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BIOTECHNOLOGY METHODS

Biofilm establishment and heavy metal removal capacity of an indigenous mining algal-microbial consortium in a photo-rotating biological contactor

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Abstract An indigenous mining algal-microbial consortium was immobilised within a laboratory-scale photo-rotating biological contactor (PRBC) that was used to investigate the potential for heavy metal removal from acid mine drainage (AMD). The microbial consortium, dominated by *Ulothrix* sp., was collected from the AMD at the Sar Cheshmeh copper mine in Iran. This paper discusses the parameters required to establish an algal-microbial biofilm used for heavy metal removal, including nutrient requirements and rotational speed. The PRBC was tested using synthesised AMD with the multi-ion and acidic composition of wastewater (containing 18 elements, and with a pH of 3.5 ± 0.5), from which the microbial consortium was collected. The biofilm was successfully developed on the PRBC's disc consortium over 60 days of batch-mode operation. The PRBC was then run continuously with a 24 h hydraulic residence time (HRT) over a ten-week period. Water analysis, performed on a weekly basis, demonstrated the ability of the algal-microbial biofilm to remove 20–50 % of the various metals in the order Cu > Ni > Mn > Zn > Sb > Se > Co > Al. These results clearly indicate the significant potential for indigenous AMD microorganisms to be exploited within a PRBC for AMD treatment.

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Keywords Acid mine drainage · Microalgae · Biofilm · Photo-rotating biological contactor · Biotreatment

Introduction

Acid mine drainage (AMD) is the wastewater resulting from mining activities, which must be treated before it can be discharged into the environment. The pH of AMD is typically around 2–4, and AMD is usually contaminated with heavy metals [13, 15, 33, 37]. Conventional AMD treatment methods are ineffective or extremely expensive. However, biotreatment may provide an efficient and cost-effective solution [5].

Living organisms require low concentrations of heavy metals as micronutrients, but elevated concentrations of heavy metals in AMD are toxic to most aquatic life [28, 56]. Additionally, the low levels of macronutrients such as organic carbon, nitrate, and phosphate in AMD restrict microbial growth. However, indigenous AMD microorganisms are resistant species that are able to survive under the extreme conditions represented by AMD [23, 24, 47]. Many investigations have reported the natural cleansing nature of these microorganisms for removing heavy metals from AMD in the environment [15, 16, 33, 38–40, 43, 53, 55]. A few species of bacteria, microalgae and fungi thrive in extensive biofilms attached to the AMD substrate; they can remove metals from AMD efficiently and accumulate them as biomass and extracellular compounds [1–3, 23, 24]. Among these microorganisms, microalgae play an important role within AMD biofilms due to their phototrophic nature and synergistic relationship with other microorganisms [4, 9, 56]. The extracellular compounds secreted by microalgae can serve as a source of carbon for bacteria [23].

In a previous study, Orandi et al. [51] isolated several species of green microalgae, fungi and bacteria from a biofilm that had formed naturally, attaching itself to the AMD substrate under acidic (pH 3–5) and heavy-metal-contaminated conditions at the Sar Cheshmeh copper mine in Iran. The microalga *Ulothrix gigas*, the fungi *Geotrichum* sp. and *Aspergillus* sp., and the bacteria *Pseudomonas* sp. and *Thiobacillus* sp. were identified in the microbial consortium. Orandi et al. [51] reported the potential for these microorganisms to remove heavy metals in situ [51]. The current study aims to replicate the same AMD biofilm, dominated by the filamentous green microalga *Ulothrix* sp., to evaluate their efficiency at removing heavy metals from AMD. To immobilise the algal-microbial consortium, a laboratory-scale photo-rotating biological contactor (PRBC) was used. Rotating biological contactors (RBC) have already been successfully used to treat municipal and industrial wastewater [18, 42, 52, 54]. The RBC facilitates the immobilisation of microorganisms as a biofilm attached to the support medium [54]. The high interfacial areas generated by the discs, its simple and feasible design and operation, low land occupancy, low energy consumption, low costs of operation and maintenance, and its high reaction rate and treatment efficiency are the principal advantages of this type of reactor [18, 35, 42].

RBCs have been evaluated in the mining industry for the removal of contaminants such as ferrous iron (Fe^{2+}), cyanide and its various species, oxalate, and selenium from mining effluents [34, 48, 49, 57]. However, the literature on the application of RBCs for the biotreatment of heavy metals is still limited, and more work is required before this opportunity can be exploited in the mining industry. Additionally, limited research has been carried out on the utilisation of indigenous AMD microorganisms in an RBC to remove heavy metals and treat AMD [48, 49, 57].

Olem and Unz [48, 49] used a pilot-scale RBC to evaluate ferrous iron oxidation in the AMD released from a coal mine. Plastic discs were selected as the support medium in the RBC and were half-immersed in the flowing mine water. During the operation of the RBC, the indigenous iron-oxidising bacteria developed a biofilm on the disc surfaces and mediated the transformation of Fe^{2+} to the less soluble ferric state Fe^{3+}. The RBC was operated for eleven months at the optimum operating speed of 10 rpm. Hydraulic loadings of 0.11 and 0.22 m^3/day-m^2 resulted in the oxidation of 240 mg/L of influent Fe^{2+}, producing 2 and 5 mg/L of effluent Fe^{2+} , respectively.

