DISCUSSION
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Indiscriminate discharge of chromium rich effluents from tanning and electroplating industries to both surface and groundwater has made chromium find its routes to biological systems (Outridge and Noller, 1991; Crowder, 1991; Tremp and Kohler, 1995; Rai, 2008c). Severe toxicity of hexavalent chromium with respect to human health including DNA-protein crosslinks-a major cause of mutagenic carcinogenesis has been the focus of concern of several modern researches (James et al., 1997; Michalski, 2004; Danadevi et al., 2004; Mattagajasingh et al., 2008). Existing chemical and physico-chemical techniques for chromium removal are unwanted either due to ecological or economic grounds. Phytoremediation for environmental clean-up has in recent years emerged as a cost effective novel green technology employing the use of macrophytes with high growth rate. *Azolla* -a free floating aquatic fern is such a highly valued macrophyte which is chosen for the present investigation due to its high multiplication rate, high pectin content, easy harvestability and its propensity to bind heavy metals.

The potential of three different species of *Azolla*, viz. *A. microphylla*, *A. filiculoides* procured from Centre for Conservation and Utilisation of Blue Green Algae, Indian Agricultural Research Institute, New Delhi, India and *A. pinnata* collected from natural habitats in and around Patiala, Punjab, India, has been tested by making comparative assessment of their growth and chromium removal efficiency in E & W medium supplemented with chromium (5 ppm and 7.5 ppm) with a view to select the species with maximum tolerance of chromium (VI) and having maximum growth and chromium removal efficiency. Optimization of process parameters for maximum chromium
removal with potential *Azolla* species was made so that a suitable application of *Azolla* for phytoremediation of chromium from polluted aquatic bodies laden with chromium rich effluents could be worked out on a large-scale. Although, *Azolla* has been reported to withstand different metal concentrations (Bennicelli *et al*., 2004; Amjad and Abraham, 2003; Pabbi *et al*., 2004; Arora *et al*., 2006; Umali *et al*., 2006; Rai, 2008c; Rai and Tripathi, 2009), yet its application for refining heavy metal-loaded industrial effluent has not been dealt with seriously. The results of the present investigation revealed that Cr (VI) caused phytotoxicity to *Azolla* species manifesting in the form of chlorosis, necrosis, decrease in plant size and root length after seven days of exposure. It has also been reported by Fargasova and Molnarova (2010) that reduction in photosynthetic pigments is an early symptom of metal toxicity. These symptoms appear due to disturbance in chloroplast development and chlorophyll synthesis (Boddi *et al*., 1995).

The results (Fig. 18) reveal the progressive reduction of freshmass of *Azolla* species in increasing concentration of chromium (VI) except at 1 ppm as compared to their respective controls. Sarkar and Jana (1985) reported that in 1 ppm of Cd, Hg, Cu, Ag, Pb and Cr treatment, *A. pinnata* showed no significant change in hill activity, chlorophyll content, protein content and dry weight. The progressive reduction in biomass in the increasing concentrations (0.1, 0.5 and 1 ppm) of Pb and Cd has, however, been reported in *A. caroliniana* by Stepniewska *et al*. (2005). Similarly, Rai (2010) reported +3.1 to -37.5% growth inhibitions in *A. pinnata* in presence of 0.5, 1.5 and 3.0 ppm of Cr (VI) in culture medium. In the present study, minimum inhibitory concentration (MIC) of Cr (VI) to *Azolla* species studied came out to be 7.5 ppm and beyond which, no growth occurred in E & W medium. This clearly indicated that *Azolla* species tolerated up to 7.5 ppm
**Fig. 18** Determination of minimum inhibitory concentration of Cr (VI) to different *Azolla* species, viz. *A. microphylla* (Am), *A. filiculoides* (Af) and *A. pinnata* (Ap) grown in E & W medium.

**Fig. 19** Comparison of freshmass of different *Azolla* species, viz. *A. microphylla* (Am), *A. filiculoides* (Af) and *A. pinnata* (Ap) on 5th, 10th and 15th day harvesting in E & W medium supplemented with different Cr (VI) concentrations.
chromium concentration in culture medium. Several possible mechanisms such as exudation of phytosiderophores-natural chelators (Takagi, 1976; Takagi et al., 1984; Zhang et al., 1991; Romheld, 1991; Hopkins et al., 1998; Shenker et al., 2001), sub-cellular compartmentalization of the metal into the vacuole by tonoplast located transporters or chelation of metal in cytosol by high affinity ligands- phytochelatins (Maitani et al., 1996; Nieboer & Richardson, 1980; Clemens et al., 1999; Ha et al., 1999) and metallothioneins particularly Azolla metallothioneins (AzMTs) (Schor-Fumbarov et al., 2005) and cell wall have been suggested that make the fern tolerant of heavy metals. In contrast to the results (Fig. 18), 15-20 ppm of Cr (VI) has been reported earlier where growth of Azolla species ceased (Arora et al., 2006). Various workers have reported different metal concentration that might be responsible for growth inhibition in Azolla. Khellaf and Zerdaoui (2009) reported EC\textsubscript{50} of Cu, Ni, Cd and Zn to Lemma as 0.47, 1.29, 0.91 and 5.64 mg/L, respectively while Eyini et al. (2000) worked out the threshold concentration of Pb as 50 ppm to Azolla.

There was a general pattern of significant reduction in growth performance of Azolla (Fig. 19-60) as observed in freshmass, drymass, moisture content, relative growth rate, doubling time, total chlorophyll and heterocyst frequency (of symbiont) in presence of chromium (5 ppm) in culture medium in comparison to control (without chromium) which further decreased when chromium concentration was increased to 7.5 ppm. Also, the severity of phytotoxicity symptoms increased with exposure time. The results are in close proximity with Phetsombat et al. (2006) in Salvinia cucullata showing significant decrease in relative growth rate, biomass productivity and total chlorophyll on increasing concentration of Pb and Cd and on increasing exposure time. Eyini et al. (2000) made comparative study of doubling time,
Fig. 20 Comparison of drymass of different *Azolla* species, viz. *A. microphylla* (Am), *A. filiculoides* (Af) and *A. pinnata* (Ap) on 5th, 10th and 15th day harvesting in E & W medium supplemented with different Cr (VI) concentrations.

Fig. 21 Comparison of moisture content of different *Azolla* species, viz. *A. microphylla* (Am), *A. filiculoides* (Af) and *A. pinnata* (Ap) on 5th, 10th and 15th day harvesting in E & W medium supplemented with different Cr (VI) concentrations.
Fig. 22 Comparison of doubling time of different *Azolla* species, viz. *A. microphylla* (Am), *A. filiculoides* (Af) and *A. pinnata* (Ap) on 5th, 10th and 15th day harvesting in E & W medium supplemented with different Cr (VI) concentrations.

