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Influence of culture density, pH, organic acids and divalent cations on the removal of nutrients and metals by immobilized *Anabaena doliolum* and *Chlorella vulgaris*

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The potential of alginate-immobilized *Anabaena doliolum* and *Chlorella vulgaris* was assessed for removal of nutrients (NO_3^- and NH_4^+) and metals ($Cr_2O_7^{2-}$ and Ni^{2+}) at different biomass concentrations (0.05, 0.1, 0.25, 0.49 and 1.22 g dry wt l^{-1}) and pH values (4 to 10). Though uptake of all these substances was higher in concentrated algal beads (0.25, 0.49 and 1.22 g dry wt l^{-1}), their rate of uptake was significantly ($P < 0.001$) lower than that of low (0.05 g dry wt l^{-1}) cell density beads. For *A. doliolum*, there was no significant difference in uptake rates for beads having densities of 0.05 and 0.1 g dry wt l^{-1}. *Chlorella vulgaris*, however, showed maximum efficiency at 0.1 g dry wt l^{-1}. Uptake of both the nutrients and the metals was maximal at pH 7 followed by pH 8, 6, 9, 10, 5 and 4. Of the different substances (organic acids and divalent cations) used, humic acid was most efficient in decreasing metal uptake. Mg^{2+} was, however, more efficient than Ca^{2+} in decreasing Ni^{2+} uptake. Immobilized algae with a cell density of 0.1 g dry wt l^{-1} were the most efficient for nutrient and metal removal at pH 6 to 8.

Key words: Anabaena doliolum, calcium, *Chlorella vulgaris*, heavy metals, immobilization, magnesium, organic acids, pH.

The major environmental health problems confronting both developed and developing countries are related to lack of clean water supply and proper facilities for disposal of sewage. Many aquatic ecosystems are being progressively contaminated by indiscriminate discharge of toxic metals and nutrients from industries and agriculture (Stokes 1983). Sustenance of life in such contaminated water bodies requires proper management and restoration by way of nutrient and metal removal. Of the various strategies used to achieve this end, immobilized cell technology has gained prominence in removing metals and nutrients (N, P) from contaminated environments (Chevalier & De la Noüe 1985; Darnall *et al.* 1986; Singh *et al.* 1989; Costa & Gomez 1991). This technology not only prevents the wash-out of biomass in continuous flow reactors, but offers a greater degree of operational flexibility and easy separation (Lakhawala *et al.* 1989). Immobilized algae are not only resistant to high concentrations of toxic metals but possess high chlorophyll content (Robinson *et al.* 1985) and greater efficiency than free cells in removing metals and nutrients over repeated cycles (Bozeman *et al.* 1989; Rai & Mallick 1992). Compared with other immobilizing materials, calcium alginate is becoming more attractive because the technique required is simple and low cost (Bajpai *et al.* 1985).

The performance of such biological reactors may be affected by a number of variables, one of which is the bead design. It is well known that an algal culture cannot maintain its optimal viability for long due to shading effects if the density of the culture is very high (Abeliovich 1980). Optimal utilization of light usually requires low algal densities (De la Noüe 1983).

Although most microbial assays are conducted in media at pH 7, the pH of natural water spans a wide spectrum. In biological systems, there is usually a range within which pH changes have little measurable effect, despite the fact that every organism has a specific pH requirement (Babich & Stotzky 1986). Since immobilized-cell reactors are mainly composed of biological organisms, it is presumed that functioning of such reactors would be affected by a change in pH of the medium. In addition to this, the presence of

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different cations and organic substances may also affect the performance of bioreactors designed for restoration of environmental quality. Hence, the objectives of this study were to find out the most effective cell density and pH for maximal operational performance of bioreactors consisting of a cyanobacterium, *Anabaena doliolum* and a green alga, *Chlorella vulgaris*. It was further intended to understand the interactive effects of naturally-occurring organic acids and divalent cations on the removal efficiency of the bioreactors.