Travieso et al. [57] used a rotary drum covered with 0.5 mm wide polyurethane bands to immobilise microalgae for the removal of heavy metals. The reactor was operated with synthesised wastewater containing 3 mg/L iron cobalt in a 20-day batch mode. A constant rotational speed of 2 rpm was used during the experiment. The authors reported the removal of 94.5 % of the cobalt after ten days.

Prior to the application of an RBC for any treatment application, it is necessary to establish a biofilm, which can take a considerable amount of time. In the current study, limiting factors on microalgal growth such as the light intensity [30, 36] and nutrient requirements [10] were considered. Additionally, maintaining a healthy biofilm in an RBC relies on parameters such as medium composition, rotational speed and dosing rates [18], which are also discussed in this paper. The current study uses a novel approach to determine the parameters required to set up and operate a PRBC to establish an AMD algal-microbial biofilm at the laboratory scale. The PBRC was then operated continuously to determine the heavy metal loading capacity and the health of the biofilm.

Materials and methods

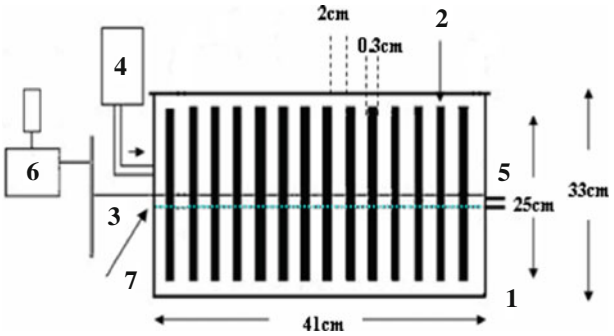
Photo-rotating biological contactor

A single-stage laboratory-scale PRBC was constructed using 16 discs that were made of polyvinyl chloride (PVC). High-density plastic sheets such as PVC, polystyrene and polyethylene have been commonly used as RBC discs [46, 52]. The surface of each disc was roughened with 5-grit-grade sandpaper. The 25 cm diameter discs were mounted on a horizontal shaft with spaces of 2 cm between them. The shaft was mounted in a Plexiglas® trough so that the discs were submerged to a level of 40 % of the disc surface area. A 3 cm wide PVC strip was used to connect the terminal discs at their outer edge, and this acted as a mixing paddle to prevent short-circuiting in the bottom of the trough. The dimensions are presented in Table 1. The PRBC trough was covered with a Plexiglas® lid.

The disc shaft was coupled to a motor (REMX, EF series three-phase induction motor) operated with a speed controller (Teco Speecon 7300 CV series inverters), which was used to maintain the rotational speed at 2 rpm during biofilm establishment and at 5 rpm during the treatment

Table 1 Dimensions of the PRBC (cm)

Length of trough	41
Width of trough	30
Depth of trough	33
Diameter of disc	25
Thickness of disc	0.3
Distance between discs	2.0
Distance between outer edge of discs and wall of trough	2.5
Distance between outer edge of discs and bottom of trough	2.0



1. Plexiglas Trough
2. Discs
3. Shaft
4. Feed tank and inlet
5. Outlet
6. Motor and speed controller
7. Level of immersion

Fig. 1 Configuration of the single-stage PRBC

period. A feed tank was connected to the trough to introduce the reactor liquid media through an inlet. A volume of 15 l was required for 40 % immersion of the discs in the trough. An outlet was placed on the trough to maintain this working volume during continuous operation with a 24 h hydraulic residence time (HRT) over a ten-week period (Fig. 1). The simulated AMD was supplied to the system at 10 mL/min using a MasterFlex (model 7521-35) peristaltic pump with an easy-load MasterFlex pump head (model 7518-00).

PRBC liquid media

The PRBC was operated with a synthesised AMD that contained various anions and cations (presented in Table 2) to mimic the natural composition of the microbial habitat. Importantly, the multi-ion composition of the simulated AMD increases the validity of the treatment results for future use in the field. The calculated value of each parameter was based on an analysis of natural AMD, as described previously [50]. The concentrations of the elements varied; some were high (20–80 mg/L) and others were low (0.005–0.5 mg/L), depending on their concentrations in actual AMD (Table 2). The synthesised AMD contained low concentrations of nitrate and phosphate, which are the major nutrients required for microalgae growth. The indigenous microalgae were initially maintained in Bold's basal medium in the laboratory, which contains high nutrient levels [50]. Subsequently, during the biofilm establishment period, the levels of the nutrients NO_3^- and PO_4^{3+} were increased in the PRBC medium to the same level as in Bold's basal medium, 180 and 90 mg/L, respectively, to encourage biofilm growth in the PRBC [14]. An additional benefit of these increased nutrient levels in this research was that it facilitated the daily measurement of nutrient concentrations in the PRBC solution during batch mode. The concentrations of nutrients were decreased to the background levels present in natural AMD during continuous operation. The PRBC was acid washed with 2 % nitric acid before introducing the synthesised AMD.