Fig. 23 Comparison of relative growth rate of different *Azolla* species viz. *A. microphylla* (Am), *A. filiculoides* (Af) and *A. pinnata* (Ap) on 5th, 10th and 15th day harvesting in E & W medium supplemented with different Cr (VI) concentrations.
Fig. 24  Comparison of total chlorophyll of different Azolla species, viz. *A. microphylla* (Am), *A. filiculoides* (Af) and *A. pinnata* (Ap) on 5th, 10th and 15th day harvesting in E & W medium supplemented with different Cr (VI) concentrations.

Fig. 25  Comparison of heterocyst frequency of symbiont of different Azolla species, viz. *A. microphylla* (Am), *A. filiculoides* (Af) and *A. pinnata* (Ap) on 5th, 10th and 15th day harvesting in E & W medium supplemented with different Cr (VI) concentrations.
Fig. 61 Comparison of chromium removal percentage by *A. microphylla* (Am), *A. filiculoides* (Af) and *A. pinnata* (Ap) in E & W medium supplemented with (a) 5 ppm and (b) 7.5 ppm Cr (VI) concentrations
Fig. 26  Comparison of freshmass of *A. microphylla* (Am) on 5th, 10th and 15th day harvesting in different culture media, viz. IRRI medium (I), Warne medium (W) and E & W medium (E) supplemented with different Cr (VI) concentrations.
Fig. 28 Comparison of moisture content of *A. microphylla* (Am) on 5th, 10th and 15th day harvesting in different culture media, viz. IRRI medium (I), Warne medium (W) and E & W medium (E) supplemented with different Cr (VI) concentrations.

Fig. 29 Comparison of doubling time of *A. microphylla* (Am) on 5th, 10th and 15th day harvesting in different culture media, viz. IRRI medium (I), Warne medium (W) and E & W medium (E) supplemented with different Cr (VI) concentrations.
Fig. 30  Comparison of relative growth rate of *A. microphylla* (Am) on 5th, 10th and 15th day harvesting in different culture media, viz. IRRI medium (I), Warne medium (W) and E & W medium (E) supplemented with different Cr (VI) concentrations.

Fig. 31  Comparison of total chlorophyll of *A. microphylla* (Am) on 5th, 10th and 15th day harvesting in different culture media, viz. IRRI medium (I), Warne medium (W) and E & W medium (E) supplemented with different Cr (VI) concentrations.
Comparison of heterocyst frequency of symbiont of *A. microphylla* (Am) on 5th, 10th and 15th day harvesting in different culture media, viz. IRRI medium (I), Warne medium (W) and E & W medium (E) supplemented with different Cr (VI) concentrations.
Fig. 62  Comparison of chromium removal percentage by *A. microphylla* in IRRI medium (I), Warne medium (W) and E & W medium (E) supplemented with (a) 5 ppm and (b) 7.5 ppm Cr (VI) concentrations
Fig. 33 Comparison of freshmass of *A. microphylla* among different variants of P, P1 (14.16 ppm), P2 (16.5 ppm), P3 (9.4 ppm) and P4 (7ppm) alongwith Psc (11.8 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days

Fig. 34 Comparison of drymass of *A. microphylla* among different variants of P, P1 (14.16 ppm), P2 (16.5 ppm), P3 (9.4 ppm) and P4 (7ppm) alongwith Psc (11.8 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days
Fig. 35 Comparison of moisture content of *A. microphylla* among different variants of P, P1 (14.16 ppm), P2 (16.5 ppm), P3 (9.4 ppm) and P4 (7ppm) alongwith Psc (11.8 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

Fig. 36 Comparison of doubling time of *A. microphylla* among different variants of P, P1 (14.16 ppm), P2 (16.5 ppm), P3 (9.4 ppm) and P4 (7ppm) alongwith Psc (11.8 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 37 Comparison of relative growth rate of *A. microphylla* among different variants of P, P1 (14.16 ppm), P2 (16.5 ppm), P3 (9.4 ppm) and P4 (7ppm) along with Psc (11.8 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days

Fig. 38 Comparison of total chlorophyll of *A. microphylla* among different variants of P, P1 (14.16 ppm), P2 (16.5 ppm), P3 (9.4 ppm) and P4 (7ppm) along with Psc (11.8 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days
Fig. 39  Comparison of heterocyst frequency of symbiont of *A. microphylla* among different variants of P, P1 (14.16 ppm), P2 (16.5 ppm), P3 (9.4 ppm) and P4 (7 ppm) along with Psc (11.8 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days
**Fig. 40** Comparison of freshmass of *A. microphylla* among different variants of Mg, Mg1 (36 ppm), Mg2 (42.2 ppm), Mg3 (24 ppm) and Mg4 (18 ppm) alongwith Mgsc (30 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

**Fig. 41** Comparison of drymass of *A. microphylla* among different variants of Mg, Mg1 (36 ppm), Mg2 (42.2 ppm), Mg3 (24 ppm) and Mg4 (18 ppm) alongwith Mgsc (30 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
**Fig. 42** Comparison of moisture content of *A. microphylla* among different variants of Mg, Mg1 (36 ppm), Mg2 (42.2 ppm), Mg3 (24 ppm) and Mg4 (18 ppm) along with Mgsc (30 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

**Fig. 43** Comparison of doubling time of *A. microphylla* among different variants of Mg, Mg1 (36 ppm), Mg2 (42.2 ppm), Mg3 (24 ppm) and Mg4 (18 ppm) along with Mgsc (30 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 44  Comparison of relative growth rate of A. microphylla among different variants of Mg, Mg1 (36 ppm), Mg2 (42.2 ppm), Mg3 (24 ppm) and Mg4 (18 ppm) along with Mgsc (30 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

Fig. 45  Comparison of total chlorophyll of A. microphylla among different variants of Mg, Mg1 (36 ppm), Mg2 (42.2 ppm), Mg3 (24 ppm) and Mg4 (18 ppm) along with Mgsc (30 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 46 Comparison of heterocyst frequency of symbiont of *A. microphylla* among different variants of Mg, Mg1 (36 ppm), Mg2 (42.2 ppm), Mg3 (24 ppm) and Mg4 (18 ppm) along with Mgsc (30 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 47  Comparison of freshmass of *A. microphylla* among different variants of Ca, Ca1 (15 ppm), Ca2 (18 ppm), Ca3 (9 ppm) and Ca4 (6 ppm) alongwith Casc (12 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

Fig. 48  Comparison of drymass of *A. microphylla* among different variants of Ca, Ca1 (15 ppm), Ca2 (18 ppm), Ca3 (9 ppm) and Ca4 (6 ppm) alongwith Casc (12 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
### Fig. 49
Comparison of moisture content of *A. microphylla* among different variants of Ca, Ca1 (15 ppm), Ca2 (18 ppm), Ca3 (9 ppm) and Ca4 (6 ppm) alongwith Casc (12 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

