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JAMES COOK UNIVERSITY
AUSTRALIA

Bioremediation of coal-fired power station waste water using freshwater filamentous algae from the genus *Oedogonium*

Thesis submitted by

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Statement of the contribution of others

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Abstract

The bioremediation of industrial waste water by macroalgae is a sustainable and renewable approach to the treatment of waste water produced by mining and mineral processing industries. The cultivation of live algae for metal bioremediation has simultaneous benefits as a form of biological carbon capture, particularly if algal growth is supported by carbon dioxide emitted from industrial facilities that also produce waste water, such as coal-fired power stations. However, few studies have tested the bioremediation of complex multi-element waste streams from coal fired power stations by live algae.

Application of algal bioremediation at scale requires an algal species that can grow in waste water while sequestering multiple contaminants. This thesis investigates the ability of multiple species of freshwater green macroalgae from the widely distributed ("cosmopolitan") genus *Oedogonium* to grow in waste water as a means of metal bioremediation. The *Oedogonium* genus is comprised of multicellular filamentous green macroalgae with an ability to grow rapidly in intensive monocultures. This thesis will expand this existing knowledge base by investigating the general ability of multiple species of *Oedogonium* to grow in industrial waste water and sequester metals. The experiments used Ash Dam water produced in the washing of residual ash from the power stations flue stacks and is contaminated by multiple regulated metals (Al, Cd, Ni and Zn) and metalloids (As and Se) in excess of Australian and New Zealand Environmental Control Council (ANZECC) guidelines.

The first experiment tested three species of *Oedogonium* for their growth and concentration of 18 elements from waste water from Tarong power station, a 1400 MW coal-fired power station in Queensland, Australia. All species had consistent growth rates in Tarong Ash Dam water, despite significant differences in their growth rates in

"clean" water. While there were differences in the temporal pattern of metal uptake by the three species, over the course of the experiment all three species accumulated the same elements preferentially and to a similar extent. These results demonstrate that the genus has a consistent ability to grow in waste water and concentrate elements. ANZECC trigger values for the key elements of Al, As, Cd, Ni, Se and Zn initially exceeded guidelines, and all three study species of the genus *Oedogonium* were able to concentrate these elements. There was, however, a much higher bioconcentration of the metals Cu, Mn, Ni, Cd and Zn, with slower accumulation of the metalloids defined here as As, Mo and Se. Bioremediation would therefore be most rapid and complete for metals.

The second experiment investigated the effect of nutrient addition on the growth of the three species of *Oedogonium* in Ash Dam water. The maintenance of high productivity is essential to a successful remediation program as growth is positively correlated with metal sequestration. Tarong Ash Dam water is a complex effluent that contains many of the constituents of commercial growth media, particularly Zn, Cu, Mn, Mo and Fe, and so the additional nutritive requirements of algae grown in Ash Dam water are not clear. I contrasted the growth of three species of *Oedogonium* in Ash Dam water and "clean" water amended with nitrogen and phosphorus, both in isolation and simultaneously, with control cultures receiving no nutrients and f/2 media.

The addition of nutrients enhanced the growth rate of all species of *Oedogonium*; however, the response was specific to each nutrient. The two nutrient regimes that coincided with the highest productivity were the addition of f/2 and the addition of phosphate, regardless of species of *Oedogonium*. The addition of nitrogen alone did not increase the growth rate of any species. Interestingly, the least variation within growth rates was demonstrated in phosphate treatments in Ash Dam water. There was little additional benefit in terms of increased growth by adding the more complex f/2

media. The three species had mean specific growth rates of 2.9 – 7.5% in the no nutrient treatment, increasing to 9.5-10.9% in the P treatment, and 7.6-14.8% in the f/2 treatment. Overall, these results strongly suggest that P is the most limiting nutrient in both Ash Dam water and, furthermore, that there are no clear differences in the nutritive requirements of the three species of algae in Ash Dam water and dechlorinated town water.

The results show that the species of *Oedogonium* evaluated in this study all have an ability to grow in complex waste water and deliver similar rates of remediation. Consequently, due to its widespread distribution, high growth rates in intensive culture and, through this study, proven ability to grow in and remediate waste water, *Oedogonium* is a key target for scaled bioremediation applications. Macroalgal bioremediation can clearly remediate a complex suite of pollutants, providing an innovative and sustainable bioremediation process. The scope for remediation is greatest for metals (Al, Cd, Cu, Mn, Ni and Zn), with slower remediation of metalloids (As, Mo and Se). These data provide a predictive context to determine the ability of live algae to remediate complex waste waters containing multiple targets of remediation.

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Chapter 1

General Introduction

1.1. Waste water production in the coal-fired energy generation industry

Coal-fired energy generation produces a complex multi-element waste water stream from the washing of flue stacks to remove combustion by-products and residual ash (Klein et al., 1975). This waste water contains a high density of ash and high concentrations of a range of metals and metalloids (Saunders et al., 2012). As such, this waste water is typically not discharged but rather stored in large catchments on site known as Ash Dam water storages. The accumulated, and eventually concentrated, dissolved metals and metalloids in the Ash Dam water cannot be biodegraded by micro-organisms into non-toxic or volatile compounds, as is frequently the case with organic pollutants (Hubbe et al., 2011; Lobban & Harrison, 1994), and therefore remain in the environment in a number of chemical states with the potential to be assimilated and accumulated into the food chain (Mane et al., 2011). This assimilation and accumulation has critical implications for the management of Ash Dam water as these storage structures often support water birds and fish and therefore are a source of diverse, and often complex mixtures, of toxicants to local vertebrate populations despite their apparent confinement (Yang et al., 2010).

Given the environmental issues associated with the storage of Ash Dam water there is a pressing need to develop and implement sustainable treatment methods. Existing treatment methods for metal and metalloid-contaminated waste include lime precipitation, coagulation and co-precipitation as metal hydroxides, electro-chemical treatment, ion exchange (e.g. resins), and membrane separation (Sawyer et al., 2003; Tchobanoglous et al., 2003). However, the inherent high capital costs for these

processes has meant that waste producing industries have often not implemented their use and instead stock-pile waste water, relying on evaporation and on-site reuse where possible to reduce the volume of water in the storage dams. This has led to the investigation of more cost-effective water treatment options, such as the use of natural materials including algal biomass to passively adsorb (dead algae) or actively absorb (live algae) metals from solution (Fu & Wang, 2011; Hubbe et al., 2011; Mehta & Gaur, 2005; Saunders et al., 2012).

1.2. Live algal culture as a bioremediation strategy

Algal cell walls are a natural ion-exchange material because they have many anionic groups on their cell surface (Kratochvil & Volesky, 1998) and this enables them to remove metal ions efficiently, mainly by means of an ion-exchange mechanism or passive electrostatic attraction (Crist et al., 1994; Hubbe et al., 2011; Mehta & Gaur, 2005; Schiewer & Volesky, 1996). The constituents of algal cell walls that contribute to this process vary depending for taxonomic grouping of algae, but are primarily alginate polysaccharides (Phaeophyceae, "brown algae") and proteins (Chlorophyceae, "green algae"), both of which provide functional groups that bind metal ions in solution (Kloareg & Quatrano, 1988). Metal cations can bind by electrostatic attractions to these anionic charged sites (Crist et al., 1994; Crist et al., 1988; Crist et al., 1981). Traditionally, research into the bioremediation of metalliferous wastewaters has focused on dried algal biomass (Hubbe et al., 2011). The removal of metals from solution by dried algal biomass through biosorption has been studied extensively at small scales. However, there are several barriers to implementing and demonstrating biosorption at scales relevant to the needs of industries (Hubbe et al., 2011; Saunders et al., 2012) with perhaps the greatest barrier being the absence of a cost-effective and sustainable source of algal biomass required to support industrial applications.

An alternative to the use of dried algal biomass that circumvents the need for an exogenous biomass supply is to culture live biomass within waste streams at industrial facilities. This approach has not been broadly investigated but potentially has widespread application for waste streams with dissolved metals and metalloids across mining, mineral processing and energy production (Saunders et al., 2012). The limited consideration of live culture of algal biomass directly in metal-contaminated waste streams is in part due to concerns about the potential toxicity of the effluents to live algae, and that divalent metal ions may show greater affinity for dried biomass than live tissues (Gadd, 2009; Kratochvil & Volesky, 1998; Volesky, 1999). Metals may induce a range of negative effects in live algae, including oxidative stress (Bray, 2007). However, there are a broad diversity of algae that can be cultured within metal-contaminated waste streams (Doshi et al., 2007; Lee & Chang, 2011; Mane & Bhosle, 2012; Saunders et al., 2012). Furthermore, some species of algae have compensatory mechanisms to deal with luxury metal supply, such as the over-production of metal-binding substrates in the cell wall that may act as a detoxifying mechanism (Andrade et al., 2010). An additional benefit of live algae is that the affinity of divalent metal ions for constituents of the algal cell wall is such that algae can assimilate metals through passive uptake, followed by facilitated internalisation as they grow (Escuder-Gilabert et al., 2001; Lavoie et al., 2012; Volland et al., 2011). Live algae therefore represent a continuously replenishing substrate for metal removal, as opposed to dried biomass which rapidly saturates and requires treatment before re-use (Lee & Chang, 2011; Mane et al., 2011).

The culture of living algal biomass is currently being implemented at pilot-scale, or utilised, for the remediation of municipal, agricultural and aquaculture wastewaters (de Silva et al., 2008; Park & Craggs, 2010, 2011; Paul & de Nys, 2008; Troell et al., 2009) with the most established practice being in land-based aquaculture, where nutrients can be recycled from waste into valuable products (Bird et al., 2011; Troell et al.,

2009). However, there is an increasing focus on the application of algal biomass in the remediation of industrial pollutants, in particular metals (Bird et al., 2011; Esmaeili et al., 2010; Hubbe et al., 2011; Mehta & Gaur, 2005). Actively growing algal biomass concentrates metals including aluminium, cadmium, chromium, copper, gold, lead, manganese, tin, silver and zinc (Gadd, 2009). The bioconcentration of metals from complex effluents is dependent upon the species and growth rate of an alga (Boullemant et al., 2009; Escuder-Gilabert et al., 2001; Genter, 1996; Ghimire et al., 2008; Stengel et al., 2005). The capacity for bioremediation also varies according to the concentration of elements in the wastewater stream and the availability of nitrogen and phosphorus for growth (Mehta & Gaur, 2005; Saunders et al., 2012). Growth rates (i.e. productivity) affect metal uptake and concentration as new growth facilitates new binding sites for metals externally and internally and biomass productivity is a key driver for the optimisation of algal bioremediation. Algae are also present in most aquatic environments and are adapted to saline, brackish and freshwater habitats (Lobban & Harrison, 1994) providing a broad biodiversity from which to select species of algae to trial. Given these properties, the direct aquaculture of algae in contaminated effluent streams has the potential to remediate inorganic nutrients, metals and organic compounds (Doshi et al., 2007; Lee & Chang, 2011; Mane & Bhosle, 2012). However, to be successful in bioremediation applications associated with coal fired power generation algal species must be able to grow and survive in wastewater effluent streams containing high concentrations of metals in solution, they must have a capacity to concentrate contaminants, through both passive (adsorption) and active metabolic processes (absorption) (Boullemant et al., 2009; Saunders et al., 2012), and they must be amenable to high productivity in intensive mono-cultures. Therefore, the selection of species for culture, the nutrient requirements for culture, and the productivity of algae in contaminated waters must be firstly determined. Secondly,

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biomass productivity must be optimised where possible, to deliver a successful bioremediation strategy (Saunders et al., 2012).

1.3. *Oedogonium* as a key target genus for scaled remediation

The genus *Oedogonium* was selected for this study as it satisfies many of these criteria (Lawton et al., 2014) and is able to survive and grow rapidly in Ash Dam water (Saunders et al., 2012). *Oedogonium* was also selected because it has simple culture requirements, has high productivity and outcompetes other freshwater species when grown in polyculture (Lawton et al., 2013). These characteristics support the ability of species of *Oedogonium* to target metal species whilst successfully growing in Ash Dam water (Saunders et al., 2012). Furthermore, the genus is cosmopolitan in its distribution and understanding its bioremediation capacity in this study also provides a template to investigate the genus more broadly across multiple contaminated environments. Notably, the factors that affect growth and overall productivity may also impact on the bioconcentration potential of the chosen algae, making it necessary to simultaneously quantify algal growth and bioconcentration to accurately quantify and understand the dynamics of bioremediation.