Table 2 Composition of the simulated AMD

Species	Concentration range (mg/L)
Ca	18–20
Na	20–25
K	4–5
Mg	85–100
Cu	80–100
Mn	35–45
Zn	18–20
Ni	2–3
Co	0.3–0.5
Fe	0.4–0.5
Si	12–15
Cr	0.1–0.2
Sb	0.005–0.007
Al	0.07–0.09
Ag	0.02–0.03
Mo	0.02–0.03
Pb	0.03–0.04
Se	0.03–0.04
CO_3	12–15
Cl	13–15
$NO_3 + NO_2$	18–20
PO_4	0.8–1.0
NH_4	0.9–1.0
$C_6H_{12}O_6$	0.8–1.0

Microbial inoculum

The inoculum used to inoculate the PRBC was obtained from the natural AMD biofilm at the Sar Cheshmeh copper mine in Iran. The microbial consortium, dominated by filamentous green microalgae, was collected in plastic tubes with some of the AMD, leaving some air. Sampling

and preservation were carried out according to standard methods [11]. A homogeniser was used to break up the field microbial consortium and prepare the inoculum. The volume of the inoculum was 1.5 L with 0.1 g/L dry weight (microbial consortium), which was added to the PRBC solution.

Light irradiance

Light is a critical parameter for microalgae reproduction and photosynthesis. Graham et al. [29] reported that 520 μmol m^{-2} s^{-1} was the optimal light irradiance for the growth of the filamentous green microalga *Ulothrix zonata*. In the current study, to provide enough light for *Ulothrix* sp. to grow in the PRBC, eight tubular cool white fluorescent lamps (Crompton F18T8/840) were installed as four pairs inside a semicylindrical cover, which was placed on top of the PRBC to distribute light from the top and sides. A light meter was used to measure the light intensity of the lamps, which was 189 μmol m^{-2} s^{-1} for each pair, giving 756 μmol m^{-2} s^{-1} in total. The light was set to a 12:12 h light:dark cycle. To reduce the effect of the heat generated by the lamps inside the cover, a fan was installed and operated during the 12 h of illumination.

Analysis of the PRBC medium

The PRBC was operated for 60 days in batch mode to establish a biofilm, and then for 70 days continuously to investigate heavy metal removal from the simulated AMD. During batch operation, the major nutrients nitrate and orthophosphate (NO_3^- and PO_4^{3+}) were measured on a daily basis using a Thermo Scientific nutrient analyser to investigate the nutrient consumption rate during biofilm development. During continuous operation, copper, nickel, manganese, zinc, antimony, selenium, cobalt, and aluminium were analysed on a triplicate basis at weekly intervals using inductively coupled plasma-mass spectroscopy (ICP-MS) to evaluate the potential of the system to remove these elements from the simulated AMD over an extended period. Sampling, preservation and analytical methods were carried out according to standard methods [11].

During PRBC operation, pH, Eh and dissolved oxygen (DO) were recorded daily with a pH-Eh-DO meter (TPS-90 FL). Additionally, the water and air temperatures were recorded.

Analysis of the algal-microbial biofilm

Tissue culture cover slips (Thermanox^{TM}) of size 30 × 24 mm were used to analyse the algal-microbial biofilm in the PRBC. The slips were roughened with 5-grit-grade sandpaper and attached to the discs with plastic clips. Before attachment, each slip was numbered and weighed. Four slips were attached symmetrically to the edge of each disc. Three cover slips were randomly removed and weighed weekly after being dried in order to evaluate the biofilm growth rate. The biofilm mass in the PRBC was calculated by multiplying the total disc surface area by the average mass per unit area of the biofilm that developed on the cover slips. To observe the microbial constituents of the biofilm structure, a number of slips were removed for scanning electron microscopy (SEM) analysis during biofilm growth.

Sample preparation for SEM analysis

The cover slips were removed, cut into small pieces (0.5 × 0.5 cm), and fixed in electron microscopy fixative (4 % paraformaldehyde/1.25 % glutaraldehyde in PBS buffer + 4 % sucrose, pH 7.2). Samples were washed in PBS buffer + 4 % sucrose for 10 min and post-fixed in 2 % aqueous osmium tetroxide. Dehydration of samples was carried out in an alcohol series (ethanol 70, 90 and 100 %, 10 min each). The samples were dried in a Bal-Tec 030 critical point dryer (CPD) mounted on metal stubs and sputter coated with platinum prior to analysis in a Philips XL30 SEM.

Efficient rotational speed for biofilm growth

The algal-microbial biofilm developed on the PRBC's discs over a two-month period, which resulted in a thin layer on each of the discs rotating at 2 rpm. To increase the thickness and strength of the biofilm, the PRBC was operated for a further four-week period to determine the optimal rotational speed. Speeds of 5 rpm and 10 rpm were applied, each for a two-week period. Biofilm growth was measured throughout this period. The literature suggests that the optimal rotational speed for establishing biofilms in RBCs ranges from 1 to 10 rpm [18, 52].

