The potential of selected macroalgal species for treatment of AMD at different pH values in temperate regions

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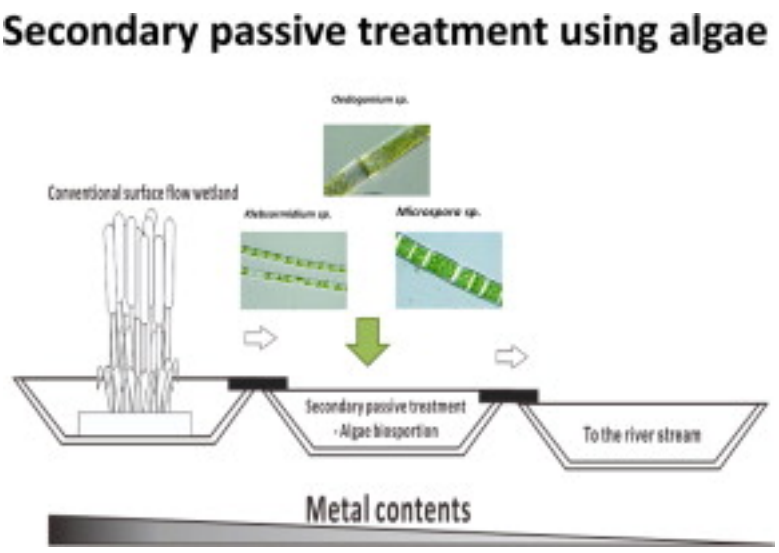
Graphical Abstract:



Abstract:

. The metal bioaccumulation potential of selected macroalgae species as different pH ranges were study for usage as part of a possible secondary passive AMD treatment technology in algae ponds in temperate regions during winter months. . Two separate studies were conducted to determine the suitability of macroalgae for passive treatment when metabolic processes in macrophytes and microrganisms in constructed wetlands decreases due to seasonal changes. In the field study, the bioconcentration of metals (mg/kg dry weight)

measured in the macroalgae mats were in the following order: site 1. *Oedegonium crassum* Al > Fe > Mn > Zn; site 2. *Klebsormidium klebsii*, Al > Fe > Mn > Zn; site 3. *Microspora tumidula*, Fe > Al > Mn > Zn and site 4. *M. tumidula*, Fe > Mn > Al > Z. In the laboratory study, cultured macroalgae *K. klebsii, O. crassum* and *M. tumidula* isolated from the field sampling sites were exposed to three different pH values (3, 5 and 7), while bioaccumulation of the metals, Al, Fe, Mn and Zn and glutathione-S-transferase (GST) activity was measured in the different algae species at a constant water temperature of 14 ˚C. . Bioaccumulation of Al was the highest for *O. crassum* followed by *K. klebsii* and *M. tumidula* ($p < 0.0001$). From the study it was evident that the highest metal bioaccumulation occurred in the macroalgae *O. crassum* at all three tested pH values under constant low water temperature.

Key words: acidophilic filamentous macroalgae, AMD, bioaccumulation, oxidative stress

1. Introduction

Properties of AMD render receiving water resources less habitable to various biotas, while waters that receive AMD are often characterized by very low biodiversity, dominated by acidophilic biota (Oberholster et al., 2013). Due to the toxicity effects accompanying AMD, sensitive species for example macroinvertebrates and fish are systematically reduced or eliminated, e.g. through a failure to reproduce, reduced feeding ability and other adverse physiological and health effects, which can also alter the ecological interaction such as prey-predator relations (Niyogi et al., 1999).

The cost to rehabilitate impacts of AMD from abandoned mines in South Africa is estimated at R30 billion (~ 3 Billion US dollars) (McGinnes, 1999; Auditor-General South Africa, 2009). Around 90% of this AMD originated from abandoned underground coal and gold

mines which was in operation more than a century ago. Since no company or individual claims responsibility for reclaiming abandoned mine lands, the treatment of any AMD source or stream becomes a public expense. However, South Africa is not alone in its lack of a national strategy to deal with AMD. Based on studies in the USA, approximately 20, 000 km of streams and rivers in the eastern United States are degraded by AMD (U.S. EPA, 1995).

Conventional AMD treatment systems, which are most commonly used at abandoned mine sites, require continual addition of expensive chemicals such as lime, which generate voluminous low density sludge (≈ 5%) (Stark et al., 1994). The disposal of this sludge becomes another environmental problem and carries an additional financial burden. Thus, conventional treatment processes are often expensive both in terms of capital and operational costs, since very high acidity of AMD water results in higher operatinal costs, associated with the shorter life-time and more frequent replacement of consumables such as filters and ion-exchange resins. During the last three decades, passive treatment of AMD using technologies such as constructed wetlands have been developed as an alternative to conventional treatment in environments with medium to low water flow regimes (Sheoran and Sheoran, 2006). Biological removal is perhaps the most important pathway for metal removal in these wetlands (Sheoran and Sheoran, 2006). Probably the most widely recognized biological processes for metal removal in wetlands is macrophyte uptake (Greenway, 1997). However, the metal storage capability of macrophytes in constructed wetlands for AMD treatment can be lost in temperate regions during winter months when plant and microbial metabolic processes are reduced due to lower water temperature and shorter day length (Kalff, 2001). According to Kadlec and Reddy (2001) many individual wetland processes, such as microbially mediated reactions, are affected by temperature. From their study it was evident that responses of microbial processes was much greater to changes at the lower end of the

temperature scale (< 15 °C) than at the optimal range of 20 to 35°C. Although macroalgae have been reported to be quite efficient in heavy metal removal from AMD water in constructed wetlands, very little is known about different macroalgal species and their potential as AMD remediation technologies at different pH values (Das et al., 2009a, b). According to Novis and Harding, (2007), adsorption of metals by algae is highly variable depending on the metals, and the algal species. Many heavy metals are necessary micronutrients of macroalgae at low concentrations, which may be lethal at high concentrations.