1.4. Thesis aims

The aim of this research is therefore to investigate the intensive culture of the cosmopolitan freshwater macroalgal genus *Oedogonium* as an innovative bioremediation tool for Ash Dam water that is contaminated from the combustion of coal for the generation of electricity. Specifically, this thesis will test variation in growth and metal and metalloid remediation by *Oedogonium* during their culture in Ash Dam water from the Tarong Energy (1400 MW) coal fired power station at Tarong, Queensland, Australia. The first data chapter of this thesis (Chapter 2) quantifies

between-species variation in the growth of three species of *Oedogonium* (Genbank: KC606914, KC701473 and KC606977) (Lawton et al., 2014) in dechlorinated town water supply (DTW) and Ash Dam water, and the bioremediation a range of metals and metalloids ($n = 18$) from Ash Dam water, through this culture process (Chapter 2). The second data chapter (Chapter 3) compares the productivity of the three species of *Oedogonium* under a range of nutrient addition regimes to understand the relationship between nutrient addition to complex waste water streams and algal growth rates. This data will provide baseline information on the application of *Oedogonium* to remediate complex wastewater.

Chapter 2

Growth of the genus *Oedogonium* and metal

accumulation in Ash Dam water

This chapter has been published as:

1.1.   Introduction

The production and harvest of algae can provide an effective mechanism to remove metals from industrial wastewater (Hubbe et al., 2011; Mehta & Gaur, 2005; Troell et al., 2009) as algae concentrate metal ions in their tissues above concentrations in solution (Muse et al., 2006; Saunders et al., 2012). The accumulation of metal ions by algal cells can be divided into two components. The first, adsorption, is rapid and metabolism independent (i.e. passive electrostatic reaction) (Volesky, 2007). The second, absorption, is slow and metabolically dependent as active facilitated internalisation. Both processes contribute to the overall bioconcentration of contaminants (Genter, 1996). Once concentrated within the algae the removal of the metal contaminants can be accomplished by harvesting the biomass (Cunningham & Ow, 1996).

Bioconcentration, and consequently bioremediation, varies depending on the species of algae, the growth rate of the species, and the characteristics and concentration of metal ions in solution (Genter, 1996). The absorption, transport and distribution of metal ions in algae are governed largely by the chemical properties of inorganic ions and the associated effects of partitioning between the lipid and aqueous phases within

the algal cells (Hubbe et al., 2011; Lobban & Harrison, 1994). Many metals (e.g. Cu and Zn) are essential trace elements for algal growth at low concentrations and algae have evolved efficient cellular mechanisms to sequester these elements from the environment. However, in metal-contaminated waste water these metals can be accumulated by these same pathways to concentrations much higher than background concentrations (Pawlik-Skowrońska, 2001, 2003). Key molecular properties of metal ions are hydrophobicity, degree of ionisation and molecular shape and size (Hubbe et al., 2011). Once metals are absorbed into the cell, intracellular partitioning and accumulation occurs. This is a complex process with interacting mechanisms based on the binding of metal ions with intracellular compounds and the precipitation of metals inside the cell (Escuder-Gilabert et al., 2001). There is a correlation between the growth of algae, producing new cells for the bioconcentration of metal ions and the total removal of metals, with higher growth rates providing more biomass for bioconcentration (Roberts et al., 2013).

Therefore, maintaining high algal growth and productivity is the cornerstone to developing bioremediation technologies that are dependent on biomass outputs (Roberts et al., 2013; Saunders et al., 2012). By increasing algal productivity, the rate of metal remediation may be enhanced through the creation of fresh algae tissue to accumulate metals from solution. Optimised productivity is determined by quantifying growth in an environment with an unlimited supply of nitrogen, phosphorous and trace elements, which are the limiting elements for algal growth at high densities (Lobban & Harrison, 1994).

This chapter quantifies the productivity and metal bioremediation of three species within the genus *Oedogonium*. In doing so, these data will quantify variation in growth and the bioconcentration of metals and metalloids to determine whether species from the genus have a consistent ability to grow in contaminated water or, in contrast,

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whether there is variability in the suitability of species for bioremediation applications. Together, these data will provide the fundamental information that is required to assess the suitability of these species for broad-scale bioremediation applications in industrial waste waters.

1.2. Materials and methods

1.2.1. Biomass and effluent collection

Three species of filamentous algae from the genus *Oedogonium* were used in the cultivation experiments. One species was collected from the Ash Dam water storage at Tarong power station in October 2012 ('Tarong *Oedogonium*'), while the other two species were collected from irrigation ditches in the Brandon sugarcane region. Attempts at species level identification of the three species were made using taxonomic keys (Yee & Entwisle, 2011), however, the lack of clear morphological characteristics meant that the species could not be identified beyond genus level using the methods of classical taxonomy. The species were then assessed using molecular techniques and each was a unique genotype, supporting its assignment as a unique species (Lawton et al., 2014). As all three species could not be matched to extant species of *Oedogonium* on the basis of classical taxonomic features they are hereafter referred to by their GenBank accession numbers. The Tarong *Oedogonium* species has the accession number KC606914, while the two species isolated from the Brandon region (19.56°S, 147.36°E) in Far North Queensland, have the accession numbers KC701473 and KC606977. All three species have been maintained in library and stock cultures at the Marine and Aquaculture Research Faculties Unit (MARFU), James Cook University (JCU), Douglas campus (19.33°S, 146 .76°E), in dechlorinated town water supply (DTW) with f/2 media addition since collection.

Ash Dam water was sourced directly from the Tarong coal-fired power station in south-east Queensland (26.76˚S, 151.92˚E) and transported to James Cook University (JCU), Townsville in clean plastic 1000 L Intermediate Bulk Containers (IBCs) in November 2012. The Ash Dam water was then stored at ambient temperature in 12,000 L storage tanks. A sub-sample of this water was taken from the storage tank for this study in December 2012 and subject to metals analysis to quantify initial conditions in the effluent (cf. results section, Table 2.1).

1.2.2. *Experimental design*

A cultivation study was conducted to comparatively assess the growth and metal and metalloid bioremediation of three species of *Oedogonium* in the Ash Dam water. The experiments were conducted within a climate controlled facility (28˚C, 100 μmol photons m^{-2} s^{-1} and a 12:12 light: dark cycle). Stock cultures of each species were maintained for five weeks in DTW with f/2 nutrient addition (0.1 g f/2 L^{-1}) to acclimate them to the experimental conditions. The aim of the acclimation period was to attain 'steady state' productivity, defined here as < 5% change in specific growth rate (SGR) for two consecutive weeks prior to initiating the experiment. This was achieved on day 35 (cf. results section, Figure 2.1), and from this time point (day = 36) onwards the three species were cultivated in either Ash Dam water or DTW with f/2 (0.1 g f/2 L^{-1}) addition as described below, for three consecutive growth periods of seven days.

The three species of *Oedogonium* were cultured in 1.0 L Schott bottles at an initial stocking density 0.5 g fresh weight (FW) L^{-1}. The FW was measured by gently blotting surface moisture from the biomass and weighing it. The Schott bottles were stocked with biomass from one of the three species and filled with either Ash Dam water or DTW amended with f/2 media, and then placed haphazardly in the climate controlled room. The bottles were aerated with compressed air delivered via a Pasteur pipette (at a flow rate of 0.2 L min^{-1}) to each replicate bottle, and they were randomly redistributed

on a daily basis to minimize the potential for light bias between the treatments. Each replicate bottle was then harvested every seven days with a complete water exchange and all replicates (n = 3) were replenished with new f/2 growth media. All replicates were harvested and the biomass dried with paper towel and weighed to the nearest 0.1 g. Subsequently, the stocking density was reset to 0.5 g FW L^{-1} from this weighed biomass, for all replicates of all species. Any remaining biomass from each harvest was then dried in a dehydrator for 48 hours at 60℃. All the dried biomass was stored at 4.0℃ for further analysis.

Growth rates for each treatment were calculated based upon the FW determined for eight consecutive growth periods of seven days (t = Day 7). The SGR (% FW d^{-1}) was calculated using the equation:

$$SGR = Ln (W_f / W_i)/T*100$$

Where W_f = the final weight (g FW) of biomass, W_i = the initial weight (g FW) of biomass, and T = the number of days in culture (7).

1.2.3. Elemental analysis

The algal biomass and the two water sources (Ash Dam water and DTW) were each analysed for the same 18 elements (Table 2.1). Water samples were collected using a 60 mL syringe and filtered (0.45 µm) to remove particulates. The concentrations of the 18 elements (Table 2.1) were also determined for all three species of *Oedogonium* grown in the Ash Dam water treatments. All biomass was prepared for the analysis by drying in a dehydrator for 48 hours at 45℃. A mini mum of 100 mg dry weight (DW) of algae was required for accurate determination of the elemental composition. For the elemental analyses, 100 mg samples of the dried algae were placed into digestion vessels with 2.5 mL SupraPure (Merck Germany) double distilled HNO_3 and 1.0 mL AR Grade H_2O_2. The mixture was left to stand in the fume-hood for two hours to allow the

reaction to complete. The vessels were then heated to 180°C in a microwave oven (Milestone Starter D) and maintained at this temperature for ten minutes. After cooling to room temperature, the digested samples were diluted to 100 mL with Milli-Q water in a volumetric flask. No further dilution was needed before elemental analysis.

For both digested biomass and water samples, elemental analysis was carried out using two instruments. Major elements (Al, Ca, K, Na and P) were measured using a Varian Liberty Series II Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). The remaining elements were measured using a Varian 820-MS Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Many of the elements present in Ash Dam water (in particular As, Se and V) may be subject to Cl^- polyatomic ion interference. To assess the potential for false positives, Ash Dam water was spiked with 1 ppb As, Se and V and measured three times for quality control with recovery between 102 and 108% indicating no significant interferences. Collisional Reaction Interface (CRI) was employed when interferences were detected. These measurements were completed by the Advanced Analytical Centre (AAC) at James Cook University. External calibration strategy was used for both instruments with a series of multi-element standard solution containing all the elements of interest and the results were reported after subtracting the procedure blanks.

1.2.4. Statistical analysis

As the aim of the experiment was to compare long-term growth rates for the three species of *Oedogonium* in Ash Dam water with DTW, the mean growth rate was determined for each replicate over the course of the three week experimental period. The mean growth rate was then analysed by two-factor analysis of variance (ANOVA) for the fixed factors of water source (Ash Dam water and DTW) and species. Where necessary the growth data were log-transformed (Quinn & Keough, 2002). Residual histograms and scatter plots of residuals vs. estimates were assessed to determine

normality and homogeneity of variance respectively (Quinn & Keough, 2002). Post-hoc comparisons for main effects and their interactions were made using Tukey's HSD multiple comparisons.

The concentrations of metals in the algal biomass were first subject to multivariate ordination with non-metric Multi-Dimensional Scaling (nMDS) to assess multivariate patterns in the temporal profile of metal bioaccumulation from Ash Dam water by the three species. The mean data for the metal concentration within the biomass (mg kg^{-1}) were reassembled in a Bray-Curtis similarity matrix, focusing specifically on elements for which there are existing Australian and New Zealand Environmental Control Council (ANZECC) water quality criteria (see Table 2.1 for ANZECC water quality criteria). Mean metal contents of the three species were then contrasted for individual metals with a one-way ANOVA as above. The elemental concentration of the algal biomass was also converted to a bioconcentration factor (BCF) for elements that have stipulated ANZECC trigger values. The BCF is the ratio of the element concentration in the water, to the final concentration in the biomass (DeForest et al., 2007). The bioconcentration factor (BCF) was calculated using the equation:

$$BCF = C_b / C_w$$

Where, C_b is the concentration of the element in the dry macroalgal biomass (mg kg^{-1}) and C_w is the initial concentration of the element in the water phase (mg L^{-1}). The BCF is therefore a dimensionless ratio that expresses uptake of elements relative to availability in the water sample.

1.3. Results

1.3.1. Elemental profile of Ash Dam water and DTW

The elemental composition of the Ash Dam water was complex with several metals and metalloids at concentrations significantly higher than the relevant ANZECC water quality criteria (Table 2.1). The concentrations of Al, As, Cd, Mg, Mo, Ni, Se, Sr, V and Zn were all significantly higher in Ash Dam water than DTW, with Al, As, Cd, Ni, Se and Zn concentrations all exceeding the ANZECC trigger values designated for the protection of aquatic life at the 95% level (Table 2.1). The Ash Dam water is therefore a complex industrial effluent with multiple metal (Al, Cd, Ni and Zn) and metalloid (As and Se) targets for bioremediation. The remainder of the results section will focus on those elements that exceed ANZECC water quality criteria as the aim of the study is to ascertain between-species variation in bioremediation potential.

1.3.2. Between-species variation in growth rates

'Steady state' productivity (defined here as < 5% change in SGR for two consecutive weeks) was attained after 5 weeks of acclimation for all three species (Figure 2.1). Acclimation to 'steady state' productivity was conducted to minimise any anomalous growth rates during the experiments as a result of changing photo-regime (µmol photons m^{-2} s^{-1}) and photoperiod associated with the biomass being sourced from outdoor stock cultures and subsequently exposed to climate controlled conditions. During this period, one of the species of *Oedogonium* (KC701473) had a significantly higher mean productivity (approximately 23% SGR) than the other two species (approximately 20% SGR for KC606977 and KC606914; Figure 2.1).

