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Accumulation of Platinum Group Elements by the Marine Microalga, *Chlorella stigmatophora*

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**ACCUMULATION OF PLATINUM GROUP
ELEMENTS BY THE MARINE MICROALGA
CHLORELLA STIGMATOPHORA**

LEYLA SHAMS

DOCTOR OF PHILOSOPHY

2010

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**Accumulation of Platinum Group Elements by
the Marine Microalga, *Chlorella stigmatophora***

by

Leyla Shams

A thesis submitted to the University of Plymouth in partial fulfilment
for the degree of

Doctor of Philosophy

School of Geography, Earth and Environmental Sciences

Faculty of Science and Technology

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Abstract

Accumulation of Platinum Group Elements by the Marine Microalga, *Chlorella stigmatophora*

Leyla Shams

Very little information exists on the marine biogeochemistry of Rh, Pd and Pt, or the platinum group elements (PGE), an emerging group of contaminants whose principal emissions are associated with the abrasion of the catalytic converter in motor vehicles and chemotherapy drugs discharged in hospital wastes. In this study, Rh(III), Pd(II) and Pt(IV) were added individually and in combination to cultures of the marine microalga, *Chlorella stigmatophora*, maintained in coastal seawater at 15°C and under fluorescence lighting both in the presence and absence of trace nutrients (e.g. Fe, Co, Zn and EDTA). The accumulation of PGE was established under varying conditions (pH, algal biomass, PGE concentration, time) by ICP-MS analysis of seawater and nitric acid digests and EDTA washes of the alga, the latter giving a measure of PGE adsorption by *C. stigmatophora*. Under all conditions the extent of accumulation was in the order: Rh > Pd >> Pt. In short-term (24-h) exposures, accumulation isotherms were quasi-linear up to PGE concentrations of 30 µg L⁻¹, although Pd displayed convexity, hence saturation of available binding sites, at greater concentrations. The pH, adjusted between about 5.5 and 9.5 by addition of acid or base, did not have a great impact on PGE accumulation, with Rh displaying a moderate increase in accumulation and Pd a moderate reduction with increasing pH. More important, all PGE displayed a significant reduction in accumulation on a weight-normalized basis with increasing concentration of algae, an effect not reported for metal-marine algal interactions previously in the literature. Longer-term experiments showed that the rates of both overall accumulation and internalization were greatest for Pd and least for Pt. Consistent with this observation, the toxicity to *C. stigmatophora* evaluated by both pigment content and growth rate, was significantly greater for Pd than for Pt. Differences in the biogeochemical behaviours among the PGE are attributed to differences in their aqueous speciation in seawater, different affinities for the algal surface, different tendencies to cross the cell membrane, and especially with regard to Pd and Pt, differences in the rates of these interactions. Thus, although the equilibrium chemistries of Pd and Pt are very similar, their differential biogeochemistries are the result of kinetic constraints on reactions involving the latter. Because the environmental concentrations of PGE are predicted to increase with increasing emissions from vehicles and hospitals, the results of this study make an important contribution to an improved understanding of the likely effects and fates of these emerging contaminants in the marine environment. The results are also more generally important to an improved understanding of the interactions of trace metals with microalgae in seawater.

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Author's declaration

At not time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

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روز بجران و شب فرقت یار آخر شد زدم این فال و گذشت اختر و کار آخر شد

سگر ایزد که به اقبال کله گوشه گل نخوت باد دی و شوکت خار آخر شد

آن پریشانی شب‌های داز و غم دل همه در سایه کیسوی نگار آخر شد

ساقیا لطف نمودی قدحت پر می باد که به تدبیر تو تشویش خار آخر شد

*The day of separation from, and the night of disunion with, the Beloved is ended,
This omen, I cast; the star passed; and the work of grief is ended.*

*Thanks to God that, by the fortune of the cap-corner of the rose,
The pomp of December's wind and the majesty of the thorn is ended.*

*That agitation of long nights and the heart's grief,
All, in the shade of the idol's tress, is ended.*

*O Saki! thou showedest kindness. Be thy goblet full wine!
For, by thy deliberation, the disquietude of wine-sickness is ended.*

Hafez Shirazi (1325-1390)

*"To My Parents,
And to Mehdi"*

تقدیم به ستارگان پرفروغ آسمان زندگیم
پدر و مادر عزیز و همسر مهربانم

CHAPTER 1

Introduction

Introduction

1.1. Platinum Group Elements: Definition and Sources

Platinum group elements (PGE), mainly known by rhodium – Rh(III), palladium – Pd(II) and platinum – Pt(II) and Pt(IV), are noble metals, which belong to the rarest elements in the upper Earth's crust. Interest in the determination of platinum group elements in the environment has increased since the introduction of automobile catalytic converters (a device fitted directly to the exhaust system of a vehicle to reduce the amount of harmful gases emitted) in the late 1970s. A typical catalytic converter for a family car contains a total of about 1.75 g of PGE (Jarvis et al., 2001; Morton et al., 2001). Platinum and Pd are employed in a converter to oxidize carbon monoxide and hydrocarbons to water and carbon dioxide, and Rh is applied to reduce nitrogen oxides to nitrogen (Haus et al., 2007; Hooda et al., 2007). Catalytic converters have resulted in the reduction of air pollution by about 90 percent (Hooda et al., 2007; Morcelli et al., 2005). While improvement in air quality is an important benefit, the use of converters has led to widespread release of PGE as fine particles in the environment. The probable emission rate of Pt from vehicles equipped with catalytic converters has been estimated as 0.5 – 5 $\mu\text{g km}^{-1}$ travelled (Jarvis et al., 2001; Morcelli et al., 2005) with particle sizes of smaller than 3 μm to larger than 10 μm (Colombo et al., 2008b).

In addition to automobile traffic, Pt emissions are also derived from effluents of hospitals which use Pt compounds as anti-cancer drugs. Cancerostatic platinum compounds concentrations ranging between 4.7 and 145 $\mu\text{g L}^{-1}$ were found in the wastewater of the

Vienna University Hospital (Lenz et al., 2005). However, platinum emitted by hospitals is only 3.3 – 12.3% (1.3 – 14.3 kg year⁻¹) of the estimated amount emitted by vehicles equipped with catalytic converters (Kümmerer et al., 1999). There are some other sources of PGE emissions, such as effluents from dental laboratories and jewellery manufacturers (Rauch & Morrison, 2001). However, there is sufficient evidence nowadays that traffic is the main source of pollution by PGE in populated areas.

Following their emission, PGE are either subjected to atmospheric transport by wind as fine particles and deposited on adjacent vegetation or are deposited on the road. Road dust is the first material that becomes contaminated by vehicle catalyst emitted PGE and a large fraction of it is likely to be delivered through runoff to rivers and consequently to coastal waters and estuarine systems, where PGE can become solubilized and be accumulated by sediments, organisms and consequently the food chain (Haus et al., 2007; Sures & Zimmermann, 2007). Thus the presence and behaviour of vehicle derived PGE in environments receiving urban runoff is of interest not only in terms of understanding PGE behaviour and mobility, but also in terms of assessing their potential risk to ecosystems.

1.2. Platinum Group Element Concentrations in the Environment

The Earth's crust contains approximately 5 µg kg⁻¹ platinum (Artelt et al., 1998), 1 µg kg⁻¹ rhodium and 0.5 µg kg⁻¹ palladium (Ayres & Hellier, 1998). Although they are amongst the least abundant elements in the Earth's crust, anthropogenic emissions (especially in connection with their use in vehicle exhaust catalysts) have considerably increased their environmental concentrations. Atmospheric pollution by PGE is mainly due to the release

of microparticles from the catalyst (Bocca et al., 2006). PGE concentrations in the air are strongly influenced by emission patterns and geographical conditions of the area. PGE are now highly enriched in roadside particles, such as soil and tunnel dust. Although the hazard of these metals to human beings has not yet been fully explored, monitoring the PGE concentrations in the soils around heavy traffic routes and urban areas was considered necessary. Some of the cities assessed for urban PGE concentrations are summarised in Table 1.1.

Elevated concentrations of PGE within a period of time (typically around a decade) have also been monitored. Figure 1.1 shows the comparison of the timescale PGE contents in soil adjacent to heavy traffic route in some European cities. It clearly indicates that the concentrations of PGE increased up to at least two fold, which is considered to be due to the increasing number of vehicles equipped with catalytic converters.

Although traffic related PGE are believed to behave in an inert manner, as most of the emitted metals are in elemental or oxidized form (bound to aluminium oxide particles; (Moldovan et al., 2001; Singer et al., 2005)), studies have revealed that emitted metals undergo environmental transformations into soluble and thus more reactive species (Colombo et al., 2008a; 2008b; Moldovan et al., 2001). These soluble PGE species are more bioavailable and have the potential to directly affect organisms and plants, and therefore they present a greater environmental risk.

Long range atmospheric transport of catalyst-emitted PGE particles can also occur. Significant concentrations of PGE have been measured at locations much further away from urban areas with no immediately apparent PGE source. Concentrations of Pd and Rh in Mont Blanc snow and ice showed a 1.5 to 2 fold increase within a century

Table 1.1. Concentrations of platinum group elements (Rh, Pd and Pt) in the soil (otherwise stated) of different locations

<i>Area</i>	<i>Pt</i>	<i>Rh</i>	<i>Pd</i>	<i>Units</i>	<i>Reference</i>
San Diego, USA	100 - 680	—	38 - 280	ng g ⁻¹	(Hodge & Stallard, 1986)
SW Germany (road dust)	1000	110	100	ng g ⁻¹	(Schäfer et al., 1998)
Germany (tunnel dust)	< 730	< 60	—	ng g ⁻¹	(Helmers & Mergel, 1998)
Frankfurt, Germany	72	18	6	ng g ⁻¹	(Zereini & Alt, 2000)
Madrid, Spain	12.8	3.3		pg g ⁻¹	(Gómez et al., 2001)
London - Imperial college and surrounded area	10 - 500	<0.21	1 - 70	ng g ⁻¹	(Jarvis et al., 2001)
Rome, Italy	11.5 ± 4.7	—	—	ng g ⁻¹	(Cinti et al., 2002)
Hong Kong	14 - 38	—	—	pg g ⁻¹	(Kan & Tanner, 2005)
Sao Paulo, Brazil	0.3 - 17	0.07 - 8.2	1.1 - 58	ng g ⁻¹	(Morcelli et al., 2005)
Oxfordshire and west London	16	22.5	120	ng g ⁻¹	(Hooda et al., 2007)
Ruhr District, Germany (Sediments nearby highways)	2.5 - 5	—	—	ng g ⁻¹	(Haus et al., 2007)
Munich, Germany (Tunnel dust)	—	311 - 516	—	ng g ⁻¹	(Leopold et al., 2008)

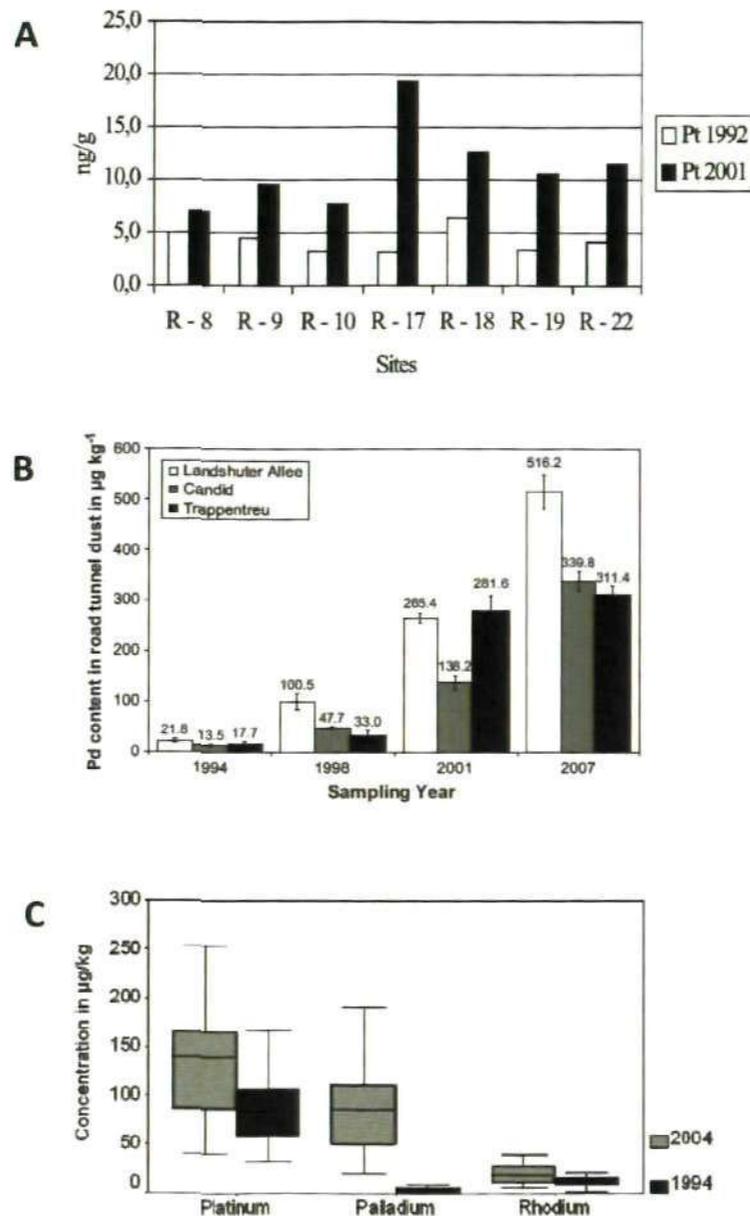


Figure 1.1. Variation of PGE concentrations, in (A) soil samples collected in Rome, Italy at 1992 and 2001 (Cinti et al., 2002), (B) tunnel dust samples collected in Munich, Germany between 1994 and 2007 (Leopold et al., 2008), and (C) soils samples collected in Frankfurt, Germany during 1994 and 2004 (Zereini et al., 2007). Error bars denote the standard deviations about the mean of three independent measurements (B) and min and max values (C).

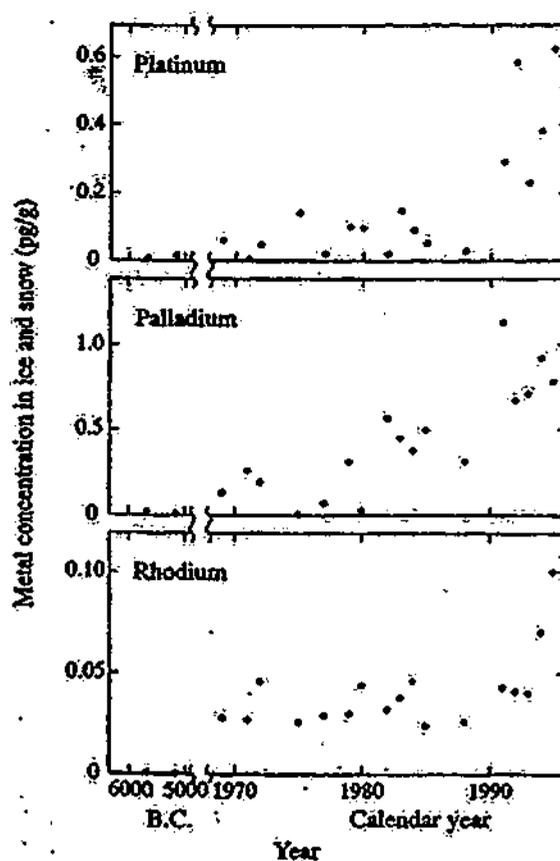


Figure 1.2. Changes in Pt, Pd and Rh concentrations in central Greenland ice and snow as a function of the age (Barbante et al., 2001).

($1.7 \pm 0.6 \text{ pg g}^{-1}$ of Pd and $0.052 \pm 0.021 \text{ pg g}^{-1}$ of Rh before 1900 compared with 2.4 ± 2 and 0.11 ± 0.08 , respectively, in 1991) (Van de Velde et al., 2000). Elevated concentrations of PGE in central Greenland snow also show that the emitted PGE are widely dispersed (Figure 1.2).

Concentrations of PGE in the aquatic system are in the picomolar range. Several studies have investigated the increasing concentration of PGE in various parts of aquatic ecosystem (Table 1.2). As mentioned above, anthropogenic emissions of PGE can be accumulated in freshwater and estuarine sediments and their effect on aquatic life will

Table 1. 2. Concentrations of platinum group elements (Rh, Pd and Pt) in the aquatic system (otherwise stated) of different locations

<i>Area</i>	<i>Pt</i>	<i>Rh</i>	<i>Pd</i>	<i>Units</i>	<i>Reference</i>
Pacific Ocean	—	—	40	pg L ⁻¹	(Lee, 1983)
Pacific Ocean	150	—	—	pg L ⁻¹	(Goldberg et al., 1986)
Indian Ocean	74	—	—	pg kg ⁻¹	(Jacinto & van den Berg, 1989)
Germany (runoff from road)	15 – 1600	—	—	ng L ⁻¹	(Laschka et al., 1996)
Mölnadal River, Sweden (sediment)	1.0	0.67	13.9	ng g ⁻¹	(Rauch et al., 2000)
Göteborg, Sweden (river sediments)	53.2 – 54.6	—	—	ng/g	(Moldovan et al., 2001)
Perth, Australia (wetland and sediments)	9 – 103.8	5.4 – 61.2	1.6 – 17.2	ng/g	(Whiteley & Murray, 2005)
California, USA (Seaweed)	0.25 – 1.75	0.09 - 0.61	—	ng/g	(Yang, 1989)
Göteborg, Sweden (river isopod)	5.1 – 119	—	—	ng/g	(Moldovan et al., 2001)
Austria (Moss)	7.07	2.8	0.6	ng/g	(Zechmeister et al., 2006)

depend on their biological availability. Moldovan et al. (2001) collected sediment samples in the river Mölnadal in Göteborg (Sweden) which receives water from surface runoff, and found Pt concentrations at about 55 ng g⁻¹ sediment *aquaticus* collected from the same in the tissue of the animal. Other studies revealed that of uptaking soluble and particle bound Rh, Pd and Pt. For example, the accumulation of PGE from road or tunnel dust has been shown for the freshwater isopod *A. aquaticus* (Moldovan et al., 2001; Rauch & Morrison,

1999), zebra mussel *Dreissena polymorpha* (Sures & Zimmermann, 2007; Zimmermann et al., 2005) and European eel *Anguilla anguilla* (Sures et al., 2001).

1.3. Toxicity of Platinum Group Elements

It is believed that emitted PGE from catalytic converters are in metallic form which are non-toxic and non-allergenic (Colombo et al., 2008a). From a toxicological point of view, soluble species are more bioavailable and have the potential to affect living organisms. Therefore, they present a greater environmental risk. The transformation of emitted PGE particles to the soluble species can take place in the air or in the soil where metals are deposited. Humic substances play an important role in the chemical transformation of PGE. Fulvic acids and other organic ligands (acetates, oxalates or phthalates) are responsible for the increased solubility of platinum in water environments (Bojanowska, 2005).

Some PGE species (especially those containing chloride) are toxic. Chloride ligands are one of the most effective compounds that increase the cytotoxicity and allergenicity of Pt (Bosch Ojeda et al., 2006; Farago et al., 1998) and the allergic response increases with increasing number of chlorine atoms.

The possible human risk of exposure to PGE from the exhaust of vehicles is still under discussion. Studies indicated the induction of micronucleus of human lymphocyte by Pt and Rh compounds (Migliore et al., 2002). The 50% lethal concentration (LC_{50}) of PGE for human bronchial epithelial cells was found 0.05 mmol L^{-1} for Pt(IV), 0.4 mmol L^{-1} for

Pd(II) and 1.2 mmol L^{-1} for Rh (Schmid et al., 2007). Some other health problems of exposure to PGE are mentioned by Bosch Ojeda et al. (2006) and Cicchella et al. (2003) such as chronic dermatitis, sneezing, rhinorrhea, bronchial asthma, watering of the eyes, tightness of the chest, eczematous and urticarial skin lesions.