![Mean moisture content (%)](Image)

### Fig. 50
Comparison of doubling time of *A. microphylla* among different variants of Ca, Ca1 (15 ppm), Ca2 (18 ppm), Ca3 (9 ppm) and Ca4 (6 ppm) alongwith Casc (12 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

![Mean doubling time (days)](Image)
Fig. 51  Comparison of relative growth rate of *A. microphylla* among different variants of Ca, Ca1 (15 ppm), Ca2 (18 ppm), Ca3 (9 ppm) and Ca4 (6 ppm) alongwith Casc (12 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

Fig. 52  Comparison of total chlorophyll of *A. microphylla* among different variants of Ca, Ca1 (15 ppm), Ca2 (18 ppm), Ca3 (9 ppm) and Ca4 (6 ppm) alongwith Casc (12 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 53 Comparison of heterocyst frequency of symbiont of *A. microphylla* among different variants of Ca, Ca1 (15 ppm), Ca2 (18 ppm), Ca3 (9 ppm) and Ca4 (6 ppm) alongwith Casc (12 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days
Fig. 54  Comparison of freshmass of *A. microphylla* among different variants of Na, Na1 (6.75 ppm), Na2 (9 ppm), Na3 (2.2 ppm) and Na4 (0 ppm) along with Nasc (4.5 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

Fig. 55  Comparison of drymass of *A. microphylla* among different variants of Na, Na1 (6.75 ppm), Na2 (9 ppm), Na3 (2.2 ppm) and Na4 (0 ppm) along with Nasc (4.5 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 56 Comparison of moisture content of *A. microphylla* among different variants of Na, Na1 (6.75 ppm), Na2 (9 ppm), Na3 (2.2 ppm) and Na4 (0 ppm) alongwith Nasc (4.5 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

Fig. 57 Comparison of doubling time of *A. microphylla* among different variants of Na, Na1 (6.75 ppm), Na2 (9 ppm), Na3 (2.2 ppm) and Na4 (0 ppm) alongwith Nasc (4.5 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 58  Comparison of relative growth rate of *A. microphylla* among different variants of Na, Na1 (6.75 ppm), Na2 (9 ppm), Na3 (2.2 ppm) and Na4 (0 ppm) alongwith Nasc (4.5 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days

Fig. 59  Comparison of total chlorophyll of *A. microphylla* among different variants of Na, Na1 (6.75 ppm), Na2 (9 ppm), Na3 (2.2 ppm) and Na4 (0 ppm) alongwith Nasc (4.5 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days
Fig. 60 Comparison of heterocyst frequency of symbiont of *A. microphylla* among different variants of Na, Na1 (6.75 ppm), Na2 (9 ppm), Na3 (2.2 ppm) and Na4 (0 ppm) along with Nasc (4.5 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days
relative growth rate and heterocyst frequency of *A. microphylla* and *A. filiculoides* in response to Pb(NO$_3$)$_2$ and observed negative response in growth and biochemical characters. Presence of Pb in culture medium affects the growth of *Azolla* through its inhibitory influence on the symbiont *Anabaena azollae* and its heterocyst frequency. Similar trend of growth inhibition by heavy metals in *A. pinnata* was noted by Amjad and Abraham (2003) in dry weight, root length, chlorophyll and protein content at higher concentration of Cd (40 - 60 ppm). A similar effect of Cr on growth and chlorophyll content of *Woffia globosa* (Boonyapookana *et al.*, 2002) and of *A. imbricata* (Dai *et al.*, 2006) was observed. The reason behind the negative response is heavy metal stress (Hagemeyer, 1999; Pandey and Sharma, 2002; Bennicelli *et al.*, 2004) which might be due to serious impairment of the uptake of mineral nutrients and water leading to the deficiency in the shoot (Rout *et al.*, 2000; Svetkova and Fargasova, 2007). Additionally, Cr may have a direct impact on cellular metabolism of the plant, contributing to reduction in biomass (Shanker *et al.*, 2005). Cr (III) and Cr (VI) treatment to *A. pinnata* resulted in increase in activation of superoxide dismutase, peroxides and glutathione reductase as well as non-enzymatic antioxidants and accumulation of H$_2$O$_2$ and oxidative stress. Also, decline in catalase activity was reported (Panda and Upadhyay, 2008). Chlorophyll content is one of the most investigative physiological characteristics used for identification of physiological disturbance. Higher concentration of Cr and Ni in washing waters (Fargasova and Molnarova, 2010), in sludge (Sinha *et al.*, 2002) and tannery wastes (Singh *et al.*, 2004) decreased chlorophyll contents which matches with the results of the present investigation. Similar observations in cauliflower leaves exposed to excess supply of Co, Cu and Cr were also made by Chatterjee and Chatterjee (2000).
Reduction in chlorophyll content may be the consequence of an inhibited photosynthetic electron transport system (Bohner et al., 1980; Clijster and Van Assche, 1985; Krupa and Baszynski, 1995) or of the decomposition of the chloroplast membrane with metal excess (Sandman and Boger, 1980). Further, Cr (VI) can replace Mg ions from active sites of many enzymes and from pyrrole ring of chlorophyll molecule preventing photosynthetic light harvest in the affected chlorophyll molecule leading to photosynthetic breakdown (Vajpayee et al., 2000). Deactivation of enzymes involved in chlorophyll biosynthetic pathway particularly 2-aminolevulinic acid hydrogenase and protochlorophyllide reductase by heavy metals has been reported (Ouzounidou, 1995; Fargasova and Molnarova, 2010).