Once the biomass had been acclimated, all three species were exposed to either Ash Dam water or DTW with nutrient addition. There was a significant 'species x water source' interaction for the mean growth rates of the three species of *Oedogonium*

(Table 2.2). The growth rate of one species of *Oedogonium* (KC701473) was significantly higher than the growth rates of the two other species in DTW (Figure 2.2). KC701473 had a mean SGR of 26% in DTW, in comparison to the other species that had mean SGR of 20% (KC606977) and 16% (KC606914) over the three week experimental period (Figure 2.2).

In contrast to the significant difference in growth in DTW, there was no significant difference in the growth rates of the three species of *Oedogonium* in Ash Dam water (Figure 2.2). The overall growth rates for each species were reduced in Ash Dam water relative to those in DTW, with the greatest reduction for KC701473 (decreasing from 26% in DTW to 7.5% in Ash Dam water) (Figure 2.2). The species of *Oedogonium* from the Brandon region (KC606977) and the species from Tarong (KC606914) had slightly higher mean SGR of approximately 15 and 12% in Ash Dam water respectively (Figure 2.2), but these differences in SGR were not statistically significant (Figure 2.2).

Table 2.1. Elemental composition of Ash Dam water and dechlorinated town water.

Element	Dechlorinated town water (µg L^{-1} ± SE)	Ash dam water (µg L^{-1} ± SE)	ANZECC trigger value (µg L^{-1})
Aluminium	10 ± 5.8	**123.3 ± 13.3**	**55**
Arsenic	1 ± 0.6	**33.7 ± 1.2**	**24**
Boron	< LOD	< LOD	370
Barium	< LOD	< LOD	ID
Cadmium	0.1 ± 0.1	**2.5 ± 0.0**	**0.2**
Chromium	< LOD	< LOD	1.0
Cobalt	< LOD	< LOD	ID
Copper	2 ± 1.2	1 ± 0.0	1.4
Iron	50 ± 58.9	50 ± 0.0	ID
Lead	1 ± 0.6	1 ± 0.0	3.4
Magnesium	2 ± 1.2	92.7 ± 0.7	ID
Manganese	1 ± 0.6	4 ± 0.0	1,900
Molybdenum	1 ± 0.6	1,280 ± 35.1	ID
Nickel	1 ± 0.6	**34.7 ± 0.3**	**11**
Selenium	10 ± 5.8	**70 ± 0.0**	**11**
Strontium	56.7 ± 32.7	2,243 ± 63.3	ID
Vanadium	10 ± 5.8	843.3 ± 17.6	ID
Zinc	8 ± 4.6	**55 ± 0.6**	**8.0**

Notes: < LOD - less than Level of Detection (1 µg L^{-1} for most elements); ID = insufficient data to calculate ANZECC 95% trigger values; all data are mean concentrations ± S.E.; bold values exceed the ANZECC trigger value.

Table 2.2. Analysis of variance for the specific growth rate of the three species of *Oedogonium* in Ash Dam water and dechlorinated town water.

Factor	df	SS	MS	F	P
Species (Sp.)	2	71.81	35.91	2.13	0.136
Water source (WS)	1	659.03	659.03	39.13	**< 0.001**
Sp. x WS	2	439.73	219.86	13.06	**< 0.001**
Residual	30	505.22	16.84		

Note: bold values are statistically significant (P < 0.05).
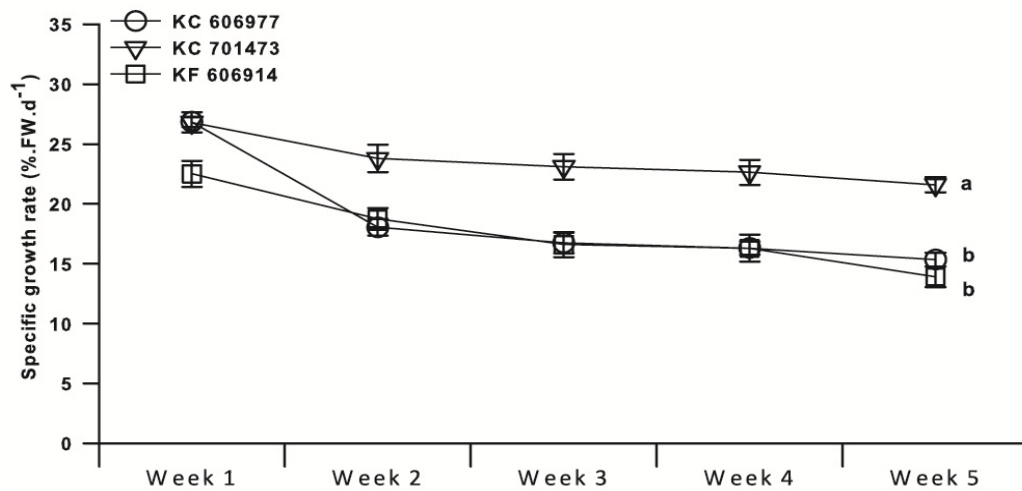
Figure 2.1. Specific growth rate of the three species of *Oedogonium* during the acclimation phase in dechlorinated town water. NB. Species that share a common letter are not significantly different (Tukeys HSD, P > 0.05). Data show means ± S.E. (*n* = 30).
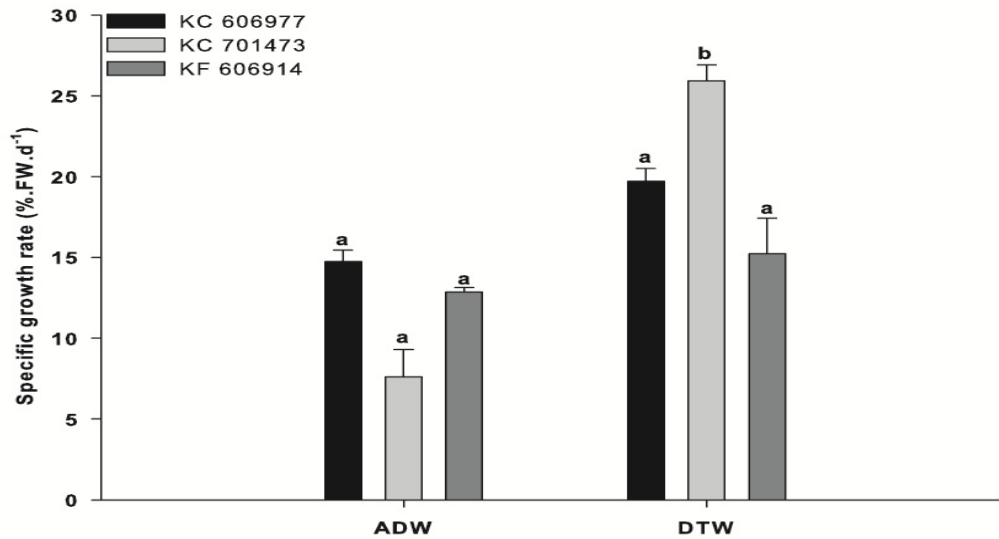
Figure 2.2. Mean specific growth rate of the three species of *Oedogonium* in Ash Dam water and dechlorinated town water. NB. Bars that share a common letter are not significantly different (Tukey's HSD, $P > 0.05$). Post-hoc comparisons contrasted differences in mean SGR for each species within water sources. Data show means ± S.E. ($n = 3$).

1.3.3. Temporal patterns in metal and metalloid bioconcentration

All three species had low concentrations of most elements at the end of the acclimation period in DTW, although KC701473 had a significantly higher K content than the other two species of *Oedogonium* at the start of the cultivation period in Ash Dam water (Figures 2.3a and b; Table 2.3). The clustering of the three species with respect to the vector orientation suggests there is a temporal sequence in metal uptake, but that this differs between the three species. In the first week following the transfer of the cultures to Ash Dam water, all three species accumulated a subset of elements from Ash Dam water (Mn, Pb and Fe, Figures 2.3a and b; Table 2.3), however, KC606914 and KC606977 accumulated the metals Cd, Ni and Zn much more rapidly in the first week than KC701473 (Figures 2.3a and b; Table 2.3). By week two, KC701473 had a similar

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element profile to the other species having accumulated the trace elements Al, Cd, Ni, Se and Zn, while KC606914 clustered with the group of element vectors of Se, Ni, Zn, Cd, Al and B and had the highest internal concentrations of these elements (Figures 2.3a and b; Table 2.3). In the third week of cultivation, the elemental profile of *Oedogonium* KC606914 was similar to its initial (post-acclimation) composition, while the other two species of *Oedogonium* (KC701473 and KC606914) continued to have higher concentrations of elements (Figures 2.3a and b; Table 2.3).

These patterns are also clearly reflected in the temporal sequence of bioaccumulation of metals (Al, Cd, Ni and Zn) and metalloids (As, Se) that exceeded ANZECC criteria. The four metals (Al, Cd, Ni and Zn) were accumulated more rapidly by *Oedogonium* KC606914 and KC606977, than KC701473. There were significantly higher internal concentrations of Al, Cd, Ni and Zn in these two species after one week of exposure to Ash Dam water (Figures 2.4a-d). However, the internal concentrations of Al, Cd and Ni in the three species converged and were not different by the end of the experiment, while Zn remained higher in KC606977 than the other two species at week 3 (Figure 2.4d; Table 2.3). In contrast, bioaccumulation of As differed between the three species, with a significantly higher contents of As in KC701473 than KC606977 and KC606914 by the end of three weeks (Figure 2.4e; Table 2.3). There were no significant differences in the rate or extent of Se accumulation by the three species of *Oedogonium* (Figure 2.4f; Table 2.3).

Despite these relatively minor differences in the temporal sequence of the bioaccumulation of some elements by the three species of *Oedogonium*, there were no statistically significant differences between the mean concentrations of summed ANZECC metals (Al, Cd, Ni and Zn) and metalloids (As and Se) in the three species of *Oedogonium* over the three experimental periods of the experiment (Figures 2.5 and 2.6, Table 2.4). Therefore, all three species of *Oedogonium* accumulated a similar total

concentration of metals and metalloids over the duration of the experiment when short-term variations in bioconcentration on immediate exposure to Ash Dam water were averaged with longer-term metal concentrations in the biomass samples.
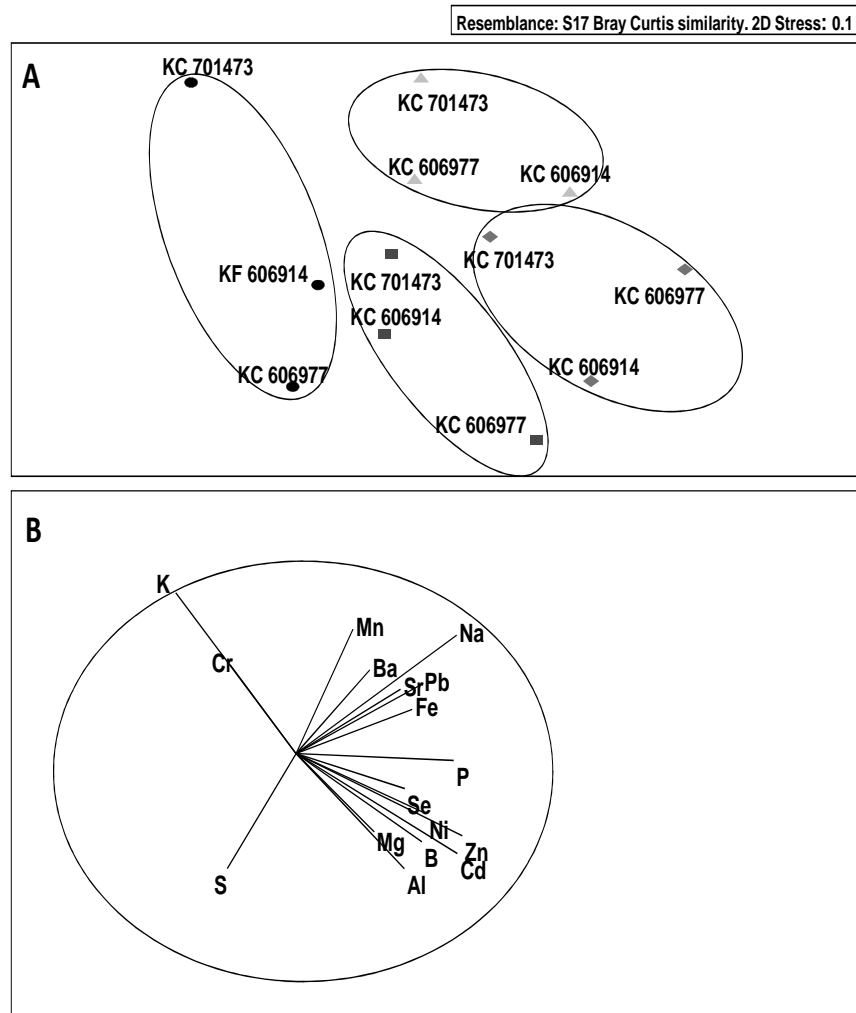
Figure 2.3. Nonmetric multidimensional scaling bi-plot of biomass elemental profiles through time. NB. Circles represent initial stock cultures, triangles represent week 1, diamonds represent week 2 and squares represent week 3.