The toxicity of metals to algae occurs by affecting either their essential metabolic processes, through protein denaturation by the blockage of functional groups, displacing an essential metal, or by rupture of cellular and organelle membrane integrity (Ford and Ryan, 1995). Under unfavourable environmental conditions, algae respond by the induction of reactive oxygen species (ROS) producing enzymes such as glutathione S-transferase, superoxide dismutase, catalase and peroxidase (Aguilera et al., 2002; Li et al., 2010). High metal concentrations lead to ROS production reaching intolerable ranges causing damage to the algae cells (Pinto et al., 2003). In the current study glutathione S-transferase (GST) was selected as a biomarker to monitor oxidative stress induced in macroalgae species under AMD conditions at different pH values over a period of 192 hours (8 days). GST is commonly used as a biomarker for its ability to inactivate toxic compounds that can induce oxidative stress in organisms (Regoli et al., 2012; Vega-López et al., 2013).

Whitton and Kelly (1995) showed in their study that algal metal content rises with the increasing metal content of the surrounding water. Therefore, the presence and tolerance of diverse benthic algal species to AMD provides an opportunity to utilize them as part of an AMD passive bioremediation technology, especially in winter months, when metal

bioaccumulation efficiency by constructed wetland plants is reduced. The objectives of this study were (1) to determine the bioaccumulation of metals in different macroalgal species under winter field conditions at AMD impacted sampling sites; (2) to perform laboratory studies on isolated macroalgal species to determine the metal bioaccumulation capacity of selected macroalgae at different pH values under constant low water temperature; (3) to determine through time trials the levels of glutathione S-transferase enzyme activity at different pH values; and (4) to establish which of the selected macroalgae is the best candidate to be used as a secondary treatment strategy in macroalgal treatment ponds.

2. Materials and Methods

2.1. Field experiment

Field study sites were located in the Mpumalanga province of South Africa where a number of defunct and flooded underground coal mines, such as the Middelburg Colliery to the west and northwest of the town of eMalahleni commenced decanting in the mid-1990's, contributing to AMD pollution of the water resources in the upper Olifants River catchment (Maree et al., 2004). In 2004, the Department of Water Affairs and Forestry (DWAF) estimated the post-closure decant from defunct coal mines at ~62 ML/d in the upper Olifants catchment (DWAF, 2004). Four AMD impacted streams with visible bright-green macroalgal mats were sampled during the winter months (March to July of 2012). The minimum water column temperature during the sampling period ranged between 10 and 14 °C. Table 1 summarizes the physicochemical habitat characteristics of each sampling site (e.g. bottom substrate).

Table 1 – Bioaccumulation and water chemistry characteristics of different sampling sites during winter.

Location		Site 1, Klip stream	Site 2, Brug stream	Site 3, Blesbok stream	Site 4, Groot stream
Co-ordinate	(Lat, long)	S 25°37.290′ E 29°12.752′	S 25°51.424′ E 29°08.139′	S 26°11.527′ E 27°72.314′	S 26°11.110′ E 27°72.273′
Substrate tape		Cobbles, boulders	Cobbles, boulders	Cobbles	Cobbles, boulders
AMD source		Decanting surface flows from coal mines	Seepage from decanting coal mines forming a stream	Decanting water from coal mine	Decanting surface flows from coal mines
Stream cross-sectional area	(m^2)	6–9	2–3	5–7	2–4
pH		3.2	3.1	3.4	2.9
Temperature	(°C)	14	13	14	14
Depth	(cm)	25	21	18	22
Al	Algae bioaccumulation (mg/kg d wt)	14 406	46 292	18 867	2143
	Water column chemistry (mg/l)	4.33	4.83	0.14	3.9
	Bioconcentration factor (BCF)	3327.02	9584.27	134 764.28	549.48
Fe	Algae bioaccumulation (mg/kg d wt)	24 325	37 280	34 051	401 739
	Water column chemistry (mg/l)	0.288	9.7	79	8.67
	Bioconcentration factor (BCF)	84 461.80	3843.30	431.03	46 336.68
Mn	Algae bioaccumulation (mg/kg d wt)	184	4195	1629	3586
	Water column chemistry (mg/l)	2.45	3.21	51	17.86
	Bioconcentration factor (BCF)	75.10	1306.85	31.94	200.78
Zn	Algae bioaccumulation (mg/kg d wt)	24	84	143	146
	Water column chemistry (mg/l)	0.23	0.633	0.26	3.9
	Bioconcentration factor (BCF)	104.34	132.70	550	37.43

2.1.1. Epilithic filamentous algae sampling and identification

Epilithic filamentous macroalgae mat samples were collected from cobbles and boulders at the selected AMD impacted sites (Oberholster, 2011). One third of the epilithic macroalgae samples were preserved in the field by addition of 2.5 % (v/v) calcium carbonate-buffered glutaraldehyde, while two thirds were used for metal bioaccumulation analyses and cultivation of axenic macroalgae strains. On site preservation of field samples was carried out according to Clesceri et al. (1998). The collected macroalgae were kept cool at 4 °C and in the dark during the transfer from the field to the laboratory. Macroalgae were identified microscopically using a Zeiss AX compound microscope at 1250 x magnification (Truter, 1987; Van Vuuren et al., 2006). Aliquots (10-100 ml) were sedimented depending on the abundance of the filamentous algae in the samples. Strip counts were made until at least 100-300 individuals of each of the dominant macroalgal species had been counted (American Public Health Association, 1992). Epilithic macroalgae abundance in the samples was determined by counting the presence of each species (as cells in a filament or equal number of individual cells).