The results (Fig. 61-66) showed increase of chromium removal with increase in exposure time and further showed higher chromium removal percentage at 7.5 ppm than at 5 ppm initial chromium concentration, which are in agreement and closely match with those of Phetsombat et al. (2006) in Salvinia cucullata who reported significant increase in accumulation of Cd and Pb with initial metal concentration and exposure time. The residual concentrations of various heavy metals tested (Fe, Cu, Ni) from solutions picked by Hydrilla, Elodea canadensis and Salvinia for a period of 10 days at 5 mg L$^{-1}$ each, deceased with exposure time (Begum and Harikrishna, 2010). Also, the residual concentrations of lead decreased with passage of time and with initial Pb concentrations (Kaur et al., 2010). Kachenko et al. (2007) investigated the effect of Cd, Cu, Ni, Pb and Zn on the growth and metal uptake in 10 fern species exposed to four levels of metal concentration, viz. 0, 50, 100 and 500 mg Kg$^{-1}$ for a period of 20 weeks and found metal accumulation increased with increasing metal concentration. However, in contrast, Rai (2010) in his 13
Fig. 61  Comparison of chromium removal percentage by *A. microphylla* (Am), *A. filiculoides* (Af) and *A. pinnata* (Ap) in E & W medium supplemented with (a) 5 ppm and (b) 7.5 ppm Cr (VI) concentrations
Fig. 62  Comparison of chromium removal percentage by *A. microphylla* in IRRI medium (I), Warne medium (W) and E & W medium (E) supplemented with (a) 5 ppm and (b) 7.5 ppm Cr (VI) concentrations
Fig. 63  Comparison of chromium removal percentage by *A. microphylla* among different variants of P, P1 (14.16 ppm), P2 (16.5 ppm), P3 (9.4 ppm) and P4 (7 ppm) along with Psc (11.8 ppm) in Warne medium supplemented with (a) 5 ppm and (b) 7.5 ppm Cr (VI) concentration at different incubation periods
Fig. 64  Comparison of chromium removal percentage by *A. microphylla* among different variants of Mg, Mg1 (36 ppm), Mg2 (42.2 ppm), Mg3 (24 ppm) and Mg4 (18 ppm) alongwith Mgsc (30 ppm) in Warne medium supplemented with (a) 5 ppm and (b) 7.5 ppm Cr (VI) concentration at different incubation periods
Fig. 65  Comparison of chromium removal percentage of *A. microphylla* among different variants of Ca, Ca1 (15 ppm), Ca2 (18 ppm), Ca3 (9 ppm) and Ca4 (6 ppm) along with Casc (12 ppm) in Warne medium supplemented with (a) 5 ppm and (b) 7.5 ppm Cr (VI) concentration at different incubation periods
Fig. 66  Comparison of heterocyst frequency of symbiont of *A. microphylla* among different variants of Na, Na1 (6.75 ppm), Na2 (9 ppm), Na3 (2.2 ppm) and Na4 (0 ppm) alongwith Nasc (4.5 ppm) in Warne medium supplemented with (a) 5 ppm and (b) 7.5 ppm Cr (VI) concentration at different incubation periods
day experiment on *A. pinnata* on phytoremediation of chromium (VI) reported that the metal contents in the residual solution decreased up to 70% (CrO$_4^{2-}$, 3.0 mgL$^{-1}$) to 88% (CrO$_4^{2-}$, 0.5 mgL$^{-1}$). Also, in the study made by Stepniewska *et al.* (2005), the decline in Pb concentration, in nutrient solution of *A. caroliniana* correlated negatively with increasing initial Pb concentration whereas it correlated positively for increasing initial Cd (II) concentration.

Of the three species studied, *A. microphylla* excelled in various growth parameters in controls as well as in presence of chromium as compared to *A. filiculoides* and *A. pinnata* (Fig. 19-25). This clearly indicates that *A. microphylla* can tolerate chromium concentrations below 7.5 ppm more effectively than the other two species. Eyini *et al.* (2000) has also shown that relative growth rate and heterocyst frequency (of symbiont) of *A. microphylla* was higher than that of *A. filiculoides* at 2.5 ppm of Pb (NO$_3$)$_2$ concentration but reverse trend was followed by doubling time. A three-fold increase in accumulation of Pb by *A. microphylla* at 50 ppm of Pb(NO$_3$)$_2$ in culture medium showed the species to be more tolerant than *A. filiculoides*. Stewart and Lee (1974) suggested the more proline accumulation to be the reason behind stress tolerance of the species. Eyini *et al.* (2000) suggested from their study that higher susceptibility of *A. filiculoides* to heavy metal stress as compared to that of *A. microphylla* might be due to drastic reduction in catalase activity resulting into more accumulation of H$_2$O$_2$ and greater inhibition of growth at higher Pb concentration. In the present study too, chromium might have induced more stress in *A. filiculoides* and *A. pinnata* resulting in drastic reduction of catalase activity in these species with consequent more accumulation of H$_2$O$_2$ in their tissues.
A. microphylla also excelled in chromium removal efficiency (Fig. 61). The presence of Cr (VI) in E & W medium caused maximum reduction in freshmass of A. microphylla as compared to that in controls leading to more necrosed biomass which is able to bind more heavy metals as compared to living biomass (Arora et al., 2004). This might be the possible reason behind outstanding chromium removal efficiency in A. microphylla. Moreover, A. microphylla also excelled in biomass production which according to Khosravi et al. (2005) is positively related to heavy metal removal efficiency. Results of the present investigation also reveal that chromium removal occurred in two stages, first rapidly within 24 hours and second slowly starting after 5th day. The rate of chromium removal increased at a slow pace during the period between these phases. Ceribasi and Yetis (2001) reported similar removal by fungus, Phanerochaete chrysogenum, first stage of removal due to rapid surface adsorption and subsequent due to slow intracellular diffusion. The initial binding and exchange of heavy metal ions to insoluble constituents in Azolla matrix, most probably involves cell wall charged groups such as carboxyl and phosphates. Additionally, pectins and cellulose are important polysaccharide constituents of plant cell wall made up of fragments of polygalactouronic acid chains which interact with Ca (II) and Mg (II) to form a three dimensional polymer by -(COO)$_2$Ca and or -(COO)$_2$Mg bindings as the ion exchanging bases (Jauneau et al., 1997; Kamnev et al., 1998 and Iijima et al., 2002).

The results of comparative analysis of growth and chromium removal efficiency of A. microphylla in different culture media, viz. IRRI medium, Warne medium and E & W medium revealed that A. microphylla excelled in Warne medium besides, the presence of same macro and micronutrients in all the three culture media used (Fig. 26-32 & 62). The difference is only of nature of chelating
agent, i.e. EDTA in Warne medium which might be responsible for better performance of A. microphylla in control as well as in presence of chromium (5 ppm and 7.5 ppm). Many researchers have studied the role of EDTA in lessening the phytotoxicity and in improving metal removal (Turgut et al., 2004; Chiu et al., 2005; Pastor et al., 2007; Zhuang et al., 2007; Marques et al., 2008a). Addition of EDTA to Pb contaminated medium was found beneficial in improving plant water relations and growth in two sunflower hybrids H-33 and H-64A93 (Huang and Cunningham, 1996; Azhar et al., 2006, Azhar et al., 2009; Krystofova et al., 2009). Synthetic chelating agents are shown to have the potential to increase the bio-availability of unavailable and exchangeable heavy metal fractions in soil/water (Sun et al., 2001; Komarek et al., 2007). On the similar grounds, it can be suggested that the better growth performance of A. microphylla in control of Warne medium as compared to control of other two culture media might be due to the role of EDTA in improving nutrient availability in culture medium. Comparatively better growth performance of A. microphylla in Warne medium led to its appreciable chromium removal efficiency (Khosravi et al., 2005).

