Charophytes as a hyperaccumulator of heavy metals: role of calcification and applicability in restoration of polluted water bodies

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Abstract: Uptake of heavy metals to produce an internal concentration greater than in the external environment appears widespread in aquatic organisms. Thus remediation of metal and heavy metal contaminated waters by aquatic plants has a prominent role in aquatic eco-systems. Here we discuss the capacity of a characean alga *Nitella pseudoflabellata* to accumulate heavy metals, in the context of calcite encrustation. Preliminary investigations based on varying concentrations of Cr^{6+} and Ca showed a positive correlation between calcification of plants and shoot elongation. Similar trend was observed between Cr levels and calcification of plants. Subsequently microcosms were maintained for a period of 1 year for two heavy metal treatments; $0.2 \ mg.L^{-1} \ Cr^{6+}$ and $0.01 \ mg.L^{-1}$ Cd with the objective of assessing the role of calcite encrustation on heavy metal accumulation. In both cases, alkaline areas contained over 50% of total heavy metals, elucidating calcification to play a significant role. Speciation of sediment showed 35 % of total Cr, in the form of carbonate-bound, whereas carbonate-bound fraction of Cd was observed 43 % of total Cd. Thick marl sediment frequently observed in charophyte habitats suggests long term storage of heavy metals in less bio-available forms could be possible. Furthermore charophytes compare well to commonly used vascular plants in their role in remediation from hydroponic environments at dilute heavy metal concentrations. Thus charophytes can be a potential tool for natural remediation of heavy metal contaminated water bodies.

Key words: Charophytes, Heavy metals, Carbonate-bound

Introduction

Charophytes the growth form of characean algae are an obvious form of aquatic vegetation in many quiescent water bodies: fresh to brackish and temporary to permanent (Coops 2002) with a world wide distribution. Existence of charophytes considers being an indication of clear water ecosystems (Gomes and Asaeda 2009). However some authors have reported it to be a nuisance plant, referring to paddy field habitats (Guha 1995). Generally charophytes are abundant world wide, however some reports list these species under extinction (Hu et al. 2009) and Japan too consider charophytes under threat. It should be noted even in Japan species like *Chara braunii* and *Nitella flexilis* are well abundant in temporary water bodies like paddy fields (personal observations, Saitama), thus the extinction could be rather the diversity.

Many forms of Charophytes are subject to calcification (Heumann 1987), which in the form of $CaCO_3$, takes place on stems, branchlets and on the surface of oogonia (Imahori 1954). Calcification accompanies the photosynthetic utilization of bicarbonate (Okazaki and Tokita 1988; McConnaughey 1991).

Charophytes have a significantly higher calcifying potential than other aquatic plants (Gomes and Asaeda 2009). Van den Berg et al. (2002) reported Charophytes to contain $CaCO_3$ at levels as high as 60% per dry weight. It is also reported that many heavily calcified Charophytes get deposited in aquatic environments leading to the formation of marl (Heumann 1987; Vymazal 1995).

Uptake of heavy metals to produce an internal concentration greater than in the external environment appears widespread in aquatic organisms (Vymazal 1995). Due to this reason aquatic plants have the potential to act as bioindicators of heavy metal contamination as well as tools of remediation. Thus remediation of metal and heavy metal contaminated waters by aquatic plants has a prominent role in aquatic ecology. However, charophytes for this purpose was not given due prominence and, we are with the opinion macro algae like charophytes could play a vital role in these aspects. Here we discuss the capacity of a characean alga *N. pseudoflabellata* to accumulate heavy metals by that to discuss the applicability as a bioindicator and as a tool for phytoremediation in natural aquatic habitats. These two aspects will be assessed in the context of inevitable calcite encrustation.

Materials and methods

Experiment 1

A laboratory experiment was carried out (repeated in duplicate) for five Cr (VI) concentrations (0, 0.1, 0.2, 0.4 and 0.8 mg.L^{-1}) for a period of 9 weeks in 1 L beakers (pyrex®). Each Cr (VI) concentration was tested for three Ca concentrations (4, 40 and 80 mg.L^{-1}).