2.1.2. Metal bioaccumulation analysis of field samples

All macroalgae samples were stored in acid washed polyethylene bottles at 4 ^{o}C and kept in the dark during transfer from the field to the laboratory. Macroalgal samples were rinsed three times with 1 N HCl and deionised water to remove surface metals and debris after which samples were dried to constant weight at 60 ^{o}C. Triplicate subsamples (50-100 mg dry weight) were digested in concentrated 69 % (v/v) nitric acid to extract metals, which were then determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Sample-based standards were used as described by Jugdaohsingh et al. (1998).

2.1.3. Physical and chemical variables

Water temperature, pH and electrical conductivity were measured *in situ* at each sampling site using a Hach sension^{TM} 156 portable multiparameter (Loveland, USA). Water samples for chemical analyses were collected in 1 litre acid cleaned polyethylene bottles. At the stream site, the bottles were rinsed once with stream water before collection of the final sample. On return to the laboratory, water samples were filtered through 1 µm Gelman glass fibre filters and preserved in 69 % (v/v) nitric acid, after which total metal concentrations were determined by ICP-OES.

2.1.4. Bioconcentration factor

The bioconcentration factor (*BCF*), which is the ratio of the chemical concentration in the organism to the water column, was calculated using the equation $BCF = C_b/C_w$ where *Cb* = concentration of elements in the dry algal biomass (mg kg^{-1}) and *Cw* = concentration of elements in the water (mg/L).

2.2. Laboratory experiments

2.2.1. Cultivation of axenic macroalgae

Based on the microscopic analyses of the macroalgae mats collected at the four field sampling sites, three dominant algae were chosen for the study, namely *K. klebsii* (site 3), *M. tumidula* (site 4) and *O. crassum* (site 1 and 2). The different macroalgae mats were centrifuged and washed 3 times with sterile phosphate buffer saline (PBS) buffer (137 mM Sodium chloride, 2.7 mM Potassium chloride, 8.1 mM Disodium hydrogen phosphate, 1.47 mM Potassium dihydrogen phosphate at pH 7.4). To establish axenic cultures, petri dishes containing the different macroalgae mats were placed under a dissecting microscope and filaments of the dominant macroalgae were isolated and washed 3 times with sterile PBS (pH

7.5; Lonza, Switzerland) buffer containing 10 mg/L germanium dioxide. After the different filamentous macroalgae were isolated and washed, they were placed in 100 ml of algae culture broth medium (Sigma-Aldrich Chemie GmbH, Switzerland) supplemented with 10 mg/L germanium dioxide to inhibit any diatom growth attached to a filamentous macroalgae. To verify if the different macroalgae cultures were axenic, the cultures were examined every 3 days using a compound microscope at 1250 x magnification. Conformation of the different cultured macroalgae species namely *K. klebsii, M. tumidula*, *O. crassum* were also done according to the physiological characteristics of the vegetative cells and morphology of the motile reproduction cells.

After axenic cultures were established, the three different algae cultures, namely *M. tumidula, O. crassum* and *K. klebsii* were filtered and their wet biomass determined prior to inoculation of the algae culture broth (Sigma-Aldrich Chemie GmbH, Switzerland). The different stock axenic macroalgae cultures (100 mg) were poured into 900 ml of sterilized algae culture broth (Sigma-Aldrich Chemie GmbH, Switzerland). Each macroalgal growth culture was prepared in triplicate. Erlenmeyer flasks were shaken at 100 rpm under eight tubular cool white fluorescent lamps, providing ~9000 µMol/m^2/s illumination. Light was set to 12:12-h light–dark cycles and water temperature was kept at 14 ^{o}C (average water column temperature in the winter months as measured at the field sampling sites). The growth rate of algal biomass was expressed relative to total chlorophyll and was measured over a 192 h incubation period according to standard procedures of Porra et al. (1989).

Exposures of the algal culture were conducted in triplicate with stock macroalgae samples exposed to AMD (pH 3) water collected from Site 4 (Table 1) and NaOH treated AMD (pH 5 and pH 7) also from Site 4. As a control, macroalgae were exposed to saline solution (154

mmol/L^{-} NaCl) at different pH levels (i.e. pH 3, 5 and 7). The experiment was conducted over a period of 192 hours with samples collected and analysed at the following exposure times: 0.0 h, 0.1 h, 1 h, 24 h, 48 h, 96 h and 192 h.

2.2.2. Glutathione S-transferase (GST) activity assays

Fresh macroalgae cultures were harvested after exposure by centrifugation at 4 000 g, and washed 3 times by re-suspending the pellets in 1 ml of ice cold PBS buffer(pH 7.4; Lonza, Switzerland) and centrifuged at 4 000 g at 4 ^{o}C. After washing, the macroalgal pellets were reconstituted with 500 ml of ice cold PBS buffer(pH 7.4; Lonza, Switzerland) and homogenized using sonication. Samples were kept on ice at 4 ^{o}C during sonication using a Banson sonifier at 3 x 60 pulse cycle. Sonicated algal tissues were centrifuged at 13 000 g for 15 min at 4 ^{o}C. The supernatant was then transferred into clean eppendorf tubes and kept on ice.

GST activity assay was performed as described by the Sigma manual (Sigma-Aldrich Chemie GmbH, Switzerland). GST activity was assessed at each time interval and at each selected pH value by monitoring the increase in absorbance at 340 nm, at 25 °C for 5 min (Mozer et al., 1983). A reaction mixture of 200 ml was obtained by adding Dulbecco's PBS Buffer; CDNB (1-chloro-2, 4-dinitrobenzene) (5 mM final concentration); reduced glutathione: GSH (10 mM final concentration) and the enzyme extract (2 µg protein). GST activity was calculated as µmol CDNB conjugate mL/min of protein (extinction coefficient, ε_{mM}: 9.6 M cm and path length was 0.552 cm) after subtracting the Δ340 min for the blank reaction from the Δ340 min for each sample reaction. Protein concentrations were determined using a Bio-Rad (Bio-Rad, Laboratories GmbH, Munich, Germany) protein assay with bovine serum albumin as the standard according to the manufacturer's instructions and adjusted to 0.1 mg/ml for all samples.