Table 2.3. Concentration (mg kg^{-1}) of elements in the three species of *Oedogonium* before (initial) and after 1-3 weeks of cultivation in Ash Dam water.

|	KC 606914				KC 606977				KC 701473			
	Initial	Week 1	Week 2	Week 3	Initial	Week 1	Week 2	Week 3	Initial	Week 1	Week 2	Week 3
Al	4.34 ± 0.38	28.4 ± 10.9	73.3 ± 25.2	28.1 ± 2.5	24.3 ± 12.8	32.2 ± 9.1	28.3 ± 5.2	40.9 ± 1.2	8.90 ± 5.67	2.09 ± 1.84	29.6 ± 9.5	41.0 ± 7.8
As	< LOD	20.1 ± 1.0	20.1 ± 1.5	15.6 ± 1.1	< LOD	17.1 ± 2.1	15.4 ± 2.2	16.7 ± 1.5	0.69 ± 0.19	21.6 ± 11.1	35.1 ± 1.8	35.8 ± 5.6
B	7.44 ± 1.69	138.3 ± 18.4	344.7 ± 51.8	231.2 ± 84.1	20.0 ± 0.9	92.3 ± 18.0	103.6 ± 26.7	192.5 ± 58.9	9.22 ± 1.14	47.5 ± 22.3	63.9 ± 2.7	84.2 ± 5.3
Ba	37.5 ± 6.0	185.0 ± 33.2	172.6 ± 38.4	64.3 ± 12.7	34.3 ± 2.9	55.1 ± 8.9	21.7 ± 3.9	16.0 ± 3.3	67.8 ± 8.4	125.7 ± 80.3	94.2 ± 59.6	69.4 ± 22.9
Ca	2,670 ± 514	5,170 ± 143	6,560 ± 147	5,003 ± 987	3,063 ± 434	2,463 ± 223	2,517 ± 404	3,100 ± 407	2,847 ± 448	3,387 ± 508	3,630 ± 236	3,617 ± 309
Cd	< LOD	1.22 ± 0.17	2.38 ± 0.43	1.21 ± 0.17	0.03 ± 0.00	1.97 ± 0.30	1.69 ± 0.23	1.82 ± 0.25	0.06 ± 0.03	0.30 ± 0.20	0.71 ± 0.07	0.98 ± 0.28
Co	0.08 ± 0.02	1.42 ± 0.03	1.45 ± 0.13	0.63 ± 0.03	0.39 ± 0.01	0.51 ± 0.01	0.49 ± 0.05	0.57 ± 0.08	0.16 ± 0.01	0.74 ± 0.35	1.69 ± 0.28	1.01 ± 0.12
Cr	0.36 ± 0.08	0.40 ± 0.04	0.47 ± 0.05	0.39 ± 0.03	0.27 ± 0.02	0.66 ± 0.15	0.35 ± 0.06	0.34 ± 0.00	0.73 ± 0.07	0.56 ± 0.10	0.39 ± 0.04	0.51 ± 0.09
Cu	5.48 ± 1.08	10.24 ± 0.32	9.57 ± 0.40	6.85 ± 0.45	5.65 ± 0.09	8.76 ± 0.70	6.72 ± 0.08	5.33 ± 0.44	9.75 ± 1.02	8.57 ± 1.81	7.39 ± 0.47	10.9 ± 1.0
Fe	590.7 ± 116.6	2,417 ± 185	2,303 ± 95	1,570 ± 85	530.7 ± 52.8	1,180 ± 85	1,147 ± 27	1,042 ± 152	940.0 ± 51.9	1,429 ± 596	1,670 ± 205	2,150 ± 386
K	13,387 ± 2,700	11,637 ± 1,309	8,593 ± 1,751	11,017 ± 1,099	10,283 ± 773	9,650 ± 793	8,447 ± 1,454	6,767 ± 315	25,533 ± 1,433	19,167 ± 3,002	15,533 ± 2,384	14,193 ± 2,678
Mg	2,283 ± 416	2,947 ± 245	4,187 ± 1,282	2,370 ± 115	2,280 ± 180	4,043 ± 979	2,610 ± 315	4,373 ± 1,075	2,900 ± 67	2,910 ± 388	3,007 ± 175	2,930 ± 568
Mn	14.1 ± 2.8	68.8 ± 5.58	50.5 ± 9.0	33.5 ± 2.39	15.0 ± 2.21	25.1 ± 2.95	25.7 ± 0.54	19.9 ± 1.74	35.33 ± 3.35	64.5 ± 8.3	75.2 ± 6.7	60.9 ± 10.9
Mo	1.00 ± 0.12	5.89 ± 0.80	7.70 ± 0.57	5.12 ± 0.77	6.73 ± 0.78	4.28 ± 0.52	3.37 ± 0.58	2.89 ± 0.26	1.43 ± 0.29	4.47 ± 1.13	4.84 ± 0.32	4.15 ± 0.42
Na	6,430 ± 1,262	1,947 ± 446	2,413 ± 225	1,650 ± 191	672.7 ± 35.5	1,497 ± 181	1,350 ± 218	1,477 ± 202	6,657 ± 1,097	7,180 ± 4,910	1,927 ± 126	1,567 ± 62
Ni	0.38 ± 0.06	31.5 ± 3.1	59.6 ± 6.4	39.7 ± 4.2	0.40 ± 0.05	28.3 ± 0.8	30.6 ± 7.9	33.6 ± 3.8	0.62 ± 0.10	8.69 ± 5.13	39.5 ± 2.6	48.5 ± 7.9
P	2,263 ± 453	5,310 ± 446	4,300 ± 374	2,913 ± 224	1,690 ± 95	3,503 ± 155	2,543 ± 71	2,157 ± 308	4,520 ± 289	6,317 ± 1,418	4,937 ± 84	4,483 ± 355
Pb	0.15 ± 0.03	1.82 ± 0.80	0.74 ± 0.25	0.47 ± 0.14	0.18 ± 0.02	0.33 ± 0.16	0.48 ± 0.22	0.22 ± 0.05	0.24 ± 0.05	0.58 ± 0.39	0.49 ± 0.25	0.24 ± 0.02
S	670.7 ± 175.5	3,463 ± 77	4,510 ± 142	3,327 ± 364	279.3 ± 51.2	2,913 ± 180	2,813 ± 651	4,067 ± 662	1,650 ± 132	2,311 ± 938	2,840 ± 207	3,387 ± 472
Se	< LOD	7.97 ± 0.60	11.8 ± 2.3	7.53 ± 0.95	< LOD	4.14 ± 0.26	4.90 ± 1.77	7.50 ± 1.20	< LOD	3.09 ± 1.37	6.07 ± 0.27	14.7 ± 3.9
Sr	28.9 ± 5.0	64.6 ± 32.2	51.0 ± 19.2	21.0 ± 6.9	26.4 ± 2.3	32.0 ± 1.8	23.0 ± 3.2	24.7 ± 2.4	19.9 ± 5.4	54.4 ± 9.6	27.3 ± 5.8	22.0 ± 4.7
V	0.73 ± 0.15	64.3 ± 2.2	51.4 ± 5.1	61.0 ± 11.6	0.81 ± 0.02	39.5 ± 4.7	24.7 ± 1.8	31.4 ± 4.0	1.42 ± 0.05	53.9 ± 27.3	81.9 ± 8.8	74.0 ± 4.8
Zn	25.5 ± 4.8	50.8 ± 10.8	87.4 ± 26.1	32.3 ± 2.2	21.3 ± 1.5	73.2 ± 1.2	78.4 ± 8.8	82.4 ± 9.4	30.3 ± 0.4	29.5 ± 2.3	51.7 ± 7.3	35.9 ± 2.3

Notes: < LOD - biomass concentrations were less than the limit of detection (1 mg/kg / 1 ppm for most elements); all data presented as mean (mg kg^{-1}) ± standard error.

Figure 2.4. Bioaccumulation of individual metals (Al, Cd, Ni and Zn) and metalloids (As and Se) by the three species of *Oedogonium* from Ash Dam water through time. Data show means ± S.E. (*n* = 3).
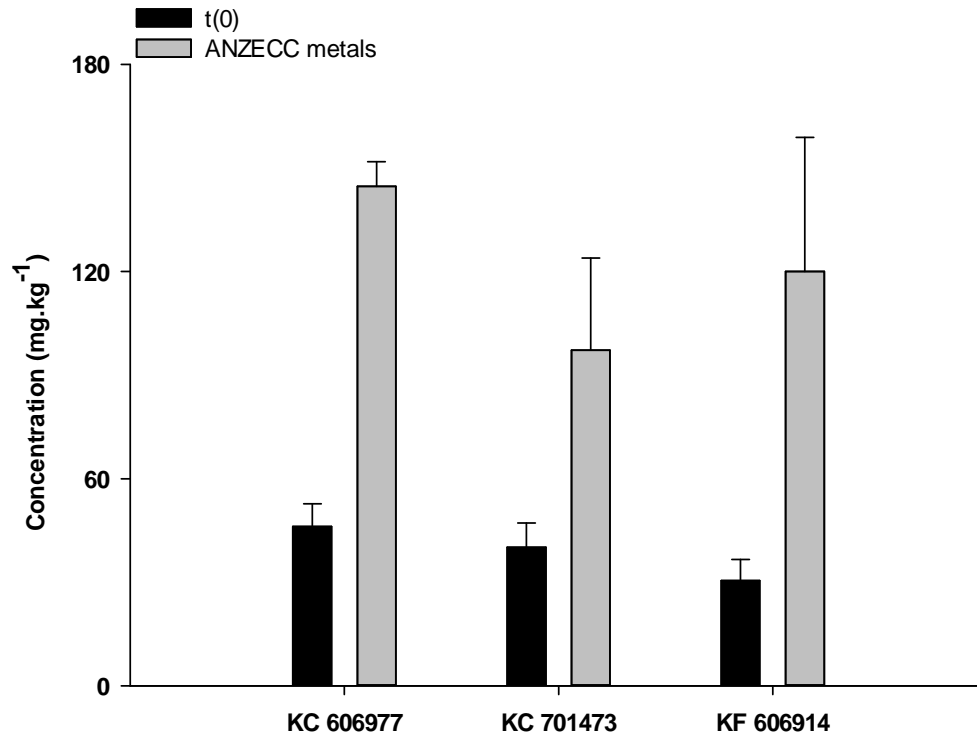
Figure 2.5. Mean concentration of summed ANZECC metals (Al, Cd, Ni and Zn) in the three species of *Oedogonium* cultured in Ash Dam water at time 0 (black bars) and after three weeks of cultivation (grey bars). NB. Bars that share a common letter are not significantly different (Tukey's HSD, P > 0.05). Data show means ± S.E. (*n* = 3).
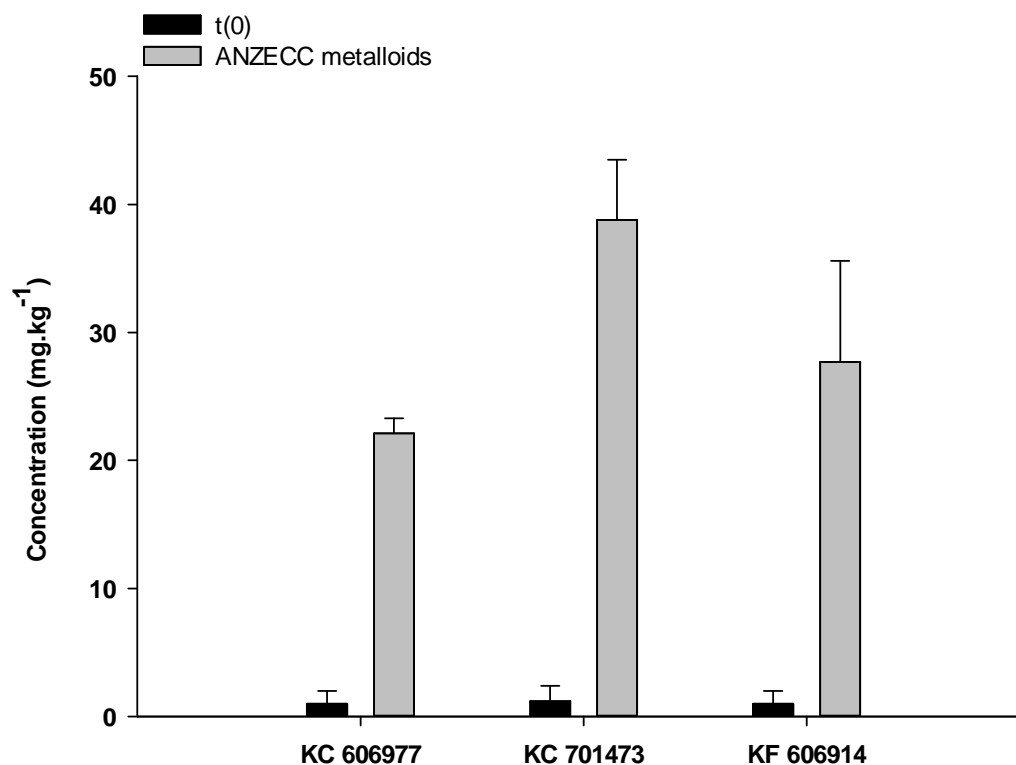
Figure 2.6. Mean concentration of summed ANZECC metalloids (As and Se) in the three species of *Oedogonium* cultured in Ash Dam water at time 0 (black bars) and after three weeks of cultivation (grey bars). NB. Bars that share a common letter are not significantly different (Tukey's HSD, $P > 0.05$). Data show means ± S.E. ($n = 3$).