Considering the fact that medium constituents do play a major role in nutrient availability and metal removal efficiency, Warne medium was selected for further experimentation and concentrations of the macronutrients were varied individually by taking two above and two below the standard concentration in Warne medium and the concentration at which the system worked at its maximum efficiency was selected for making combinations of nutrients. Maximum values of freshmass, drymass, moisture content, relative growth rate, chlorophyll content and heterocyst frequency (of symbiont) and minimum values of doubling time were recorded in controls (without chromium) when defined.
concentration of P, Mg, Ca and Na (11.8 ppm, 30 ppm, 12 ppm and 4.5 ppm) in Warne medium were increased to 16.5 ppm, 42 ppm, 18 ppm and 9 ppm, respectively (Fig. 33-60).

Optimized concentration of P, i.e. P2 (16.5 ppm) was combined with two varying concentrations of Mg, i.e. Mg1 (36 ppm) and Mg2 (42 ppm) alongwith Mgsc (30 ppm). Growth and chromium removal efficiency of *A. microphylla* were tested among three combinations, viz. P2Mgsc, P2Mg1 and P2Mg2. The results revealed that P2Mg2 combination supported maximum growth and chromium removal efficiency. Similarly, other two sets with varying concentration of Ca and Na were tested and the medium with P, Mg, Ca and Na containing 16.5 ppm, 42 ppm, 18 ppm and 9 ppm, respectively supported maximum growth of *A. microphylla* (Fig. 67-84). Culture medium with P, Mg, Ca and Na consisting of 16.5 ppm, 42 ppm, 18 ppm and 4.5 ppm, respectively supported maximum chromium removal efficiency (Fig. 85-87). Although, there has been no systematic and targeted studies on culture media optimization for growth as well as metal removal but various workers have investigated the role of certain essential macro or micronutrients on growth and/or metal removal. Growth of *A. microphylla* was greatly affected by removal of any of macronutrients such as P, K, Mg and Ca as well as Fe as micronutrients from culture medium (Zaki and Ghazal, 2000). Concentration of P (105 µM) proved to be an effective approach for maximum biomass production in *Pteris vittata* in Hoagland solution and arsenic removal (Santos *et al.*, 2010). Jefferson *et al.* (2001) studied that nutrient addition can stimulate the microbial activity and in turn be helpful for the treatment of grey waters. Their study demonstrated that macronutrients could significantly influence the rate of metal accumulation. Chromium accumulation in macroalgae increased significantly with increasing phosphate
Fig. 67 Comparison of freshmass of *A. microphylla* in combinations of varying concentrations of magnesium with variant P2 (16.5 ppm), viz. P2Mgsc (16.5 ppm + 30 ppm), P2Mg1 (16.5 ppm + 36 ppm) and P2Mg2 (16.5 ppm + 42 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

Fig. 68 Comparison of drymass of *A. microphylla* in combinations of varying concentrations of magnesium with variant P2 (16.5), viz. P2Mgsc (16.5 ppm + 30 ppm), P2Mg1 (16.5 ppm +36 ppm) and P2Mg2 (16.5 ppm + 42 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 69  Comparison of moisture content of A. microphylla in combinations of varying concentrations of magnesium with variant P2 (16.5), viz. P2Mgsc (16.5 ppm + 30 ppm), P2Mg1 (16.5 ppm + 36 ppm) and P2Mg2 (16.5 ppm + 42 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days

Fig. 70  Comparison of doubling time of A. microphylla in combinations of varying concentrations of magnesium with variant P2 (16.5), viz. P2Mgsc (16.5 ppm + 30 ppm), P2Mg1 (16.5 ppm + 36 ppm) and P2Mg2 (16.5 ppm + 42 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days
Fig. 71 Comparison of relative growth rate of *A. microphylla* in combinations of varying concentrations of magnesium with variant P2 (16.5), viz. P2Mgsc (16.5 ppm + 30 ppm), P2Mg1 (16.5 ppm + 36 ppm) and P2Mg2 (16.5 ppm + 42 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

Fig. 72 Comparison of total chlorophyll of *A. microphylla* in combinations of varying concentrations of magnesium with variant P2 (16.5), viz. P2Mgsc (16.5 ppm + 30 ppm), P2Mg1 (16.5 ppm + 36 ppm) and P2Mg2 (16.5 ppm + 42 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 73 Comparison of heterocyst frequency of symbiont of *A. microphylla* in combinations of varying concentrations of magnesium with variant P2 (16.5), viz. P2Mgsc (16.5 ppm + 30 ppm), P2Mg1 (16.5 ppm + 36 ppm) and P2Mg2 (16.5 ppm + 42 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days
**Fig. 74** Comparison of freshmass of *A. microphylla* in combinations of varying concentrations of calcium with variant P2Mg2 (16.5 ppm + 42 ppm), viz. P2Mg2Casc (16.5 ppm + 42 ppm + 12 ppm), P2Mg2Ca1 (16.5 ppm + 42 ppm + 15 ppm) and P2Mg2Ca2 (16.5 ppm + 42 ppm + 18 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

**Fig. 75** Comparison of drymass of *A. microphylla* in combinations of varying concentrations of calcium with variant P2Mg2 (16.5 ppm + 42 ppm), viz. P2Mg2Casc (16.5 ppm + 42 ppm + 12 ppm), P2Mg2Ca1 (16.5 ppm + 42 ppm + 15 ppm) and P2Mg2Ca2 (16.5 ppm + 42 ppm + 18 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 76  Comparison of moisture content of *A. microphylla* in combinations of varying concentrations of calcium with variant P2Mg2 (16.5 ppm + 42 ppm), viz. P2Mg2Casc (16.5 ppm + 42 ppm + 12 ppm), P2Mg2Ca1 (16.5 ppm + 42 ppm + 15 ppm) and P2Mg2Ca2 (16.5 ppm + 42 ppm + 18 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

Fig. 77  Comparison of doubling time of *A. microphylla* in combinations of varying concentrations of calcium with variant P2Mg2 (16.5 ppm + 42 ppm), viz. P2Mg2Casc (16.5 ppm + 42 ppm + 12 ppm), P2Mg2Ca1 (16.5 ppm + 42 ppm + 15 ppm) and P2Mg2Ca2 (16.5 ppm + 42 ppm + 18 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 78 Comparison of relative growth rate of *A. microphylla* in combinations of varying concentrations of calcium with variant P2Mg2 (16.5 ppm + 42 ppm), viz. P2Mg2Casc (16.5 ppm + 42 ppm + 12 ppm), P2Mg2Ca1 (16.5 ppm + 42 ppm + 15 ppm) and P2Mg2Ca2 (16.5 ppm + 42 ppm + 18 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.