The effects of PGE on other organisms have also been investigated. Eight to 12 % decrease in leaf length of barley after one week exposure to $50 \mu\text{M L}^{-1}$ Pd was shown by Battke et al. (2008). One ppm of combined PGE solution inhibited nitrate reductase activity in lettuce *Lactuca sativa* to up to 50% and reduced the protein content of the cells from 0.45 mg g^{-1} to 0.39 mg g^{-1} (Odjegba et al., 2007). PGE concentration of $500 \mu\text{g L}^{-1}$ could induce heat shock protein (hsp70, an important biomarker for a wide range of stressors including metal pollution) in zebra mussel *D. polymorpha* after 18 days of exposure (Singer et al., 2005).

Considering the large number of vehicles that circulate in large cities, and that the increasing number of cars using catalytic converters, it is essential to carry out further studies on the environmental impact of PGE as well as their transformation reactions in different ecological materials.

1.4. Platinum Group Elements in the Aquatic Environment

Platinum group elements are relatively inert in their metallic state, but investigations suggest that they are mobilised in the presence of natural complexing agents such as humic acids through oxidation and complexation (Sures & Zimmermann, 2007). Water solubility

is probably one of the most important factors that determines the bioavailability of PGE for aquatic organisms. For example accumulation of Pt by *A. aquaticus* was shown to be higher from river sediments which had Pt concentrations of 5 times lower than tunnel dust (Moldovan et al., 2001). This fact suggests that Pt present in the river sediments, although in lower concentration level, exist in a more bioavailable form than that in the tunnel dust.

The extent of bioaccumulation of PGE as well as other metals is a function of its speciation in water. The predominant oxidation state of PGE in aqueous form is +III for rhodium, +II for palladium and +II and +IV for platinum (Cosden et al., 2003; Goldberg et al., 1986). The metals are all strongly hydrolyzed in water. Increasing concentration of chloride in water is accompanied by an increasing proportion of chloride species of metal. At low pH, when chloride concentration is high ($>10 \text{ mmol L}^{-1}$), anionic species such as PdCl_3^- and PdCl_4^{2-} are predominant, whereas non-ionic or cationic species such as PdCl_2 and PdCl^+ are predominant at low chloride concentration ($\sim 0.5 \text{ mmol L}^{-1}$) (Vargas et al., 2004). In sea water $\text{PtCl}_5\text{OH}^{2-}$ is the dominant inorganic form of Pt(IV), while the dominant inorganic forms of Pd(II) and Pt(II) in seawater are PdCl_4^{2-} and PtCl_4^{2-} , respectively (Cosden et al., 2003). Rhodium is mainly present as cationic species in seawater, e.g. $[\text{RhCl}(\text{H}_2\text{O})_5]^{2+}$ and $[\text{RhCl}_2(\text{H}_2\text{O})_4]^+$ (Turner, 2007). Significantly, the proportion of the free ion of each PGE in seawater is extremely low. Stability constants for PGE complexation with natural organic ligands are lacking, but Pd and Pt appear to have an exceptional affinity for nitrogen- and sulphur-bearing model ligands (Cosden et al., 2003; Li & Byrne, 1990). Rhodium and Pt are also kinetically inert relative to Pd. The equilibrium chemistries of Pt and Pd are very similar, but rearrangements in coordination spheres of Pt are sufficiently slow that Pt may appear much less reactive toward surfaces than Pd. Therefore, dissolved Pt and Pd are

likely to have considerably different pathways and exhibit substantial fractionation in natural waters. PGE have different mobilities in natural systems. Colombo et al. (2008b) showed that in catalytic converters, concentration ratios are about 5 for Pt/Pd and 8 for Pt/Rh and they decrease further in the food chain, which indicates a higher mobility and biological uptake for Pd and Rh compared to Pt. Reedjik (2008) showed that platinum is less soluble and consequently less bioavailable than Pd and Rh.

1.5. Metals and Phytoplankton

The biosorption or accumulation of metals by algae and other microorganisms has been of interest for a variety of reasons. Microorganisms, and microalgae in particular, are the first organisms affected by metal discharges in aquatic environments, because they are directly in contact with the medium, separated only by the cytoplasmic membrane and the cell wall. Unicellular algae are an important part of aquatic systems as they are the foundation of most food chains and account for much of the production of freshwater and marine ecosystems. As primary producers, algae fix a major portion of the Earth's carbon and generate, via photosynthesis, much of the oxygen in the atmosphere. Any adverse impact on algae is likely to affect organisms at higher trophic levels and consequently the whole aquatic ecosystem. Microalgae also represent good model systems for investigating mechanisms and processes controlling metal accumulation at the cellular level. They present a high affinity to metal ion binding (Ribeiro et al., 2010; Tripathy et al., 1981), are relatively cheap to process and provide a low cost adsorbent. Since microalgae and their debris are major components of natural particulate matter (Fisher, 1986), they can play

significant roles in mediating the vertical transport of metals, consequently affecting their oceanic residence times. Therefore, microalgae are sensitive indicators of environmental change and are important test species for the regulatory assessment of metals.

Growth of microalgae (i.e. increase in cell numbers) in a batch culture follows a characteristic course which usually follows four phases (Figure 1.3):

I) *Lag Phase*: an adaptation phase in which no increase in cell numbers is apparent.

II) *Exponential Growth Phase*: in which cell multiplication is rapid and cell number increase exponentially.

III) *Stationary Phase*: in which the ratio of cell division to mortality is about 1.

IV) *Death Phase*: in which cell division is minimum and more likely occurs due to the lack of essential nutrients.

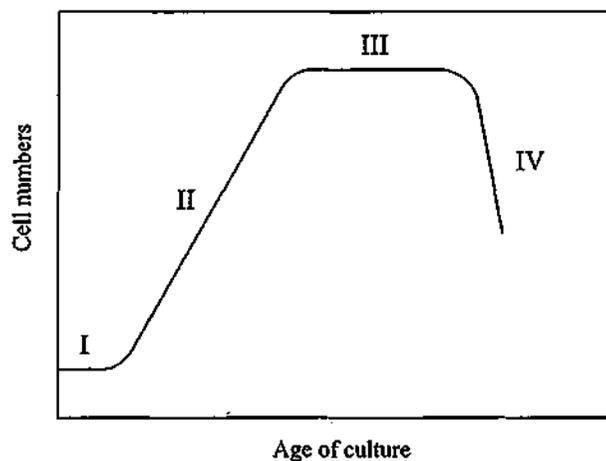


Figure 1.3. The characteristic pattern of unicellular alga growth in a batch culture. (I) Lag Phase, (II) Exponential Growth Phase, (III) Stationary Phase and (IV) Death Phase.

The results of both culture and field studies indicate that trace metals can affect the productivity (Capelo et al., 1993; Franklin et al., 2000; Lin et al., 2007) and species composition of algal communities (Gold et al., 2003; Gustavson & Wångberg, 1995). These communities in turns exert important controls on metal chemistry. Trace metal speciation strongly impacts their availability to phytoplankton. Redox cycling affects the speciation, bioavailability, and biogeochemical behaviour of metals and it has been evidenced for at least four essential metals (Fe, Mn, Cu, and Co) which exist in two or more oxidation states (Sunda, 2000; Sunda & Huntsman, 1998). As another example, stable or metastable forms of Mn (Mn(III) or Mn(IV)) are shown to be insoluble and thus not directly available for algal uptake. Unstable Mn(II) can persist in waters below pH ~ 8.5 because of slow oxidation kinetics. Regarding the PGE, Pd does not have a stable tetravalent oxidation state for conditions in the aquatic environment (Goldberg et al., 1986). Platinum (II) was found to be less reactive relative to Pt(IV), thus less bioavailable for accumulation towards surfaces (Rauch & Morrison, 1999).

Microalgal cells can regulate the concentration of dissolved metals in the water by surface reactions. The surface of algal cells contain a number of functional groups with high affinity for metal ions and carry a negative charge, mainly due to carboxylic, sulphhydryl and phosphatic groups (Franklin et al., 2002a). These groups are binding sites which can be involved in metal binding as carboxyl, hydroxyl, sulphate, phosphate, amino groups and others that can transport metal ions across the cell membrane and into the cell. These binding sites mainly adsorb and transport the essential trace metals. However, non-essential metals can enter a cell by binding to carrier molecules for essential elements or by making metal complexes with other essential nutrients such as amino acids or proteins (Haus et al., 2007). The accumulation of toxic metals by algal cells is inversely related to

the specific growth rate, and hence increases as growth rate declines (Sunda, 2000). In other words, increasing cellular concentrations of the toxic metal will cause the growth rate to decrease, which further increases cellular metal accumulation. Such a relationship can result in large increases in accumulated metal and associated adverse effects on the growth rate.

The ability of microalgae to accumulate metals from aqueous solution is well documented. However, very little is known about the accumulation of platinum group elements by microalgae. To date, PGE uptake by microalgae has been only studied on a fresh water green alga *Chlorella vulgaris* (Godlewska-Zylkiewicz, 2003), in order to investigate the ability of microalgae for selective biosorption of trace amounts of Pt and Pd, and the accumulation process was shown to be time and pH dependent. Due to the great abundance of marine phytoplankton species and their role as the first link of the marine food chain, studies of the mechanisms and interactions of marine microalgae with PGE are of particular interest in the investigations of biogeochemical processes in the environment.

1.6. Aims and Objectives

Despite the abundant studies about trace metal uptake and their toxicity on microalgae, no particular investigation has been undertaken on PGE accumulation by phytoplankton. This is perhaps surprising since PGE are metals of increasing environmental concern. Therefore, in this study the accumulation of PGE is studied by a simple unicellular marine microalga, *Chlorella stigmatophora*, under controlled experimental conditions. The overall aim is to investigate the ability of marine microalga to accumulate Rh(III), Pd(II) and

Pt(IV) from seawater. In order to achieve this aim, the following objectives will be investigated:

- The ability of *Chlorella stigmatophora* to accumulate Rh(III), Pd(II) and Pt(IV) from seawater
- The tendencies of Rh(III), Pd(II) and Pt(IV) for either surface adsorption and / or internalisation into the microalgal cells
- The impact of environmental variables, such as pH, microalgal cell concentration, Fe(III) concentrations and the essential trace metals concentrations, on the accumulation of Rh(III), Pd(II) and Pt(IV) by the microalga
- The adsorption and biosorption isotherms defining uptake of Rh(III), Pd(II) and Pt(IV) by microalga when exposed to both combined and individual metal solutions
- The accumulation kinetics of Rh(III), Pd(II) and Pt(IV) by microalgal cells over a period of time
- The potential toxicological and growth inhibitory effects of Rh(III), Pd(II) and Pt(IV) on microalgal cells, as a combined and as an individual metal solution

The results of this study will provide a valuable basis for improving our understanding of the biogeochemical behaviour and potential impacts of these metals in the marine environment.

CHAPTER 2

General Experimental Methods

General Experimental Methods

This chapter describes the overview of the general methodology which is followed throughout the thesis. In Chapters 3, 4, 5 and 6 the variations of this methodology are specified for different experiments investigating the accumulation of PGE by algal cells.

2.1. Equipment Preparation

Immediately prior to use, all plastic- and glass-ware needed for the experiments and sample storage were soaked in 5% HCl for 24 to 48 hours and rinsed afterwards 3 times with double distilled water.

2.2. Algal Species

A uni-algal phytoplankton stock culture of genus *Chlorella* was obtained from the Marine Biological Association of the UK (MBA). Among the three species of *Chlorella* available at the MBA (*C. saccharophila*, *C. stigmatophora* and *C. salina*), the marine species *C. stigmatophora* with the classification detailed in Table 2.1 was chosen for this study (Fig. 2.1). It is a fast growing species and reaches a high biomass in a short period of time compared with the other two species. Moreover, unlike *C. saccharophila*, it does not adhere to the bottom of the culture container which causes poor estimation of biomass and imperfect sampling. *C. stigmatophora* cell size ranges between 2 to 5 μm and is smaller

than the other two species. This is not convenient for counting cell numbers under a light microscope, however, since this species has less mobility relative to the other two species, in particular *C. salina* (4 – 8 μm), counting its cell on a haemocytometer is less challenging.

Table 2.1. Classification of *Chlorella stigmatophora* provided from the MBA

Order	Chlorophyta
Class	Chlorophyceae
Genus	<i>Chlorella</i>
Species	<i>stigmatophora</i>
Area collected	Irish Sea
Date isolated	June 1935



Figure 2.1. Light microscopic image of *Chlorella stigmatophora* provided from the MBA.

2.3. Algal Culture Maintenance

A common and widely used enriched seawater medium is *f/2* Guillard which is designed for growing marine algae. It contains 5 main components: (i) nitrate; (ii) phosphate; (iii) silicate; (iv) trace metals; (v) vitamins. The solutions were added into filtered seawater according to the concentrations in Table 2.2 under aseptic conditions. The silicate solution is eliminated from the recipe in this study as it is not essentially required by non-diatom species of phytoplankton.

To make the *f/2* trace metals solution, EDTA and other components detailed in Table 2.3 were dissolved into 950 mL of distilled water and the final volume were brought to 1 L. In order to make *f/2* vitamins solution, thiamin.HCl, biotin and cyanocobalamin were dissolved in 950 mL of distilled water, according to the concentrations detailed in Table 2.3 and final volume were brought to 1 L with distilled water.

Chemicals used for preparation of culture medium were of cell culture reagents and supplied by Sigma or BDH AnalaR[®]. Seawater used in the experiments was supplied to the laboratory via polymer piping and double filtered through a 5 µm pore size filter followed by a 0.6 µm carbon filter (Genuine Matriks[®] Extruded Carbon, +CTO[®]/2/) from a darkened, fiberglass-lined storage tank at the University. It is routinely collected from Plymouth Sound, south-west England, at high water.

The stock cells of *C. stigmatophora* were acclimatized after being transferred to the laboratory prior to the main experiment. To this end, 5 mL of stock cells were transferred to 400 mL of *f/2* Guillard culture media (pH 7.4; salinity 34.3) in 500 mL borosilicate bottles and cultivated in a Snijders Scientific culture cabinet (Figure 2.2) at 15°C, under

Table 2.2. f/2 Guillard culture medium for growing *C. stigmatophora* (Andersen, 2005)

<i>Solution</i>	<i>Component</i>	<i>Stock Solution</i> (g L ⁻¹ distilled H ₂ O)	<i>Quantity Used</i> (L ⁻¹ filtered seawater)	<i>Concentration in</i> <i>Final Medium (M)</i>
<i>Nitrate</i>	NaNO ₃	75	1 mL	8.82 × 10 ⁻⁴
<i>Phosphate</i>	NaH ₂ PO ₄ · H ₂ O	5	1 mL	3.62 × 10 ⁻⁵
<i>Silicate</i>	Na ₂ SiO ₃ · 9H ₂ O	30	1 mL	1.06 × 10 ⁻⁴
<i>Trace Metals</i>	See Table 2.3		1 mL	—
<i>Vitamins</i>	See Table 2.3		0.5 mL	—

Table 2.3. The components and concentrations of trace metals and vitamins solutions used in f/2 Guillard culture medium for growing *C. stigmatophora* (Andersen, 2005)

<i>Solution</i>	<i>Component</i>	<i>Stock Solution</i> (g L ⁻¹ distilled H ₂ O)	<i>Quantity Used</i> (L ⁻¹ distilled water)	<i>Concentration in</i> <i>Final Medium (M)</i>
<i>Trace Metals</i>	FeCl ₃ · 6H ₂ O	—	3.15 g	1.17 × 10 ⁻⁵
	Na ₂ EDTA · 2H ₂ O	—	4.36 g	1.17 × 10 ⁻⁵
	MnCl ₂ · 4H ₂ O	180.0	1 mL	9.10 × 10 ⁻⁷
	ZnSO ₄ · 7H ₂ O	22.0	1 mL	7.65 × 10 ⁻⁸
	CoCl ₂ · 6H ₂ O	10.0	1 mL	4.20 × 10 ⁻⁸
	CuSO ₄ · 5H ₂ O	9.8	1 mL	3.93 × 10 ⁻⁸
	Na ₂ MoO ₄ · 2H ₂ O	6.3	1 mL	2.60 × 10 ⁻⁸
<i>Vitamins</i>	Thiamin · HCl (B ₁)	—	200 mg	2.96 × 10 ⁻⁷
	Biotin (H)	1.0	1 mL	2.05 × 10 ⁻⁹
	Cyanocobalamin (B ₁₂)	1.0	1 mL	3.69 × 10 ⁻¹⁰

cool-white fluorescent light with photon flux density of $60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a light:dark cycle of 14:10 h. In order to provide essential carbon dioxide for algal photosynthesis and to keep the phytoplankton cells suspended in solution, the containers were continuously aerated using a Pasteur pipette vertically placed in the containers, connected to an air pump via polyethylene airline tubing. A porous bung of non-absorbent cotton was placed inside the pipette in order to minimize the risk of culture contamination via aeration (Figure 2.3). Following few successive subcultures, a reasonable acclimatized biomass of *C. stigmatophora* was obtained and used further for the PGE exposure experiments.



Figure 2.2. Culture cabinet used for cultivation and maintenance of PGE exposed *C. stigmatophora* cultures under controlled culture conditions.

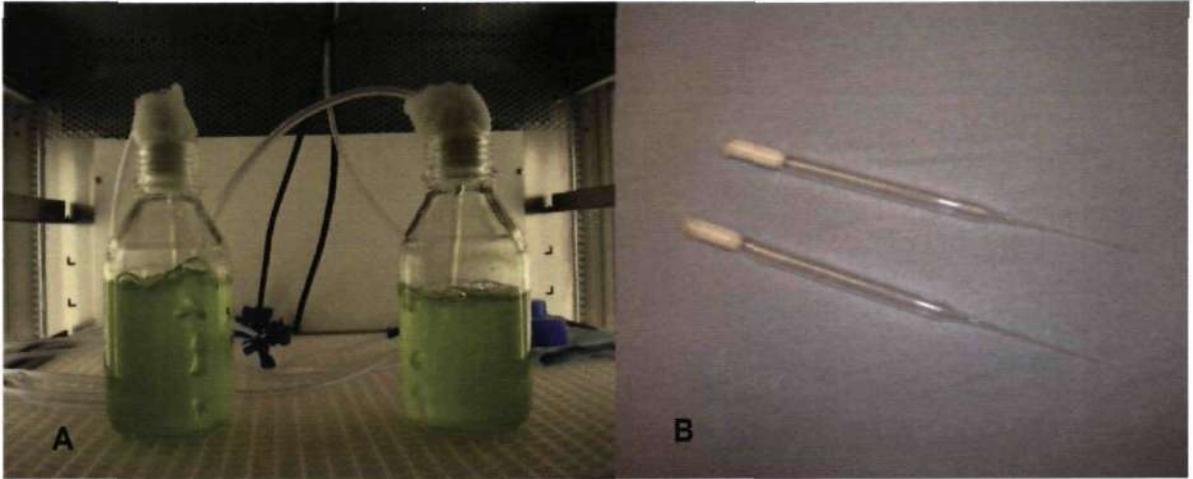


Figure 2.3. Stock culture of *C. stigmatophora* inside a culture cabinet growing in *f/2* medium, at 14:10 (L:D), $60 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ and 15°C (A) and filter inserted Pasteur pipette used for aeration of algal culture (B).