Results and discussion

An appropriate immobilisation technique is required to maintain and exploit microbial biomass for metal absorption during a continuous industrial process [25]. The current research successfully developed a novel approach to immobilising indigenous AMD microorganisms in a PRBC. The application of growing microbes, particularly mixed consortia in suitably configured reactors for heavy metal removal, has significant advantages compared with dead/pretreated biomass in bioremediation. However, achieving adequate microbe immobilisation and development as well as finding appropriate carbon and nutrient

sources have remained challenging tasks, which need to be resolved before the technology is a relevant and economically viable treatment option [39]. The algal-microbial biofilm reported here was successfully established on PRBC discs over a 60-day batch operation period and maintained during a ten-week biotreatment period using low-substrate concentrations.

As discussed previously, despite the fact that the indigenous mining microalgae are adapted to the low nutrient availability in the AMD environment, in the PRBC the nutrient concentration was increased during biofilm development to encourage microbial growth. The available nutrients and daily concentrations are presented in Figs. 2 and 3 for the study reported here. The daily concentration of orthophosphate (PO_4^{3+}) is presented in Fig. 2, where three distinct stages with three different consumption rates of orthophosphate are apparent. The orthophosphate concentration decreased gradually over the first 16 days during the first stage, marked (a). The average daily consumption rate was 1.90 mg/L over this period ($R^2 = 0.9401$). This was due to the microbial consortium adapting to the new environment, commonly known as the lag phase. The concentration reduced at a faster rate in the second stage, marked (b), and the orthophosphate was depleted within ten days. The consumption rate during this period was 4.90 mg/L ($R^2 = 0.9733$). The orthophosphate was replenished after 27 days to the initial concentration. The consumption rate decreased to 1.50 mg/L during the third stage, marked (c) ($R^2 = 0.972$). Clearly, the results indicate that the algal biofilm required more orthophosphate during establishment (at the beginning of growth). However, the indigenous microalgae adapted to the low nutrient levels, as can be seen in the third stage of growth.

The changes in the concentration of nitrate over the 60 days of the experiment are illustrated in Fig. 3, and two distinct stages can be observed. Similar to the orthophosphate data, nitrate concentration increased slightly after mixing the PRBC solution with the inoculum. However, after two days it was corrected to the initial concentration and decreased gradually at a consumption rate of 0.95 mg/L (stage a) ($R^2 = 0.7132$). This period corresponded to the lag phase. A significant decrease was observed over the rest of experiment at a consumption rate of 1.90 mg/L, marked (b) ($R^2 = 0.9394$).

During the first week, filaments of green microalgae became attached to the rough surfaces of the discs. After that, the biofilm gradually developed and covered the discs within 60 days. Using the three cover slips that were randomly removed each week, the dried weight of the total biofilm was measured, using the surface area of each cover

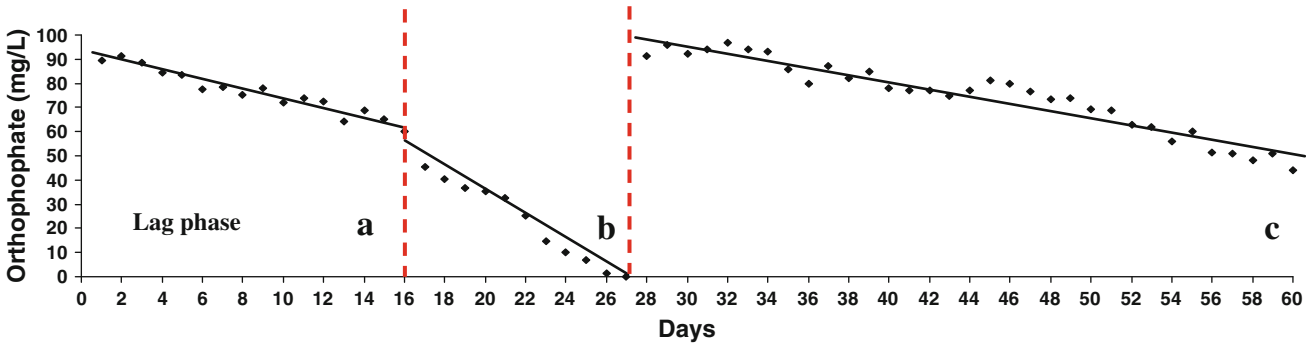


Fig. 2 Orthophosphate concentration in PRBC solution: *a* first stage, *b* second stage, *c* third stage