N. pseudoflabellata was cultured axenically in a 50 L tank for approximately 1 year at a controlled temperature of 24–25 °C. Apical tips of uncalcified *N. pseudoflabellata* (2–3 internodes, 2–3 cm length) with similar morphological features were harvested and planted in 1 L beakers, for each 10 tips, positioning 2–2.5 internodes ($\sim$2 cm) above the substrate.

The substrate in the experimental beakers consisted of commercially available river sand (90% < 1 mm) (DIY, Doite®, Japan); approximately for each up to a height of 2 cm. Sand was washed extensively with tap water several times and finally with distilled water. Desired Ca concentrations were achieved by adding $CaCl_2 \cdot 2H_2O$ (analytical reagent grade; Sigma–Aldrich®) and $K_2Cr_2O_7$ (analytical reagent grade; Sigma–Aldrich®) was used as the Cr (VI) source. However, water in these units contained acute levels of Ca ($\sim$4 mg.L^{-1}) due to mixing with the substrate. Total phosphorous (TP) and total nitrogen (TN) in the water were $\sim$75 and $\sim$100 μg. L^{-1}, respectively. All units were kept in a water bath at a constant temperature of 24 °C. Three heaters (IC AUTO NEO type 180, NISSO, Japan) were used to maintain the desired temperature, and the water was mixed mechanically to provide a homogeneous temperature. Illumination was supplied using 4 × 20 W fluorescent lamps (Kyushu Denki Hanabai Corporation, Japan) with a photoperiod of 12 h light and 12 h dark.

Experiment 2

Five sets of microcosms were maintained for a period of 1 year. Three microcosms with plants; no heavy metals (control), 0.2 mg.L^{-1} Cr^{6+} and 0.01 mg.L^{-1} Cd. The other two without plants were given the same heavy metal treatments; 0.2 mg.L^{-1} Cr^{6+} and 0.01 mg.L^{-1} Cd. All units contained 40 mg.L^{-1} Ca. After 1 year, plants and sediments were sampled. Plants were analyzed for relevant heavy

metals for alkaline and acidic regions of the main thalli. A sequential fractionation procedure to determine Cr or Cd speciation as exchangeable, carbonate-bound, organic-bound, bound to iron and manganese oxides and residual was carried out according to Tessier et al. (1979) for sediment.

Statistical analysis

All data are presented in the paper as mean ± SD. The homogeneity of variance test and Levene's check for equality of variances were carried out on data sets prior to the statistical analysis, in order to verify the assumptions of normal distribution and homogeneity of variances. Differences amongst treatments were analysed by one-way ANOVA to check the significant differences ($P<0.05$). For this purpose SPSS for windows (release 13, SPSS INC., Chicago, IL) statistical software package was used.

Results and discussion

At concentrations ≤ 0.2 mg.L^{-1} Cr (VI), the plant growth was affected slightly; however elongation of shoots measured at the end of 9th week was significantly less relative to plants grown in beakers without Cr (VI), but $F_V/F_m > 0.75$. Usually stress-free plants give F_V/F_m values over 0.8 (Maxwell and Johnson 2000) and some reports indicate this threshold to be 0.76 (Mohammed et al 2003). Thus it is fair to state that none of the treatments causing significant stress to plants. However at concentrations ≥ 0.4 mg.L^{-1} Cr (VI)

plant growth was observed to be affected significantly, as shoot elongations were observed to be significantly less relative to plants grown in beakers without Cr (VI) as well as F_V/F_m observed to be between 0.39 to 0.48.

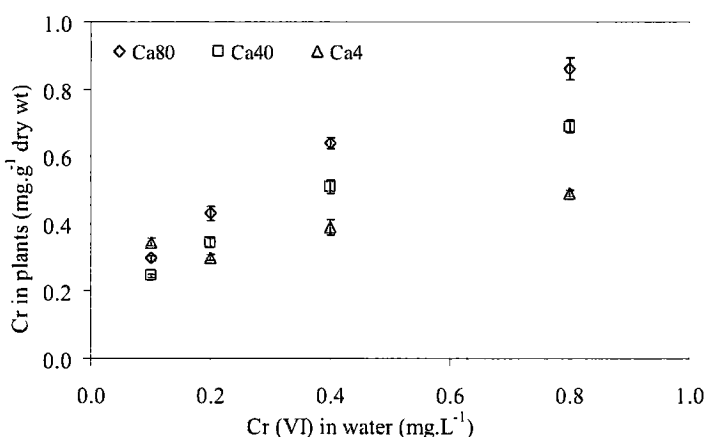According to Figure 1, with increase in Cr (VI) in water,