2.4. Effects of Nutrient Trace Metals

In order to investigate the possible effects of nutrient trace metals in the seawater medium and PGE uptake by algal cells, experiments were carried out in two types of *f/2* culture media. One contained all the solutions described in Table 2.2 (hereafter defined as +TM), and the other contained nitrate, sulphate and vitamins but not the trace metals and EDTA (hereafter defined as -TM). In the beginning, the stock cells of *C. stigmatophora* were separately sub-cultured in the two different media of +TM and -TM. The cells grown in these two media were separately exposed to experimental concentrations of PGE after they reached the mid-exponential phase of their growth. The growth rate (μ) was calculated from equation 2.1 (Fogg & Thake, 1987):

$$\mu = \frac{\ln N - \ln N_0}{t - t_0} \quad (2.1)$$

where N and N_0 are cell concentrations at time t and t_0 , respectively. Cell concentration was estimated by measuring cell counts using a haemocytometer. The results indicated that cell concentration in -TM culture was considerably lower than +TM culture (Figure 2.4) and one-way analysis of variance (ANOVA) revealed a significant difference between the growth rate of the two cultures ($p < 0.05$). Therefore, an unequal number of cells were to be exposed to PGE in +TM and -TM. Further investigations determined that the number of surfaces available in the environment may affect PGE accumulation (see Chapter 3). Therefore, the cultivation method of algae was modified. In the new method, the batch culture was grown in the original $f/2$ media containing all solutions of phosphate, nitrate, vitamins and trace metals. Cells in mid-exponential growth phase were centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and the pellet, containing *C. stigmatophora* cells, was resuspended in new +TM and -TM culture media separately. They were left for 24 hours to acclimatize before being exposed to PGE solutions for the experimental studies. Subsequent microscopic observations indicated no damage of the cells due to centrifugation.

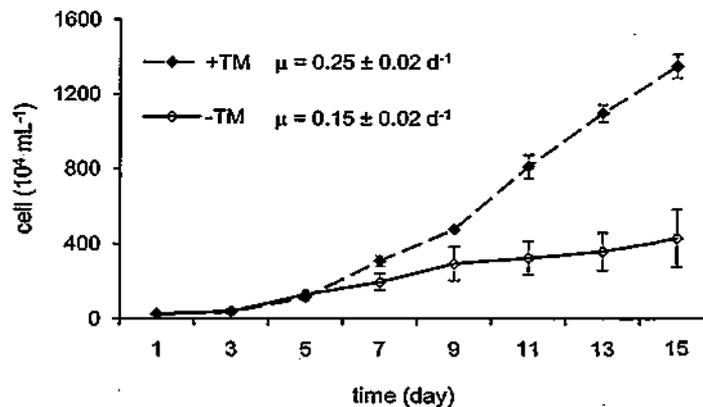


Figure 2.4. Growth curves of *C. stigmatophora* grown in +TM and -TM cultures for two weeks. μ is the specific growth rate of algae between days 5 and 15 \pm standard deviation. Error bars denote the standard deviations about the mean of three independent replicates.

2.5. PGE Exposure

Mixed and individual solutions of 200 mg L^{-1} of Rh(III), Pd(II) and Pt(IV) were prepared by dilution of individual $10,000 \text{ mg L}^{-1}$ plasma emission standards (BDH Aristar) in 0.4 M HCl (32%, analytical reagent, Fisher). Working solutions were prepared immediately before being used in each set of experiments by serial dilutions of the 200 mg L^{-1} stock solution in seawater. *C. stigmatophora* cells in their mid-exponential growth phase were centrifuged at 4000 rpm for 10 min as explained in Section 2.4, and resuspended in +TM and -TM cultures media separately. After 24 h acclimatization, 120 mL of cells in each medium were added to 150 mL styrolux (crystal polystyrene) containers and spiked with the desired concentrations of the mixed solution of Rh(III), Pd(II) and Pt(IV), resulting in a working concentration of $20 \text{ } \mu\text{g L}^{-1}$ for each element, assuming no background PGE concentration exist. The concentration of PGE was chosen after a few pilot studies of exposure of algae to various concentration of PGE. Twenty $\mu\text{g L}^{-1}$ was found to be the lowest concentration of PGE which present the most reliable results in the initial stage of this research. However, by improving the methods and techniques at later stages, lower concentration of PGE was also applied. In some experiments different concentrations of PGE solution were applied which will be explained where required. Each experiment was carried out using three or four independent replicates.

To eliminate the risk of contamination, the containers were covered with lids which were left open slightly to provide CO_2 exchange required for photosynthesis. The containers were gently agitated on a Denley orbital mixer or a Heidolph UNIMAX2010 shaker, depending on the number of the containers (Figure 2.5), at 85 rpm and $15 \text{ }^\circ\text{C}$ and under the light conditions specified above for 24 h (unless otherwise stated).



Figure 2.5. *C. stigmatophora* cultures exposed to PGE, agitating on a Denley orbital mixer (left) and a Heidolph UNIMAX2010 shaker (right), at 85 rpm, 15 °C and $60 - 70 \mu\text{mol m}^{-2} \text{s}^{-1}$ 14:10 (L:D).

The pH values of the cultures were measured at time intervals during the experiments with a precision of 0.01 pH units using a Denver Instrument pH Meter, which was regularly calibrated using buffers at pH 4.0, 7.0 and 9.0. Alterations of pH in the solutions due to the utilization of CO_2 by the algae were adjusted to the required pH by drop wise addition of either 1 M of HCl or NaOH.

Control experiments were also performed using cultures of *C. stigmatophora* in +TM and -TM amended seawater without addition of PGE in order to check for molecular interferences during subsequent analysis of the samples.

2.6. Filtration, Algal Digestion and EDTA Washing

At the end of the exposure period, 15 mL aliquots from each replicate of each treatment

were vacuum filtered through pre-weighed 0.2 μm pore size polycarbonate (Cyclopore®, 47 mm diameter, Whatman) filter membranes using a polysulphone filtration unit. Ten mL filtrates were transferred to clean 60 mL polystyrene containers, acidified with 40 mL of 1 M HNO_3 (70%, trace analytical grade, Fisher) in order to stabilize PGE in solution and dilute the salt matrix. Filter membranes were rinsed three times with 10 mL double distilled water in order to remove seawater salt, followed by an EDTA wash using 15 mL of 5 mM ethylenediaminetetra-acetic acid disodium ($\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$, Sigma) for 20 min to separate adsorbed PGE from the surface of phytoplankton cells. Among all techniques for extracting cell surface bound metals, EDTA appears to be the most effective ligand for metal extraction (binding to adsorbed metal and desorbing it from the surfaces) with rare secondary effects (Hassler *et al.*, 2004). After the extraction, the algal cells in solution were re-filtered and 10 mL of EDTA solution were stored in clean 30 mL polypropylene universal tubes. Filter membranes were allowed to air dry at room temperature for 24 h before being reweighed and then were placed in 30 mL polypropylene universal tubes. They were immediately digested using 2 mL of concentrated HNO_3 , while rolling on a roller mixer (SRT2, BIBBY Stuart Scientific) for 40 min. Digested samples were then diluted with 20 mL of 1 M HNO_3 . All samples were stored in a cold room at 4°C until analysis was performed. The dry cell weight content was calculated from the increase of weight of the filter membranes.

2.7. PGE Analysis

Inductively coupled plasma-mass spectrometry (ICP-MS) was employed for the

determination of Rh (as ^{103}Rh), Pd (as ^{105}Pd , ^{106}Pd and ^{108}Pd) and Pt (as ^{194}Pt and ^{195}Pt and ^{196}Pt) in acidified filtrate samples, EDTA washes and acid digests of cells, using a Thermo Elemental PlasmaQuad PQ2+ with a Meinhard dissolved solids nebuliser. ICP-MS is often used for the determination of PGE in environmental samples. Due to the low detection limit and multi-element analysis capability of ICP-MS, this method is considered as the most appropriate for direct determination of PGE in various environmental samples (Lesniewska *et al.*, 2006).

The instrument was calibrated over the range 0 to $7 \mu\text{g L}^{-1}$ using multi-element standards for mixed PGE exposure and single-element standards for individual Pt, Pd and Rh exposure. The standards were prepared by dilution of individual plasma emission standards in 1 M HNO_3 for digested cells samples, in a 1:4 ratio of *f/2* media:1 M HNO_3 for acidified filtrate samples, and in 5 mM EDTA for EDTA washed samples. To compensate for instrumental drift and variations in plasma conditions, $10 \mu\text{g L}^{-1}$ of internal standards were added to all samples and standards. Due to the similar atomic masses to those of the respective PGE, ^{115}In was used for the determination of Pd and Rh and ^{191}Ir for the determination of Pt. During the analysis a standard was inserted after every seven samples as a check, and the instrument was flushed between samples and standards with 0.2 M HNO_3 . Each sample was read three times during the analysis, except blanks of standard solutions which were read nine times for calculating the limits of detection. The instrument limit of detection was determined as 3 times the standard deviation of the blank solutions ($n = 9$) and is given for each matrix (i.e. 1 M HNO_3 , *f/2* medium + 1 M HNO_3 , 5mM EDTA solution) and for each set of results in Appendix 1.

2.8. Data Presentation and Analysis

Although different isotopes of Pt and Pd were initially analysed, Pt concentrations were determined from ^{196}Pt which gave identical results compared to ^{195}Pt and appeared to be the least interfered of the three isotopes. Concentrations of Pd were derived from the heavier isotope (^{108}Pd) because of its greater analytical precision. Molecular ion interferences were detected in the filtrates, EDTA washed and digested algal cells of controls with no added PGE (see Appendix 2.2) and were corrected for in the samples by subtracting the mean value of metal concentration from that of the corresponding exposure experiment.

Using the data obtained from the samples analyses, the following parameters were calculated and considered in each experiment:

(i) *Recovery* describes the total analytical concentration of metal relative to the amount added in the experiment:

$$\text{Recovery (\%)} = \frac{\text{mass balance}}{C_{\text{initial}}} \cdot 100 \quad (2.2)$$

where C_{initial} is the initial (added) PGE concentration ($\mu\text{g L}^{-1}$) spiked in the solution, and mass balance is calculated from following equation:

$$\text{Mass balance} = C_{\text{int}} + C_{\text{ads}} + C_{\text{aq}} \quad (2.3)$$

where C_{int} is PGE concentration internalised by the cell, C_{ads} is PGE concentration bound to the surface of the cell and C_{aq} is PGE concentration remaining in solution (all in $\mu\text{g L}^{-1}$).

(ii) *Accumulation Factor (AF)* is equivalent to the partition coefficient, K_D , and reflects the partitioning or distribution of PGE between the cells and solution (Sures et al., 2005). It is calculated as follows:

$$AF (L kg^{-1}) = \frac{C_{alga}}{C_{aq}} \cdot 1000 \quad (2.4)$$

where C_{alga} is total PGE accumulated by algae which is $C_{int} (\mu g g^{-1}) + C_{ads} (\mu g g^{-1})$, and 1000 is a unit conversion factor.

(iii) *Removal* defines the transfer of PGE from the aqueous phase to the algae and is calculated from the accumulated concentration by algae relative to total analytical concentration as follows:

$$Removal (\%) = \frac{C_{alga}}{C_{alga} + C_{aq}} \cdot 100\% \quad (2.5)$$

C_{alga} is total PGE accumulated by the algae in $\mu g L^{-1}$.

(iv) *Internalisation* is the percentage of PGE taken up by the algae that is internalised.

$$Internalization (\%) = \frac{C_{int}}{C_{int} + C_{ads}} \cdot 100\% \quad (2.6)$$

Significant differences among the data were identified using a one-way analysis of variance (ANOVA) and Tukey follow up test. The significance levels were tested at the $p = 0.05$ level. Comparisons were performed using StatGraphics software (Version 5.1).

CHAPTER 3

Environmental Variables Influencing the Accumulation of Platinum Group Elements by *Chlorella stigmatophora*

Environmental Variables Influencing the Accumulation of Platinum Group Elements by *Chlorella stigmatophora*

3.1. Introduction

The bioavailability of trace metals relates to the accumulation and transfer across the plasmalemma (i.e. cell membrane) of an organism. The uptake process mainly depends on the internalization pathways and the speciation of the metal, which is determined by the physical and chemical properties of the external medium. Studies show that the chemical speciation of metals has a major impact on their cellular uptake (Sunda, 2000; Sunda & Huntsman, 1998; Zimmermann et al., 2005).

There are key parameters such as pH, metal concentration and metal ion chemistry that influence both the speciation of the metal in solution and the binding of metals to cell membranes (Vargas *et al.*, 2004), consequently affecting metal accumulation and internalization by organisms. pH is one of the most important environmental factors that influences not only by affecting metal uptake, but also by affecting the chemical speciation and bioavailability of the dissolved metal to organisms (Fisher, 1986). The dependence of metal biosorption on pH has been documented for variety of metals such as Cu, Zn, Cd, Ni, Mn and Zn and it is shown that, generally, an increase in pH would lead to an increase in uptake due to the protonation of the metal binding sites. (Fraile *et al.*, 2005; Gonzalez-Davila, 1995; Melčáková & Růžovič, 2010; Worms *et al.*, 2006; Zhou *et al.*, 1998). Microalgal cells also affect metal chemistry in natural and oceanic waters by surface reaction, metal uptake and production of extracellular organic matter with metal complexing properties (Garnham *et al.*, 1992; Santana-Casiano *et al.*, 1995; Worms *et al.*, 2006).

In the presence of exudates and natural organic materials the adsorption characteristics of metals can change and hence, their bioaccumulation by cells will be affected. Micronutrients are another factor which impact the accumulation of metals. A number of studies have examined the influence of metal-metal interaction on metal accumulation in marine phytoplankton (Franklin et al., 2002b; Vasconcelos & Leal, 2001; Wang & Dei, 2001a). The variability of micronutrient metals in different parts of water body might affect the accumulation of other metals, because the uptake of nutrient and toxic metal ions is a function of the concentration of other metals and this can be synergism, antagonism or non-interaction behaviour (Gonzalez-Davila, 1995). The aim of this part of the study is to increase our understanding of how the accumulation of platinum group elements by marine microalgae *C. stigmatophora* will be influenced by the environmental parameters explained above. The specific objectives are to:

- A) Investigate PGE accumulation by algae as a function of pH over the range 5.5 – 9.5 which reflects the pH range in different aquatic systems
- B) Investigate PGE accumulation as a function of algal concentration over the range 1×10^6 to 10×10^6 cell mL⁻¹, representing the average microalgal cell concentration in different waters from coastal and estuarine waters to open oceans
- C) Investigate the effect of Fe(III), as an the most important micronutrient (Sunda et al., 2005), on PGE accumulation by algae
- D) Investigate the competition between uptake of PGE and essential trace metals

3.2. Methodology

3.2.1. Dependence of PGE Accumulation on pH

Chlorella stigmatophora cells in their mid-exponential growth phase, grown in *f/2* media, were centrifuged at 4000 rpm for 10 min and the pellets then were resuspended separately in both +TM and -TM cultures as explained in Chapter Two (Section 2.4). Five nominal pH of 5.5, 6.5, 7.5, 8.5 and 9.5 was selected to be investigated. This range was chosen to reflect the pH values of the aquatic systems, including rain water with pH = 5.6 (O'Neill, 1998) to seawater with pH = 8 – 8.4 (Chester, 2003). The greatest pH of 9.5 in this experiment was chosen based on the results of the pilot studies which showed an increase of pH during the algal culture growth. In the most of the culture containers, pH frequently shifted to values above 9. Therefore to investigate the possible effect of this increase on the amount of PGE during the experiment, a series of cultures were set for investigation of PGE uptake at pH = 9.5. Experiments were carried out in triplicates by transferring 120 mL of algal suspension into fifteen separate 150 mL styrolux (crystal polystyrene) containers for each cultures of +TM and -TM. All containers were spiked with a cocktail of Rh, Pd and Pt each with a concentration of 20 $\mu\text{g L}^{-1}$ and incubated under the conditions detailed in Table 3.1. The containers were constantly agitated on a Denley orbital mixer at 85 rpm with the lids ajar to allow air exchange. The pH were recorded initially and at the time of filtration, as well as at 3 intervals throughout the 24 h of exposure and adjusted by adding microlitre volumes of 1M HCl or 1M NaOH as required. Phytoplankton cells were filtered after 24 h exposure and processed as explained in Chapter Two and analysed by ICP-MS to determine the intra-cellular, extra-cellular and aqueous PGE concentrations. Limits of detection of Rh, Pd and Pt in unspiked acidified seawater, 5 mM EDTA and diluted HNO_3 are indicated in Appendix 1.

Table 3.1. Incubation conditions of *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a mix solution for 24 h at various pH. (+TM: cultures included nutrient trace metals and EDTA; -TM: cultures with no added nutrient trace metals and EDTA)

<i>culture</i>	<i>Light</i> ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	<i>Temperature</i> ($^{\circ}\text{C}$)	<i>salinity</i>	<i>pH</i>	<i>Initial</i> <i>Cell concentration</i> ($\sim \text{mL}^{-1}$)	<i>No. of</i> <i>replicates</i>
+TM	60	16 \pm 1	33.3	5.5 – 9.5*	6,900,000	3
-TM	60	16 \pm 1	33.9	5.5 – 9.5*	5,960,000	3

* pH values are described in the text.

3.2.2. Dependence of PGE Sorption on Algal Concentration

Chlorella stigmatophora cells in their mid-exponential growth phase, grown in *f/2* media, were isolated from their original *f/2* culture media as described in Chapter Two and were resuspended separately in both +TM and -TM cultures and left for 24 h to acclimatise. The stock culture of microalga was maintained to achieve 10.8×10^6 and 11.4×10^6 cells mL^{-1} in +TM and -TM cultures, respectively, which reflect the cell concentration of phytoplankton in an algal bloom (maximum algal biomass) (Boney, 1989). The treatments were made from serial dilutions of the stock culture (2, 5 and 10 times) to obtain cultures with various concentrations of *C. stigmatophora* reflecting the lower concentrations of microalgae in various water bodies such as coastal water and oceanic waters (with the lowest concentration of microalgae) (Reynolds, 1991). Therefore the treatments were named based on their dilutions as follows: 1/1 (initial cell concentration – not diluted), 1/2 (2 times dilution), 1/5 (5 times dilution) and 1/10 (10 times dilution). For each of +TM and -TM culture types, 120 mL of each dilution of algae was transferred to 3 replicates each of 150 mL styrolux (crystal polystyrene) containers. All containers were spiked with $20 \mu\text{g L}^{-1}$

¹ of mixed solution of PGE and incubated under the conditions detailed in Table 3.2. Other incubation conditions and pH adjustments (see appendix 2.1) were carried out as explained in Section 3.2.1. Limits of detection of acidified seawater, EDTA and diluted HNO₃ for Rh, Pd and Pt are shown in Appendix 1.

