis* although capable of reducing chromate and easily grown on a relatively simple medium, would not be a good candidate for such a process since its rate of chromate reduction is approximately 200 times slower than that described for other bacteria such as Pseudomonads [10]. The low levels of chromate biosorption observed by both cyanobacteria in this study also suggest that cyanobacteria would not be useful for a biological treatment process for the removal of chromate from waste effluent unless uptake by the cells was improved by manipulation of cell or external conditions.

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