Materials and Methods

Anabaena doliolum and *Chlorella vulgaris* were raised axenically, respectively, in the medium of Allen & Arnon (1955) at pH 7.5 and the medium Chu-10 of Gerloff *et al.* (1950) at pH 6.8. Incubation was at 24 ± 2°C under 72 μmol photon m^{-2} s^{-1} light intensity with a photoperiod of 14:10 h. Stock solutions of $NiCl_2.6H_2O$ and $K_2Cr_2O_7$ were filter-sterilized by passing through membrane filters (0.45 μm) before adding to the culture medium. Non-inhibitory concentrations (2 μg ml^{-1}) of organic acids (citric, humic and oxalic) and cations (Ca^{2+} and Mg^{2+}) were used for this study.

Immobilization of Algal Cells

Algal cells were suspended in 5% (w/v) solution of sodium alginate prepared in the growth medium. Different algal cell densities were maintained by measuring their absorption at 678 nm and converted to dry wt using the equation of Lavoie & De la Noüe (1983). The sodium alginate/algal mixture was then added dropwise, from a syringe, into 0.2 M $CaCl_2$. The alginate beads (diameter: 3.0 ± 0.15 mm) formed by cross-linking with Ca^{2+} were harvested and washed with culture medium and resuspended in fresh growth medium. Removal of nutrients and metals at different biomass concentrations was estimated after 24 h. Beads were also withdrawn from the experimental flasks after 24 h and resuspended in fresh medium containing nutrients/metals.

Removal efficiency was tested at selected pH values adjusting medium with MES or borax/boric acid (5 nM).

Uptake of NO_3^-, NH_4^+ and Metals

The monolayer of beads spread over the bottom of the flask was incubated in the culture room with light intensity of 72 μmol photon m^{-2} s^{-1} at 24 ± 2°C. NO_3^- and NH_4^+ were estimated colorimetrically by the brucine/sulphuric acid method (Nicholas & Nason 1957) and Nessler's reagent (Herbert *et al.* 1971), respectively. Ni and Cr were measured by the method of Martin (1979) with an atomic absorption spectrophotometer. All the experiments were performed in triplicate in the growth medium. Uptake of nutrients and metals by immobilized algae was defined as the value obtained after subtracting the amount of metals or nutrients taken up by alginate beads (without algae) from the amount taken up by the alginate-immobilized algae.

Estimation of Carbon Fixation and O_2-evolution

Carbon fixation was estimated by measuring the depletion of $^{14}CO_2$ ($NaH^{14}CO_3$; sp. act. 6.4 × 10^{10} Bq/mol) from the medium as described by Rai & Raizada (1986). Gross photosynthesis (O_2 evolution + consumption in dark) was measured with a polarographic oxygen electrode enclosed in a 10-ml reaction vessel and connected to an oxygen analyzer (Digital Oxygen System, Model 10; Rank Brothers, Cambridge, UK).

Statistical Analysis

The results were analysed using analysis of variance (ANOVA) and Student's *t*-tests.

Results

Uptake of NO_3^-, NH_4^+, Ni^{2+} and $Cr_2O_7^{2-}$ by Immobilized Algae

Data presented in Figure 1 demonstrate a density-dependent removal of NO_3^- and NH_4^+ by the immobilized algae. However, the rate of uptake was greater in low cell density beads (0.05 and 0.1 g dry wt l^{-1}) than those with high cell density (0.25, 0.49 and 1.22 g dry wt l^{-1}; Table 1). Compared with the 0.05 g dry wt l^{-1} of *A. doliolum*, there was a 27%, 42% and 65% decrease in the dichromate uptake rate at cell density of 0.25, 0.49 and 1.22 g dry wt l^{-1}, respectively. For Ni^{2+} uptake, the corresponding decreases were, however, 18%, 45% and 62%, respectively (Table 1). *Chlorella vulgaris* also exhibited a similar pattern with corresponding decreases of 32%, 42% and 71% for dichromate and 42%, 54% and 69% for Ni^{2+} uptake, respectively. Although the amount of metal taken up by 0.1 g dry wt l^{-1} of *A. doliolum* was almost double that by 0.05 g dry wt l^{-1}, there was no significant difference in uptake rates between these two densities of beads. However, *C. vulgaris* showed better efficiency at a culture density of 0.1 g dry wt l^{-1} (Table 1). It is also clear from Table 1 that the rate of Ni^{2+} uptake was generally higher than that of dichromate. With *A. doliolum*, Ni^{2+} uptake was approximately 30%, 34%, 47%, 26% and 44% higher than $Cr_2O_7^{2-}$ for cell densities of 0.05, 0.1, 0.25, 0.49 and 1.22 g dry wt l^{-1}, respectively. For *C. vulgaris*, the corresponding values were, respectively, 55%, 53%, 33%, 22% and 66% higher. *Chlorella vulgaris* had a faster initial uptake of nutrients than *A. doliolum* (Figure 1). A similar pattern was also noticed for metal uptake (data not shown).