Table 3.2. Incubation conditions of *C. stigmatophora* exposed to 20 µg L⁻¹ of mixed solution of Rh, Pd and Pt for 24 h at various concentrations of phytoplankton cells (+TM: cultures included nutrient trace metals and EDTA; -TM: cultures with no added nutrient trace metals and EDTA)

Culture	Treatment (dilution)	Initial Cell concentration (~ mL ⁻¹)	Light (µmol m ⁻² s ⁻¹)	Temperatur e (°C)	Salinity	Initial pH	No. of replicates
+TM	1/1	10,760,000	65	16±1	34.1	7.0	3
	1/2	5,380,000	65	16±1	34.0	7.0	3
	1/5	2,150,000	65	16±1	34.0	7.0	3
	1/10	1,070,000	65	16±1	34.0	7.0	3
-TM	1/1	11,410,000	65	16±1	34.3	7.2	3
	1/2	5,700,000	65	16±1	34.3	7.2	3
	1/5	2,280,000	65	16±1	34.3	7.2	3
	1/10	1,140,000	65	16±1	34.3	7.2	3

3.2.3. Dependence of PGE Accumulation on Fe (III) Concentration

Iron is the most important micronutrient for phytoplankton life cycle. It plays a key role in growth and community structure of microalgae (Muggli & Harrison, 1997; Tovar-Sanchez et al., 2003). Iron is required for photosynthetic and respiratory electron transport, nitrate reduction, chlorophyll synthesis, and detoxification of reactive oxygen species (Sunda &

Huntsman, 1995; Sunda et al., 2005). Fe concentration in *f/2* cultures media reflects the coastal water concentration of it, while Fe occurs at lower concentrations in open waters (Sunda & Huntsman, 1995). In order to investigate the possible interactions of Fe at different environmental concentrations with the accumulation of platinum group elements, PGE contaminated cultures of microalgae were exposed to various concentrations of Fe. To this end *Chlorella stigmatophora* in the mid-exponential growth phase, growing in *f/2* media were centrifuged at 4000 rpm for 10 min and the pellets were resuspended in *f/2* culture media with no added $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and left for 24 h to acclimatise to the new growth medium. Experiments were carried out in triplicate at five $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ concentrations (0, 0.8, 1.6, 2.1 and 3.2 mg L^{-1}) by transferring 120 mL of algae into fifteen separate 150 mL styrolux (crystal polystyrene) containers. The desired concentrations of Fe(III) were made based on the final concentration of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in the original *f/2* media ($3.2 \text{ mg L}^{-1} = 1.17 \times 10^{-5} \text{ M}$) and by diluting the initial concentration of Fe(III) to 1/1, 2/3, 1/2, 1/4 and 0. Algae, therefore, were exposed to 3.2, 2.13, 1.6, 0.8 and 0 mg L^{-1} (or 1.17×10^{-5} , 0.78×10^{-5} , 0.59×10^{-5} , 0.29×10^{-5} and 0 M, respectively) of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. All three replicates of each treatment were spiked with $20 \mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a combined solution and incubated under the conditions detailed in Table 3.3. Three replicates of *C. stigmatophora* cultures in the original *f/2* medium with no added PGE were used as controls to compare the accumulation of Fe(III) in the presence and absence of PGE. The containers were constantly agitated on two shakers (Heidolph UNIMAX2010) and the pH was adjusted as described in Section 3.2.1 (see Appendix 2.1)

Inductively coupled plasma–optical emission spectrometry (ICP-OES) was used for the determination of Fe (as ^{56}Fe) in acidified filtrate samples, EDTA wash solutions and acid digests of the cells, using a Varian 725ES, Yarnton, UK. The instrument was calibrated

Table 3.3. Incubation conditions of *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a mixed solution for 24 h at various concentrations of Fe

<i>Initial Cell concentration</i> ($\sim \text{mL}^{-1}$)	<i>Light</i> ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	<i>Temperature</i> ($^{\circ}\text{C}$)	<i>Salinity</i>	<i>Initial pH</i>	<i>No. of replicates</i>
4,860,000	65	16 ± 1	33.8	7.07	3

over the range of 0.1 to 4 mg L^{-1} of Fe. The standards were prepared by dilution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10% HNO_3 for digested cell samples, in a 1:4 ratio of Fe eliminated $f/2$ media: 10% HNO_3 for acidified filtrate samples, and in 5 mM EDTA for EDTA wash samples. Ten $\mu\text{g L}^{-1}$ of ^{89}Y was added to all samples and standards as an internal standard to compensate for instrumental drift and variations in plasma conditions. During the analysis a standard solution was inserted after every eight samples as a quality check, and the instrument was flushed between samples and standards with 2% HNO_3 . Limits of detection of each matrix for Rh, Pd and Pt are indicated in Appendix 1.

3.2.4. Dependence of PGE Accumulation on Essential Trace Metals

The concentrations of trace metals vary widely in aquatic systems due to the differences in rates of input, loss and internal cycling. Their dissolved concentrations in surface waters are often lower than those at depth due to the uptake of metals by phytoplankton in the euphotic zone (Sunda, 2000), while because of inputs from terrestrial sources such as rivers, groundwater, dust and sediments, the concentration of trace metals decrease considerably along the surface from coastal and estuarine waters to oceanic waters. The

variation of the trace elements concentrations in different aquatic environment may affect the accumulation of metal due to the antagonistic and/or synergistic interactions amongst metal (Franklin et al., 2002b; Gonzalez-Davila, 1995) during their accumulation by aquatic organisms. To investigate the assumption of the uptake interactions between PGE and other essential trace metals exist in the *f/2* culture media by algae, various concentrations of nutrient trace metals solutions were considered to be applied during the PGE exposure of algae. To this end, *Chlorella stigmatophora* cells in the mid-exponential growth phase, grown in *f/2* media, were centrifuged at 4000 rpm for 10 min and the pellets were resuspended in a new *f/2* media with no added trace metal solution. After 24 h acclimatisation, 120 mL of algae was transferred to 150 mL styrolux (crystal polystyrene) containers and different concentrations of trace metals were added to them. These concentrations were made by diluting the original trace metal solution to 1/1, 2/3, 1/2, 1/4 and 0. The original concentrations of trace metals in the final culture media are detailed in Table 3.4.

Table 3.4. Concentrations of nutrient trace metals in the original *f/2* media

<i>Component</i>	<i>Concentration</i>	
	<i>M</i>	<i>mg L⁻¹</i>
FeCl ₃ .6H ₂ O	1.17×10 ⁻⁵	3.150
MnCl ₂ .4H ₂ O	9.10×10 ⁻⁷	0.180
ZnSO ₄ .7H ₂ O	7.65×10 ⁻⁸	0.022
CoCl ₂ .6H ₂ O	4.20×10 ⁻⁸	0.010
CuSO ₄ .5H ₂ O	3.93×10 ⁻⁸	0.009
Na ₂ MoO ₄ .2H ₂ O	2.60×10 ⁻⁸	0.006
Na ₂ EDTA.2H ₂ O	1.17×10 ⁻⁵	4.400

All three replicates of each treatment were spiked with $20 \mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a mixed solution and incubated under the conditions detailed in Table 3.5. The containers were constantly agitated on two Heidolph UNIMAX2010 shakers and the pH was adjusted as described in Section 3.2.1 (Appendix 2.1). Limits of detection of Rh, Pd and Pt in unspiked acidified seawater, 5 mM EDTA and diluted HNO_3 are indicated in Appendix 1

Table 3.5. Incubation conditions of *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of mixed solution of Rh, Pd and Pt for 24 h at various concentrations of nutrient trace metals solution

<i>Initial Cell concentration (mL^{-1})</i>	<i>Light ($\mu\text{mol m}^{-2}\text{s}^{-1}$)</i>	<i>Temperature ($^{\circ}\text{C}$)</i>	<i>Salinity</i>	<i>Initial pH</i>	<i>No. of replicates</i>
4,160,000	65	16 ± 1	33.5	7.32	3

3.3. Results and Discussion

3.3.1. Recovery of Platinum Group Elements

In all experiments the algal cultures were spiked with $20 \mu\text{g L}^{-1}$ of mixed solution of PGE. The results of calculated recovered metal concentrations showed that Rh and Pd and to a lesser extent Pt were lost from the aqueous phase over the period of incubation and it is assumed that PGE adsorbed to the walls of the containers.

3.3.1.1. Rhodium

The effects of various factors (pH, algal concentration, Fe and nutrient trace metals) on recovery of $20 \mu\text{g L}^{-1}$ of Rh are shown in Figure 3.1. Sixty to eighty percent of Rh was

recovered overall. Rh recovery decreased from 85% at pH~6 to 60% at pH~7 ($p < 0.05$) where after it remained relatively constant ($7 < \text{pH} < 10$) in both +TM and -TM cultures. According to previous studies, at high pH, Rh appears to be significantly removed from seawater by means of precipitation, and possibly co-precipitates with iron or magnesium hydroxides (Cobelo-Garcia et al., 2007). In this experiment, due to the presence of unicellular algal cells in the containers, observation of precipitates was not possible. Therefore, there are uncertainties about whether the loss of Rh at high pH was due only to adsorption to container walls or also to precipitation.

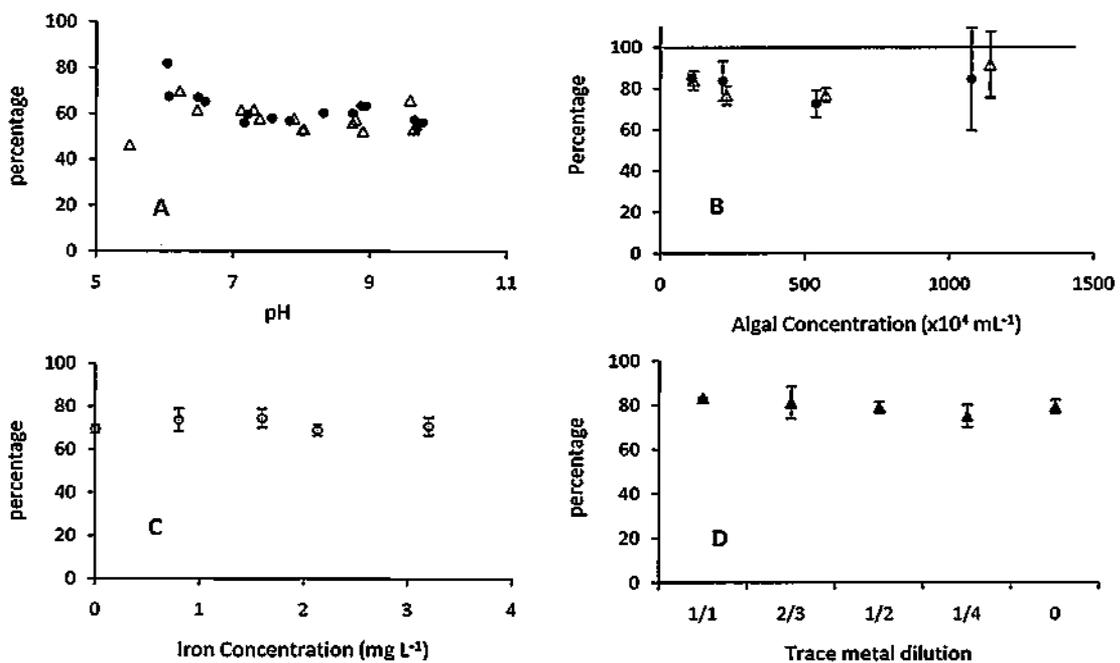


Figure 3.1. Recovery of $20 \mu\text{g L}^{-1}$ Rh in the presence of *C. stigmatophora* as a function of pH (A), *C. stigmatophora* cell concentration (B), iron concentrations (C) and nutrient trace metals concentration (D). (●) represents data for +TM cultures, (△) represents data for -TM cultures, (○) represents data for cultures with various concentrations of Fe and (▲) represents data for cultures with various concentrations of nutrient trace metals of $f/2$ media. Initial algal cell concentrations were $6.9 \times 10^6 \text{ cell mL}^{-1}$ for (A) +TM, $5.9 \times 10^6 \text{ cell mL}^{-1}$ for (A) -TM, $4.9 \times 10^6 \text{ cell mL}^{-1}$ for (C) and $4.2 \times 10^6 \text{ cell mL}^{-1}$ for (D). pH alterations in each experiment is detailed in Appendix 2.1. Error bars denote the standard deviations about the mean of three independent measurements. Dashed line indicates 100% value.

Increasing the number of algal cells resulted in a reduction of Rh recovery from 84% to 72% ($p < 0.05$), while at the highest cell concentration the recovery increased. This increase, which was not statistically different, is presumably occurred due to the pH alteration in this group of cultures. At the time of filtration, pH was recorded as 4.94 ± 2.80 for +TM cultures and 5.12 ± 2.42 for -TM cultures and this possibly caused the reduction in Rh recovery. In dense cultures of algae, higher values of pH are expected due to a greater extent of CO₂ consumption by algae and, therefore, these low values of pH recorded for the cultures are unexpected. Since the samples were randomly selected for pH adjustment, it is assumed that mis-estimation of the required volume of HCl for adjusting pH resulted in the reduction of pH in some of the replicates in these treatments.

Recovery of Rh was shown to be independent of Fe and nutrient trace metal concentrations (Figure 3.1. C and D) and ranged between 69% to 83%, although the recovery of PGE was slightly higher in Fe experiments ($p > 0.05$).

3.3.1.2. Palladium

The recovery of added palladium was the lowest among the three metals and ranged between 40% and 60% (Figure 3.2). At $\text{pH} \geq 8$ the recovery of Pd increased to above 50% with a gradual rise up to $\text{pH} \sim 10$, although not significantly, and this is in disagreement with previous findings by Cobelo-Garcia *et al* (2007). They studied the recovery of PGE from different contact surfaces in different types of water, and reported that recovery of Pd in English Channel waters exceeded 90% with a small increase at pH above 6. This discrepancy might indicate the contribution of algae on recovery of metals. This is

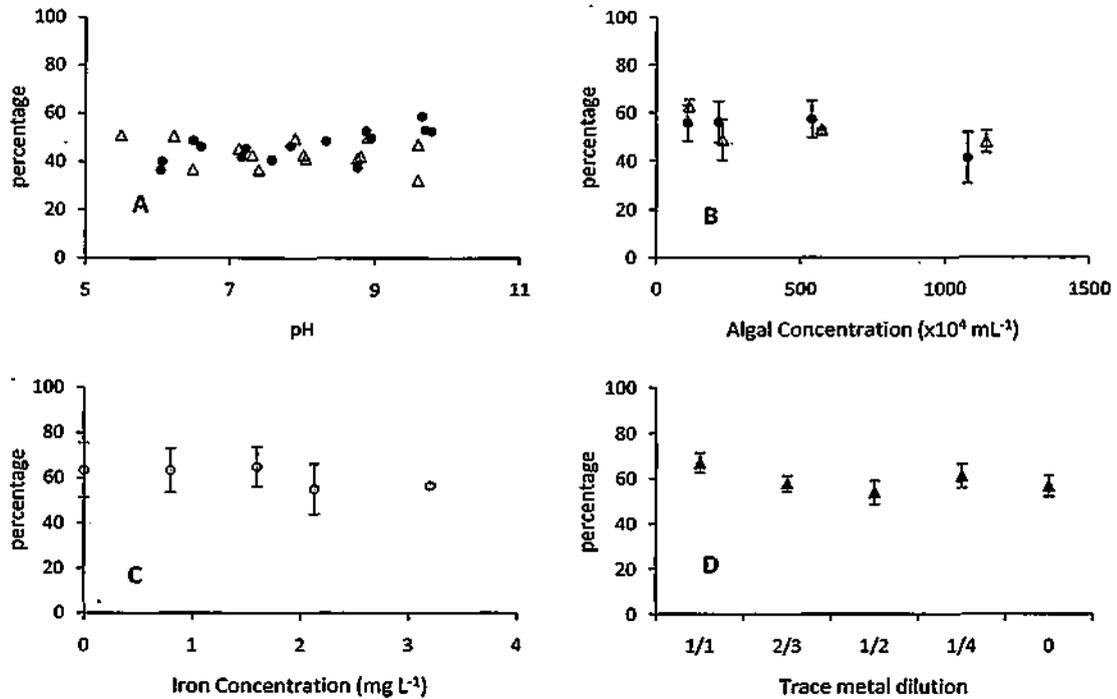


Figure 3.2. Recovery of $20 \mu\text{g L}^{-1}$ Pd in the presence of *C. stigmatophora* as a function of pH (A), *C. stigmatophora* cell concentration (B), iron concentrations (C) and nutrient trace metals concentration (D). (●) represents data for +TM cultures, (Δ) represents data for -TM cultures, (○) represents data for cultures with various concentrations of Fe and (▲) represents data for cultures with various concentrations of nutrient trace metals of *f/2* media. Initial algal cell concentrations were $6.9 \times 10^6 \text{ cell mL}^{-1}$ for (A) +TM, $5.9 \times 10^6 \text{ cell mL}^{-1}$ for (A) -TM, $4.9 \times 10^6 \text{ cell mL}^{-1}$ for (C) and $4.2 \times 10^6 \text{ cell mL}^{-1}$ for (D). pH alterations in each experiment is detailed in Appendix 2.1. Error bars denote the standard deviations about the mean of three independent measurements.

consistent with other findings in this research that indicate increasing concentration of algal cells results in a reduction of Pd recovery (Figure 3.2.D). This inverse proportional relationship between cell concentration and Pd recovery might show the impact of algal cell exudates on the speciation of Pd. Iron did not show any noticeable effect on Pd recovery ($p > 0.05$), while dilution of essential trace metal concentration in the media resulted in a 10% decrease in Pd recovery ($p < 0.05$), but this reduction did not vary signif-

icantly across the different concentrations of nutrient trace metals. This is likely to be attributed to the greater concentration of EDTA in solution. As described in Chapter Two, nutrient trace metal solution in *f/2* media contains $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ as a chelator for controlling the availability of metals in the solution to algae. It seems that Pd and, to a lower extent, Rh can be solubilized by a complexing agent such as EDTA. Therefore, these metals are less adsorbed to container walls, and became more available in solution.

3.3.1.3. Platinum

Platinum appeared to show the lowest extent of loss from the dissolved phase and above 90% of the metal was recovered in all experiments (Figure 3.3). Platinum recovery was found to be independent of pH, while increasing number of cells resulted in a greater loss of Pt from the solution with a reduction of recovered Pt from 90% to less than 80% ($p < 0.05$). Platinum is kinetically inert relative to Rh and Pd complexes (Cosden *et al.*, 2003) and has stronger stabilization in seawater through complex formation with such ligands as chloride (Hårdstedt-Roméo, 1982). This makes Pt less reactive towards surfaces (as well as container walls). Therefore, Pt is not expected to be lost to as high an extent as either Pd or Rh.

In some samples, mass balance calculations revealed recovery values moderately greater than 100%. It is assumed that minor artefacts arising from the formation of molecular ions in the plasma of the ICP-MS or imperfect dispensation of the Pt stock are responsible for high values.

Iron concentrations did not influence the recovery of Pt significantly, while dilution of

nutrient trace metals resulted in an increase in Pt recovery ($p < 0.05$ in trace metal dilutions of $1/2$, $1/4$ and 0) which is not consistent with the data obtained for Rh and Pd.