Table 2.4. Analysis of variance for summed ANZECC metals (Al, Cd, Ni and Zn) and metalloids (As and Se).

Factor	df	ANZECC metals			ANZECC metalloids		
		MS	F	P	MS	F	P
Species	2	1,815.7	1.83	0.24	216.1	3.34	0.11
Residual	6	990.1			64.7		

Table 2.5. Analysis of variance for individual ANZECC metals (Al, Cd, Ni and Zn) and metalloids (As and Se).

Factor	df	Aluminium			Cadmium			Nickel			Zinc			Arsenic			Selenium		
		MS	F	P	MS	F	P	MS	F	P	MS	F	P	MS	F	P	MS	F	P
Species	2	271.8	0.73	0.52	1.14	5.82	**0.04**	156.9	0.77	0.50	1,141.6	3.66	0.09	178.1	7.41	**0.02**	10.1	0.67	0.55
Residual	6	372.8			0.20			203.4			312.7			24.0			14.9		

Note: bold values are statistically significant (P < 0.05).

1.3.4. Individual ANZECC metals and metalloids

As for summed metals, there were no clear differences between the three species of *Oedogonium* with respect to mean bioaccumulation of individual elements over the course of the experiment. The only elements for which there was a significant difference between species of *Oedogonium* were Cd and As. Cd was accumulated to a significantly higher concentration in KC606977 and KC606914 than KC701743 (Figure 2.7b, Table 2.5), while As was accumulated to a significantly higher concentration in KC701743 than the other two species (Figure 2.7e, Table 2.5). The remaining elements showed no significant differences in their bioaccumulation by the three species of *Oedogonium*. (Figure 2.7, Table 2.4). The same pattern was also seen for the majority of elements that did not initially exceed ANZECC criteria, most of which had similar concentrations in the three species of *Oedogonium* at the conclusion of the experiment (Appendix Figure A1, Appendix Table A1).

1.3.5. Metal and metalloid bioconcentration factors

The bioconcentration factors of elements were very consistent between the three species of *Oedogonium*. For all species, the highest BCF was attained for Cu, Mn, Zn and Ni with values ranging from 709-1418 for Zn, up to 5892-16719 for Mn (Tables 2.6 and 2.7). Conversely, Al, Se and Mo all had the lowest BCF with values of 3-4 for Mo up to 259-351 for Al (Tables 2.6 and 2.7). The BCFs attained in the current study were, on average, significantly lower than those previously reported for green macroalgal species in Tarong Ash Dam water (Saunders et al., 2012, Tables 2.6 and 2.7). The ranking of the BCF amongst elements was, however, relatively consistent. In previous reports, Cu, Mn and Zn also had a high BCF, while Se and Mo had a low BCF (Tables 2.6 and 2.7).
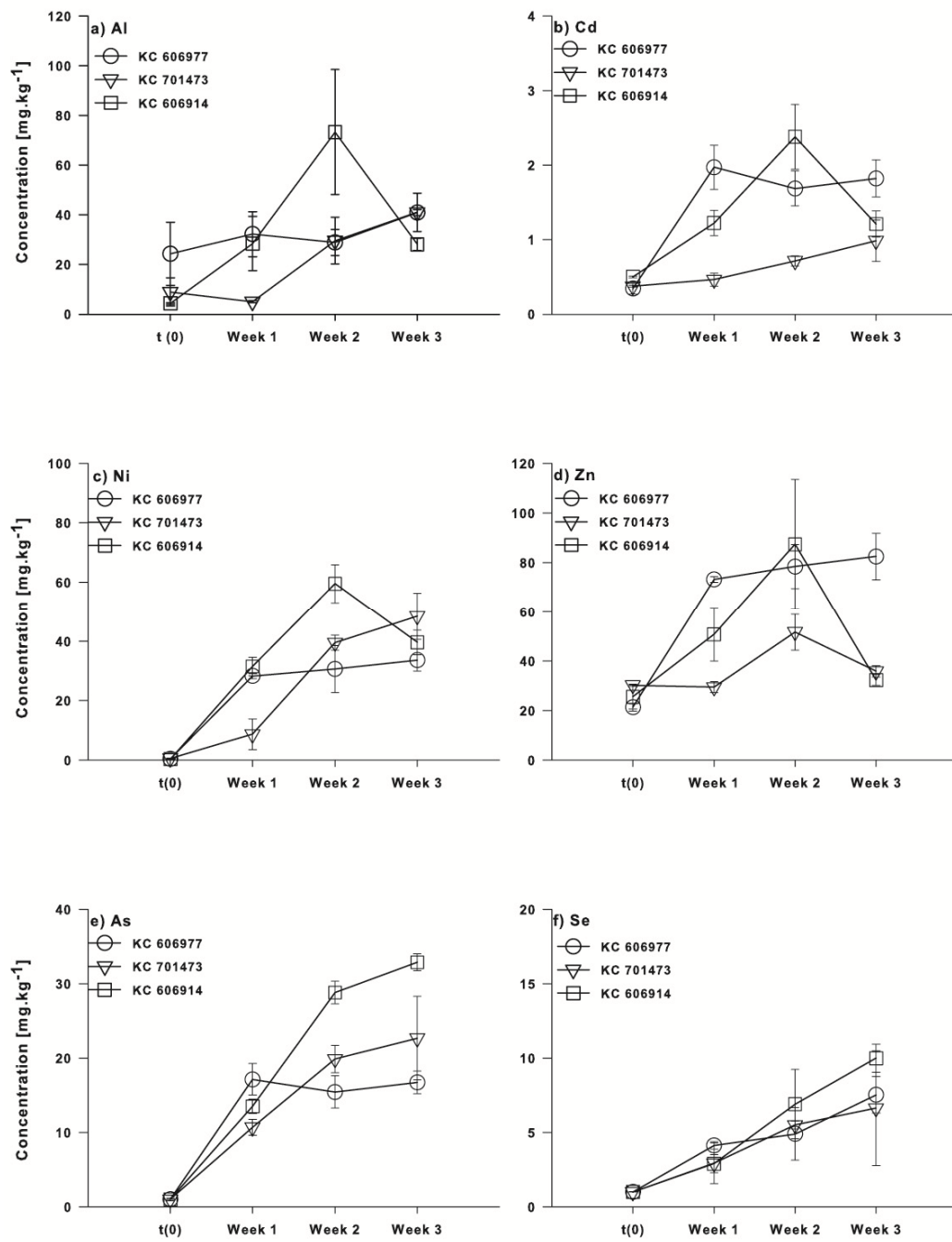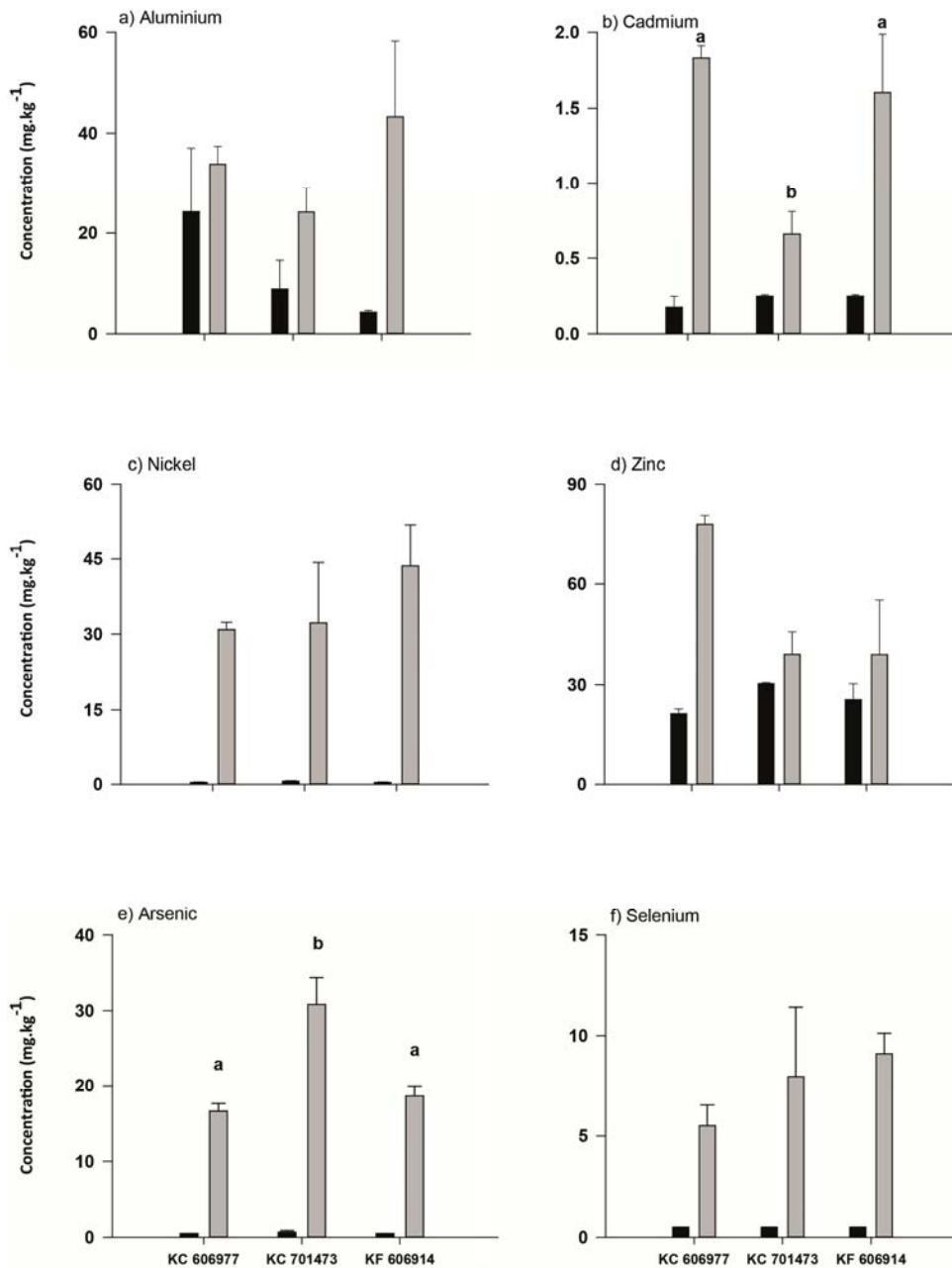
Figure 2.7. Individual metals (Al, Cd, Ni and Zn) and metalloids (As and Se) in the three species of *Oedogonium* in Ash Dam water at time 0 (black bars) and after three weeks of cultivation (grey bars). NB. Bars that share a common letter are not significantly different (Tukey's HSD, P > 0.05). Data show means ± S.E. (*n* = 3).

Table 2.6. Bioconcentration factors for ANZECC-listed elements in Ash Dam water.

Element	Ash dam water (mg L^{-1})	Bioconcentration factor			
		KC606977	KC701473	KC606914	Saunders et al. (2012)
Al	0.1233	276	259	351	17,111
As	0.0337	481	504	721	6,341
Cd	0.0025	730	288	642	1,036
Cu	0.001	6,938	8,943	8,884	3,537
Pb	0.001	346	438	1,009	NA
Mn	0.004	5,892	16,719	12,739	17,116
Mo	1.28	3	3	3	15
Ni	0.0347	889	929	1,256	112
Se	0.07	83	79	93	837
Zn	0.055	1,418	709	1,033	2,879

Notes: data from Saunders et al. (2012) is the mean of three species; NA – not applicable, Pb < LOD in Saunders et al. (2012).

Table 2.7. Bioconcentration factors in order of decreasing magnitude.

Species	Bioconcentration factor
KC 606977	Cu > Mn > Zn > Ni > Cd > As > Pb > Al > Se > Mo
KC 701473	Mn > Cu > Ni > Zn > As > Pb > Cd > Al > Se > Mo
KC 606914	Mn > Cu > Ni > Zn > Pb > As > Cd > Al > Se > Mo
Saunders et al. (2012)	Mn > Al > As > Cu > Zn > Cd > Se > Ni > Mo

1.4. Discussion

To be a suitable candidate for bioremediation applications, a target group of macroalgae should be widely distributed, be robust to variable environmental conditions, be able to out-compete other species in intensive culture, and be able to grow at fast rates. The genus *Oedogonium* meets all of these criteria (Lawton et al., 2013, 2014) in individual studies investigating species within the genus. However, it remains unresolved as to whether individual species within the genus also have a similar capacity to grow and accumulate metals and metalloids from waste water to provide a bioremediation service. My results clearly demonstrate that the individual species within the genus *Oedogonium* that were assessed in this study are equally versatile and robust in terms of growth in a complex industrial waste stream, and in their ability to sequester metals and metalloids from this waste stream to provide a bioremediation service.