Fig. 79 Comparison of heterocyst frequency of symbiont of *A. microphylla* in combinations of varying concentrations of calcium with variant P2Mg2 (16.5 ppm + 42 ppm), viz. P2Mg2Casc (16.5 ppm + 42 ppm + 12 ppm), P2Mg2Ca1 (16.5 ppm + 42 ppm + 15 ppm) and P2Mg2Ca2 (16.5 ppm + 42 ppm + 18 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 80  Comparison of freshmass of *A. microphylla* in combinations of varying concentrations of sodium with variant P2Mg2Ca2 (16.5 ppm + 42ppm + 18ppm), viz. P2Mg2Ca2Nasc (16.5 ppm + 42ppm + 18ppm + 4ppm), P2Mg2Ca2Na1 (16.5 ppm + 42ppm + 18ppm + 6.75 ppm) and P2Mg2Ca2Na2 (16.5 ppm + 42ppm + 18ppm + 9 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days

Fig. 81  Comparison of drymass of *A. microphylla* in combinations of varying concentrations of sodium with variant P2Mg2Ca2 (16.5 ppm + 42ppm + 18ppm), viz. P2Mg2Ca2Nasc (16.5 ppm + 42ppm + 18ppm + 4ppm), P2Mg2Ca2Na1 (16.5 ppm + 42ppm + 18ppm + 6.75 ppm) and P2Mg2Ca2Na2 (16.5 ppm + 42ppm + 18ppm + 9 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days
Fig. 82 Comparison of moisture content of *A. microphylla* in combinations of varying concentrations of sodium with variant P2Mg2Ca2 (16.5 ppm + 42ppm + 18ppm), viz. P2Mg2Ca2Nasc (16.5 ppm + 42ppm + 18ppm + 4ppm), P2Mg2Ca2Na1 (16.5 ppm + 42ppm + 18ppm + 6.75 ppm) and P2Mg2Ca2Na2 (16.5 ppm + 42ppm + 18ppm + 9 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days

Fig. 83 Comparison of doubling time of *A. microphylla* in combinations of varying concentrations of sodium with variant P2Mg2Ca2 (16.5 ppm + 42ppm + 18ppm), viz. P2Mg2Ca2Nasc (16.5 ppm + 42ppm + 18ppm + 4ppm), P2Mg2Ca2Na1 (16.5 ppm + 42ppm + 18ppm + 6.75 ppm) and P2Mg2Ca2Na2 (16.5 ppm + 42ppm + 18ppm + 9 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days
Fig. 84 Comparison of relative growth rate of *A. microphylla* in combinations of varying concentrations of sodium with variant P2Mg2Ca2 (16.5 ppm + 42ppm + 18ppm), viz. P2Mg2Ca2Nasc (16.5 ppm + 42ppm + 18ppm + 4ppm), P2Mg2Ca2Na1 (16.5 ppm + 42ppm + 18ppm + 6.75 ppm) and P2Mg2Ca2Na2 (16.5 ppm + 42ppm + 18ppm + 9 ppm) in Warne medium supplemented with different Cr (VI) concentrations on different harvesting days.
Fig. 85  Comparison of chromium removal percentage of *A. microphylla* in combinations of varying concentrations of magnesium with variant P2 (16.5), viz. P2Mgsc (16.5 ppm + 30 ppm), P2Mg1 (16.5 ppm + 36 ppm) and P2Mg2 (16.5 ppm + 42 ppm) in Warne medium supplemented with (a) 5 ppm and (b) 7.5 ppm Cr (VI) concentration at different incubation periods
Fig. 86  Comparison of chromium removal percentage of *A. microphylla* in combinations of varying concentrations of calcium with variant P2Mg2 (16.5 ppm + 42 ppm), viz. P2Mg2Casc (16.5 ppm + 42 ppm + 12 ppm), P2Mg2Ca1 (16.5 ppm + 42 ppm + 15 ppm) and P2Mg2Ca2 (16.5 ppm + 42 ppm + 18 ppm) in Warne medium supplemented with (a) 5 ppm and (b) 7.5 ppm Cr (VI) concentration at different incubation periods
Comparison of chromium removal percentage of *A. microphylla* in combinations of varying concentrations of sodium with variant P2Mg2Ca2 (16.5 ppm + 42ppm + 18ppm), viz. P2Mg2Ca2Nasc (16.5 ppm + 42ppm + 18ppm + 4ppm), P2Mg2Ca2Na1 (16.5 ppm + 42ppm + 18ppm + 6.75 ppm) and P2Mg2Ca2Na2 (16.5 ppm + 42ppm + 18ppm + 9 ppm) in Warne medium supplemented with (a) 5 ppm and (b) 7.5 ppm Cr (VI) concentration at different incubation periods.
concentration (Lee and Wang, 2001). The present investigation, also reveals that increasing concentration of macronutrients like P, Mg, Ca enhanced Cr (VI) removal in Warne medium (Fig. 63-66 & 85-87). These nutrients individually as well as in combination in Warne medium affected the growth of *A. microphylla* in controls as well as in presence of chromium (5 ppm and 7.5 ppm). The results also match with the work of Shun-Xing *et al.* (2007) who studied the effect of major nutrients on adsorption and absorption of heavy metals in *Dunaliella salina*. Reduction in relative growth rate, root biomass and chlorophyll content in *Salvinia herzogii* on exposure to heavy metals, viz. Zn, Ni and Cr got attenuated by addition of nutrients (Hadad *et al.*, 2007). Thus, increase in chromium removal percentage in *A. microphylla* in Warne medium having supplemented doses of macronutrient concentration individually as well as in combination can be explained on the basis of stimulation in biomass increase which has a critical role in the removal of metal. Jankong *et al.* (2007) found an increase in biomass and accumulation of arsenic in silver oak fern, *Pityrogramma calomelanos* fertilized with phosphorus. Meanwhile, Barrutia *et al.* (2009) observed an increase in plant biomass and tolerance when treated with fertilizers in Pb, Cd and Zn contaminated soils. In contrast, Marques *et al.* (2008b) showed a reduction in the accumulation of Zn in *Solanum nigrum* from the soil amended with manures. Khosravi *et al.* (2005) also found that the increasing concentration of Ca has caused reduction in biomass in controls but increase was observed in presence of different heavy metal ions, viz. Pb, Cd, Ni and Zn in culture medium supplemented with increasing doses of Ca. Shailza (2010) reported decreased removal percentage of metals by *Synechocystis pevalekii*, *Lyngbya spirals*, *Oscillatoria chlorina*, *Phormidium molle* and *Anabaena torulosa* in presence of different nutrients. This
could be a consequence of competitive inhibition as suggested by Schecher and Driscoll (1995), Yun et al. (2001) and Mehta et al. (2002). The decrease of chromium removal on increasing NaCl concentration in Warne medium (Fig. 66 & 87) is due to higher mobility of Na\(^+\) and Cl\(^-\) ions as compared to that of Cr ions (Rai and Rai, 2003). The antagonistic effect of NaCl on chromium biosorption has also been studied by Donmez and Aksu (2002) who suggested it to be due to inhibitory effect of salt on the permeability of cell membrane for Cr (VI) ions and relative competition between chloride and chromate anions on active centers of Dunaliella species.