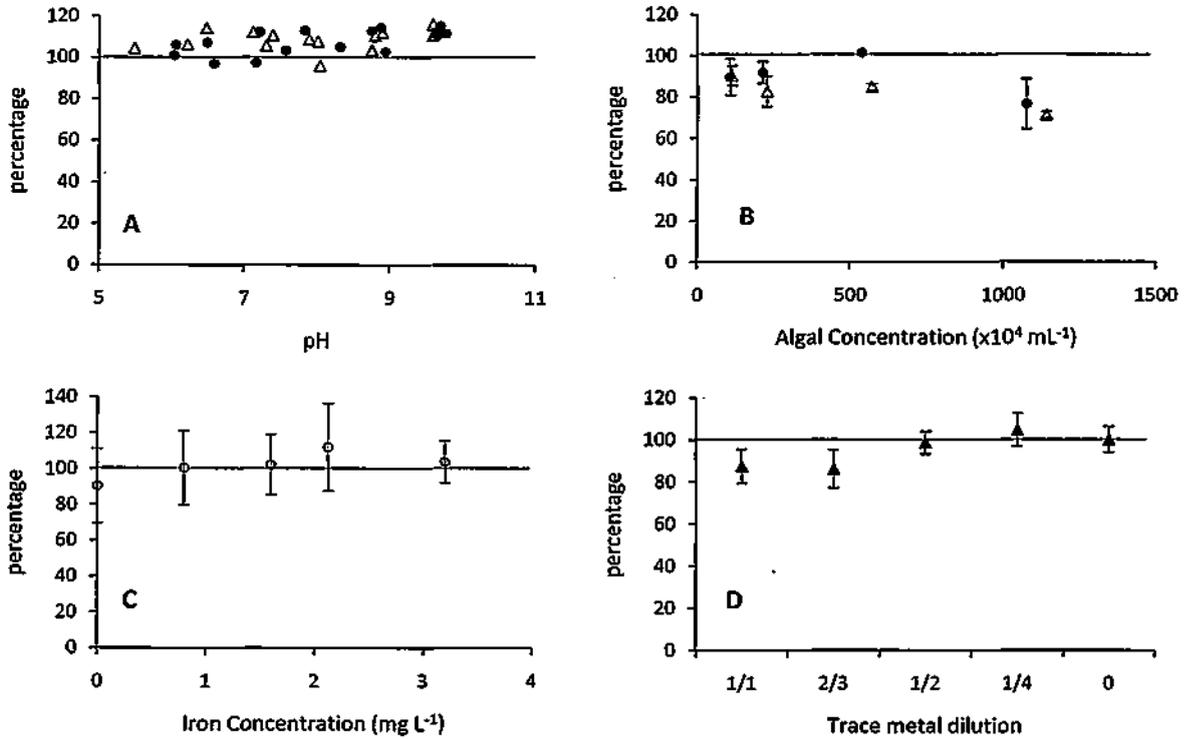


Figure 3.3. Recovery of $20 \mu\text{g L}^{-1}$ Pt in the presence of *C. stigmatophora* as a function of pH (A), *C. stigmatophora* cell concentration (B), iron concentrations (C) and nutrient trace metals concentration (D). (●) represents data for +TM cultures, (Δ) represents data for -TM cultures, (\circ) represents data for cultures with various concentrations of Fe and (\blacktriangle) represents data for cultures with various concentrations of nutrient trace metals of f/2 media. Initial algal cell concentrations were $6.9 \times 10^6 \text{ cell mL}^{-1}$ for (A) +TM, $5.9 \times 10^6 \text{ cell mL}^{-1}$ for (A) -TM, $4.9 \times 10^6 \text{ cell mL}^{-1}$ for (C) and $4.2 \times 10^6 \text{ cell mL}^{-1}$ for (D). pH alterations in each experiment is detailed in Appendix 2.1. Error bars denote the standard deviations about the mean of three independent measurements. Dashed line indicates 100% value.

3.3.2. Accumulation of Platinum Group Elements by *C. stigmatophora*

3.3.2.1. Dependence of PGE Accumulation on pH

Increasing phytoplankton cell density in a batch culture causes the rate of inorganic carbon uptake and fixation to exceed the rate of CO₂ supply from the atmosphere (Sunda *et al.*, 2005). Therefore, the CO₂ levels in the medium decrease and lead to a rise in pH. In this study, pH was observed to increase significantly during the cultivation period. Since pH elevation in the medium can affect the chemistry and/or speciation of metals and the net charge on the algal surface, the accumulation of PGE by the algae was studied over a range of pH values to help to elucidate how Pt, Pd and Rh behave or react towards algal surfaces in seawater. The pH was stabilized as much as possible by interval adjustments during the experiment. It, however, increased in all cultures, presumably shortly after every adjustment. At the end of the incubation, recorded pH values varied from the initial adjusted pH. The mean value of pH measured for each replicate, shown in Table 3.6, was used in the analysis and the alterations of pH during the exposure are detailed in Appendix 2.1.

Figure 3.4 shows that accumulation factors (AF) of Rh, Pd and Pt for *Chlorella* as a function of pH. Rhodium AF was the highest of the three metals tested and Pt AF was the lowest. By increasing pH within the range of 5.5 to 9.8, Rh AF was significantly ($p < 0.05$) greater in +TM cultures compared with -TM cultures, and it exhibited an increase of one order of magnitude, from $33 \times 10^3 \text{ L kg}^{-1}$ to $300 \times 10^3 \text{ L kg}^{-1}$ in +TM cultures and from $10 \times 10^3 \text{ L kg}^{-1}$ to $110 \times 10^3 \text{ L kg}^{-1}$ (pH~9) in -TM cultures. Platinum AF by *C. stigmatophora* ranged about 1 to 1.5×10^3 and displayed no clear dependence on pH, although a small decrease in its accumulation was seen in -TM cultures. Overall, no

significant differences were observed for algal accumulation of Pt between +TM and -TM cultures ($p > 0.05$). Palladium AF in +TM cultures showed a gradual reduction ($p < 0.05$) from $11 \times 10^3 \text{ L kg}^{-1}$ at pH = 6.5 to $2 \times 10^3 \text{ L kg}^{-1}$ at pH = 9.7. However, no significant difference in AF between +TM and -TM was observed ($p > 0.05$).

Table 3.6. pH alterations in *C. stigmatophora* cultures exposed to $20 \mu\text{g L}^{-1}$ of mixed solution of PGE for 24 h. Data presented as mean value \pm standard deviations of 6 to 9 measurement of pH (detailed in Appendix 2.1)

Treatment (Initial pH)	Replicate	Mean pH \pm SD	
		+TM	-TM
5.5	a	6.50 \pm 1.86	6.22 \pm 1.60
	b	6.04 \pm 1.84	5.49 \pm 1.27
	c	6.11 \pm 1.51	6.38 \pm 1.54
6.5	a	7.16 \pm 1.73	7.12 \pm 1.16
	b	6.54 \pm 1.80	7.30 \pm 1.32
	c	7.63 \pm 1.30	7.27 \pm 1.21
7.5	a	8.32 \pm 0.91	7.89 \pm 1.00
	b	8.15 \pm 0.98	8.01 \pm 0.80
	c	7.93 \pm 1.08	7.94 \pm 0.82
8.5	a	8.94 \pm 0.50	8.75 \pm 0.54
	b	8.87 \pm 0.52	8.80 \pm 0.47
	c	8.79 \pm 0.56	8.83 \pm 0.49
9.5	a	9.65 \pm 0.24	9.59 \pm 0.07
	b	9.67 \pm 0.28	9.59 \pm 0.08
	c	9.76 \pm 0.28	9.64 \pm 0.18

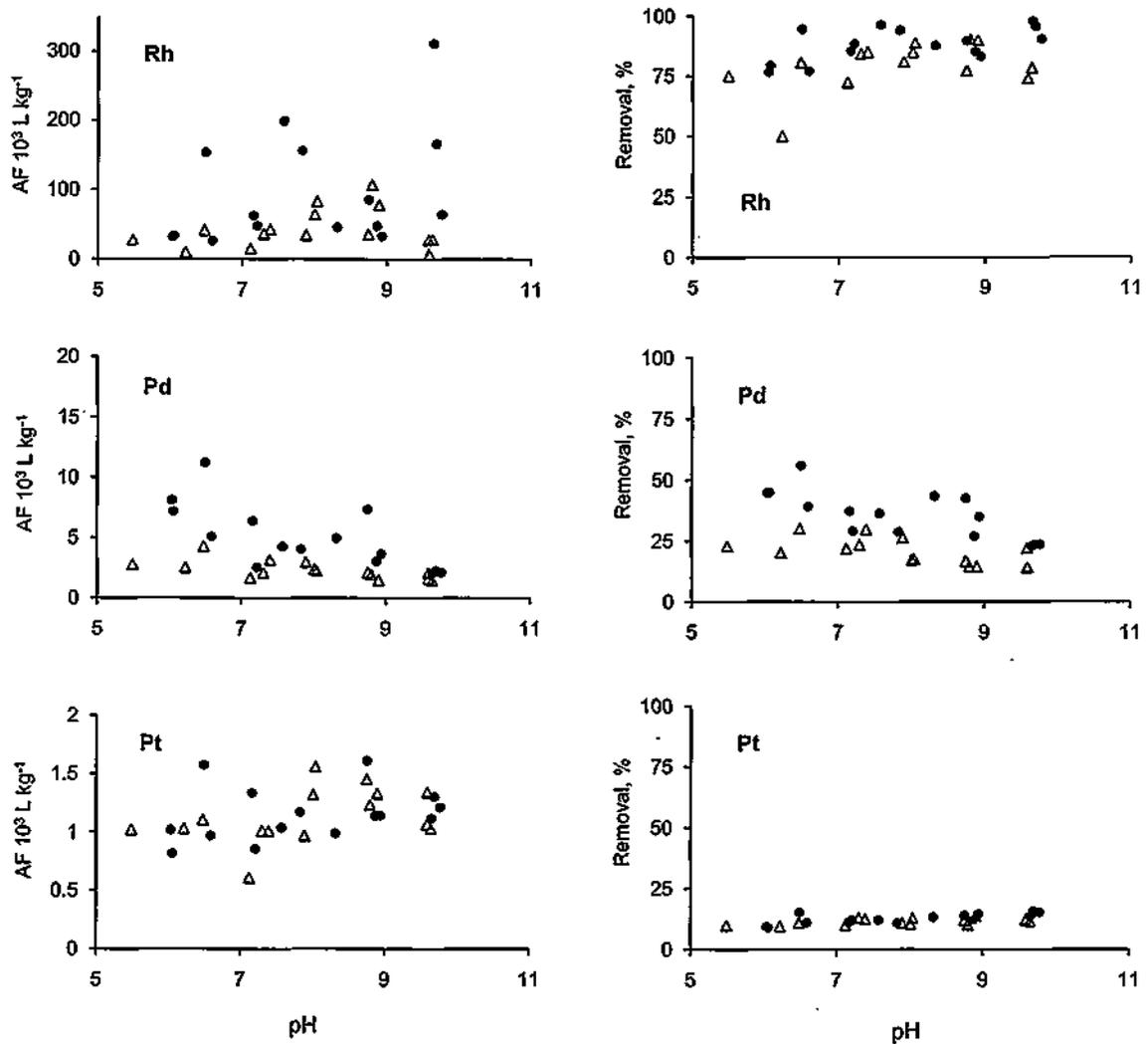


Figure 3.4. Uptake of Rh, Pd and Pt by *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of mixed solution of PGE for 24 h, shown in terms of accumulation factor – AF (left column) and percentage of removal from water (right column), as function of pH. Removal was calculated from the percentage of PGE concentration in algae relative to total analytical concentration. Initial cell concentrations of algae were $6.9 \times 10^6 \text{ cell mL}^{-1}$ for +TM cultures (●) and $5.9 \times 10^6 \text{ cell mL}^{-1}$ for -TM cultures (Δ). pH values for each data point are detailed in Table 3.6.

Changes in the pH of the medium can influence the accumulation of metal by affecting metal speciation and/or the biological surface (Simkiss & Taylor, 1995). The surface of algal cells contain a number of functional groups with high affinity for metal ions and carry a negative charge, mainly due to carboxylic, sulphhydryl and phosphatic groups

(Franklin et al., 2002a). When the pH of the medium increases, an increasing number of negatively charged sites are formed on cell surfaces (Franklin et al., 2000). As Rh exists predominantly in cationic form ($[\text{RhCl}(\text{H}_2\text{O})_5]^{2+}$ and $[\text{RhCl}_2(\text{H}_2\text{O})_4]^+$; (Turner et al., 2007)) and Pd and Pt in anionic form (PdCl_4^{2-} and $\text{PdCl}_3\text{OH}^{2-}$; PtCl_6^{2-} and $\text{PtCl}_5\text{OH}^{2-}$ in seawater; (Cosden et al., 2003; Turner, 2007), it is predicted that increased pH results in increased Rh adsorption by the cell surface. This leads to a greater accumulation of cationic metal species and/or less accumulation of anionic forms, which are Pd and Pt in this study. In contrast, at low pH when more H^+ is available, the surface of phytoplankton cells presents protonated groups (Vargas et al., 2004) and hence anionic palladium species are electrostatically attracted and subsequently coordinated at the cell surface. Although Pd and Pt in seawater are anionic predominantly, they still exist in cationic forms as free ions, though in very low abundance. Therefore their interaction with algae can be similar to Rh, but because of their small contribution to speciation they are accumulated to a lesser extent. Thus, in other words, Pd^{2+} and Pt^{4+} might strongly bind to the binding sites on the algal surface, but due to their very low abundance in seawater, less overall accumulation of these metals occurs. Moreover, the results also show a lower Pd accumulation, although not significant, when no nutrient trace metals or EDTA were added to the solution (-TM culture). This suggests that other mechanisms may also be involved in Pd accumulation. For example, it is not known if Pd accumulation by *C. stigmatophora* is assisted by nutrient trace metals in solution, since toxic metals typically enter cells through the transport systems of nutrient metals (Sunda, 2000; Sunda & Huntsman, 1998).

The results obtained regarding the pH dependence of PGE accumulation are qualitatively consistent with equivalent results obtained using the marine macroalga *Ulva lactuca* (Turner et al., 2007) and sediment-seawater suspensions (Turner, 2007). Accumulation of

Rh by *U. lactuca*, exposed to $10 \mu\text{g L}^{-1}$ of PGE for 100 h, increased considerably with increasing pH (above 8.3). In contrast Pd accumulation displayed no systematic dependence on pH across the range studied (7.9–8.4), while Pt exhibited a small increase in accumulation. The increase in Rh removal by estuarine sediment with increasing pH in both river and estuarine waters has been shown to be associated with a reduction in protonation of the particle surface which implies that particle–water interactions involve the adsorption of cationic species of Rh (Turner, 2007).

The accumulation of PGE by *Chlorella* is also shown as the percentage of PGE removal from the aqueous phase by the algae (Figure 3.4). Percentage was calculated from the algal metal concentration relative to total analytical concentrations at each pH. In all cases metal removal in –TM cultures was relatively insensitive to pH, but in the presence of nutrient trace metals (+TM cultures), removal of both Rh and Pt slightly increased with increasing pH throughout the range studied, but removal of Pd decreased from 45 – 55% at pH ~ 6 to 23% at pH > 9. The increasing removal of Rh and Pt with pH has also been observed by estuarine sediments (Turner, 2007) although the extent of removal of PGE is greater in the presence of algae which indicates the important role of algal surface interactions with PGE.

3.3.2.2. Dependence of PGE Accumulation on Algal Concentration

Results of the experiment in which various concentrations of *C. stigmatophora* were exposed to $20 \mu\text{g L}^{-1}$ of PGE are shown in Figure 3.5. With the exception of Rh accumulation in –TM cultures, AF for all PGE exhibited a significant reduction ($p < 0.05$) with increasing concentration of phytoplankton cells in the medium. The AF of Pt was 5

times lower than Pd and Rh and decreased from about $3.5 \times 10^3 \text{ L kg}^{-1}$ to $0.9 \times 10^3 \text{ L kg}^{-1}$ by increasing the algal cell concentration. Rh accumulation in -TM cultures was not dependent on algal cell concentrations and was about $5 \times 10^3 \text{ L kg}^{-1}$ across the various cell concentrations of *Chlorella*.

The effect of particle concentration on weight normalized metal accumulation by a solid is generally defined by equation 3.1 (Schwarzenbach et al., 1993):

$$K_D = a\text{SPM}^{-b} \quad (3.1)$$

where K_D is the distribution coefficient and is equivalent to the accumulation factor (AF), SPM is suspended particulate matter, equivalent to algal concentration, a is the partitioning normalised to a particle concentration of 1 g L^{-1} , and b is the slope of the effect. Both a and b are empirical constants and are derived from non-linear regression analyses of the data. This equation is commonly observed in the literature for metal-sediment interactions (Turner & Wu, 2007). This type of equation can be applied here for microalgal cells instead of SPM. This is the very first time that the effect is demonstrated for the interaction of any metal with marine microalgae. In the present context, we modify the equation to:

$$\text{AF} = a[\text{alga}]^{-b} \quad (3.2)$$

where AF is the accumulation factor and [alga] is concentration of phytoplankton cells in solution. The values of a and b for Rh, Pd and Pt are reported in Table 3.7.

Table 3.7. Empirical constants defining the relationship between AF and phytoplankton concentration (Eq. 3.1). p is statistical significance between AF in +TM and -TM cultures

		a ($L\ kg^{-1}$)	b	R^2	P value
Rh	+TM	268000	0.619	0.855	0.01
	-TM	4360	0.021	0.003	
Pd	+TM	53100	0.259	0.505	0.74
	-TM	182000	0.484	0.882	
Pt	+TM	45900	0.581	0.894	0.90
	-TM	31700	0.494	0.903	

An inverse dependence of PGE concentration on a w/w basis of the concentration of *C. stigmatophora* is an expected result because for a given PGE concentration, increasing cell concentration is accompanied by increasing number of uptake sites. However, in this case, dissolved and accumulated metal should decrease proportionally and, therefore, AF (which reflects the partitioning of metal between algal cells and the solution) should remain constant. The results of this experiment, however, show that the AF of PGE and algal cell concentration have an inverse relationship. A consequence of this relationship is that the increase in the percentage removal of PGE as a function of cell concentration is offset to some extent dependent on the value of the parameter b (Figure 3.5). In the case of Pt, the value of b is so high that removal of the metal from the aqueous phase does not increase with increasing algal concentration.

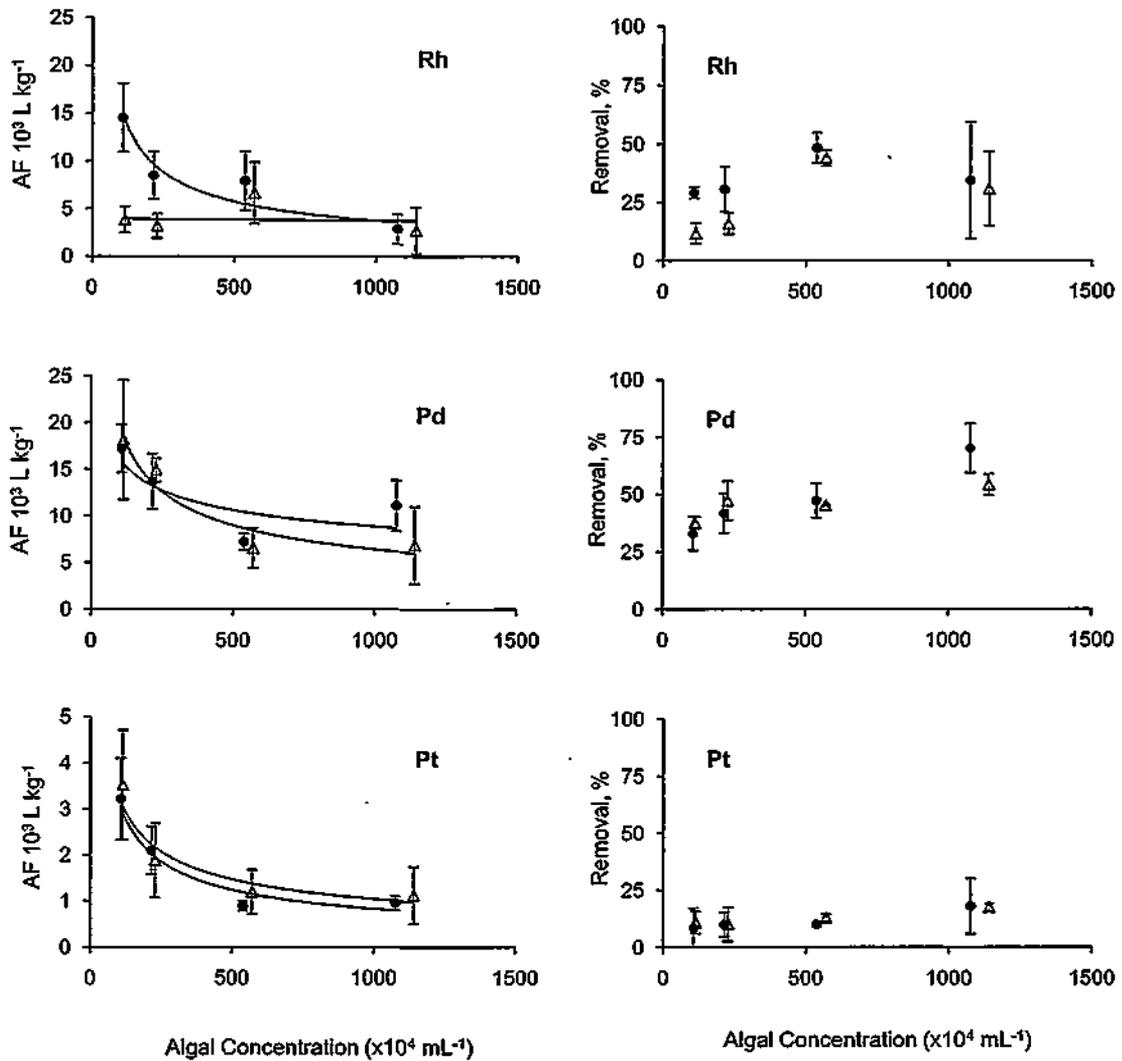


Figure 3.5. Uptake of Rh, Pd and Pt by *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of mixed solution of PGE for 24 h, shown in terms of accumulation factor – AF (left column) and percentage of removal from water (right column), as function of various microalgal concentrations. (●) represents data for +Tm cultures and (Δ) represents data for –TM cultures. pH alterations in each experiment is detailed in Appendix 2.1. Error bars denote the standard deviations about the mean of three independent measurements.