2.2.3. ICP-MS analysis

For ICP-MS analysis, both control and exposed algae were centrifuged at 13 000 g for 2 min at 4 ^{o}C. Macroalgae pellets were collected and washed with sterile Milli Q water and spun at 13 000 g for 1 min at 4 ^{o}C. The washing step was repeated three times, while excess supernatant were removed using a sterile pipette.

The macroalgae samples were weighed before adding 2 ml concentrated 69 % (v/v) HNO_3 to each vial and left for cold digestion for 24 hours. After 24 hours, 1 ml of concentrated 37 % (v/v) HCl acid was added. The vials were placed in a water bath at 60 ^{o}C until all visible particles dissolved. Samples were allowed to cool to room temperature (25 ^{o}C). The digested macroalgae were decanted into an ICP tube and made up to a final volume of 10 ml with Milli Q water. The vials were cleaned, dried and weighed. The mass of the macroalgae was calculated by subtracting the mass of the empty vial from the mass of the vial containing the macroalgae. The digested macroalgae samples were filtered using a 0.45 µm syringe filter into an ICP-MS sample cup. The sample was analysed using an Agilent 7500cx quadrupole Inductively Coupled Mass Spectrometer equipped with Mass hunter software version G7200. Calibration standards ranged from 1 ppb to 100 ppb prepared in 2% acid.

A modified method of Jones and Laslett (1994) was applied for the analysis of selected trace metals in biological tissue. Data was calculated and processed automatically using the Analysis Batch Mass hunter software version G7200. The mass and volume of the macroalgae was used to converts the results from µg/L to µg/kg.

2.2.4. Statistical analysis of cultured macroalgal experiments

All variables, except pH, were log-transformed to normalise distributions. Two way ANOVA (site and time) was used to determine physicochemical and biological differences: (i) among algae, (ii) between time and (iii) different pH values having different temporal variations. Homogeneity of variances and normality of data were checked prior to data analysis. If significant differences were observed ($p < 0.05$), the ANOVA analysis was followed by a Tukey-b test. Pearson correlations were performed in order to explore the relation between GST and metals. All these statistical analyses were done with SPSS v15.0 software.

Multivariate analyses were performed on the macroalgae differences based on GST activity, metal bioaccumulation and the corresponding time measured. This was done using the CANOCO soft-ware version 4.5 (Ter Braak and Smilauer, 2002).

Parameters for various algae had different magnitudes and scales of measurement, which needed to be normalized (Davis, 1973). Dimensionality and information ordination of the data set were converted to the numerical mean and a variance of one, by subtracting from each variable the mean of the data set and dividing by the standard deviation without reducing or minimizing the meaning. Principal component (PC) analyses were used to identify which parameters were responsible for the variation in the analyses using the programme R (version 3.0.1; 2013). The characteristics roots (eigenvalues) of the PCs were a measure of their associated variances, and the sum of eigenvalues coincides with the total number of variables (Samsudin et al. 2011).

3. Results and Discussion

3.1 Field analysis: bioaccumulation and water chemistry characteristics of locations samples in winter

Three dominant algae were microscopically identified from the macroalgae mats collected at the four field sampling sites, namely *K. klebsii* (cells cylindrical with cross walls surrounded by H-shaped pieces, each with a pariental gridle shaped chloroplast); *M. tumidula* (cells are cylindrical with lamellated walls each with a parietal chloroplast) and *O. crassum* (cells are cylindrical, slightly broader at the anterior end and characterized by one or more ring-like caps immediately below the cross wall).

The bioaccumulation of the different selected metals by the macroalgae mats in the field study, as revealed by the bioconcentration factor (*BCF*) in Table 1, was not related to the concentrations measured in the water column, indicating that certain benthic macroalgae may have a greater preference for certain metals**.**, The concentration of metals measured in the different benthic filamentous green algae at the different sites was as follow: site 1. *O. crassum* Al > Fe > Mn > Zn; site 2. *K. klebsii*, Al > Fe > Mn > Zn; site 3. *M. tumidula*, Fe > Al > Mn > Zn, and site 4. *M. tumidula*, Fe > Mn > Al > Zn. A previous study by Kepler (1986) reported on filamentous blue-green algae *Oscillatoria* sp. collected from a constructed wetland where Mn concentrations were effectively reduced, and found that the sampled algae contained up to 56 g Mn/kg (dry weight) of algae. While the filamentous green algae *M. tumidula*, sampled by the US Bureau of Mines in a constructed wetland contained up to 30-90 g Mn/kg. The Mn concentrations in the later was much higher in comparison to the Mn (1.6 g Mn/kg dry weight) reported in the current study for the same algal species (Table 1) (Hedin and Hyman, 1989).