Figure 1 Total Cr in plants verses Cr (VI) concentration in water for different Ca concentrations (mg.L^{-1}) of water

Cr content of plants increased at all levels of Ca in water. Where as, increase in Ca in water Ca content observed in plants increased, irrespective of the level of Cr (VI) in water (Figure 2). Similarly with increase in Ca in water Cr in plants also increased at all levels of Cr (VI) in water (Figure 3). Thus it is conspicuous for the Cr accumulation by plants not only Cr (VI) in water, but also Ca in water shows a positive correlation. Over 98 % of the measured Ca in plants was observed to be from externally precipitated calcite (data not shown). Thus Ca in water and

Figure 2 Ca in plants verses Cr (VI) concentration in water for different Cr (VI) concentrations (mg.L^{-1}) of water
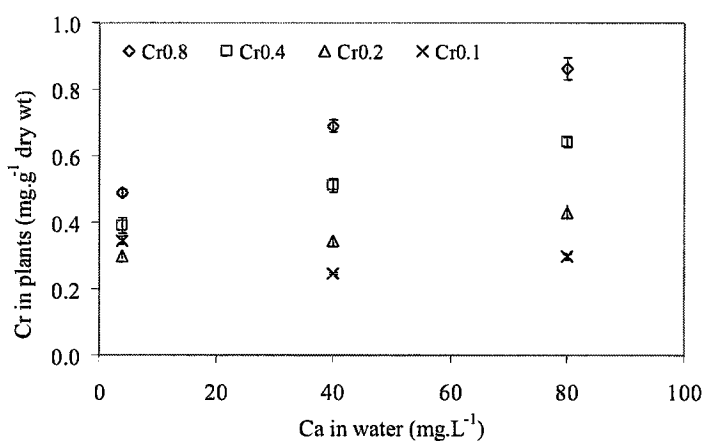
Figure 3 Total Cr in plants verses Ca concentration in water for different Cr (VI) concentrations (mg.L^{-1}) of water

subsequent calcification seemed to be aiding Cr accumulation by plants. At this point the observations are not enough to derive a firm conclusion. However Ca in water can provide several advantages for plants. According to Imahori (1954) Ca in water and subsequent calcification is correlated with the assimilation by Charophytes. The carbon dioxide contained in the calcium hydrogen carbonate is utilized by the Charophytes during assimilation (Imahori 1954; Van den Berg et al. 2002). Ca is definitely required by Charophytes and absence of it resulted in collapse of plants as reported by Anderson (1958) for *Chara zeylanica*. Furthermore Ca is known to aiding cell wall maintenance for integrity (Starling et al. 1974). Thus intensified growth of plants could be advantageous in heavy metal accumulation process.
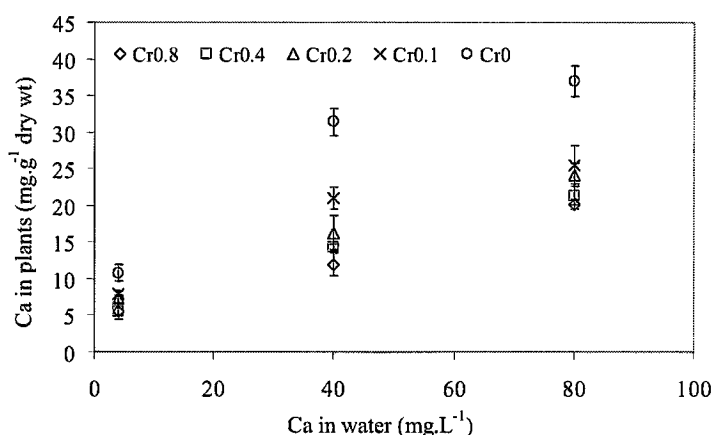
Heavy metal levels in alkaline and acidic areas

Table1 Chromium (Cr) and calcium (Ca) levels measured in alkaline and acidic areas. Parentheses are for SD.