There are various possible reasons for the reduction in the partitioning of metals to sediment as a function of particle concentration that have been discussed in the literature (Benoit et al., 1994; Gschwend & Wu, 1985; Turner & Wu, 2007). The higher concentration of sediments can cause sediment to aggregate and reduce the exposed surface area or number of binding sites. Also, an increase in sediment concentration may be accompanied by an increase in the number of filterable colloidal forms which add to the aqueous phase pool and reduce the apparent partitioning. Neither of these mechanisms applies to living microalgae, however. Firstly, they are not able to aggregate because, during the mid-exponential growth phase, intracellular compounds are released that prevent coagulation. Secondly, the microscopic monitoring in this experiment showed that *Chlorella* cells did not rupture through the filtration process, indicating that smaller filterable particulate entities were not formed. However, it is possible that exudates generated by the algae may act as a third, filterable phase that cause or contribute to the relationship between AF and algal concentration in the present experiment.

A mechanism which is considered to be involved in PGE accumulation and regulation is the release of exudates by microalgal cells. The production of exudates with metal binding capacity is one of the mechanisms by which algae regulate their internal cell environment as well as their immediate surroundings. Exudates are known to be electron donors (Gerringa et al., 2000), and therefore the release of such organic ligands (exudates) to the external medium, may significantly alter the trace metal speciation and bioavailability and, as a consequence, alter the equilibrium between the cell surface sites and bulk solution (Vasconcelos & Leal, 2001). Studies on microalgal species have indicated that some species are capable of producing ligands with a strong binding capacity for Cu (Levy et al., 2008) and in the freshwater microalgae *Chlamydomonas reinhardtii*, exudates were found

even more significant in altering Cu speciation than binding to algal cell surfaces (Xue & Sigg, 1990).

Some species of green algae produce a mucilaginous capsule composed of polysaccharides. Polysaccharide production is more pronounced during the stationary phase of growth. However, significant release of polysaccharides into the medium during the logarithmic phase of growth has also been reported (Kaplan *et al.*, 1987). These polysaccharides act as natural metal chelators and thus reduce metal toxicity by altering the speciation of metal and hence inhibiting their accumulation. Kaplan *et al.* (1987) found that the metal complexing capacity of polysaccharides produced by *C. stigmatophora* was dependent on the metal and the concentration of released polysaccharides. The dissolved polysaccharide of *C. stigmatophora* was capable of binding Cd^{2+} , Cu^{2+} , Zn^{2+} and Pb^{2+} , although to different extent of complexing capacity. The binding capability is reported to be due to the negative surface charges of the polysaccharide that possibly result from the presence of uronic acids or sulphate. It seems that the free carboxylic groups of uronic acids in the polysaccharide of *C. stigmatophora* play a major role in metal complexing, while sulphate ions apparently play only a minor role. Only 25% of *C. stigmatophora* polysaccharides are neutral and weakly charged, whilst the major portion of the polymer mixture (75%) is charged and there is a correlation between highly anionic charged polymers and metal-complexing capacity.

As mentioned above, the amount of metal bound to polysaccharides depends on the metal as well as the concentration of polysaccharides. At greater concentrations of algal cells, more polysaccharides are released into the water, and as a result, metal speciation in the solution will be affected for a given concentration of aqueous metal. In the present

experiments, this can stabilize PGE in solution and reduce the concentration binding to the algae. Therefore, with increasing cell concentration, less PGE is accumulated by algae and more remains in the water and as a consequence the accumulation factor, i.e. the ratio of algal PGE concentration to aqueous PGE concentration, diminishes. The extent of this effect is expected to be dependent on the affinity of the metal for the exudates. The binding of PGE by aqueous organic ligands is unknown, but based on their chemistries Pd and Pt are predicted to be complexed to greater extents than Rh, and Pt binding may be constrained because its reaction kinetics are relatively slow compared with those of Pd. In this experiment, no analysis was carried out on the release of algal exudates and their capability to bind with PGE. However, it is known that dissolved polysaccharides of *C. stigmatophora* are capable of binding Cd^{2+} , Cu^{2+} and Zn^{2+} (Kaplan et al., 1987), although the metal complexing capacity of these polysaccharides was found to be different for each metals. Further investigation is required to evaluate the extent of exudates released by *C. stigmatophora* as well as their capability in complexing with PGE in seawater.

The cell concentration or particle concentration effects of the marine macroalga *Ulva lactuca* exposed to $10 \mu\text{g L}^{-1}$ of PGE are different to the results of this study, but are more consistent with their complexation chemistries mentioned above (Turner et al., 2007). Thus, Pt AF appeared to be insensitive to the biomass of *U. lactuca*, but accumulation of Rh increased and Pd diminished with increasing algal concentration. The Rh response was also attributed to an increase in pH that was due to the increasing algal concentration. In this experiment, alteration of pH was a concerning issue particularly in cultures with high concentration of algal cells. Despite the effort for maintaining pH by interval adjustments, a decrease of pH in some of the cultures occurred which resulted in an increase at Pd accumulation and a decrease in Rh accumulation in those particular cultures.

3.3.2.3. Dependence of PGE Accumulation on Fe (III) Concentration

Accumulation of platinum group elements by *C. stigmatophora* was studied under various concentrations of Fe(III), based on serial dilutions of FeCl₃ in *f/2* culture media. After 24 h of exposure, Rh was accumulated the most (AF = $16.6 \times 10^3 \pm 1.4 \times 10^3$ L kg⁻¹) and Pt the least (AF = $0.8 \times 10^3 \pm 0.09 \times 10^3$ L kg⁻¹). Palladium AF was $7.6 \times 10^3 \pm 0.6 \times 10^3$ L kg⁻¹ at all Fe(III) concentrations and its accumulation was not dependent on the concentrations of Fe(III) in the media, while Rh and Pt accumulation factor exhibited a small decrease with increasing Fe(III) concentration (Figure 3.6), although statistical analysis (ANOVA) did not indicate a significant difference for this reduction ($p > 0.05$).

The percentage of PGE removal from solution, shown in Figure 3.6, also indicates that accumulation of Rh, Pd and Pt is independent of Fe(III) and that variation in iron concentration does not affect the accumulation of PGE. Across the various concentrations of Fe, 60% to 70% of Rh was removed by *C. stigmatophora*, while consistent with previous results, Pd removal was about 50% and Pt removal did not exceed 20% of the total Pt. While Fe(III) did not interfere with PGE accumulation by *C. stigmatophora*, it may have stimulated its growth since iron is known to play a critical role in growth of phytoplankton. Studies on iron enrichment experiments have shown that a low iron concentration can limit productivity and control species diversity in many algal communities (Sunda & Huntsman, 1995). Iron performs essential metabolic functions in photosynthetic and respiratory electron transport, nitrate reduction, nitrogen utilization and metabolism, chlorophyll biosynthesis, and detoxification of reactive oxygen species (Chen *et al.*, 2003; Sunda *et al.*, 2005). The growth rate of *C. stigmatophora* was not calculated at

the end of the exposure and, therefore, it is not known to what extent growth or productivity of algae in cultures was influenced by the concentration of Fe(III).

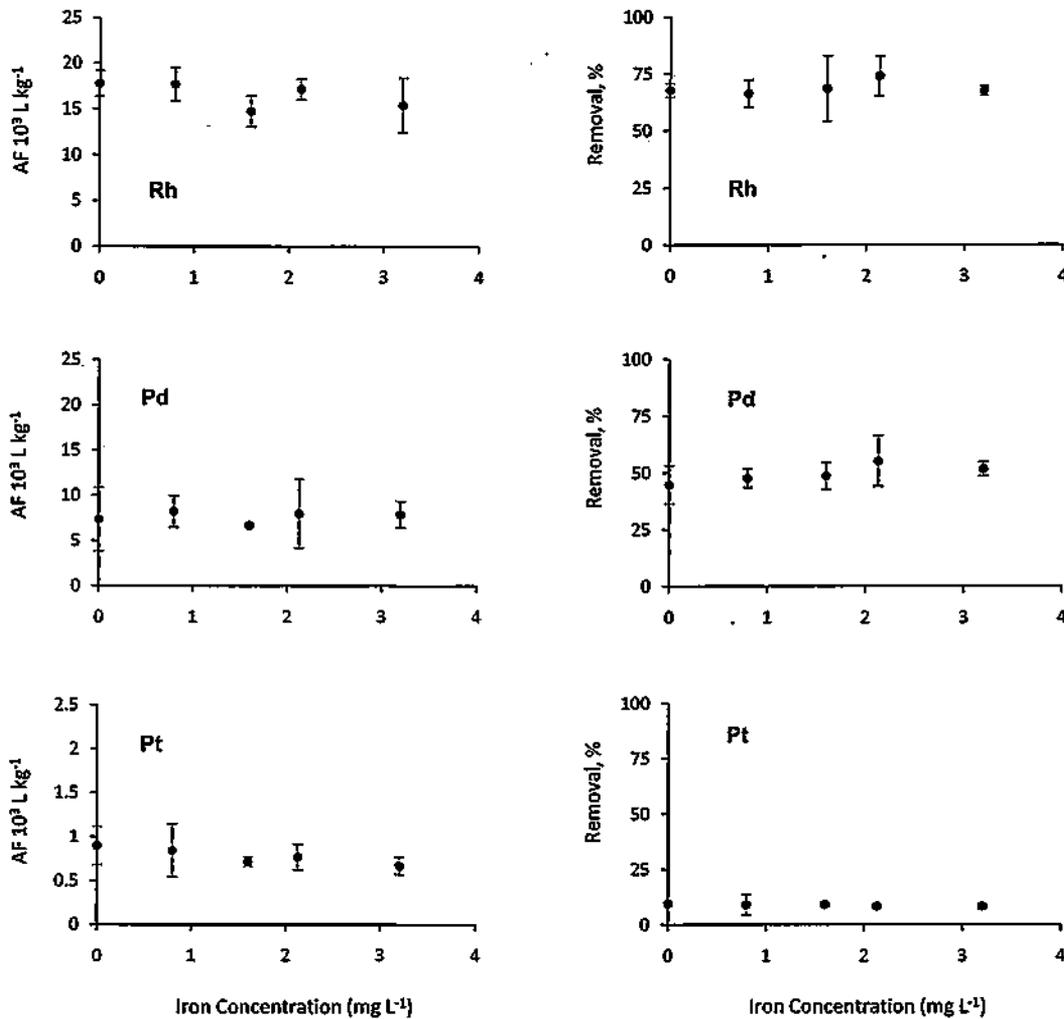


Figure 3.6. Uptake of Rh, Pd and Pt by *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of mixed solution of PGE for 24 h, shown in terms of accumulation factor – AF (left column) and percentage of removal from water (right column), as function of concentration of Fe(III). Removal was calculated from the percentage of PGE concentration in algae relative to total analytical concentration. Initial cell concentrations of algae were $4.9 \times 10^6 \text{ cell mL}^{-1}$. pH alterations in each experiment is detailed in Appendix 2.1. Error bars denote the standard deviations about the mean of three independent measurements.

Macronutrients e.g. N, P and Si, in general have a proportional relationship with Fe(III) accumulation and an increase in their concentration results in a greater accumulation of Fe(III). However, with regard to toxic trace metals, a common mode of action is the inhibition of nutrient metal accumulation and intracellular interference with nutrient metal metabolism (Sunda *et al.*, 2005). As a consequence, antagonistic interactions often exist between toxic and nutrient metals. This type of interaction was also expected for PGE and nutrient metals of the medium. However, the findings indicated contrary results and accumulation of Rh, Pd and Pt by algae was not shown to be dependent on Fe(III). The small increase of the AF for Rh at Fe concentrations of 0.8 and 0 mg L⁻¹ is attributed to the higher levels of pH, which was due to the insufficient volume of HCl added to solution for pH adjustment as explained in Section 3.3.1.1.

The interference of Fe and PGE accumulation by *C. stigmatophora* was also studied and the results indicated that iron AF in control cultures, with no added PGE, was considerably ($p < 0.05$) lower than iron accumulation in the PGE spiked cultures (Figure 3.7). This discrepancy can be attributed to the aqueous concentration of Fe(III), since the internalized and surface bound concentrations of Fe(III) (Table 3.8) were not significantly different between the two cultures. The dissolved iron in the aqueous phase was one order of magnitude greater in control cultures than in PGE spiked algal cultures. The considerable difference of iron AF between the control algae and PGE spiked algae is due to, firstly, the extent of iron removal from the medium, and secondly, the differences in the extent of Fe(III) recovery in these two cultures. Fifty four percent of Fe(III) was recovered in control cultures, whereas only 34% was recovered in PGE spiked treatments. Therefore, more Fe(III) was available to be accumulated by algae in the control cultures, whilst the results showed a similar extent of intra-cellular and extra-cellular iron was accumulated by *C.*

stigmatophora in the two cultures. The amount of $8.6 \pm 2.9 \text{ mg g}^{-1}$ and $7.5 \pm 1.4 \text{ mg g}^{-1}$ Fe(III) was accumulated by algal cells from the control (containing 3.2 mg L^{-1} Fe(III)) and the treatment (containing 3.2 mg L^{-1} Fe(III) and $20 \mu\text{g L}^{-1}$ PGE), respectively, and about 68% of total Fe was found internalized in both cultures. As a result, the lower extent of Fe(III) AF in control cultures does not necessarily mean that less amount of Fe(III) was accumulated by the algae. In other words, PGE and Fe(III) accumulation by *C. stigmatophora* do not appear to interfere. However, Fe(III) is more available in the absence of PGE due to the greater extent of its recovery.

Table 3. 8. Concentrations of Fe accumulated by *C. stigmatophora* exposed to 3.2 mg L^{-1} Fe for 24 h in the presence (treatment) and in the absence (control) of $20 \mu\text{g L}^{-1}$ of PGE in the culture

	Internalized (mg g^{-1})	Surface adsorbed (mg g^{-1})	Total accumulated (mg g^{-1})	Dissolved (mg L^{-1})
control	5.61 ± 0.81	3.08 ± 2.46	8.69 ± 2.95	0.60 ± 0.15
treatment	5.00 ± 0.51	2.46 ± 0.94	7.46 ± 1.42	0.06 ± 0.03

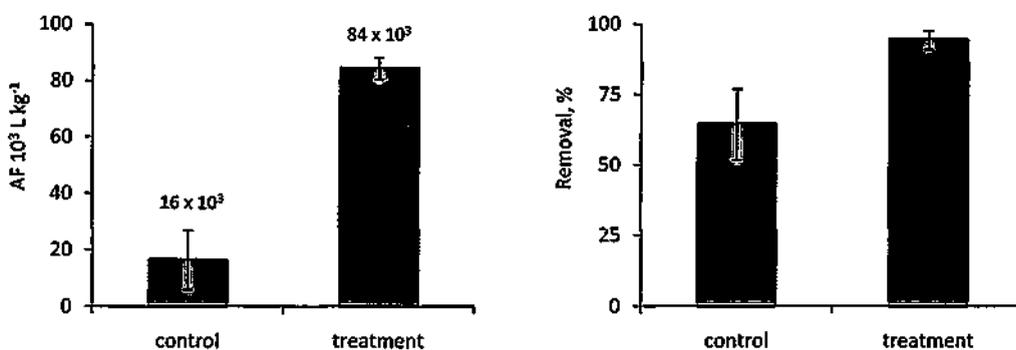


Figure 3.7. Comparison of Fe(III) accumulation by *C. stigmatophora* between the control cultures (algae exposed to 3.2 mg L^{-1} Fe(III)) and treatments (algae exposed to 3.2 mg L^{-1} Fe(III) and $20 \mu\text{g L}^{-1}$ of mixed solution of PGE) for 24 h, shown in terms of accumulation factor – AF (left) and percentage of removal from water (right). Initial cell concentration of algae was $4.9 \times 10^6 \text{ cell mL}^{-1}$. Error bars denote the standard deviation about the mean of three independent measurements.

3.3.2.4. Dependence of PGE Accumulation on Essential Trace Metals

Figure 3.8 shows the AF of Rh, Pd and Pt by *C. stigmatophora* cells as a function of various dilutions of nutrient trace metals in the media. Consistent with previous results, the percentage of PGE removed from total recovered PGE was about 70% for Rh, about 45% for Pd and less than 20% for Pt. Palladium AF did not follow a clear trend with different dilutions of the essential trace metal concentrations, while with decreasing concentration of nutrient trace metals, Pt and Rh showed a gradual decline of about 30% and 23%, respectively ($p < 0.05$).

Toxic metals are typically accumulated by cells through the transport systems of nutrient metals. For instance, by binding to carrier molecules for essential metals or by building metal complexes with other essential nutrients (Haus et al., 2007; Sunda et al., 2005). Thus, to understand the mechanisms and factors regulating the accumulation and effects of toxic metals, the competitive interactions between these metals and nutrient metals must be considered. Interactions between nutrient and toxic metals are quite common and have been identified in a variety of microalgal species (Sunda, 2000; Sunda & Huntsman, 1998). It appears to be one of the major factors in metal inhibition of growth and metabolism. Whether accumulation of combinations of essential and non-essential metals is synergistic or antagonistic depends on whether one metal facilitates the accumulation of the other and/or whether they compete for the same transport sites on the cell membrane. For example, studies have shown that accumulation of Cu by *Chlorella* sp. increased in the presence of Cd, whereas accumulated Cd reduced in the presence of Cu. Moreover, accumulation of Cu by *Chlorella* sp. was not affected by Zn, whereas, Zn accumulation in the presence of Cu was diminished (Franklin et al., 2002b).