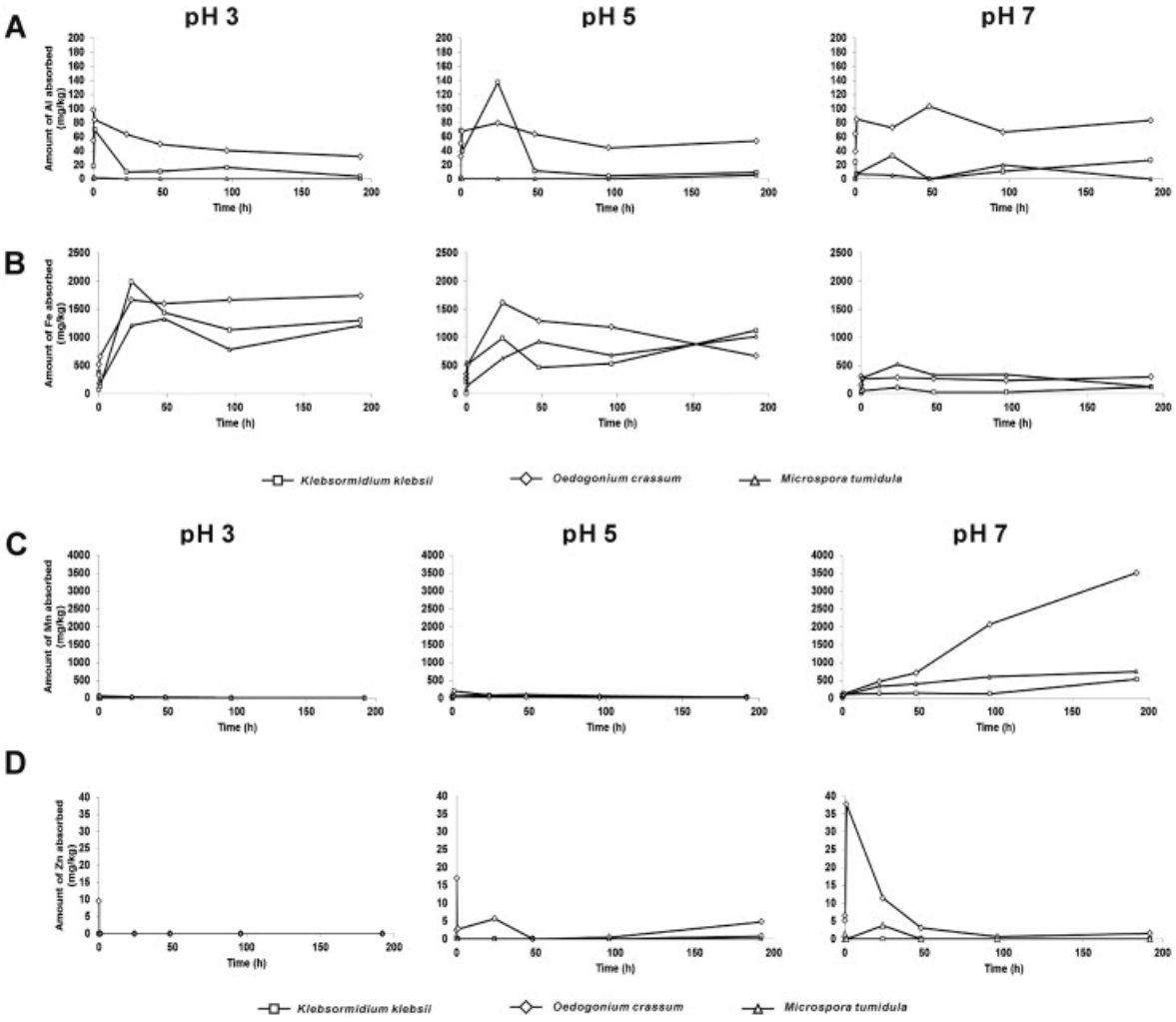


Fig. 1 Concentration of metals bioaccumulated by various algae at three selected pH values. A) Bioaccumulation of Al, B) bioaccumulation of Fe, C) bioaccumulation of Mn, D) bioaccumulation of Zn.

3.2 Concentrations of metals bioaccumulation by various algae under laboratory conditions

A significant correlation was observed between metal concentrations among macroalgae and water, and among different metal concentrations in the algae. The Fe concentrations in the macroalgae correlated negatively ($p \leq 0.003$) with` the Fe in the water column suggesting bioaccumulation of metals (Fig. 1B). There were no significant statistical differences in GST activity among algae species (*K. klebsii*, *M. tumidula* and *O. crassum*) at the pH values 3, 5 and 7. However, a decreased GST activity was observed in the three different macroalgae at