One of the most critical points of metal removal via phytoremediation is the phytoavailability of heavy metals in soils/water (Lombi et al., 2001). Based on the uptake by plants, heavy metals in abiotic part of ecosystem could be classified into three groups which include "available" fraction (easily absorbable forms including free ions and chelating ions), "exchangeable" fractions (bound to organic matter, carbonates or Fe-Mn oxides) and "unavailable" fractions (residual forms which are most difficult to be absorbed) (Zhou and Song, 2004; Wei et al., 2008). Nevertheless, there are other techniques which can be used to increase the bioavailability of heavy metals such as decreasing pH by adding sulphuric acid or organic fertilizers (Warton and Matthiessen, 2005; Roy and Singh, 2006). Sappin-Didier et al. (2005) showed increase in accumulation of Cd in transgenic tobacco as pH decreased, whereas Singer et al. (2007) proved an increase in Ni concentration in Alyssum lesbiacum paralleled with increase in pH. In this study, inoculum size, incubation period, initial chromium concentration and pH of the medium were chosen in the present investigation to increase bioavailability of chromium and maximize the chromium removal by A. microphylla
from modified Warne medium. Inoculum size significantly affected the removal percentage as observed during 10 hours from the start of the experiment (Fig. 88). The results revealed that chromium removal increased with increasing inoculum size from 1g to 4g and it became constant thereafter in OWM supplemented with 5 ppm and 7.5 ppm chromium concentration. At higher inoculum size increased metal removal is due to greater availability of metal binding sites. The chromium removal percentage became static beyond a particular inoculum size due to limited free surface of medium in the beakers which seems to be the most plausible reason for constancy in chromium removal efficiency (Kumar and Singh, 2010). Sanyahumbi et al. (1998) in their experiment on *A. filiculoides* studied the effect of biomass concentration (drymass) by increasing it from 1 to 4 mg/L and removed up to 90% of Pb. Also, the contact time for complete removal of Cr (VI) decreased from 192 to 33 hours with increase in biomass concentration from 5 to 20 g /L (Park *et al.* 2005b) which again matches with the results of the present investigation.

In the present study, the chromium removal remained apparently static at 7th hour from the start of the experiment in 5 ppm initial chromium concentration and 24th hour of incubation period in 7.5 ppm initial chromium concentration. It is clear from the results that metal removal was rapid in the beginning, during 1st hour and then went on at slow pace and became static after 6th hour (Fig. 88 & 89). With the passage of time, number of sequestering sites lessened resulting into slow pace of the metal removal process. The diminishing removal with increasing time may be due to intraparticle diffusion process dominating adsorption (Volesky, 2001). Similar results were obtained by Mehta *et al.* (2002) while studying Cu (II) removal by *Chlorella vulgaris*. In their study, main fraction of Cu (II) was removed
Fig. 88 Effect of inoculum size on Cr (VI) removal percentage by *A. microphylla* at different incubation periods in optimized Warne medium (OWM) supplemented with two different initial chromium concentrations (a) 5 ppm and (b) 7.5 ppm
Fig. 89  Effect of incubation period on Cr (VI) removal percentage by *A. microphylla* at different incubation periods in optimized Warne medium (OWM) supplemented with two different initial chromium concentrations.

Fig. 90  Effect of initial chromium concentration on Cr (VI) removal percentage by *A. microphylla* from optimized Warne medium (OWM) at different incubation periods.
within first 15 minutes. The removal of metals by the fern, thus, appears a biphasic process having quick phase adsorption followed by the slower uptake phase.

The fact that rate of metal uptake by the plants depends upon the initial metal concentration in the solution, is supported by several workers: Miretzky et al., 2004 (Pb, Fe, Cu, Zn, Mn, and Cr by *Pistia stratiotes*, *spirodela intermedia* and *Lemna minor*), Park et al., 2005b (Cr (III) by *A. caroliniana*), Mishra et al., 2009 (Hg (II) by *A. pinnata* and *Pistia stratiotes*), Hasan et al., 2010 (Cr (VI) by *Eichhornia crasspies*) and Kaur et al., 2010 (Pb by *Lemna minor*). In the present investigation too, there is increase in chromium removal percentage with increasing initial chromium concentration in OWM but it became stable beyond 7.5 ppm (Fig. 90). The metal removal efficiency of the fern at particular inoculum size is due to binding of metal ions on all available sequestering sites of the fern biomass and beyond that, total amount of metal removed was almost the same but percent removal decreased due to unavailability of the binding sites. Facar and Malkoc (2004), however, reported decrease in percentage adsorption on increasing Cr (VI) concentration in aqueous solutions using *Fagus orientalis*.

The variation in chromium removal efficiency by *A. microphylla* at different pH of culture medium (Fig. 91) might be attributed to the pH changes which affect the chemistry of sequestering groups on biomass surface (Crist et al., 1981). In the present study, maximum chromium removal was found to be at pH 6, beyond and below which it decreased. Park et al. (2005b) also observed that the contact time for complete removal of Cr (VI) decreased with decrease in pH. The complete removal of Cr (VI) is possible at highly acidic pH (Bai and Abraham, 2001; Nourbaksh et al., 1994; Merrin et al., 1998) but this range of pH below 4 inhibits the growth of *Azolla* (Subudhi and Singh, 1979). Kaur et
Fig. 91  Effect of pH of optimized Warne medium (OWM) supplemented with two different initial chromium concentrations (a) 5 ppm and (b) 7.5 ppm on Cr (VI) removal percentage by *A. microphylla* at different incubation periods.
*al. (2010)* observed a positive correlation between accumulation of Pb (mg/kg) in *Lemna minor* tissues and each of the different variables, viz. initial Pb concentration in the culture medium, incubation period (7, 14, 21 and 28 days) and pH (from 4 to 5) of the culture medium but the accumulation decreased from pH 6 to 10 in each initial chromium concentration and on every harvesting day. Lead hydroxide precipitation of Pb occurs at higher pH which prevented the uptake (Sen and Bhattacharya, 1993).