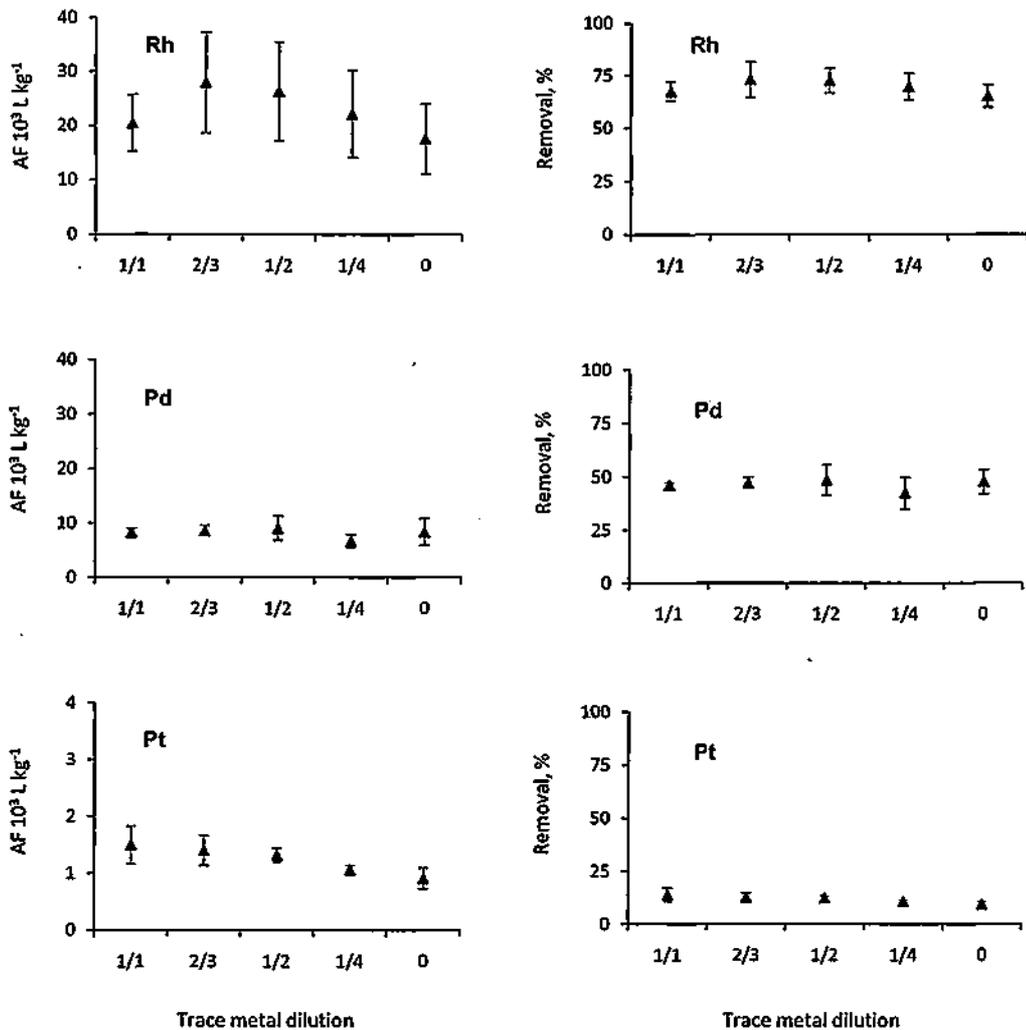


Figure 3.8. Uptake of Rh, Pd and Pt by *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of mixed solution of PGE for 24 h, shown in terms of accumulation factor – AF (left) and percentage of removal from water (right), as function of concentration of nutrient trace metals in *f/2* media. Initial cell concentrations of algae were $4.2 \times 10^6 \text{ cell mL}^{-1}$. pH alterations in each experiment is detailed in Appendix 2.1. Error bars denote the standard deviations about the mean of three independent measurements.

The results of this study showed that the removal of PGE from seawater was not inhibited by nutrient trace metals of the medium within the 24 h period of exposure. It is however not known whether PGE were in competition for other metals at the concentrations added and whether the exposure period was adequate for interactions between metals to take

place. Other studies have shown that short term accumulations of Cd and Zn by *C. vulgaris* do not interact, whereas long-term accumulation of zinc was inhibited by the presence of cadmium (Ting *et al.*, 1990). Further investigations are required to study the competitive uptake of PGE and nutrient trace metals in longer term exposures.

3.3.3. PGE Internalization

Uptake of metals into algal cells, in general, is considered to be a two-part process (Levy *et al.*, 2008; Yan & Pan, 2002). First, fast metal adsorption to the cell membrane occurs, with the metal-binding sites consisting of both metabolically active sites and non-active sites. The second step is the internalisation of metal across the cell membrane. Metal internalisation occurs via ion pores, channels or transporters in the algal cell membrane (Levy *et al.*, 2008). Platinum group elements, as well as other metals, can influx across the *C. stigmatophora* cell membrane and be accumulated internally. Figure 3.9 shows the percentage of intracellular PGE (defined as the analyzed cellular PGE after washing with EDTA) accumulated by the algae. Among the three studied metals and over the experimental conditions studied, Pd shows the highest affinity (~ 80%) to be absorbed intra-cellularly. Internalized Pd in +TM cultures slightly decreased with pH above 8.5 ($p > 0.05$). This reduction was not noticeable in -TM cultures. Rhodium internalization did not exceed 40% of the total accumulated and in neither +TM and -TM cultures was there a clear trend across the range of pH studied. Internalized Pt, both in +TM and -TM cultures, rarely exceeded 20% of the total Pt accumulated by the algae, although internalization was less at pH > 9 ($p > 0.05$). The internalization of Pd was consistent with its affinity for the

surface as a cation and transport through some specific channels, while the process is delayed for Pt because of its slow coordination kinetics (Cosden et al., 2003).

Consistent with the results obtained from the pH experiment, Pd exposed to different concentrations of *C. stigmatophora* cells exhibited the greatest uptake into algal cells which is due to its faster reaction kinetics and greater overall affinity towards surfaces relative to Rh and Pt (Cosden et al., 2003). Its internalization was independent of cell concentration and is similar to Rh in the +TM culture. The decline in algal cell concentration results in fewer surfaces for metal adsorption, which consequently leads to availability of more metal per unit surface area. Regarding Pt, it seems that increasing concentration of Pt bound to the cell surface results in the saturation of binding sites on the cell membrane and this becomes an inhibitor to the metal transporters of cell membrane and therefore Pt cannot be transferred into the cell. As a result, less Pt will be internalized with decreasing algal cell concentration.

Internalized PGE as a function of various Fe(III) concentrations is also shown in Figure 3.9. Palladium, as expected, showed the most internalization (82% - 86%). Internalized Pt did not exceed 27% of the total and neither Pd nor Pt showed any dependence on Fe(III) concentration in the medium. Intra-cellular Rh increased ($p < 0.05$) from 16% at the lowest Fe(III) concentration to 30% at 3.2 mg L⁻¹ of Fe(III) and the results showed that its internalization is proportional to Fe(III) internalization. According to Wells et al. (1995) phytoplankton utilize a variety of strategies for accumulating iron from the surrounding environments, such as uptake of Fe by membrane bound carrier sites, uptake by Fe-siderophore chelates and extra-cellular reduction. Production of highly specific ligands such as siderophore is one of the strategies to facilitate the transport of essential cations

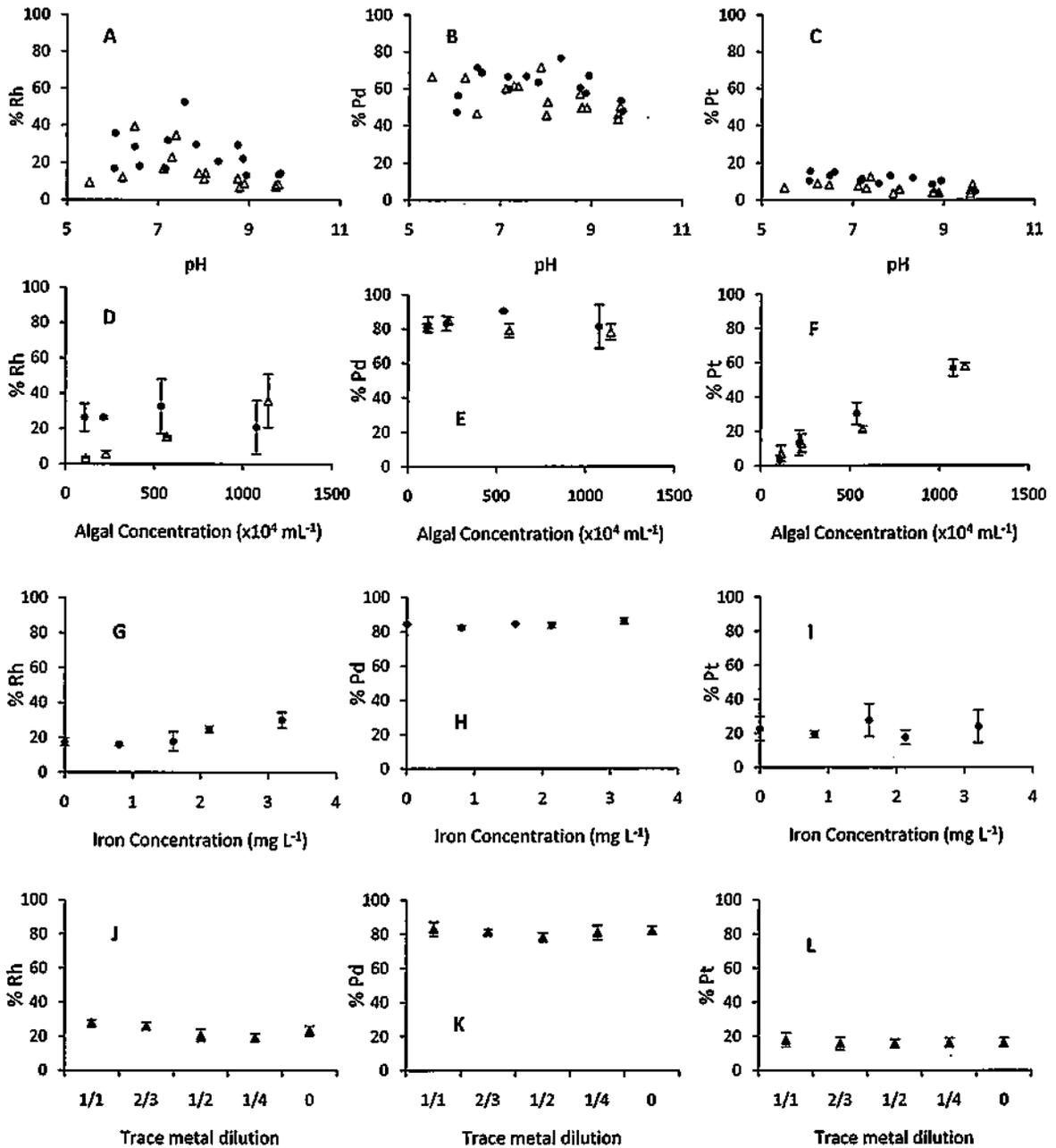


Figure 3.9. Internalization of Rh (left), Pd (middle) and Pt (right) by *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of combined solution of PGE as a function of pH (A - C), *C. stigmatophora* cell concentration (D - F), iron concentrations (G - I) and nutrient trace metals concentration (J - L). (●) represents data for +TM cultures, (Δ) represents data for -TM cultures, (○) represents data for cultures with various concentrations of Fe and (\blacktriangle) represents data for cultures with various concentrations of nutrient trace metals of *f/2* media. Initial algal cell concentrations were $6.9 \times 10^6 \text{ cell mL}^{-1}$ for +TM (A - C), $5.9 \times 10^6 \text{ cell mL}^{-1}$ for -TM (A - C), $4.9 \times 10^6 \text{ cell mL}^{-1}$ for (G - I) and $4.2 \times 10^6 \text{ cell mL}^{-1}$ for (J - L). Error bars denote the standard deviations about the mean of three independent measurements.

that are present at low concentrations in the environment (such as Fe, Co) (Worms et al., 2006). Iron in the Fe(III) (stable oxidation state) has a low solubility and tends to precipitate as oxyhydroxides. Reduction of Fe(III) can increase its biological availability since the resulting Fe(II) is much more soluble and forms much weaker and more labile coordination bonds with ligands (Sunda, 2000) and this makes it more available to phytoplankton. Iron oxidation rate is strongly dependent on $[\text{OH}^-]$. At pH below 7, Fe(II) is the more dominant species, while at higher pH, Fe(III) is present in the solution (Sunda, 2000). Since in phytoplankton cultures with insufficient supply of CO_2 pH is usually above 7, it results in the dominance of Fe(III) which is the less available oxidation state to algae. Under this situation, it is reasonable to assume that *C. stigmatophora* produces siderophore chelates in order to acquire Fe. Rhodium, as a more abundant cation in seawater than Pd and Pt, can presumably bind to siderophores and be transferred into the cell. Therefore Rh is assumed to be internalized by a non-Rh specialised excess method of transfer.

The presence of nutrient trace metals in the media at different concentrations did not significantly affect the internalization of PGE with the exception of Rh (Figure 3.9). Nutrient metals are generally transported into cells by specialized membrane proteins, and their uptake is related to the external concentration of free metal ions. Metal binding sites on the surface of algae are not entirely specific for a single metal, and thus the sites designed to bind an intended nutrient metal will also bind competing metals with similar ionic radii and coordination geometry. Such competitive binding can occur for nutrient metal transport sites, active sites of metalloproteins, or intracellular feedback control sites such as those regulating the number or activity of specific membrane transport proteins (Sunda, 2000). Toxic metals typically are taken up into cells by nutrient metal transport systems (Haus et al., 2007; Sunda & Huntsman, 1998). PGE internalization may also take

place through the same method, but because the concentrations of PGE added to the media were lower than those of the other nutrient metals no competitive effects on internalization of Pd and Pt were observed. The small increase in Rh internalization by increasing the concentration of nutrient trace metals is again assumed to be attributed to the increased concentration of iron and its facilitated internalization.

3.4. Summary

Marine microalgae, *Chlorella stigmatophora* were exposed to $20 \mu\text{g L}^{-1}$ of Rh, Pd and Pt as a combined PGE stock solution and the influence of pH, algal concentrations, Fe(III) and essential trace metals on PGE accumulation was studied. The overall outcome of the experiments indicated:

- The greatest loss (up to 60%), mainly due to the adsorption to the containers, was observed for Pd, while Pt showed the greatest recovery.
- Total accumulation of PGE by *C. stigmatophora* followed the order of Rh > Pd >> Pt, however, the extent of metal internalization within the exposure time was Pd > Rh > Pt, which mainly was due to the greater affinity of Pd to contribute in reactions.
- Rhodium was the most sensitive species to pH and its removal showed a proportional relationship, while Pd accumulation was inversely related to the pH of seawater. This was mainly due to the ionic charge of Rh (negative) and Pd (positive) along with the proportional relationship between pH and negative charge

of the algal cell surface. Pt accumulation did not show a clear dependence on the pH of the medium possibly due to its slow reaction kinetics.

- Algal biomass exerted a significant impact on PGE partitioning between algal cells and the dissolved phase; namely, an increase in algal concentration resulted in a reduction of the accumulation factor for all metals, although to different extents. It is assumed that production of cell exudates play a key role in controlling the metal speciation and hence accumulation by algae.
- Iron and other essential trace metals did not clearly influence PGE accumulation; however, Rh accumulation factors showed an enhancement in the presence of other trace metals in the solution, which is assumed to be attributed to a non-specialized excess methods of transfer.

CHAPTER 4

Isotherms Defining Platinum Group Elements

Accumulation by *Chlorella stigmatophora*

Isotherms Defining Platinum Group Elements Accumulation by *Chlorella stigmatophora*

4.1. Introduction

Trace metal concentrations vary widely in aquatic systems due to the differences in rates of input, loss and internal cycling. Their concentrations in surface waters of oceans and seas are often lower than those at depth due to the uptake of metals by phytoplankton in the euphotic zone (Sunda, 2000), while because of inputs from terrestrial sources such as rivers, groundwater, dust and sediments, the concentrations decrease considerably along the surface from coastal and estuarine waters to oceanic waters

Platinum group elements are now highly enriched in roadside particulate matter due to inputs from catalytic converters and there is evidence that runoff can carry roadside particles into rivers and estuaries, where PGE can be solubilized and accumulated in aquatic systems (Cosden *et al.*, 2003; Haus *et al.*, 2007; Moldovan *et al.*, 2001). Similar to other trace metals, PGE distributions in aquatic systems are predicted to vary with different depths in different water bodies (Goldberg, 1987; Lee, 1983) and are expected to decrease from coastal and estuaries towards oceanic waters (Santana-Casiano *et al.*, 1995). Therefore, depending on their habitats, organisms are exposed to various concentrations of PGE. It is important to understand how PGE sorption would change by alterations of the available quantity of metal to these organisms. In order to evaluate or predict PGE accumulation at different parts of water body with different concentrations of PGE, this

chapter contains the results of the accumulation behaviour of PGE by the marine phytoplankton *C. stigmatophora* as a model. To this aim algal cells are exposed to various concentrations of Rh, Pd and Pt in both combined and individual metal solutions. Accumulation of each metal by the alga across a range of concentrations and under different treatments will be evaluated and discussed.

4.2. Methodology

Chlorella stigmatophora cells in their mid-exponential growth phase, were centrifuged at 4000 rpm for 10 min and the pellets were resuspended separately in both +TM and -TM cultures as explained in Chapter Two (Section 2.4). Three sets of experiments were performed, covering a range of Rh, Pd and Pt concentrations. Experiments were carried out in quadruplicate by transferring 120 mL of algae into separate 150 mL styrolux (crystal polystyrene) containers. In the first experiment (Experiment A), twenty containers of algae for each of +TM and -TM cultures were spiked with five concentrations of 5, 10, 15, 20 and 30 $\mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a combined PGE stock solution. In the second experiment (Experiment B), twenty containers for each of +TM and -TM cultures were spiked with 5, 10, 15, 20 and 30 $\mu\text{g L}^{-1}$ of Rh, twenty containers for each of +TM and -TM cultures with Pd, and twenty containers for each of +TM and -TM cultures with Pt. The third experiment (Experiment C) was carried out in +TM cultures only and concentrations of 5, 10, 15, 20, 30, 50, 60, 70, 80, 90, 100, 150 and 200 $\mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a combined PGE stock solution were spiked into the containers. Higher concentrations were used here to be consistent with the toxicity studies described in Chapter Six.

Quadruple cultures of algae with no added PGE were set for each experiment as control cultures (+TM and -TM). The containers were incubated under the conditions detailed in Table 4.1 and constantly agitated on a Denley orbital combiner and two shakers (UNIMAX2010) at 85 rpm. Containers were covered with clear lids to prevent evaporation and any contamination. The lids were slightly left open for air exchange. The pH was recorded initially and at the time of filtration, as well as at 3 intervals throughout the 24 h of exposure and adjusted by adding microlitre volumes of 1 M HCl or 1 M NaOH as required.

Phytoplankton cells were filtered after 24 h and processed for analysis as explained in Chapter Two (Section 2.6). To determine the intra-cellular, extra-cellular and aqueous PGE concentrations, samples in Experiments A and C were analyzed by a Thermo Elemental ICP-MS and samples of Experiment B were analyzed by a XSeries2, Thermo Scientific ICP-MS. Limits of detections of the instruments based on unspiked acidified seawater, 5 mM EDTA and diluted HNO₃ are summarized in Appendix 1.