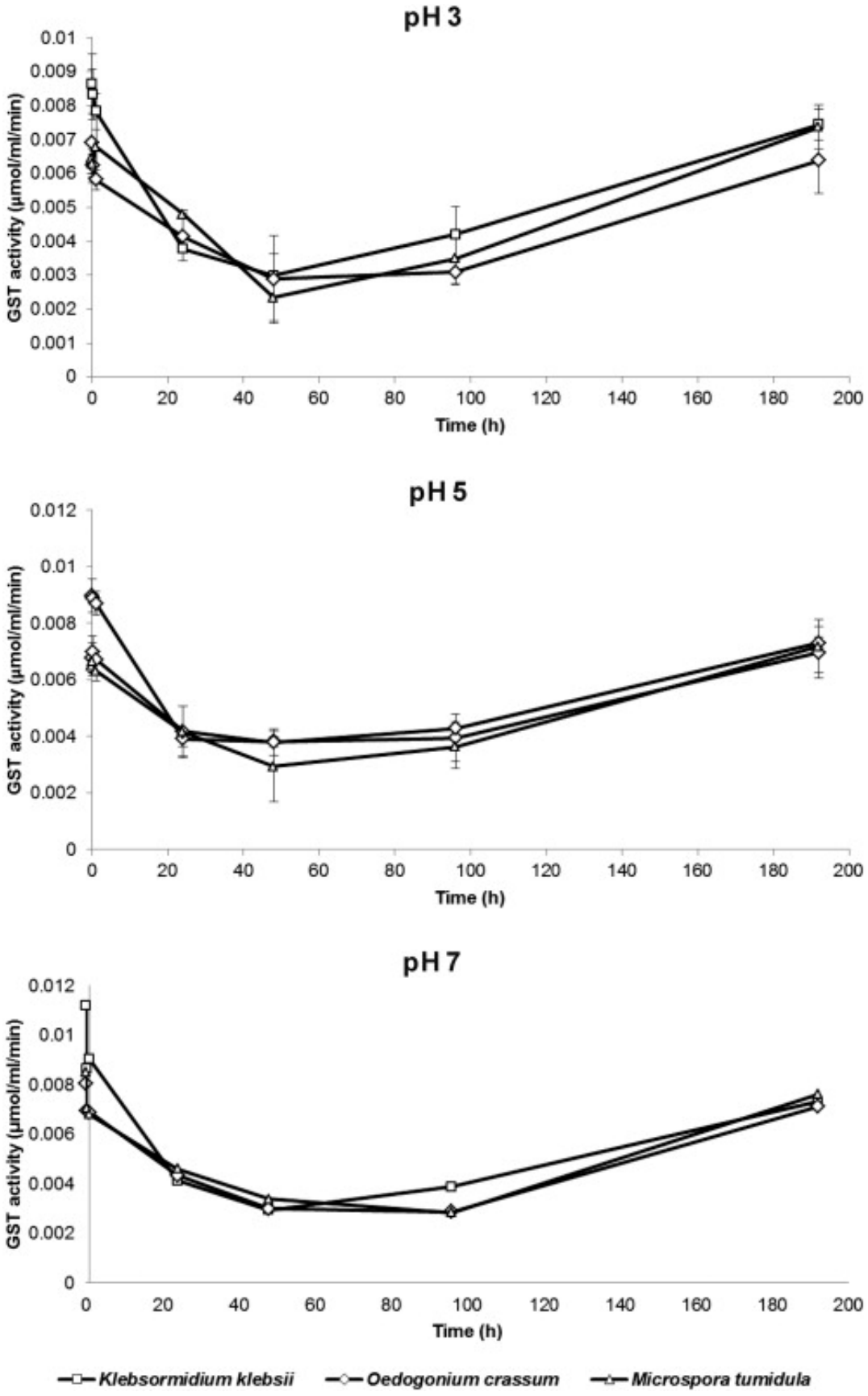


Fig. 2 GST activity of various algae at selected pH values.

all three selected pH values within the first 48 hours of exposure to AMD, while GST activity increased after 48 hours to 192 hours in all three macroalgae (Fig. 2). The reduced GST activity which was observed within the first 48 hours; was possibly due to pre-existing anti-oxidants produced inside the macroalgae to maintain innate oxidative homeostasis (Jamers et al., 2009). However, the increase in GST activity after 48 hours, suggested that a protection mechanism had been activated to produce more anti-oxidative metabolites to ensure survival under AMD conditions. In the course of biotic stress such as under osmotic stress and extreme temperatures GST responses are known to invoke plant defence reactions (Marrs, 1996). Rees (1993) has shown that GST activity increases with water pollution, while a study by Nagalakshmi and Prasad (2001) showed that GST activity increase in the freshwater algal *Scenedesmus bijugatus* after exposure to copper. In the saline control (absence of metals), *K. klebsii, M. tumidula and O. crassum* were subjected to identical exposure conditions as mentioned above, and AMD exposure, as well as GST activity, was monitored. As shown in Fig. 2 no significant difference was observed between the GTS activity of *K. klebsii, M. tumidula* and *O. crassum* under the three different pH values. However, there was a substantial increase in GST activity after 48 to 96 hours ($p < 0.00001$) between the AMD exposures and the saline exposure (results not shown). The impacts of both treated AMD (pH 5 and 7) and untreated AMD water (pH 3) in relationship with the GST activity in the macroalgae, *K. klebsii, O. crassum* and *M. tumidula*, suggests regulation of GST activity in these three macroalgae under the selected pH values.

Although metal bioaccumulation by algae has been reported in the literature, it was found to be highly variable and difficult to compare (Akhtar et al., 2008; Bayramoglu et al., 2006). Many studies have shown that the mechanism of metal bioaccumulation is a complex process

and is dependent on various environmental conditions such as pH, light, flow of water and water temperature, the initial concentration of metal ions, algal biomass and competition of ions for binding sites (Das et al., 2009a, b). In contrast to our field study, the laboratory study did indicate a much higher variation of metal bioaccumulation of the three selected algae at a low pH of 3 (Table 1, Fig. 1). This phenomenon was possibly due to the fact that the algae mats collected in the field study were a collection of different algae species with various metal accumulation capabilities in comparison with the laboratory study where pure three algal strains were studied.

After 192 h exposure to AMD at different selected pH values, the most efficient macroalgae to sequestrate the selected metals (Al, Fe, Mg, Mn, Zn) in the laboratory study were determined and found to be in the following order: *O. crassum* > *K. klebsii* > *M. tumidula*. The order of bioaccumulation of Al by macroalgae at a pH of 3, 5 and 7 was as follows: *O. crassum* > *K. klebsii* > *M. tumidula* (Fig. 1A). However, it was evident that as pH decreased, metal bioaccumulation in the macroalgae decreased as well. This suggests that pH adjustment to neutrality may have affected the chemistry of the metals. This phenomenon has previously been reported by Das et al. (2008), which found that due to metabolic processes the functional metal groups changed, as well as the competition of the metallic ions among themselves for adsorption sites. Gyure et al. (1987) reported that low external pH reduced both surface binding and intracellular influx, which leads to the abundance of hydrogen ions which in turn compete with other cations for binding sites, resulting in poor metal bioaccumulation (Mehta and Gaur, 2005; Orandi and Lewis 2012). Trace metal uptake by macroalgae under acidic conditions in our study was substantially less than that observed at at neutral pH conditions (pH 7). This may be ascribed to the fact that under acidic conditions release of trace metals by hydrogen ion occurred; while at a higher pH, trace metals have the

tendency to render their chelation as observed in other studies (Warren and Haack, 2001; Orandi and Lewis, 2012).