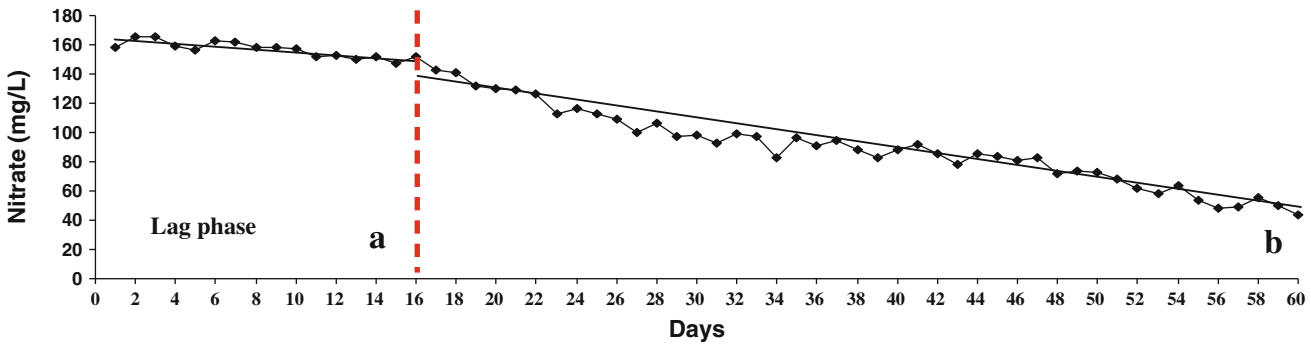


Fig. 3 Nitrate concentration in PRBC solution: *a* first stage, *b* second stage

slip (6.84 cm^2) and the total surface area of 16 discs in the PRBC (15,976.3 cm^2). The maximum, minimum and average dry weights of the biofilm are shown in Fig. 4. The data show only very minor levels of attachment and growth of biofilm during the first two weeks, which reflects the low nutrient consumption during this period (lag phase) (Figs. 2, 3). The mass of the biofilm increased after this period, as the average dried weight increased to 4.2 and 40 g after the fourth and eighth weeks, respectively.

Previous studies have highlighted that the rotational speed of the RBC discs is a critical influence on biofilm thickness and strength, as it has a direct impact on nutrient and gas mass transfer in the biofilm [42, 52, 54]. Increasing the rotational speed may lead to the detachment of the microorganisms into the RBC media, whilst decreasing the rotational speed has been also recognised to be a reason for biofilm detachment, due to nutrient starvation [18]. In the reported research, the operating speed was maintained at 2 rpm, based on the results of a previous study [57], to facilitate the attachment and growth of the indigenous AMD microalgae in the PRBC. However, in this study, the biofilm developed slowly and was relatively thin and easily detached during the first two months of batch operation. The PRBC was operated for a further four weeks to investigate the effect of increased rotational speed. The total masses of biofilm that developed at different rotational speeds are shown in Fig. 5. The results show a 50 % increase in biofilm mass at a rotational speed of 5 rpm. It

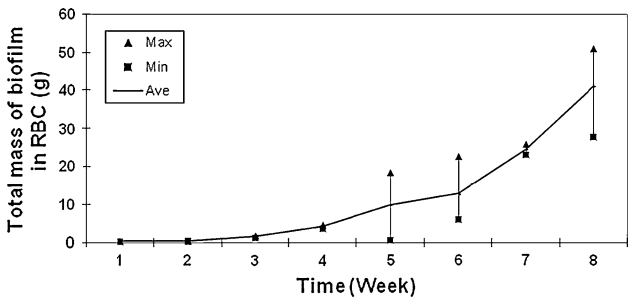


Fig. 4 Weekly maximum, minimum and average dried weights of the total algal-microbial biofilm in the PRBC

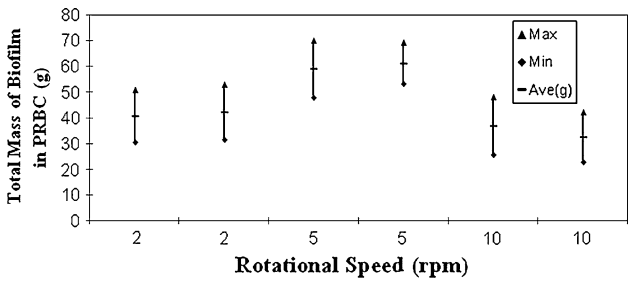


Fig. 5 Comparison of the total masses of biofilm in the PRBC that developed under three different rotational speeds: 2, 5 and 10 rpm

was assumed that optimal mass transfer occurred at this rotational speed due to the thickness and stability of the biofilm. At 2 and 10 rpm the biofilm was not as thick or robust. Thus, the PBRC was operated at 5 rpm for the continuous biotreatment period.

Biofilm samples were imaged using SEM in order to monitor biofilm development and structure. The SEM images shown in Fig. 6a, b are of a roughened bare cover slip (a) and the developed biofilm (b). The filamentous microalga *Ulothrix* sp. is depicted in Fig. 6c, d, before and after the treatment period, respectively. The microalgal filaments were deformed after treatment, which was attributed to the precipitation of metals on the cell walls [19]. The adsorption and absorption characteristics of the biofilm are discussed below.

A number of studies have reported the metal removal capacities of immobilised live microorganisms in a continuous system such as an RBC or in other media with only 1–3 cations in solution [6, 12, 19–22, 26]. The efficiencies of these different systems for metal removal vary widely and are difficult to compare. The mechanism of metal biosorption is a complicated process and depends on a variety of factors, including the ambient/environmental conditions (such as the pH, temperature and DO), the initial concentration of metal ions and biomass, and the competition among the ions for binding sites [24, 38, 42]. These factors were considered in the present study and are discussed below.

During the operational period of the PRBC, the DO fluctuated between 3.5 and 5.5 mg/L due to the microbial activity in the biofilm, but the system remained oxygenated. High DO levels can decrease treatment efficiency as this causes photooxidative damage to microalgal cells [44]. Matsumoto et al. [41] reported a 98 % decrease in the photosynthetic O_2 production rate when DO increased from 0 to 29 mg/L. In the reported study, the DO did not exceed 5.5 mg/L, which was attributed to continuous O_2 consumption by heterotrophic bacteria in the biofilm.