The data presented here have shown that the three species of *Oedogonium* assessed have the ability to grow in metal-contaminated waste water, with specific growth rates averaging 12% d^{-1} in complex waste water containing multiple elements for remediation. Interestingly, all three species of *Oedogonium* had similar specific growth

rates in Ash Dam water, despite quite significant differences in specific growth rates in DTW, during both the acclimation and post-acclimation cultivation periods. The growth rates in DTW in this experiment also closely resemble those previously documented for a wide range of *Oeodognium* species in clean water culture (Lawton et al., 2014). The consistently lower growth rates of *Oedogonium* in Ash Dam water demonstrate that the growth rates of individual species in clean-water cultures are not an appropriate proxy for their productivities in metal-contaminated waste waters. Furthermore, the species-level traits that confer more rapid growth rates in DTW do not confer more rapid growth rates in industrial effluent. The specific growth rate of all species was higher in DTW, and for this reason site-specific evaluations of potential growth rates in industrial effluents will be required.

In general, the data support that the species of the genus *Oedogonium* are excellent candidates for metal-bioremediation programs given their ability to deliver consistent growth in complex effluents while accumulating metals and metalloids over time. However, individual species will always require trials to ensure their efficacy in complex waste streams as bioremediation of waste streams will always be site-specific due to the composition of the waste stream and the endemic flora.

In addition to having similar growth rates, the three species of *Oedogonium* also had very similar profiles of metal bioconcentration. While there were minor differences in the temporal pattern of the accumulation of elements between the three species, the mean bioaccumulation over the course of the experiments did not differ for the majority of elements. Each of the species was able to accumulate internal metal and metalloid concentrations by orders of magnitude above their concentrations in the Ash Dam water. The three species also had very similar elemental composition over the course of the experiment, and delivered consistent element-specific bioconcentration factors (BCFs) for each of the ANZECC-listed elements. While the BCFs attained in the

current study were less than those previously recorded in Ash Dam water cultures with species of green algae (Saunders et al., 2012), the relative ranking of BCFs were consistent. The metals Cu, Mn, Zn and Ni were concentrated to a significantly higher degree by the species of *Oedogonium* than the metalloids Mo and Se, as previously documented (Roberts et al., 2013; Saunders et al., 2012). This evidence adds to a clear consistency amongst existing studies that live algal cultures are effective means of sequestering metal cations, but are less effective at sequestering metalloids that occur as oxyanions, particularly Se (as SeO_4^{2-}), As (as AsO_4^{3-}) and Mo (as MO_4^{2-})(Roberts et al., 2013).

The only significant differences with respect to the bioaccumulation of elements were seen for the ANZECC metalloid As and the ANZECC metal Cd. KC701473 had a significantly higher As content than the other two species, but a lower Cd content. Previous research has highlighted the relatively slow rates of metalloid bioaccumulation by green algae relative to the rate at which metals are taken up (Roberts et al., 2013). The slight variance between the three species of *Oedogonium* with respect to As accumulation suggests it may be possible to identify species that are more effective at accumulating these metalloids. Regardless, all species had low BCFs for metalloids, and accumulated them at a slow rate relative to their availability in the effluent. Therefore, the three species of *Oedogonium* delivered equal growth rates, equal rates of metal bioaccumulation, and accumulated the same elements preferentially. The genus *Oedogonium* therefore has a consistent ability to sequester a range of metals and metalloids from complex effluents for broad-spectrum remediation applications.

The ability of freshwater algae, and particularly green algae, to grow in metal-contaminated water is likely a direct result of their ability to sequester and detoxify metals through a variety of passive and active cellular mechanisms (Bray, 2007;

Oberholster et al., 2014; Pawlik-Skowrońska, 2001; Pawlik-Skowrońska, 2003). While not all macroalgae can tolerate high metal concentrations in waste water, most freshwater green macroalgae can produce metal-binding phytochelatins (Pawlik-Skowrońska, 2001) and polyphosphate bodies (Nishikawa et al., 2003) to sequester dissolved metals into storage granules in which state the metals are less toxic to the cell. Green algae can also sequester metals in storage vacuoles, isolating them from sensitive cellular processes (Hanikenne et al., 2005). As a result, green freshwater macroalgae are perhaps the best candidates for large-scale bioremediation.

There has been great interest in the ability of algae to treat industrial waste waters and the use of dried macroalgae as a passive biosorbent for waste water has been well documented (Volesky, 1999, 2007; Volesky, 1994; Wase & Foster, 1997; Yang & Volesky, 1999; Yun et al., 2001). However, the vast majority of this research has focused on understanding the kinetics and mechanisms of metal biosorption by dried algae. Previous research also highlights that the lack of availability of biomass for the production of algal biosorbents is a barrier to industrial applications of biosorption using dead algae (Gadd, 2009). Interestingly, the production of an endemic and fast growing species of macroalgae on-site at industrial facilities would firstly provide the initial step in the bioremediation process by removing metals and metalloids from the waste water. Second, it would negate one of the most problematic and costly components to the use of algal-based biosorbents, which is the source and transport of the biomass (Volesky, 2007).

The ability to culture macroalgal biomass onsite and then convert this biomass to a dried or treated biosorbent would also provide a major incentive to developing a holistic biologically based treatment for complex industrial waste streams by utilizing both living and dead and dried biosorbents for improved waste water treatment (Roberts et al., 2013). Importantly, this study has demonstrated that species within the

cosmopolitan algal genus *Oedogonium* have a consistent ability to concentrate metals from waste water. One could therefore isolate native *Oedogonium* from waste water sources requiring remediation and use these species to remediate waste water in intensive on-site cultivation. Notably, the *Oedogonium* KC606914 from Tarong used during this study is endemic at the Tarong power station (Stanwell Energy) and this provides an example of the capacity to use a native species for culture within the industrial waste stream (Ash Dam water from Tarong) to deliver the first step in a potentially larger integrated waste management process.

Chapter 3

The influence of nutrients on growth rates of *Oedogonium* in Ash Dam water of a coal-fired power station

2.1. Introduction

Nitrogen and phosphorus are the two most commonly limiting nutrients for the growth of algae (DeBoer, 1981; Lapointe, 1987). Both nitrogen and phosphorus may be encountered in various forms in aquatic systems, but nitrate (NO_3^{2-}) and phosphate (PO_4^{3-}) are the forms most readily assimilated by photosynthetic organisms and, therefore, nitrogen and phosphorus are typically added to growth media in these forms. Nitrogen is typically considered to be the main limiting nutrient in temperate oceans both for phytoplankton (Twomey & Thompson, 2001) and macroalgal communities (Harrison & Hurd, 2001; Lobban & Harrison, 1994). Nitrate fertilisation increases the photosynthetic activity and growth of macroalgae. In contrast, phosphorous is typically the main limiting nutrient for freshwater autotrophs (Correll, 1999; Pedersen et al., 2004).

Maximising the productivity of algae is critical in a bioremediation context because the rate of metal sequestration and carbon capture from waste water and flue gas, respectively, are positively correlated with the growth of algae (Roberts et al., 2013). Increasing the growth of algae results in the production of new metal binding sites and increased carbon draw-down from water. Consequently, maximising algal productivity is critical to a successful bioremediation program and this will likely necessitate the use of growth nutrients to facilitate increased growth in marginal waste waters. The use of growth nutrients is, however, a critical constraint to large-scale algal production, both in terms of cost and sustainability.

Growth nutrients constitute one of the largest operational costs in intensive large-scale algal culture and also represent a barrier to sustainability (Slade & Bauen, 2013). Consequently, the use of nutrient for enhanced growth is a significant barrier to the feasibility of large-scale cultivation for the purposes of bioremediation. Interestingly, however, despite concerns regarding the potential toxicity of the Ash Dam water, the elemental profile of Ash Dam water and "clean" water fertilised with f/2 growth media share some similarities. For example, the main constituents of f/2 growth media (in addition to nitrogen and phosphorus) are trace elements including Cu, Zn, Mo, Mn and Fe, each of which are present in Ash Dam water. Consequently, Ash Dam water may actually represent a culture medium that is similar to f/2 media, with the exclusion of nitrogen and phosphorus, making these the main limiting nutrients for algal growth. In addition to requirements for growth, nutrients also pose a sustainability barrier to large-scale algal culture. One of the key constituents of growth media (phosphorus) is a finite resource. While nitrogen can be synthesised through the Haber-Bosch process, phosphorus is a nutrient that has limited global supply, and there are already concerns that "peak phosphorus" is approaching (the point at which global production of phosphorus is maximised and demand will outstrip supply [Gilbert, 2009]).

Chapter 2 demonstrated that species from the genus *Oedogonium* have a consistent ability to grow in Ash Dam, while capturing metals and metalloids from the water. Given the similarity in the growth rates of species of *Oedogonium* in Ash Dam water, and the consistent elemental profile and bioaccumulation of elements from Ash Dam water, the genus appears to be an ideal candidate for large-scale bioremediation applications. Chapter 3 builds upon this information by examining the nutrient requirements for *Oedogonium* in Ash Dam water, quantifying the effect of nutrient addition on the growth rate of three species of *Oedogonium* in Ash Dam water and dechlorinated town water. The experiment will also quantify the relative increase in growth as a result of the addition of nitrogen, phosphorus and f/2 addition in Ash Dam

water and dechlorinated town water. This information will identify key nutritive constraints on biomass productivity in bioremediation applications, addressing a key data gap in the application of large-scale bioremediation.

2.2. Materials and methods

2.2.1. Biomass

The same three species of *Oedogonium* that were used in Chapter 2 were cultured in Ash Dam water and dechlorinated town water under different nutrient regimes to assess the effects of nutrient addition in waste water on the productivity of the algae. A description of the original biomass sources, maintenance of stock cultures, and the Genbank accession numbers for each of the three species can be found in section 2.2.1. As previously described, all three species were maintained in library cultures at MARFU, JCU. The cultures were held in dechlorinated town water with f/2 media addition until their use in the cultivation experiments. The three species were acclimated to experimental conditions for 5 weeks before the experiment began as described in Chapter 2.

2.2.2. Experimental design

A cultivation study was conducted to comparatively assess the growth of three species of *Oedogonium* under a range of nutrient addition treatments in Ash Dam water and dechlorinated town water. The experiments were conducted within a climate controlled facility (28°C, 100 µmol photons m^{-2} s^{-1} and a 12 h light: 12 h dark photoperiod). Stock cultures of each species were maintained for five weeks in dechlorinated town water with f/2 nutrient addition (0.1 g f/2 L^{-1}) in the facility. The aim of the acclimation period was to attain 'steady state' productivity, defined here as < 5% change in specific growth rate for two consecutive weeks before the experiment began. This was

achieved on day 35 (cf. results section, Figure 2.1), and from this time point (t = 36) onwards the biomass was cultivated in either Ash Dam water or dechlorinated town water under one of five different nutrient regimes.

The five nutrient regimes were a) no nutrient addition (un-manipulated Ash Dam water or dechlorinated town water, hereafter denoted "NN"), b) nitrate only (hereafter "N"), c) phosphate only (hereafter "P"), d) nitrate and phosphate (hereafter "NP"), and e) f/2 addition (hereafter "f/2"). For the N, P and NP treatments, nitrogen and phosphorus were added to Ash Dam water and dechlorinated town water at a concentration consistent with that in f/2 culture media stocked with 0.1 g f/2 media L^{-1} (12 mg L^{-1} NO_x and 1.5 mg L^{-1} filterable reactive phosphorus). The f/2 culture media used in these experiments was produced from Aquasonic commercial media. In all cases the N and P content of stock solutions were confirmed in sub-samples of the culture media.

The three species of *Oedogonium* were cultured in 1.0 L Schott bottles at an initial stocking density 0.5 g FW L^{-1}. The Schott bottles were placed randomly in the climate controlled room and rotated daily to avoid a light bias. The bottles were aerated with compressed air delivered via Pasteur pipettes (0.2 L min^{-1}) to each replicate bottle. Each replicate was harvested every seven days and the stocking densities were reset to 0.5 g FW L^{-1} for all species and treatments, a complete water exchange occurred and all replicates replenished with nutrients. At this time all replicates were harvested and dried with paper towel and weighed to the nearest 0.1 g. Growth rates for each treatment were calculated using the fresh weight (FW) determined for three consecutive growth periods every seven days (t = Day 7). The specific growth rate (SGR, % FW d^{-1}) was calculated using the equation:

$$SGR = Ln\ (W_f / W_i)/T*100$$

Where W_f = the final weight (g FW) of biomass, W_i = the initial weight (g FW) of biomass, and T = the number of days in culture (7).