	Alkaline band		Acidic band	
	Cr	**Ca**	**Cr**	**Ca**
Fraction (%)	55.1	99.8	44.9	> 0.2
Concentration (mg.g^{-1})	0.75 (0.09)	74.8 (1.1)	0.61(0.13)	0.1(0.0)

Table 1 illustrates the levels of Cr and Ca observed in alkaline and acidic areas of plants (experiment 2). Alkaline areas contained 0.75 mg.g^{-1} Cr, whereas 0.61 mg.g^{-1} Cr in acidic areas. Thus alkaline areas contained about 55% of the total Cr of plants. Similar values were observed for Cd treated units (Table 2).

Table 2 Cadmium (Cd) and calcium (Ca) levels measured in alkaline and acidic areas. Parentheses are for SD.

	Alkaline band		Acidic band	
	Cd	**Ca**	**Cd**	**Ca**
Fraction (%)	46.4	~100	53.5	n/d
Concentration (mg.g^{-1})	0.26 (0.07)	65.4 (1.9)	0.30(0.10)	0.3(0.0)

Alkaline areas of Cd treated units contained 46.4 % of total Cd. The Cd concentration was measured to be 0.26 mg.g^{-1} in alkaline areas whereas 0.30 mg.g^{-1} in acidic areas. Less accumulation of Cd is likely due to the heavy toxicity relative to Cr (VI) (Manusadzianas et al. 2002). It should be noted the alkaline areas had an ash content of > 90 % from its dry weight, compared to < 0.1 % in acidic areas. The ash content is comparable to the Ca levels of the respective regions and they can be used as alternatives when only one is available (*in sensu* Kufel and Kufel 2002). Thus after correcting for ash alkaline regions will give significantly high (t-test, $P < 0.05$) Cr content. In other words the Cr reservoir (Xu and Jaffe 2006) is very high in alkaline areas.

Sequential fractionation of sediment for heavy metals

Table 3 Sequential fractionation of sediments carried out for the sediments of Cr and Cd treated units.

	Cr		Cd	
	%	mg.g^{-1}	%	mg.kg^{-1}
Exchangeable	19.3	0.013(0.001)	20.5	1.25(0.11)
Carbonate bound	35.4	0.025(0.009)	43.1	2.63(1.30)
Organic bound	34.5	0.024(0.010)	23.4	1.42(0.21)
Residual	10.8	0.007(0.000)	13.0	0.79(0.11)

Table 3 shows sequential fractionation of sediments of Cr and Cd treated units. The carbonate-bound heavy metals observed to be the highest fraction for both treatments. Where as carbonate-bound Cd observed to be significantly high relative to other fractions in the Cd treated unit. In both treatments carbonate-bound and organic bound content is high. These two fractions certainly have some contribution from the plant detritus. These two values of both treatments were significantly high (ANOVA, $P < 0.05$) from carbonate-bound and organic-bound fractions of units without any plants (i.e. heavy metal treated, but no plants).

Applicability of Charophyte calcification as bio-indicator and bio-marker

Aquatic plants are useful when assessing pollution levels of water bodies (Ferrat et al. 2003). Charophytes able to accumulate high Ca content due to calcite precipitation. This makes analytical measurement of total Ca in charophytes easy and accurate. Furthermore the response in total Ca in plants (due to change in calcification) was observed to be showing a linear response to Cr (VI) in water. Thus charophytes Ca content could be use as a biomarker. Incorporating Ca content in plants has several advantages; easy to measure unlike some commonly use biomarkes like phytochelatins, phenolic compounds, antioxidant enzymes etc (Ferrat et al. 2003).

However the Ca in plants, its response to Cr (VI) in water, depends upon Ca in water. Thus a calibration is needed for each Ca level. All these relationships observed to be representative by simple linear functions.

Concluding remarks

Ca in water observed to be a contributing factor of heavy metal accumulation of charophytes. Calcification of plants resulted in high heavy metal accumulation. Subsequent analysis showed alkaline areas to contain over 40 % of plants HM. High carbonate-bound HM fraction in sediment elucidates the fact that charophytes has the potential of accumulating HM in redox-insensitive forms. This capability will give charophytes a superioirity as considerable portion of HM will not get re-enter upon plants senescence. Thus this method can be used to rehabilitate polluted aquatic ecosystems.

Acknowledgments

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