The efficiency of microalgae-based treatments normally decreases at low temperatures [44]. Munoz et al. [45] observed that the removal efficiency doubled upon increasing the temperature from 25 to 30 °C when using a symbiotic microcosm formed by the *Chlorella sorokiniana* and *Ralstonia basilensis* strains. However, Aksu et al. [8] reported that temperature does not influence the biosorption process in the range 20–35 °C. In the present study, the daily recorded air temperature fluctuated between 16 and 20 °C, whereas the water temperature was between 19 and 23 °C. Light irradiance was the only source of heat in the PRBC during the light period, and the PRBC was effectively run at ambient temperature.

The pH of the PRBC, which is reported to be a critical variable that influences biosorption [20], was maintained at

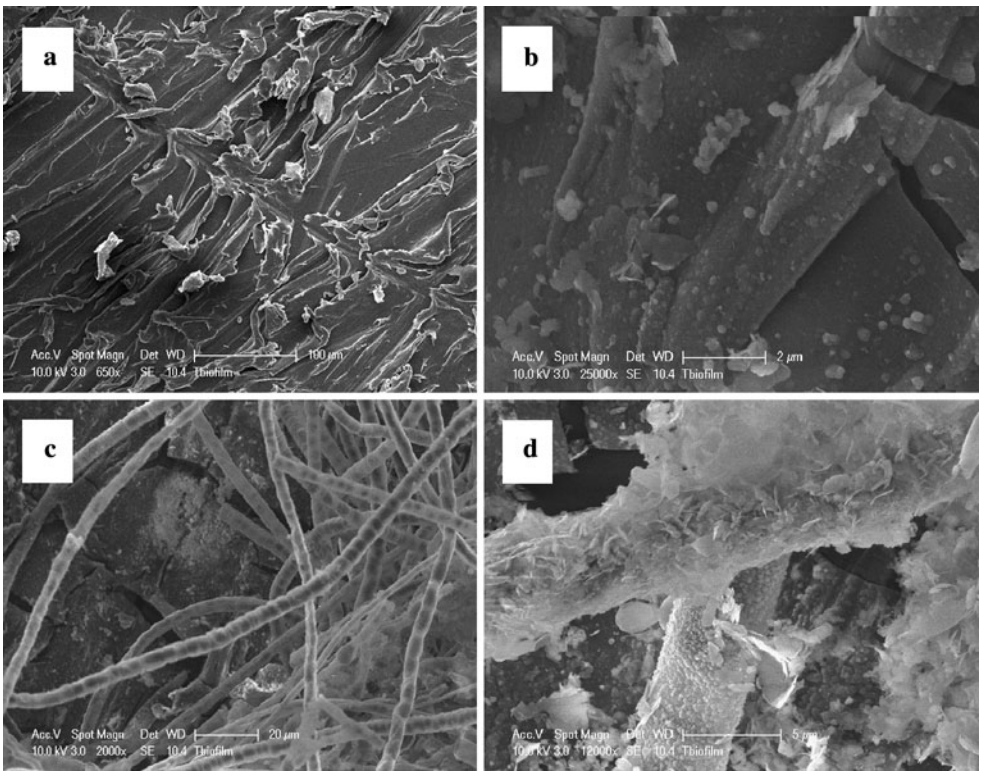


Fig. 6 SEM images of the biofilm and its constituents in both the early and late stages of biofilm development: **a** roughened bare cover slip; **b** biofilm during the early stages of development; **c** filamentous green microalga *Ulothrix* sp.; **d** deformed algal filaments after treatment

3.5 ± 0.4 during continuous operation. The pH affects the solution chemistry of metals, the activity of the functional groups in the biomass, and the competition among the metal ions [1, 25, 39]. A low external pH reduces both surface binding and intracellular influx [30] due to the presence of hydrogen ions, which compete successfully with other cations for binding sites and hence occupy many potential metal-binding sites, resulting in poor metal biosorption results [43]. The metal uptake of biofilms under acidic conditions is substantially less than the uptake under neutral conditions [27]. However, the reported system was designed for heavy metal removal from AMD, which is usually very acidic. The PBRC effectively removed metals from a solution at pH 3.5 ± 0.4, which is the typical average pH of AMD. The redox potential was measured as Eh, and this fluctuated between 212 and 375 mV, and when considering the pH-Eh relationship with elemental species in solutions, these values indicated that the cations were in a soluble form and mobile prior to absorption onto the biofilm and did not precipitate in the system [17].

After biofilm development, the PRBC was operated continuously to evaluate its efficiency at removing heavy metals from a multi-ion synthetic AMD containing 18 elements. A previous review [39] stated that a biotreatment based on laboratory studies that demonstrated high efficiency for metal removal using synthesised wastewater did not perform well in field trials. However, those investigations used synthesised wastewater containing only a few selected metals that did not truly represent actual industrial effluents such as AMD. In the reported study, when the PRBC biofilm was applied to the multi-ion synthetic AMD, it was able to remove Cu, Ni, Mn, and Zn at initially high concentrations (20–80 mg/L) and Sb, Se, Co and Al at lower concentrations (0.005–0.5 mg/L) from the influent under continuous operation. The weekly percentage removal of each element is presented individually in Fig. 7. The results showed that the maximum removal for Cu and Ni was up to 50 % of the initial concentration in the influent. The maximum removal for Mn was up to 40–45 % and for Zn up to 35 % over the ten-week experimental period.