While exploring the possibility of repeated use of immobilized algae, a significant decrease (F significant at $P < 0.001$) in removal efficiency from first to second and second to third cycles was noticed (Table 1). The reduction was, however, more pronounced for beads of low culture density (0.05 and 0.1 g dry wt l^{-1}) than the more concentrated ones.

Effect of Culture Density on Photosynthesis

Figure 2 represents the rate of carbon fixation and O_2-evolution by immobilized algae at different culture densities. Compared with 0.05 g dry wt l^{-1}, an increase in the rate of both processes was noticed at a cell density of 0.1 g dry wt l^{-1}. At higher cell densities (0.25, 0.49 and 1.22 g dry wt l^{-1}), however, the rate of O_2-evolution of *A. doliolum* was reduced, respectively, by 13%, 42% and 54%. The corresponding reductions in carbon fixation were

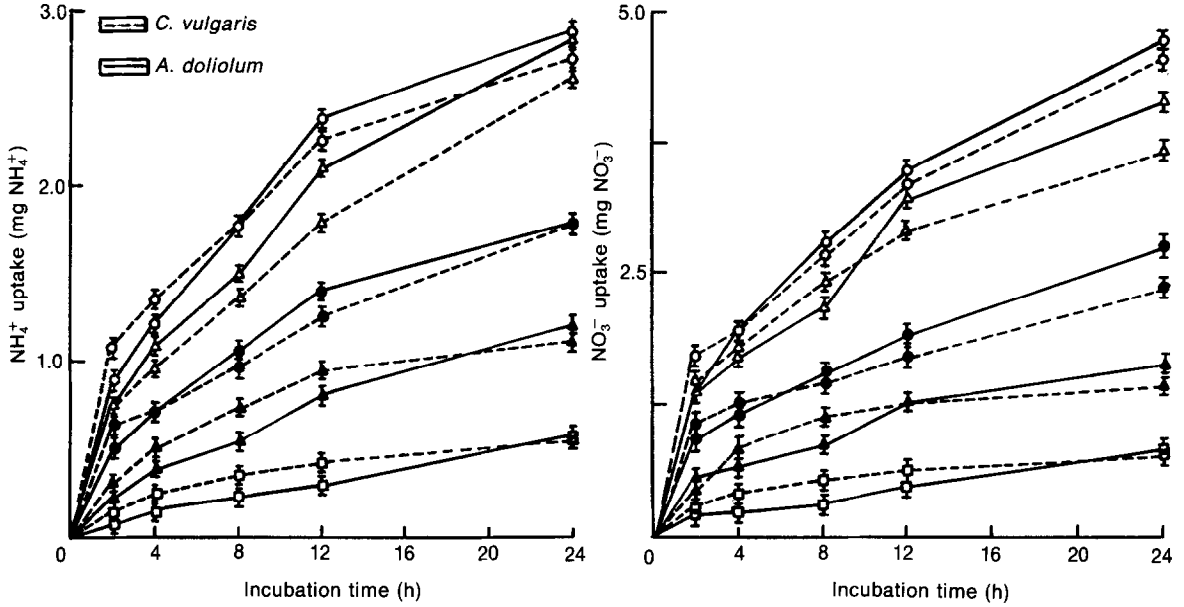

Figure 1. Uptake of NH_4^+ and NO_3^- by algal beads of different cell densities, i.e. with dry weights of 1.22 (○), 0.49 (△), 0.25 (●), 0.10 (▲) and 0.05 (□) g l^{-1}.