2.2.3. Statistical analysis

As the aim of the experiment was to assess whether each of the nutrient addition treatments could increase the growth of the three species in Ash Dam water and dechlorinated town water (rather than to compare mean growth rates under each condition in the two water sources), planned comparisons were performed for the Ash Dam water and dechlorinated town water datasets separately. The SGR of the three algal species were contrasted separately in Ash Dam water and dechlorinated town water using a two-factor analysis of variance (ANOVA). This analysis included the between subjects factors "species" and "nutrient treatment". The ANOVA was then followed with planned comparisons to quantify differences between control (no nutrient) and experimental treatments for each species in Ash Dam water and dechlorinated town water. This was done to minimise the number of comparisons and the Type I error rate associated with multiple post-hoc comparisons. Residual histograms and scatter plots of residuals vs. estimates were assessed to determine normality and homogeneity of variance respectively for the two-factor ANOVA (Quinn & Keough, 2002).

2.3.   Results

2.3.1. The effects of nutrients on SGR in Ash Dam water cultures

The three species of *Oedogonium* had variable growth rates in Ash Dam water with no nutrient addition, ranging from a minimum of 2.9% for KC701473 to a maximum of 7.5% for KC606914 (Figure 3.1a). There was a significant "species x nutrient" interaction in the ANOVA, indicating the effects of nutrient addition on SGR differed

amongst the three species (Table 3.1). Planned comparisons revealed that there was no significant increase in the SGR of any species when nitrate alone was added to the Ash Dam water, with SGR in this treatment ranging from a minimum of 1.6% for KC701473 to 6.7% for KC606914 (Figure 3.1; Table 3.2). The three species showed the same ranking of SGR in the nitrate treatment as the no nutrient treatment, with KC701473 having a lower SGR than the other two species (Figure 3.1a).

The SGR of all three species increased relative to the no nutrient control when phosphorus was added to the Ash Dam water (KC701473 increased from 2.9% to 10.9%, KC606977 from 6.7% to 9.9% and KC606914 from 7.5% to 9.4%, Figure 3.1a). These differences were only statistically significant for KC606977 and KC701473 (Table 3.2). KC606977 attained a similar SGR in the nitrogen and phosphate treatment and was significantly higher than in the control (11.2%, Figure 3.1a, Table 3.2). However, the SGR of the other two species did not differ significantly between the control and the NP treatment (Figure 3.1a, Table 3.2). All three species had significantly higher growth in f/2 treatment relative to the control treatment (Figure 3.1a, Table 3.2).

2.3.2. The effects of nutrients on SGR in dechlorinated town water cultures

The growth rates of the three species of *Oedogonium* showed a very similar pattern in response to nutrient treatments in the dechlorinated town water cultures (Figure 3.1b). All three species had consistent growth rates in the no nutrient control, ranging from 10.3% for KC606977 to 12.0% for KC701473 (Figure 3.1b). There was, again, a significant "species x nutrient" interaction in the ANOVA, demonstrating the three species did not respond to the nutrient treatments in the same manner (Table 3.1). As for the Ash Dam water cultures, there was no increase in SGR when nitrate was added to the cultures in isolation (Figure 3.1b, Table 3.2), but the addition of phosphorus significantly increased the growth rate of KC606977 and KC701473 relative to the

control, averaging 12.6 and 17.3% respectively (Figure 3.1b, Table 3.2). Interestingly, the SGR of KC606914 did not differ between the phosphorus treatment and the control, in accordance with the pattern seen in the Ash Dam water cultures of the same treatment (Figure 3.1b, Table 3.2).

The addition of nitrogen and phosphorus together increased the SGR of KC606977 relative to the control, but had no significant effect on the SGR of the other two species (Figure 3.1b, Table 3.2). Again, this pattern was in agreement with the response of the three species to N and P supplementation in Ash Dam water cultures. When f/2 media was added to dechlorinated town water, a significant increase in SGR was detected for KC606977 and KC701473, averaging 19.7 and 26.0% respectively, the highest SGR measured in any of the experimental treatments (Figure 3.1b).

Table 3.1. Analysis of variance of mean specific growth rate for the three species of *Oedogonium* grown in Ash Dam water and dechlorinated town water under the various nutrient addition regimes.

| Source | df | Ash Dam water | | | Dechlorinated town water | | |
		MS	F	P	MS	F	P
Species	2	122.93	17.75	*< 0.001*	146.74	11.29	*< 0.001*
Nutrient	4	121.44	17.53	*< 0.001*	213.03	16.39	*< 0.001*
Species x Nutrient	8	19.73	2.85	**0.014**	32.36	2.49	**0.027**
Residual	39	6.93			13		

Notes: italicised P values are significant (P < 0.05) but not interpreted due to significant interactions; bold P values are statistically significant (P < 0.05).

Table 3.2. Planned comparisons of mean specific growth rate for the three species grown in Ash Dam water (ADW) and dechlorinated town water (DTW) under the various nutrient addition regimes.

Species	Contrast	ADW	DTW
		P	P
KC606977	NN vs. N	0.209	0.132
	NN vs. P	**0.002**	**0.020**
	NN vs. NP	**0.006**	**0.003**
	NN vs. f/2	**< 0.001**	**< 0.001**
KC701473	NN vs. N	0.487	0.384
	NN vs. P	**0.005**	**0.022**
	NN vs. NP	0.219	0.368
	NN vs. f/2	**0.017**	**< 0.001**
KC606914	NN vs. N	0.400	0.352
	NN vs. P	0.191	0.319
	NN vs. NP	0.081	0.246
	NN vs. f/2	**0.050**	0.165

Note: bold P values are statistically significant ($P < 0.05$).
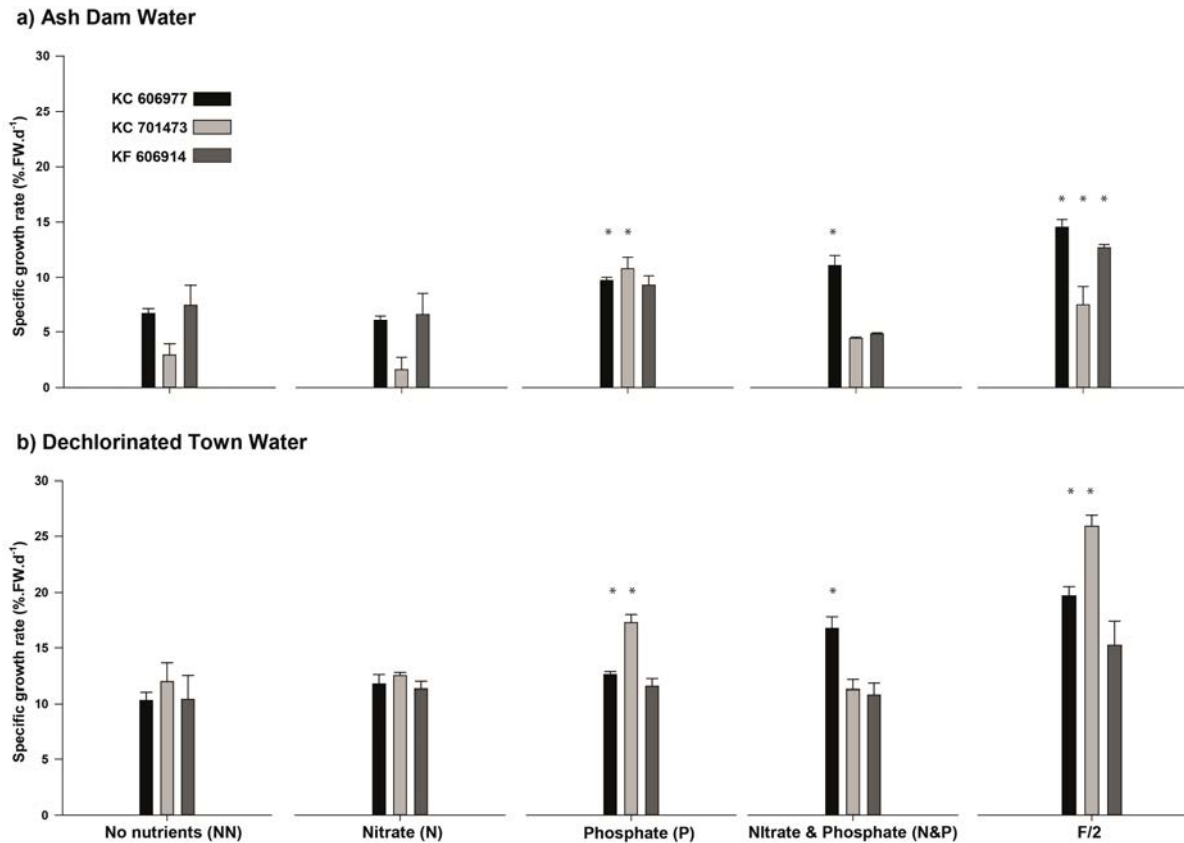
Figure 3.1. Specific growth rate for three species of *Oedogonium* cultured in Ash Dam water and dechlorinated town water under several nutrient addition treatments. NB. Asterisks above bars indicate a statistically significant difference between that treatment and the no nutrient control according to planned comparisons (P > 0.05). Data show means ± S.E. (*n* = 3).

2.4. Discussion

The addition of nutrients significantly enhanced the growth of all species of *Oedogonium*, however, the response was specific to each nutrient and not all nutrients had equal effects. All three species of *Oedogonium* had similar growth responses to nutrient addition in dechlorinated town water and Ash Dam water, however the three species, surprisingly, had different nutrient requirements. None of the species showed a significant increase in growth when nitrogen alone was added as a nutrient, and this was true of cultures in both Ash Dam water and dechlorinated town water. Both KC606977 and KC701473 had significantly higher growth in Ash Dam water and dechlorinated town water when phosphorus was added in isolation, but KC606914 had no improvement in growth. Somewhat surprisingly, the addition of nitrogen and phosphorus together yielded increased growth for only one of the three species (KC606977). The only differences in response to nutrient addition between the two water sources were detected for the f/2 treatment, which significantly increased growth of all species in the Ash Dam water, but only increased growth of KC606977 and KC701473 in the dechlorinated town water.

Overall, these results strongly suggest that phosphorus is the most limiting nutrient in both Ash Dam water and dechlorinated town water cultures and, furthermore, that there are no clear differences in the nutritive requirements of the three algal species in the two water sources. The two nutrient regimes that coincided with the highest productivity were f/2 addition and phosphate addition in Ash Dam water and dechlorinated town water, regardless of the species of *Oedogonium*. Interestingly, the least variation within specific growth rates was demonstrated in the phosphate addition treatments in Ash Dam water. There is little additional benefit in terms of increased growth by adding f/2 media. The three species delivered mean specific growth rates of

2.9 – 7.5% in the no nutrient treatment, increasing to 9.5-10.9% in the phosphorus treatment, and 7.6-14.8% in the f/2 treatment.

The nutrient regimes in this experiment were of short duration and determined under controlled laboratory conditions. Further experiments that expose the three species of *Oedogonium* utilised in this study to conditions that would be expected when grown on an industrial scale for bioremediation should be investigated. Studies for longer time periods will provide further insight into the nutrient requirements and species productivity, confirming the suitability of the genus *Oedogonium* as a bioremediation tool for Ash Dam water. Regardless, these results indicate that any of the three study species could be a candidate for bioremediation of Ash Dam water, and further, that the addition of phosphorus alone can deliver significantly higher growth of *Oedogonium* in Ash Dam water.

Overall, these results support the existing paradigm that phosphorus is the most commonly limiting nutrient for the production of freshwater macroalgal biomass (Correll, 1999; Pedersen et al., 2004). Furthermore, the fact that phosphorus addition alone delivered similar increases in growth rates as the f/2 treatment suggests that amendment of phosphorus alone may deliver the most cost effective approach to fertilization. However, it does not exclude the addition of other nutrient and trace elements beyond phosphorus as the addition of the complex f/2 media did deliver an incremental increase in growth relative to the addition of phosphorus. Further research over a longer period of time will be required to determine whether it is worth incurring the additional expense of using commercial f/2 media. Regardless, my results provide support for the use of waste streams from agriculture and municipal waste that naturally contain high concentrations of phosphorus, which is clearly an important limiting nutrient in Ash Dam water cultures (Mulbry et al., 2005).

In conclusion, my results clearly indicate that nutrient addition is necessary to increase *Oedogonium* productivity in Ash Dam water, despite the apparent bioavailability of trace elements in Ash Dam water such as Cu, Mn and Mo that are common constituents of f/2 media. The key limiting element appears to be phosphorus, with the greatest gains in productivity for most species seen when phosphorus was added as a growth nutrient. There are small additional gains in productivity when f/2 media is used and a cost-benefit analysis based on productivity over the longer-term is necessary to determine whether the additional costs of f/2 utilisation deliver meaningful increases in productivity to justify their use.

Chapter 4

General discussion

My results clearly indicate that the genus *Oedogonium* has several defining characteristics that make it a strong candidate for large-scale bioremediation programs. *Oedogonium* has previously been shown to be a robust genus of algae that grows rapidly in intensive monoculture, can outcompete other species, and is cosmopolitan, meaning it can be found and grown in almost all ecotypes (Lawton et al., 2013). My research adds further support to the widespread use of *Oedogonium* in bioremediation by demonstrating the genus has a ubiquitous ability to grow in waste water while sequestering a wide range of metals from solution.