Chromium removal percentage from OWM as observed by immobilization of *Azolla* matrix in sodium alginate beads, was maximum with beads of category B3 (sodium alginate beads prepared by using 3 g freshmass of *Azolla microphylla*), in 7.5 ppm initial chromium concentrations at 5th hour of incubation period and minimum with sodium alginate beads of category Bc (Beads prepared without *Azolla microphylla* control), in 5 ppm initial chromium concentrations at 1st hour of incubation period. It is obvious from the results that chromium removal percentage increased significantly among different categories of beads, viz. Bc, B1 (sodium alginate beads prepared by using 1 g freshmass of *Azolla microphylla*), B2 (sodium alginate beads prepared by using 2 g freshmass of *Azolla microphylla*) and B3 with increasing *Azolla* matrix immobilized in sodium alginate beads. Also, the chromium removal percentage increased significantly with increasing incubation period among different categories of sodium alginate beads in OWM supplemented with 5 ppm and 7.5 ppm chromium concentration. The chromium removal percentages were higher in 7.5 ppm chromium concentration than those in 5 ppm chromium concentration as observed among different categories of sodium alginate beads at 1st, 2nd, 3rd, 4th, 5th and 6th hours of incubation period (Fig. 92).
Fig. 92 Effect of immobilized *Azolla* matrix (freshmass) in sodium alginate beads (Bc=beads without *Azolla* matrix, control), B1, B2 and B3= beads with 1g, 2g and 3g freshmass of *A. microphylla* matrix, respectively on Cr (VI) removal percentage from optimized Warne medium (OWM) supplemented with two different initial chromium concentrations (a) 5 ppm and (b) 7.5 ppm at different incubation periods
Electroplating process is the deposition of a thin protective layer (usually metallic) onto a prepared metal surface using electrochemical processes. The process involves pretreatment, plating, rinsing, passivating and drying. The cleaning and other pre-treatment stages involve a variety of solvents (often chlorinated hydrocarbons whose use is discouraged) and surface stripping agents including caustic soda and a range of strong acids, depending upon the metal surface to be plated. Any or all of the substances used in electroplating such as acidic solutions, toxic metals (Ni, Cr, etc.), solvents and cyanides can be found in the wastewater either via rinsing of the product or from spillage and dumping of the process baths. The present study evidenced the presence of toxic metals particularly chromium (VI) in effluents from the target industries, viz. Tanneries, Malerkotla (Sangrur); Kangaroo Industries Ltd., Focal Point, Ludhiana; Kanda Brothers, Focal Point, Ludhiana and Union Brothers, Focal Point, Ludhiana was above and pH below the permissible limits (Bureau of Indian Standards, 1983; CPCB, 1998; US EPA, 2002). From the study of optimization of macronutrients, individual as well as in combinations, it was inferred that macronutrients counteract the deleterious effects of chromium to *Azolla microphylla* as well as increase its chromium removal percentage. The study was extended to the effluents of target industries and comparison of growth and chromium removal efficiency was made from the effluents diluted (x50 & x100) using OWM and SWM. Results presented in (Fig. 93-100) reveal that growth performance and chromium removal percentage of *A. microphylla* in effluents diluted by OWM and SWM increased with exposure time (5 to 10 days) and dilution (x50 to x100). Among effluents diluted by OWM, *A. microphylla* showed better growth performance and higher chromium removal percentage values than among effluents diluted by SWM. The results, clearly suggest the role of macronutrients in better performance of *A. microphylla* incubated in effluents, as well
Fig. 93  Comparison of freshmass of *A. microphylla* in Cr (VI) rich effluents of different electroplating industries (E2, E3 and E4) diluted with optimized (OWM) and standard Warne medium (SWM) on different harvesting days.

Fig. 94  Comparison of drymass of *A. microphylla* in Cr (VI) rich effluents of different electroplating industries (E2, E3 and E4) diluted with optimized (OWM) and standard Warne medium (SWM) on different harvesting days.
Fig. 95 Comparison of moisture content of *A. microphylla* in Cr (VI) rich effluents of different electroplating industries (E2, E3 and E4) diluted with optimized (OWM) and standard Warne medium (SWM) on different harvesting days.

Fig. 96 Comparison of doubling time of *A. microphylla* in Cr (VI) rich effluents of different electroplating industries (E2, E3 and E4) diluted with optimized (OWM) and standard Warne medium (SWM) on different harvesting days.
**Fig. 97** Comparison of relative growth rate of *A. microphylla* in Cr (VI) rich effluents of different electroplating industries (E2, E3 and E4) diluted with optimized (OWM) and standard Warne medium (SWM) on different harvesting days.

**Fig. 98** Comparison of total chlorophyll of *A. microphylla* in Cr (VI) rich effluents of different electroplating industries (E2, E3 and E4) diluted with optimized (OWM) and standard Warne medium (SWM) on different harvesting days.
Fig. 99 Comparison of heterocyst frequency of symbiont of *A. microphylla* in Cr (VI) rich effluents of different electroplating industries (E2, E3 and E4) diluted with optimized (OWM) and standard Warne medium (SWM) on different harvesting days.
Fig. 100 Comparison of chromium removal percentage of *A. microphylla* in Cr (VI) rich effluents of different electroplating industries (a)-E2, (b)-E3 and (c)-E4) diluted with optimized (OWM) and standard Warne medium (SWM) on different harvesting days
as in its chromium removal efficiency. Su et al. (2005) and Sridhar et al. (2007) noted a significant decrease in freshmass and relative water content in *Pteris vittata* with exposure to Cr (VI). The results of the present study also match with Bennicelli et al. (2004) in *A. caroliniana* and Rai and Tripathi (2009) in *A. pinnata* and *Vallisneria spirals* to purify waters polluted by Hg where the growth performance was negatively correlated with the concentration of the respective heavy metal studied.

Relative growth rate, root biomass and chlorophyll concentration in *Salvinia herzogii* were measured in green house when exposed to different Zn, Ni and Cr concentrations and compared with simultaneous treatments enriched with nitrogen and phosphorus (Hadad et al., 2007). These parameters were negatively correlated with increasing concentrations of metals and the deleterious effects of these heavy metals were attenuated with nutrient addition. The enhancement of heavy metal (Fe) removal has also been documented by several researchers from nutrient rich wastewaters (Tripathi and Upadhyay, 2003; Sooknah and Wilkie, 2004; Jayaweera et al., 2007; Jayaweera et al., 2008). Furthermore, various studies showed that the positive correlation occurs among the concentrations of the metal in the effluent, reservoir and tissues of phytoemediating plant (Qian et al., 1999; Rai, 2008c, 2009). The results of the present study also confirm the positive correlation between the concentration of the total metal removed and initial concentration of the metal in the diluted effluent.

Thus, successful employment of the water fern, *Azolla* for maximizing in situ chromium removal from water bodies can be achieved by altering the conditions like adequate biomass, optimum contact time, suitable initial chromium concentration and pH of aqueous solution which would prove to be ecologically as well as economically acceptable.