Table 1. Uptake rate of NO_3^-, NH_4^+, $Cr_2O_7^{2-}$ and Ni^{2+} by different biomass concentrations of immobilized *A. doliolum* and *C. vulgaris* in cycles 1, 2 and 3.*

Cell density (g dry wt l^{-1})	NO_3^- uptake (μg NO_3^- mg dry wt^{-1} h^{-1})			NH_4^+ uptake (μg NH_4^+ mg dry wt^{-1} h^{-1})			$Cr_2O_7^{2-}$ uptake (μg Cr mg dry wt^{-1} h^{-1} × 10^{-1})			Ni^{2+} uptake (μg Ni mg dry wt^{-1} h^{-1} × 10^{-1})		
	1	2	3	1	2	3	1	2	3	1	2	3
A. doliolum												
0.05	3.83	2.64	1.95	10.5	7.1	4.8	0.26	0.16	0.06	0.34	0.18	0.14
	(–)	(31)	(49)	(–)	(32)	(54)	(–)	(38)	(77)	(–)	(48)	(59)
0.10	3.78	2.59	2.04	10.6	7.2	5.4	0.26	0.13	0.07	0.35	0.20	0.13
	(2)	(32)	(47)	(–1)	(31)	(49)	(0)	(50)	(73)	(–)	(41)	(62)
0.25	2.93	1.53	1.08	7.4	4.2	2.9	0.19	0.09	0.04	0.28	0.14	0.10
	(24)	(60)	(72)	(30)	(60)	(72)	(27)	(65)	(85)	(18)	(59)	(71)
0.49	2.01	1.51	0.93	5.3	3.1	2.0	0.15	0.08	0.04	0.19	0.12	0.08
	(48)	(60)	(76)	(50)	(70)	(81)	(42)	(69)	(85)	(45)	(65)	(74)
1.22	0.93	0.58	0.42	2.1	1.9	1.3	0.09	0.04	0.02	0.13	0.07	0.07
	(76)	(85)	(89)	(80)	(82)	(88)	(65)	(85)	(92)	(62)	(79)	(79)
C. vulgaris												
0.05	3.01	2.45	2.01	9.10	7.21	5.01	0.31	0.25	0.19	0.48	0.39	0.26
	(–)	(19)	(33)	(–)	(21)	(45)	(–)	(19)	(39)	(–)	(19)	(46)
0.10	3.19	2.39	2.03	9.32	6.98	5.22	0.34	0.24	0.15	0.52	0.39	0.28
	(–6)	(21)	(33)	(–11)	(23)	(43)	(–9)	(23)	(52)	(–8)	(19)	(42)
0.25	2.63	1.95	1.75	8.63	6.21	4.03	0.21	0.14	0.13	0.28	0.21	0.18
	(13)	(35)	(42)	(5)	(32)	(56)	(32)	(55)	(58)	(42)	(56)	(62)
0.49	1.95	1.13	0.81	7.45	4.03	4.01	0.18	0.13	0.11	0.22	0.15	0.14
	(35)	(63)	(73)	(18)	(56)	(56)	(42)	(58)	(65)	(54)	(69)	(71)
1.22	0.81	0.62	0.44	5.03	2.10	2.00	0.09	0.06	0.06	0.15	0.11	0.10
	(73)	(79)	(85)	(45)	(77)	(78)	(71)	(81)	(81)	(69)	(77)	(79)

* Standard errors ranged between 0.02 and 0.07. Data in parentheses denote % reduction in uptake rate; a negative value denotes stimulation over control. F (ANOVA) values significant at $p < 0.005$.

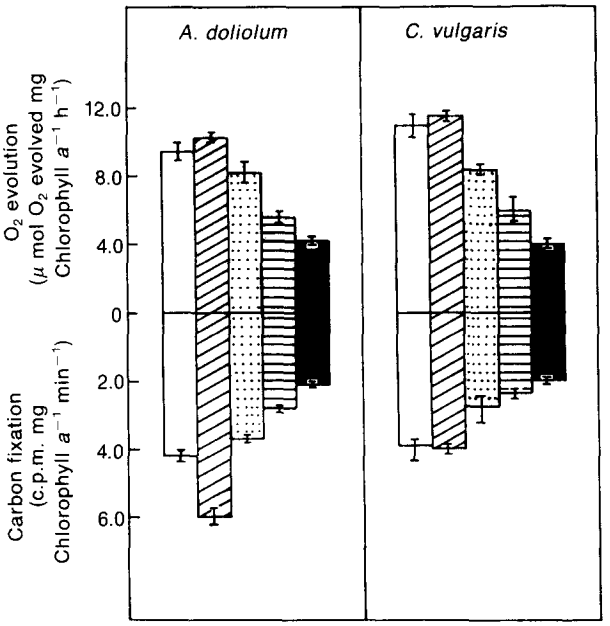

Figure 2. Rate of photosynthetic O_2-evolution and carbon fixation by beads of different biomass, i.e. with dry weights of 1.22 (■), 0.49 (▤), 0.25 (▦), 0.10 (▨) and 0.05 (□) g l^{-1}.