As mentioned previously, the initial metal concentrations and the competition among the ions for binding sites affect the metal selectivity of the biofilm towards a multi-ion solution [12]. In this study, Cu and Ni uptake did not appear to be affected by the presence of other metal ions. These metals outcompeted other ions for available adsorption sites. Many studies have reported that Cu was preferentially adsorbed in the presence of other ions [19, 20]. In the reported study, the indigenous algal-microbial consortium was obtained from a copper mine, and grew in AMD that contained >80 mg/L of copper. The

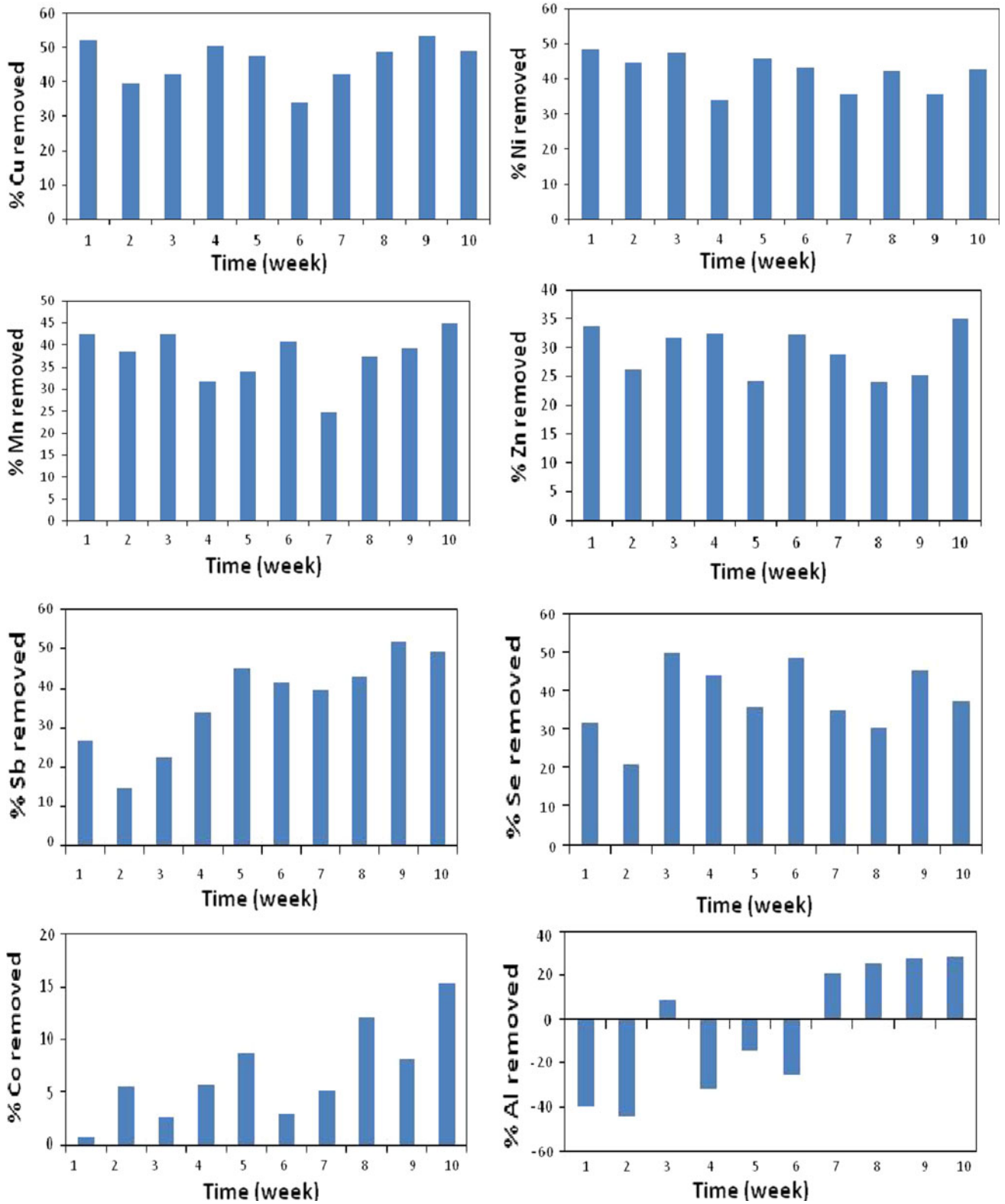


Fig. 7 The capacity of the PRBC to remove the elements Cu, Ni, Mn, Zn, Sb, Se, Co, and Al during continuous operation

establishment of this copper-selective population could favor the multilayered adsorption of Cu, and the removal efficiency was not adversely affected by the presence of other ions. The removal percentage of Mn was similar to those of Cu and Ni. The higher initial concentrations of Cu and Mn in the influent were attributed to a higher removal