At a pH of 7, the most efficient bioaccumulation of Fe was observed within the first 96 hours, and again after 192 hours by the macroalgae *M. tumidula*. Both *O. crassum* and *K. klebsii* showed an increase in bioaccumulation as time progressed (Fig. 1B). At a pH value of 5, Fe bioaccumulation increased in all three macroalgae in comparison to that observed at a pH of 7. Initial bioaccumulation of Fe, within the first 96 hours was in the following order: *O. crassum* > *M. tumidula* = *K. klebsii*, while changes in algae bioaccumulation occurred after 96 hours: (i.e., absorption was as follows: *K. klebsii* = *M. tumidula* > *O. crissum*) (Fig. 1B). Bioaccumulation of Fe at a pH of 3 increased after 48 hours of exposure, reaching a maximum algal bioaccumulation in the following order: *O. crassum* > *K. klebsii* > *M. tumidula* (Fig. 1B). From the laboratory analyses it was evident that Fe bioaccumulation efficiency in all 3 algal species increased significantly as the pH decreased. This was possibly due to precipitation of Fe at pH values ≥ 4.0 making it inaccessible to algae, whereas at lower pH the dissolved Fe speciation changed due to microbial oxidation/reduction processes which make it more readily available for bioaccumulation by macroalgae. This phenomenon was previously reported by Oberholster et al. (2013) in a study on the occurrence of benthic algae in the Bloubank stream, South Africa. Also, Fe is known to be important in photosynthesis and binds to the redox-active metal (a cofactor) found in proteins (Jeong et al., 2008; Kosman, 2010), which functions as protective mechanisms for survival of algae.

The amount of Mn bioaccumulation by macroalgae at pH of 7 increased considerably when compared to pH values of 5 and 3 (Fig. 1C). At a pH 7, the bioaccumulation was in the following order: *O. crassum* > *M. tumidula* ~ *K. klebsii* ($p < 0.003212$, Fig. 1 C). Whereas at

pH 3 and 5, no significant differences in bioaccumulation of Mn in the three macroalgae species were observed (Fig. 1C). This may indicate that Mn is more readily absorbed under neutral pH ranges in the selected macroalgae and that biological mechanisms possibly modify the Mn speciation at increasing pH. In the case of Zn bioaccumulation, efficiencies among the macroalgae species were as follow: *O. crassum* > *K. klebsii* ~ *M. tumidula* ($p < 0.000001$). As pH decreased the amount of Zn bioaccumulation also decreased in all three algal species ($p = 0.037992$, Fig. 1D). The observed Zn concentration was below detection limit and was therefore found to be not as readily absorbed in all three macroalgae when compared to other metals measured. In large quantities, Zn may have detrimental effects on algae by causing chlorophyll destruction through the inhibition of biosynthesis pathways of pigments (Battah, 2010). It was apparent that under all three tested pH values the selected macroalgae expressed a protective mechanism that prevented large quantities of Zn from entering the cells compared to the uptake of other metal ions.

To date, little is known about Al and its relation to macroalgae metabolic and cellular function. Studies have shown that Al can be toxic to algae (Wang et al., 2011). However, results from this study showed that *O. crassum* may display resistance mechanisms to withstand high concentrations of Al, and hence exhibit low bioaccumulation capacity in comparison to *K. klebsii* and *M. tumidula* (Fig. 1A). The resistance mechanism employed may either prevent initial uptake of the ion or alternatively may provide a means to drive out the ion from the algal cell (Costley and Wallis, 2001). In the macroalgae *O. crassum* the low pH value of 3 contributed significantly to the lower bioaccumulation of Al when compared to that observed in *K. klebsii* and *M. tumidula.* This demonstrated that the pH-dependent binding of metal ions in *O. crassum* differed from that in *K. klebsii* and *M. tumidula*. However, the Al binding capacity in *O. crassum* increased with increasing pH values (Fig. 1A). This is

probably due to metal competition with the hydronium ion for the same binding site, as observed by Say et al. (2003).