10%, 32% and 48%, respectively. *Chlorella vulgaris* depicted a similar trend.

Effect of pH on the Uptake of Nutrients and Metals

Uptake of NO_3^- and NH_4^+, as influenced by different pH values, is summarized in Table 2. *Anabaena doliolum* had maximal uptake of both the nutrients at pH 7.0. For NO_3^- the decrease in uptake was about 2%, 22%, 36%, 39%, 55% and 60% for pH values of 8, 9, 10, 6, 5 and 4, respectively. For NH_4^+ uptake, the corresponding decreases were, however, 2%, 19%, 29%, 40%, 55% and 58%, respectively. In contrast, *C. vulgaris* gave maximal removal efficiency in the range of pH 6 to 7. However, both the organisms had maximal efficiency for removal of Cr and Ni at pH 7. At all the pH values tested, the amount of Ni^{2+} absorbed was much higher than dichromate.

Interaction of Organic Acids (Humic, Citric and Oxalic) and Divalent Cations (Ca^{2+} and Mg^{2+}) with Metal Uptake

Impact of organic acids and divalent cations on regulation of metal uptake is given in Table 3. In the case of immobilized *A. doliolum*, Ni uptake was decreased by 67%,

Table 2. Influence of pH on the uptake of nutrients and metals by immobilized *C. vulgaris* and *A. doliolum* after 24 h of treatment.*

pH	NO_3^- uptake (μg NO_3^- mg dry wt^{-1} h^{-1})		NH_4^+ uptake (μg NH_4^+ mg dry wt^{-1} h^{-1})		$Cr_2O_7^{2-}$ uptake (μg Cr mg dry wt^{-1} h^{-1} × 10^{-1})		Ni^{2+} uptake (μg Ni mg dry wt^{-1} h^{-1} × 10^{-1})	
	A. doliolum	*C. vulgaris*	*A. doliolum*	*C. vulgaris*	*A. doliolum*	*C. vulgaris*	*A. doliolum*	*C. vulgaris*
4.0	1.28 ± 0.05	1.01 ± 0.04	3.75 ± 0.03	4.38 ± 0.04	0.07 ± 0.003	0.04 ± 0.004	0.31 ± 0.004	0.32 ± 0.003
5.0	1.45 ± 0.02	1.28 ± 0.03	4.02 ± 0.02	5.91 ± 0.03	0.14 ± 0.005	0.10 ± 0.004	0.44 ± 0.003	0.39 ± 0.004
6.0	1.96 ± 0.04	2.93 ± 0.02	5.35 ± 0.02	7.93 ± 0.03	0.18 ± 0.002	0.15 ± 0.003	0.52 ± 0.002	0.43 ± 0.005
7.0	3.21 ± 0.03	3.02 ± 0.04	8.92 ± 0.03	8.08 ± 0.05	0.23 ± 0.004	0.20 ± 0.003	0.61 ± 0.005	0.53 ± 0.003
8.0	3.14 ± 0.03	2.81 ± 0.03	8.78 ± 0.04	7.82 ± 0.03	0.21 ± 0.004	0.19 ± 0.002	0.54 ± 0.002	0.47 ± 0.005
9.0	2.50 ± 0.02	2.08 ± 0.04	7.23 ± 0.02	6.43 ± 0.02	0.10 ± 0.002	0.07 ± 0.004	0.38 ± 0.004	0.39 ± 0.002
10.0	2.05 ± 0.05	1.92 ± 0.03	6.33 ± 0.03	6.11 ± 0.04	0.09 ± 0.003	0.05 ± 0.004	0.38 ± 0.004	0.35 ± 0.003

* Values are means ± SD. 't' (students' *t*-tests) significant at $p < 0.05$.