rate. Ni is a recalcitrant pollutant, and many microorganisms have a relatively low Ni-binding capacity [58]. Mn is another recalcitrant ion that cannot be removed by chemical treatment in traditional systems [39]. The 45–50 % removal rate of Ni and Mn in the current system under acidic conditions highlights the effectiveness of using an indigenous algal-microbial biofilm in the PRBC. Less Zn was removed than other metals with higher initial concentrations, namely Cu, Ni and Mn. A similar study was carried out by Costely and Wallis [19–22], who investigated the removal of Cu, Zn and Cd separately and collectively in synthetic wastewater by immobilised activated sludge in an RBC. Their results showed that the individual metal uptakes of Zn and Cd were up to 94 and 91 % of their initial concentrations, respectively. The presence of Cu in the complex solution hampered the uptakes of both Cd and Zn considerably, as they decreased to 15 and 22 %, respectively [20]. Additionally, an investigation into the use of a three-stage sequential RBC to remove Cu, Zn and Cd showed a high level of Cu removal in reactor 1, which resulted in decreased competition from this ion in reactors 2 and 3, thus allowing increased removal of Zn in the remaining reactors. Therefore, Zn removal can be adversely affected by the presence of competing ions [31].

Metal biosorption by living cells is a two-step process. In the dominant first step, metal ions are adsorbed onto the surfaces of cells due to interactions between metals and functional groups on the cell surface [25]. The second step is related to the active uptake of metals, which coincides with the metabolic function of live cells. The relatively stable and high removal percentages for Cu, Ni and Mn in the reported study could be due to the well-known metabolic requirements of microalgae for these elements [39]. Cu, Zn, Mn, Co and Ni are essential micronutrients for the growth and metabolism of all aquatic microalgae [10].

Among the metals with low initial concentrations in the influent, the removal efficiency of Sb was low during the first few weeks. However, removal increased up to 50 % after the first four weeks and remained between 40 and 50 % during the final six weeks of the experiment. This was attributed to multilayer metal adsorption on cell walls, corresponding to biofilm growth. The removal of Se fluctuated between 35 to 50 %, which was attributed to the toxic effect of Se on the biofilm, which absorbed and desorbed spontaneously to maintain its integrity and health.

Among the elements studied, Co and Al were not readily assimilated. Co removal gradually increased up to 15 % from its initial concentration. Al initially accumulated in the system (as shown by a negative percentage), but a consistent removal pattern for aluminium ($\sim$20 %) was observed during the later weeks of the experiment. The lower removal percentages of these ions could be due to their lower initial concentrations and the biofilm's selectivity for these elements in the synthesised AMD. Additionally, some of elements such as Al and Co do not play a significant role in metabolic function, and the toxic effects of these elements can adversely affect cellular function [20]. Consequently, cells may exhibit resistance mechanisms that enable them to withstand high concentrations of such metals, so they exhibit low sorption capacities [20]. The resistance mechanism employed may either prevent the initial uptake of an ion or it may provide a way to expel the ion from the cell [20]. In a previous study, the treatment process utilising sulfate-reducing bacteria in a packed bed reactor was ineffective at removing Al and Mg from mildly acidic and multimetal (Cu, Zn, As, Ni, Fe, Mg, Al)-contaminated wastewater [32]. The removal of Al was achieved through hydrolysis, which resulted in the formation of insoluble $Al(OH)_3$ at around pH 10.5 [26, 32].

As explained previously, a low pH results in a low efficiency of metal removal by biofilms [7, 27]. The low pH (3 ± 0.5) in the reported study was attributed to the low removal percentages of Zn, Sb, Se, Co and Al. However, the increased removal percentages of Al and Co in the later weeks of the experiment demonstrated the need to consider a sequential RBC that could increase treatment efficiency, which is beyond the scope of the reported research. These results illustrate the selectivity of the removal of certain elements by the algal-microbial biofilm, which removed doubly charged ions in the order $Cu > Ni > Mn > Zn$, and trace metals were removed in the order $Sb > Se > Co > Al$. This study demonstrates the potential ability of a PRBC inoculated with indigenous AMD microorganisms to remove multiple metals that are present at differing concentrations, which cannot be achieved practically with the traditional chemical precipitation processes usually adopted in the mining industry.

Conclusion

An indigenous microbial consortium dominated by the filamentous green microalga *Ulothrix* sp. was collected from the AMD at the Sar Cheshmeh copper mine in Iran. A laboratory-scale PRBC was constructed and used as the substrate for the development of an algal-microbial biofilm, and a simulated AMD was used to mimic the natural composition of the microbial habitat. The biofilm gradually developed through the accretion of the filamentous green microalgae, fungi and bacteria, and the total attached dried mass of the biofilm reached 40 g in the PRBC after 60 days of batch operation. The PRBC was then operated continuously over a ten-week period to evaluate the heavy metal removal capacity of the biofilm. Up to 50 % of the initial concentrations of the metals were removed in the order

Cu > Ni > Mn > Zn > Sb > Se at pH 3.5. Up to 15 and 20 % of the Co and Al were removed, respectively. The results demonstrate the potential of PRBCs and algal biofilms to be exploited for heavy metal removal from AMD. Future work is required to evaluate the potential of algal-microbial biofilm to remove heavy metals from natural AMD at the pilot scale using various PRBC configurations (e.g. sequential), in order to determine the potential for this technology to be used commercially in the mining industry.

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