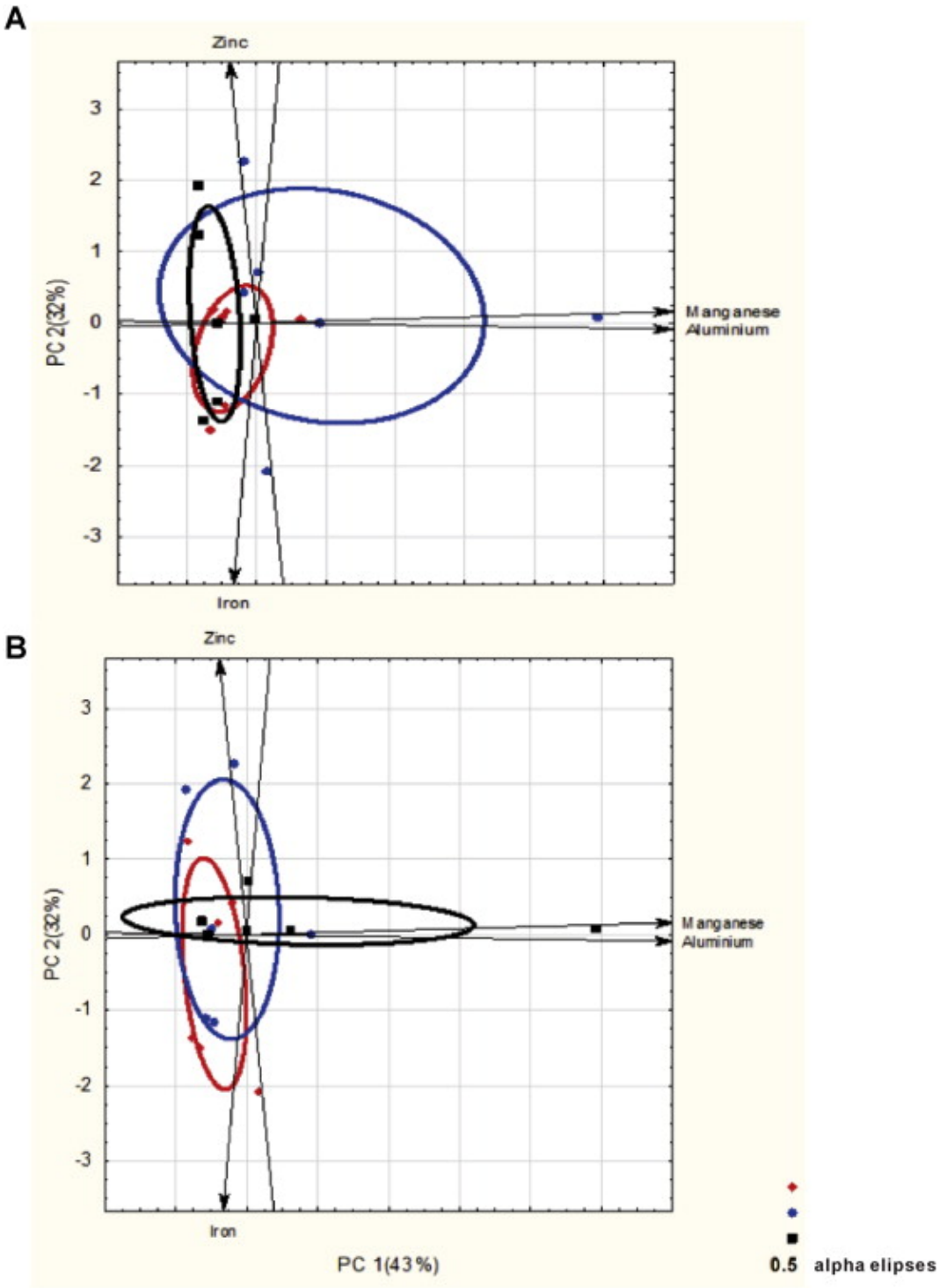


Fig. 3 Principal component analysis plot of metal bioaccumulation. A) Algal bioaccumulation with different metals where red = K. klebsii ; blue = O. crassum ; black = M. tumidula , B) metal bioaccumulation under selected pH values where red represents p...

3.3. PCA of different algae at different pH conditions

The association phenomena between algae species to metals at different pH conditions are unique and specific (Fig. 1). Association phenomena were analysed through principal component analysis. The ordination plot describes 75% of the variation in the data, with 32% on the first axis and 43% on the second axis. In this study, *O. crassum* was found to be more closely associated with Mn and Al, and less associated with iron and zinc compared to *K. klebsii* and *M. tumidula* (Fig. 3A). Similarly PCA analyses were employed to study the impact of metal bioaccumulation by algae at pH 3, 5 and 7 as shown in Fig. 3b. It was observed that bioaccumulation of Mn and Al by algae was preferred at pH 7, while bioaccumulation of Zn by algae was optimal at pH 5. Also, absorption of Fe by all three algae species was preferred at a pH 3.

It is evident from our observations in the current study that the macroalgae *O. crassum* out performed the other algal species with regards to its bioaccumulation capacity of the different metals tested at different pH levels. Therefore the macroalgal *O. crassum* can be used in High-Rate Algal Ponds (HRAP) as secondary passive treatment option following constructed wetlands to treat AMD during the winter months when the metabolic processes in macrophytes and microrganisms in constructed wetlands decreased due to seasonal changes (Fig. 4). Although, AMD treatment by algae is a significant low cost alternative to complex expensive treatment systems, the disadvantage of such treatment is the risk of metals been released when the algae start to die off. Furthermore, the reuse of the algae biomass after recovering from such AMD treatment ponds are also limited to specific uses e.g. biofuel, since it contain high concentrations of metals that may be hazardous for the food web if used as animal or fish food.

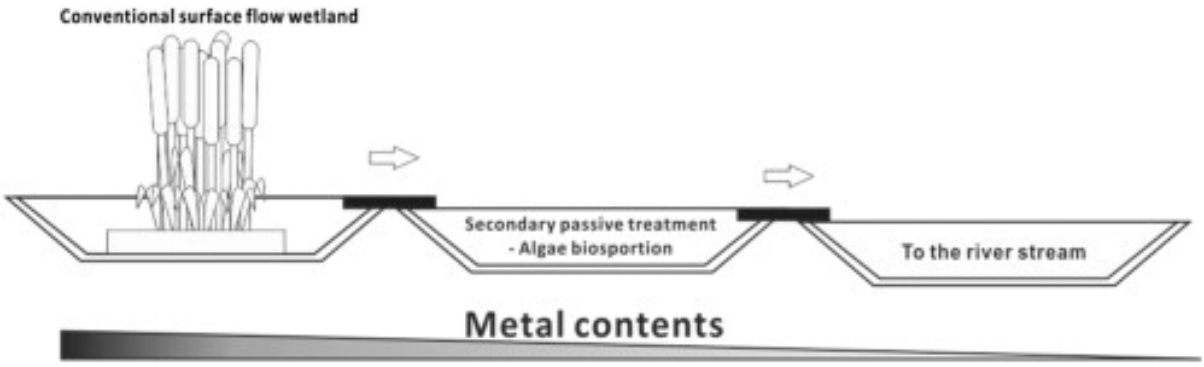


Fig. 4 Constructed wetland with an algae pond as secondary passive treatment.

5. Conclusion

Although macroalgae have been reported to be quite efficient in metal removal from AMD water it was evident from the current study that certain species of benthic filamentous algae e.g. *O. crassum* can be used as part of passive treatment technology by absorbing metals effectively under different pH values from algal ponds during winter months when environmental conditions are unfavourable for macrophytes and microbials in constructed wetlands.

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