Table 3. Interaction of organic acids and divalent cations with $Cr_2O_7^{2-}$ and Ni^{2+} uptake by test algae after 24 h of treatment.*

Combination	$Cr_2O_7^{2-}$ uptake (μg Cr mg dry wt^{-1} h^{-1})		Ni^{2+} uptake (μg Ni mg dry wt^{-1} h^{-1})	
	A. doliolum	*C. vulgaris*	*A. doliolum*	*C. vulgaris*
Control	0.028 ± 0.002	0.023 ± 0.002	0.039 ± 0.002	0.029 ± 0.002
C + oxalic acid	0.015 ± 0.003	0.016 ± 0.001	0.021 ± 0.001	0.015 ± 0.001
C + citric acid	0.013 ± 0.001	0.016 ± 0.003	0.019 ± 0.002	0.013 ± 0.002
C + humic acid	0.011 ± 0.002	0.012 ± 0.003	0.013 ± 0.001	0.011 ± 0.003
C + Ca^{2+}	0.021 ± 0.003	0.016 ± 0.001	0.031 ± 0.001	0.019 ± 0.002
C + Mg^{2+}	0.021 ± 0.001	0.015 ± 0.004	0.027 ± 0.002	0.015 ± 0.002

* Values are means ± SD. 't' (students' *t*-tests) significant at $p < 0.05$.
C—control.

51% and 46%, and $Cr_2O_7^{2-}$ uptake decreased by 61%, 54% and 66%, by humic, citric and oxalic acids, respectively. Similarly, humic acid was more efficient in decreasing the uptake of metals by *C. vulgaris*. Of the divalent cations, Mg^{2+} was more efficient (10% for *A. doliolum* and 15% for *C. vulgaris*) than Ca^{2+} in decreasing the uptake of Ni. However, no significant difference was noticed between Ca^{2+} and Mg^{2+} in decreasing dichromate uptake.

Discussion

Results presented in Table 1 showed a slow uptake rate for nutrients (NO_3^- and NH_4^+) and metals (dichromate and Ni^{2+}) in high cell density beads (0.25, 0.49 and 1.22 g dry wt l^{-1}). This decrease in removal efficiency of concentrated algal beads may be due to self-shading, i.e. algae enclosed in the centre of the beads are unable to get sufficient light and, therefore, cannot perform optimal metabolic activity. A decrease in the rate of O_2 evolution (Figure 2) in high density beads further indicates that light limitation may be considerable and lead to ATP depletion which could then affect nutrient uptake (Oelmuller *et al.* 1988; Rai *et al.* 1992).

A very fast initial uptake of nutrients and metals, as noticed from the present results, may be due to availability of free binding sites. A fast initial uptake by *C. vulgaris* may be attributed to its unicellular nature, which facilitates absorption by the entire cell surface. However, a gradual fall in uptake rate from first to subsequent cycles emphasized the significance of using fresh algal beads for nutrient and metal removal.

Table 2 demonstrates a substantial decrease in metal and nutrient removal efficiency by these biological reactors, both at acidic and alkaline pH compared with that at pH 7. A decrease in uptake of metals at high pH due to their precipitation as metal hydroxides (Borgmann 1983), on the one hand, and competition between metal and hydrogen ions for binding at low pH (Ali & Deo 1991), on the other, cannot be ruled out. Of various organic acids used, humic acid was the most effective in reducing metal uptake (Table 3), apparently because of its multiple binding sites for metal cations (Hongve *et al.* 1980). High concentrations of Ca^{2+} and Mg^{2+} are characteristic features of hard and eutrophic waters. Decreased metal uptake in the presence of Ca^{2+} and Mg^{2+} (Table 3) is a consequence of competitive inhibition (Schecher & Driscoll 1985). Mg^{2+} was, however, found more efficient in decreasing the uptake of Ni^{2+}. This could be due to the similarity in the chemistry and ionic radius of Mg^{2+} and Ni^{2+}; Ni^{2+} is known to bind in place of Mg^{2+} (Hughes & Poole 1989).

This study recommends the use of low cell density (0.1 g dry wt l^{-1}) beads and a pH range of 6 to 8 for maximal operational performance of immobilized algae. However, before using such algae for metal and nutrient removal, the presence of organic acids and divalent cations (which may impede the removal efficiency because of their greater affinity for metals) should be ascertained.

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