Adaptation of native populations to local environmental conditions has been demonstrated across a variety of photosynthetic organisms including terrestrial plants (Angert & Schemske, 2005; Byars et al., 2007), aquatic plants (Dennison & Alberte, 1986), and algae (Pakker et al., 1996; Voskoboinikov et al., 1996). In a metal-bioremediation context, green freshwater macroalgae isolated from polluted environments have been shown to have greater tolerance to metals than species isolated from "clean" environments (Pawlik-Skowrońska, 2003). The increased tolerance may be due to an ability of acclimated species to overproduce metal-binding chelates to sequester and detoxify the accumulated metals in intercellular storage vacuoles (Pawlik-Skowrońska, 2003). In this way, endemic strains of algae isolated from Ash Dams might be expected to have higher productivity than non-endemic species under local conditions, and therefore, higher bioremediation potential.

However, endemic species do not always demonstrate a comparative advantage to introduced species (Bergström & Kautsky, 2006; Bischoff et al., 2006; Santamaría et al., 2003; Thompson et al., 1991). One of the species tested in these experiments was

a native species taken from Tarong Ash Dam, and so had a history of exposure to the effluent in which I conducted the cultivation experiments. This species did not, however, either show a significantly greater growth rate in Ash Dam water, or a consistent ability to accumulate metals from Ash Dam water more rapidly than species not originally sourced from the Ash Dam water. The lack of a clear difference in tolerances of species in my study suggests the levels of metals in the Ash Dam water may not be sufficiently high to impose a selective pressure on the algal species or, in other words, that the dissolved metal contents in Ash Dam water are within the typical tolerances of species of *Oedogonium*. Green freshwater macroalgae possess many metal detoxification mechanisms including the production of metal-binding phytochelatins (Pawlik-Skowrońska, 2001), polyphosphate bodies (Nishikawa et al., 2003), and sequestration of metals in storage vacuoles (Hanikenne et al., 2005). These metal detoxification mechanisms have been shown to be both ubiquitous and rapidly inducible in response to metal exposure (Le Faucheur et al., 2005). This provides further support for the widespread ability of green freshwater macroalgae to grow in marginal waste waters as a means of metal bioremediation.

Despite the relatively complex elemental composition of Ash Dam water, my results indicate that the nutritive requirements of *Oedogonium* are consistent between Ash Dam water and dechlorinated town water. My data clearly support the notion that phosphorus is the key limiting nutrient in Ash Dam water, as the addition of phosphorus alone delivered the greatest relative increase in productivity in comparison to the no nutrient control treatments. In contrast, the addition of nitrogen (either alone or in combination with phosphorus) had no effect on biomass productivity for most species. This finding suggests that phosphate-rich waste waters from other industries, such as agriculture, could help remove some of the critical constraints to algal productivity in waste water.

In summary, the genus *Oedogonium* is a key candidate for scaled bioremediation programs due to a consistent ability to grow in marginal waste waters and sequester metals from solution. The bioremediation potential for complex industrial effluents such as the Tarong Ash Dam is clearly greatest for metals (Cu, Mn, Al, Zn and Ni) which had the greatest bioconcentration factors. In contrast, the metalloids As, Mo and Se have relatively slow rates of uptake. The maintenance of high productivity is essential for an effective remediation program, and will clearly require the addition of growth nutrients to support algal productivity *in situ*. Despite the complex nature of the Ash Dam water, the nutritive requirements of the algae are consistent with cultures in "clean" water. Phosphorus is a key limiting nutrient in Ash Dam water, where the greatest gains in productivity are associated with phosphorus addition. There are incremental improvements in productivity associated with amendment of full f/2 media, and further research at scale is required to ascertain whether the benefits of f/2 application warrant its use. The integration of macroalgal cultivation with industries requiring waste water remediation is an innovative and sustainable means of treating waste water. The scope of integrated algal culture will be greatest with industries that are co-located with a source of inorganic carbon (in the form of CO_2) and/or nutrient waste streams (particularly those that contain phosphorus) to minimise exogenous inputs of these essential requirements.

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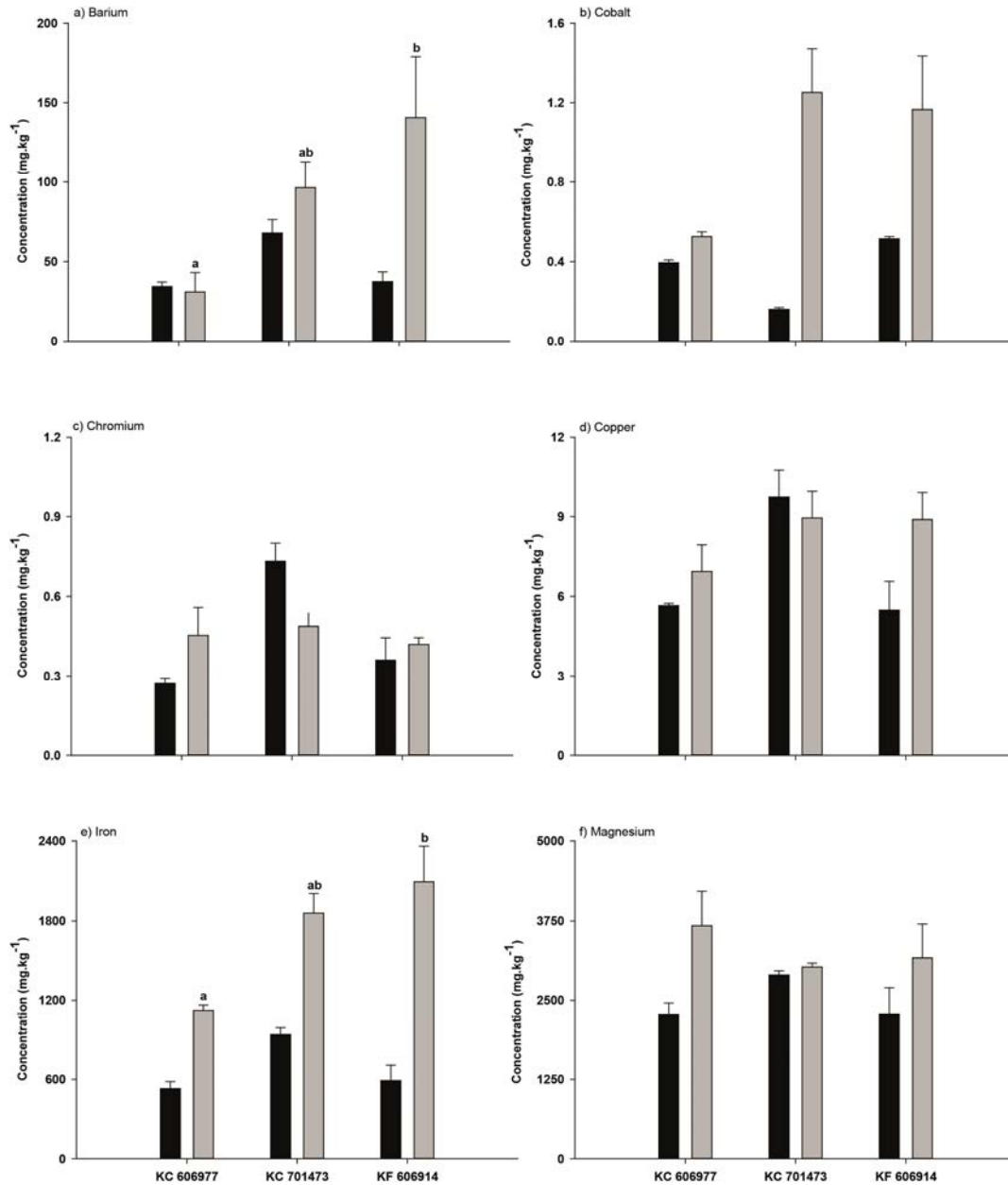
Appendix

Figure A.1. Individual elements not in excess of ANZECC trigger values in the three species of *Oedogonium* grown in Ash Dam water at time 0 (black bars) and after three weeks of cultivation (grey bars). NB. Bars that share a common letter are not significantly different (Tukey's HSD, $P > 0.05$).
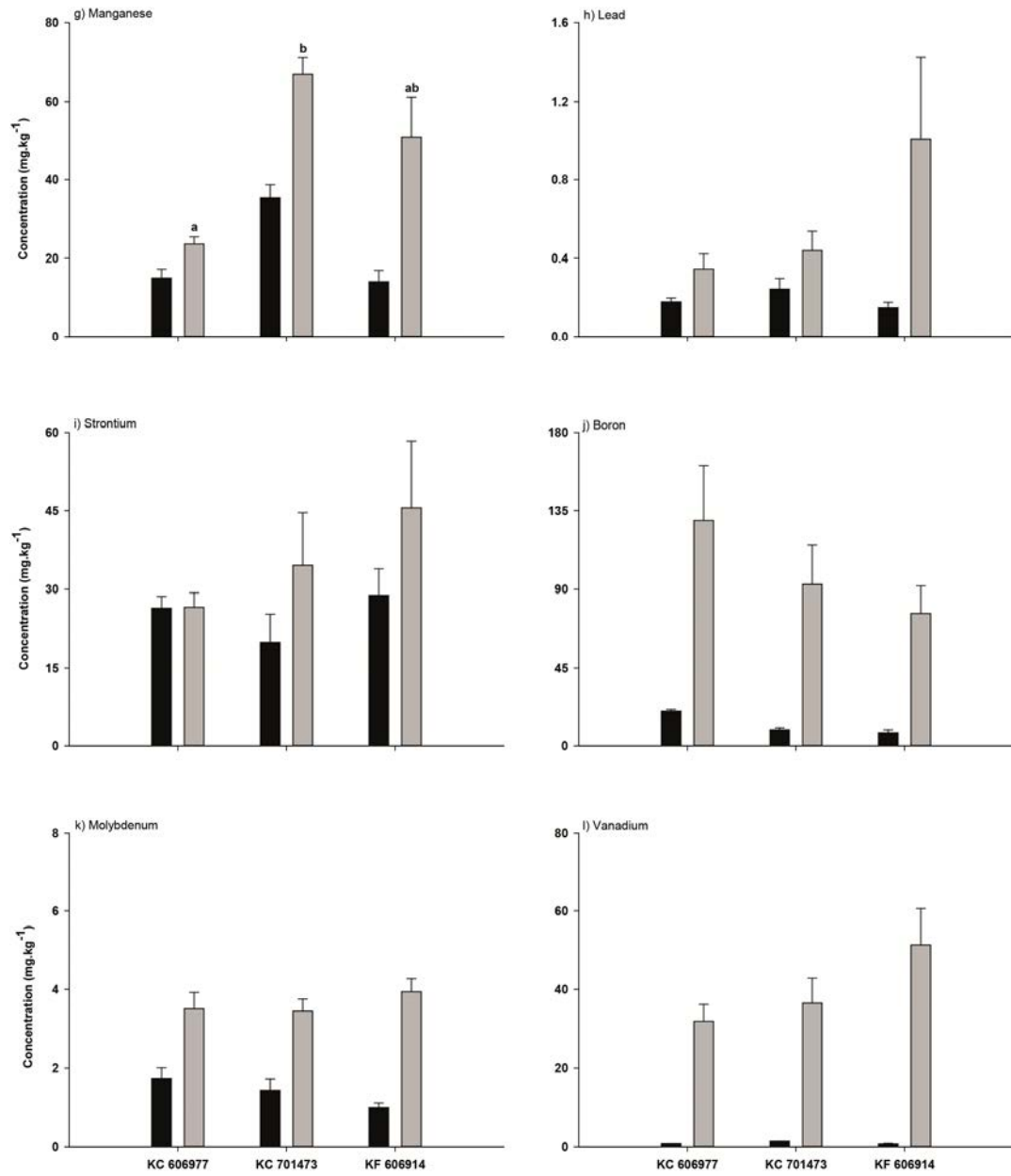
Figure A.1. continued.

Table A.1. Analysis of variance for individual elements in biomass that do no exceed ANZECC trigger values.

Factor	DF	Barium MS	P	Cobalt MS	P	Chromium MS	P	Copper MS	P	Iron MS	P	Magnesium MS	P
Species	2	9,137	0.60	0.47	0.08	0.003	0.80	3.92	0.35	774,369	**0.01**	349,400	0.58
Residual	6	1,883		0.12		0.010		3.11		93,775		584,065	

Factor	DF	Manganese MS	P	Lead MS	P	Strontium MS	P	Boron MS	P	Molybdenum MS	P	Vanadium MS	P
Species	2	1,439.7	**0.01**	0.39	0.20	270.8	0.43	2,226.9	0.35	0.21	0.59	310.4	0.20
Residual	6	125.7		0.19		274.7		1,755.6		0.37		145.1	

Note: bold P values are statistically significant (P < 0.05).