Table 4.1. Incubation conditions of *C. stigmatophora* exposed to various concentrations of combined and individual solution of Rh, Pd and Pt for 24 h (+TM: cultures included nutrient trace metals and EDTA; -TM: cultures with no added nutrient trace metals and EDTA)

Experiment	culture	Light ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	Temperature ($^{\circ}\text{C}$)	salinity	pH	Cell concentration ($\sim \text{mL}^{-1}$)	No. of replicates
A	+TM	65	15 \pm 1	34.1	7.55	5,130,000	4
	-TM	65	15 \pm 1	34.0	7.51	5,160,000	4
B	+TM	65	15 \pm 1	33.7	7.52	4,720,000	4
	-TM	65	15 \pm 1	33.9	7.48	4,190,000	4
C	+TM	65	15 \pm 1	34.1	7.55	5,130,000	4

4.3. Results and discussions

4.3.1. Recovery of Platinum Group Elements

Recovery of Rh, Pd and Pt at different PGE concentrations was calculated from the ratio of mass balance to the initial metal concentration and the results are shown in Figure 4.1. Rhodium recovery in Experiment A (PGE concentrations of 5 – 30 $\mu\text{g L}^{-1}$ in a combined metal solution) and Experiment C (PGE concentrations of 5 – 200 $\mu\text{g L}^{-1}$ in a combined metal solution) ranged between 70% and 90%. There was a slight and non-significant increase of about 10% in recovery of Rh by increasing concentration of PGE up to 15 $\mu\text{g L}^{-1}$, while at higher concentrations (Experiment C) no clear trend was observed. However, when Rh was individually exposed to algae, more metal was recovered in the solution (85% to 95% in -TM cultures and about 100% in +TM cultures). This higher extent could partly be due to the application of a different ICP-MS instrument for analysing the samples of this experiment. The XSeries2 ICP-MS is highly sensitive relative to the other ICP-MS used for Experiments A and C with limits of detection of up to 45 times lower and therefore metals could be detected at their lower concentrations. Rhodium analysis was the most interfered metal among PGE and this was mainly due to spectral overlap with ^{40}Ar ^{40}Ar ^{23}Na . The lower detection limit of XSeries2 ICP-MS resulted in a more precise detection of Rh and consequently less interference from other ions. Therefore, the higher values of recovered Rh are more likely to be due to the greater sensitivity of the instrument in detection of metals. Increasing concentrations of Rh in seawater has been shown to be accompanied by a reduction in metal recovery (Cobelo-Garcia *et al.*, 2007). However, the presence of algae in solution results in the release of exudates which can bind metals and retain them in water. Therefore the recovery of Rh is not subject to significant changes by

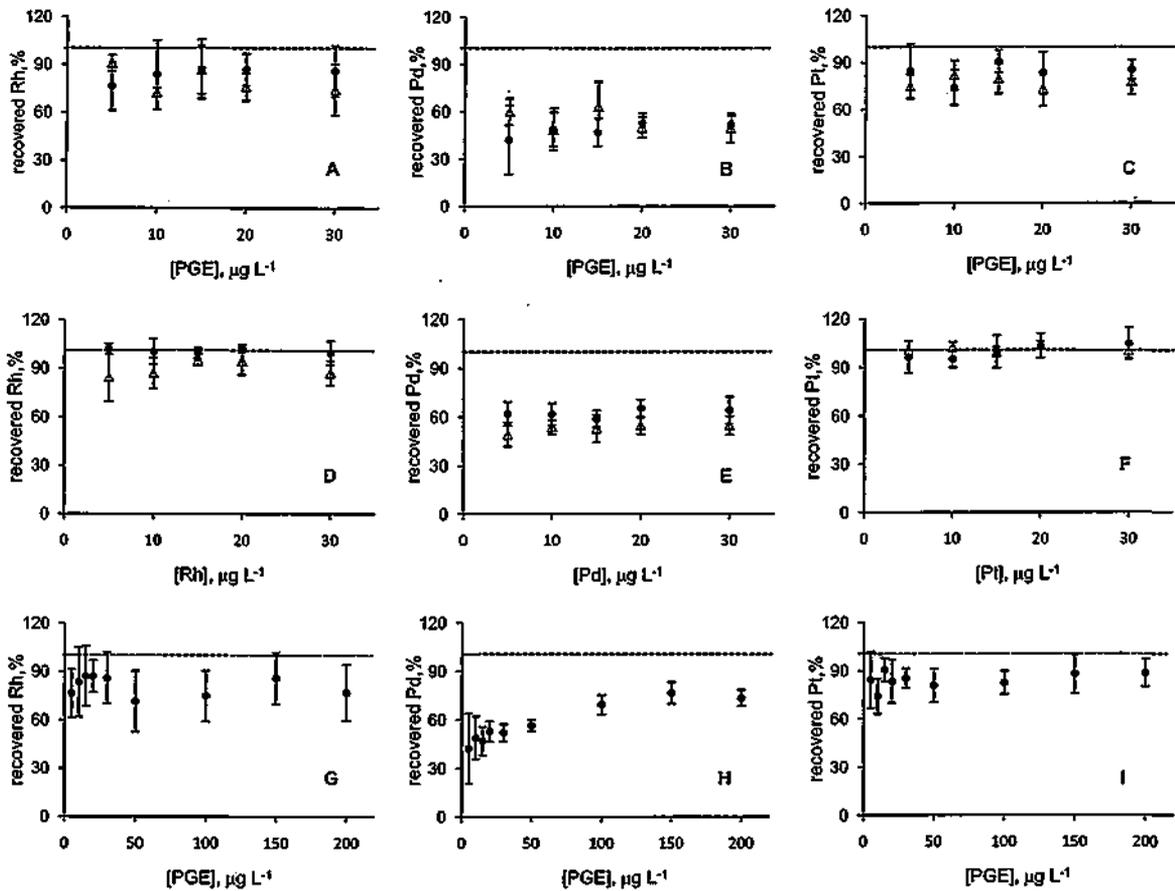


Figure 4.1. Recovery of Rh, Pd and Pt in the presence of *C. stigmatophora* as a function of PGE concentrations of 5 – 30 $\mu\text{g L}^{-1}$ of each metal in a combined solution (A – C), 5 – 30 $\mu\text{g L}^{-1}$ of each metal individually (D – F) and 5 – 200 $\mu\text{g L}^{-1}$ of each metal in a combined solution (G – I). (●) represents data for +TM cultures, (Δ) represents data for –TM cultures. Initial algal cell concentrations are detailed in Table 4.1. pH alterations in each experiment is detailed in Appendix 2.1. Error bars denote the standard deviations about the mean of four independent measurements. Dashed line indicates 100% value.

altering the concentration in the solution.

In terms of Pd, consistent with previous results, the fraction of metal which is adsorbed to the walls of the container is significantly greater than that for Rh and Pt both in +TM and –TM cultures ($p < 0.05$). Recovered Pd ranged between 40% and 60% with no significant differences between individual and combined metal exposures. There was, however, an

increase in Pd recovery by increasing its concentration in the combined PGE solution which was more significant at concentrations above $100 \mu\text{g L}^{-1}$ (Figure 4.1). Presumably, the effect of Pd itself on algae results in less metal adsorption to the container walls. Accumulation of Pd by algae takes place faster than Rh and Pt and the algal cell response to metal accumulation would consequently be faster. As a response to accumulation of a potentially toxic metal, algae are likely to release exudates which bind aqueous metal ions and stabilise them in solution, a mechanism previously suggested in Chapter 3. Moreover, there might be a saturation concentration for Pd adsorption on the surface of containers walls which limits the extent of the loss.

Recovery of Pt was found to be independent of Pt concentration in the solution. More than 80% of Pt added to the media as a combined PGE solution was recovered, while in Experiment B, when Pt was individually added, almost all of the metal was recovered, although this greater recovery may also be attributed to the higher sensitivity of XSeries2 ICP-MS used in these experiments.

4.3.2. Removal of Platinum Group Elements by *C. stigmatophora*

Removal of combined or individual solutions of Rh, Pd and Pt from seawater by *C. stigmatophora* was calculated from the ratio of algal metal concentration to total analytical metal concentration in the alga and in the dissolved phase. Removal of PGE by the microalga was shown to be largely independent of metal concentration across the range of $5 - 30 \mu\text{g L}^{-1}$ (Figure 4.2). Less Rh in +TM, and Pd in both +TM and -TM cultures were removed from seawater when metal was individually added. The differences in Rh and Pd

removal between Experiments A and B was possibly due to the lower dissolved concentration of metal in Experiment A because of the greater container loss. Therefore, despite the comparable concentration of Rh (in +TM) and Pd accumulated by the algae, a lower percentage of these metals was removed when individually added.

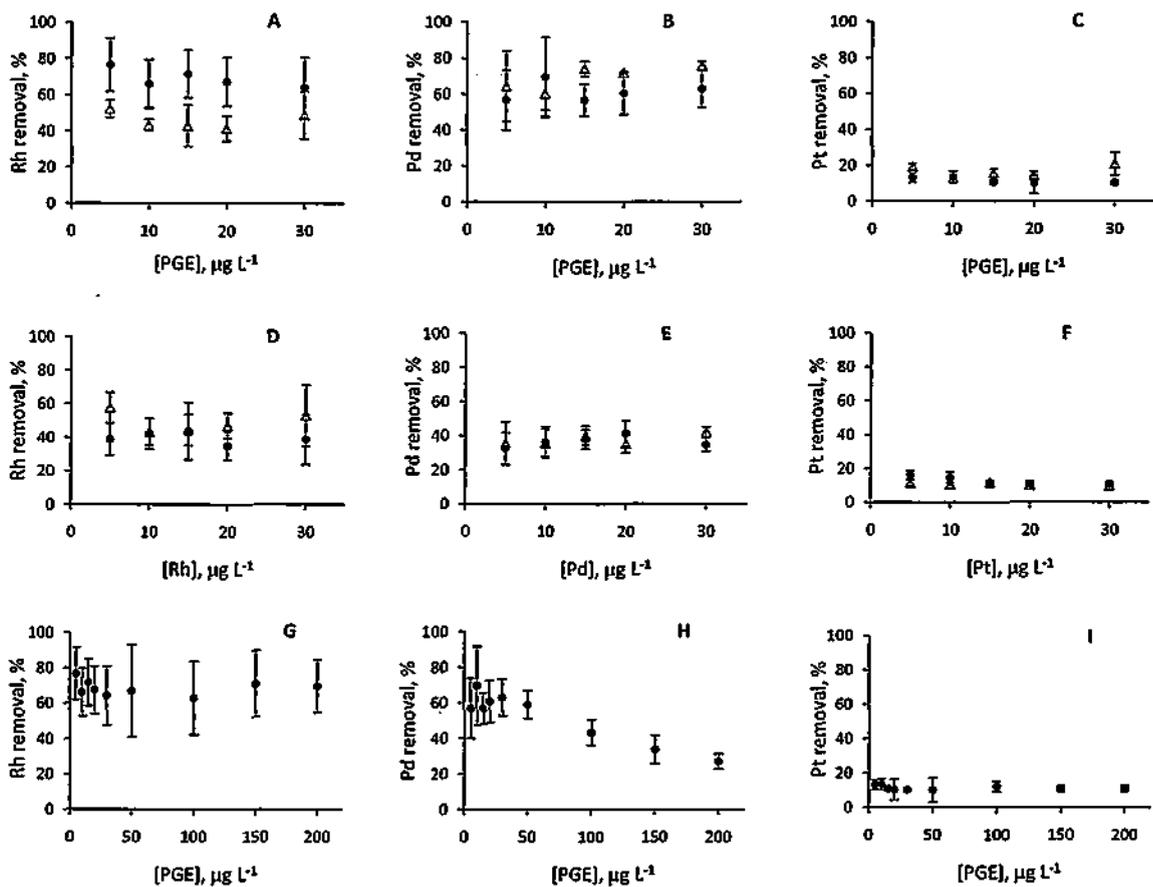


Figure 4.2. Percentage of Rh, Pd and Pt removed from the aqueous phase by *C. stigmatophora* exposed to 5 – 30 $\mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a combined solution (A – C), 5 – 30 $\mu\text{g L}^{-1}$ of each of Rh, Pd and Pt individually (D – F) and 5 – 200 $\mu\text{g L}^{-1}$ of each metal in a combined solution (G – I) after 24 h under culture conditions detailed in Table 4.1. (●) represents data for +TM cultures, (Δ) represents data for –TM cultures. pH alterations in each experiment is detailed in Appendix 2.1. Error bars denote the standard deviations about the mean of four independent measurements.

Other studies have also shown that percentage removal of PGE is independent of the aqueous PGE concentration added. When the freshwater isopod *Asellus aquaticus* was exposed to a combined PGE solution, no interaction among the three metals occurred when they were added together compared to individually added (Moldovan *et al.*, 2001). However, in that study, metal concentrations added as combined solutions were lower than of the individual solutions. Accumulation factors of PGE by *A. aquaticus*, exposed to 5 to 500 $\mu\text{g L}^{-1}$ of PGE for 24 h, followed the order: Pd > Pt > Rh; and was independent of the concentration of the standard solution, although at higher concentrations of Pt (1 mg L^{-1}) there was a different type of reaction. It is likely that at low concentrations *A. aquaticus* could partly eliminate the accumulated Pt, whereas at higher concentrations excessive Pt was accumulated so that the elimination system might have been saturated and therefore did not function (Rauch & Morrison, 1999). Studies on the removal of PGE by estuarine sediments have also confirmed that partition coefficients (equivalent to accumulation factors, AF) of PGE are independent of their aqueous concentrations over the range of metal concentrations employed in the experiment.

Exposure of *C. stigmatophora* to higher concentrations of PGE (Experiment C) also revealed that the removal of Rh and Pt across the studied concentration range (5 – 200 $\mu\text{g L}^{-1}$) is independent of their dissolved concentration. Consistent with previous results and its slow kinetics of coordination and complexation, lower removal of Pt than Rh and Pd is observed. Removal of Pd showed a dependency on metal concentration at the higher dissolved concentrations added and decreased from 60% to 20% at metal concentrations >50 $\mu\text{g L}^{-1}$ (Figure 4.2). Increasing recovery of Pd with increasing dissolved concentration may affect the amount of Pd removed by the alga (which was obtained from the ratio

between algal Pd concentration relative to total analytical concentration of Pd). However, cellular mechanisms of Pd accumulation are more likely to be an effective factor in this mechanism. An implication of these results is that the binding sites on the cell surface would be saturated by increasing the concentration of Pd in the solution and therefore no more Pd would be accumulated by the algae. The accumulation of Pd will be further discussed in the following sections.

4.3.3. Internalization of Platinum Group Elements by *C. stigmatophora*

Internalized Rh, Pd and Pt by *C. stigmatophora* across the various concentrations of PGE in solution were calculated as the percentage of analysed cellular PGE concentration after an EDTA wash relative to total metal accumulated by the cells, and the results are shown in Figure 4.3. Consistent with previous results, Pd and Pt showed, respectively, the highest and the lowest tendencies to be internalized. Across the studied PGE concentrations, internalization of Rh did not show a clear trend. However, its extent varied between different experiments and treatments. In Experiments A and C, in the presence of essential trace metals, 60% to 80% of Rh was found internalized. Its internalization in the -TM treatment ranged between 28% and 45% and was lower when Rh was added in combined solution with Pt and Pd ($p > 0.05$). However, less Rh was internalized when added individually ($p < 0.05$).

The extent of internalized Pd increased up to 95% across the lower concentrations of aqueous PGE ($5 - 50 \mu\text{g L}^{-1}$). The Pearson's correlation coefficient showed a significant relationship ($p < 0.05$) between the percentage of internalized Pd and total concentration of

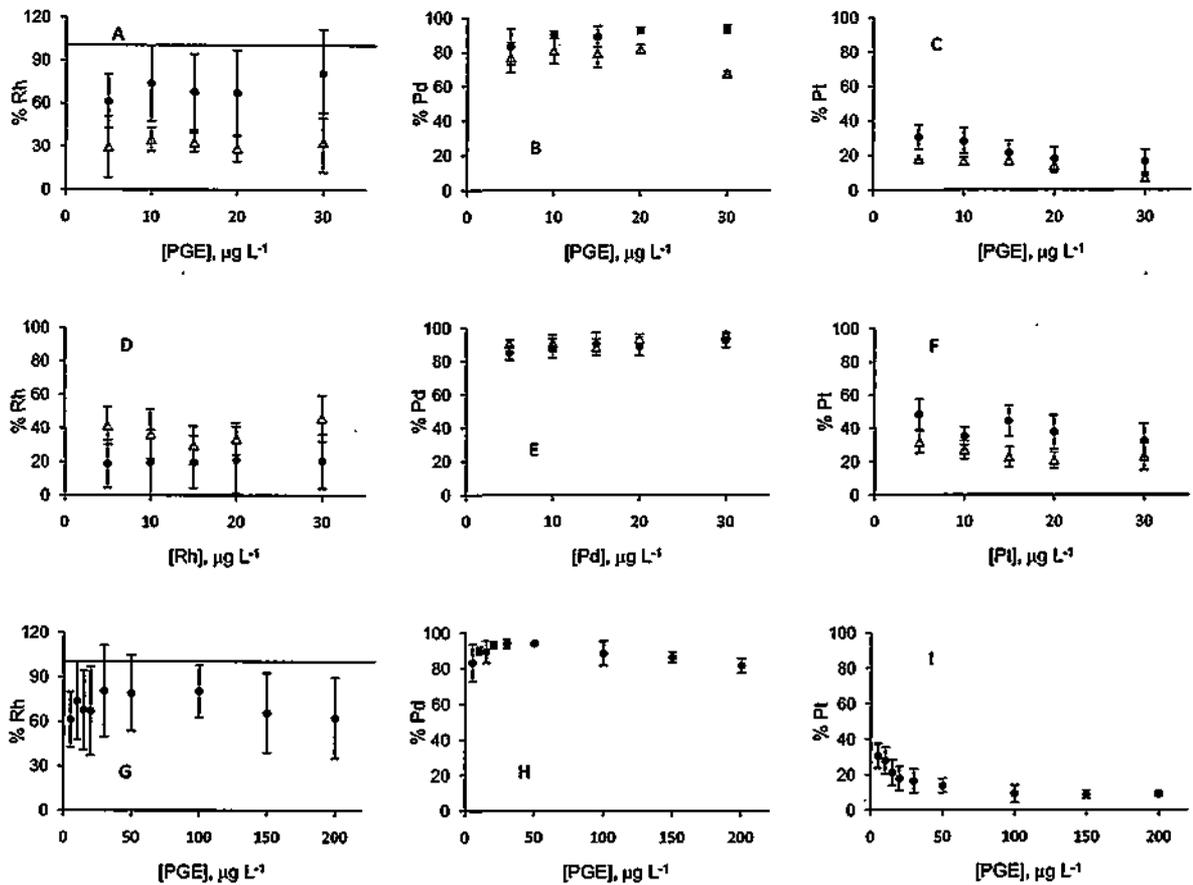


Figure 4.3. Percentage of internalized PGE by *C. stigmatophora* exposed to 5 – 30 $\mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a combined solution (A – C), 5 – 30 $\mu\text{g L}^{-1}$ of each of Rh, Pd and Pt individually (D – F) and 5 – 200 $\mu\text{g L}^{-1}$ of each metal in a combined solution (G – I) for 24 h under culture conditions detailed in Table 4.1. (●) represents data for +TM cultures, (Δ) represents data for –TM cultures. pH alterations in each experiment is detailed in Appendix 2.1. Error bars denote the standard deviations about the mean of four independent measurements. Dash line indicates 100% value.

Pd accumulated by the algae (Table 4.2). However, by increasing the aqueous concentrations of up to 200 $\mu\text{g L}^{-1}$, the internalized Pd declined to about 80%, which suggests that the saturation of specific binding sites at higher dissolved concentrations of Pd inhibits further internalisation of the metal. Also, by increasing the concentration of accumulated Pd, *C. stigmatophora* may efflux the internalized metal in order to reduce its

toxicity. In other words, effluxing accumulated metal by the algae is a defence mechanism for reducing the effects of toxicity (See Chapter 6). As explained previously, the production of extracellular compounds by phytoplankton depends on the physiological state of the cell as well as on environmental factors and the presence of toxic compounds in the medium. Some species such as *C. vulgaris* (Worms et al., 2006), cyanobacteria (Gonzalez-Davila, 1995) and *Dunaliella tertiolecta* (Levy et al., 2008) are reported to exclude toxic metals by excreting them through production of exudates. In addition to decreasing toxicity, efflux will reduce the metal internalization and also modify the speciation of the metal in the surrounding environments of algae (Worms et al., 2006). Perhaps by prolonging the exposure time, less internalized Pd and other metals would be observed. Regarding Pt, increasing the concentration of accumulated Pt by *C. stigmatophora* seemed to inhibit its internalization and an inverse relationship was found between total accumulated and ratio of internalization with correlation coefficients between -0.783 and -0.976 (Table 4.2). By increasing the concentration of Pt in the aqueous phase, there was less tendency for the metal to be internalized into the cell and at concentrations above $100 \mu\text{g L}^{-1}$ the ratio of internalized Pt to the total Pt accumulated reduced to less than 10%. This could be due to the slow reaction kinetics of Pt in complexing with other compounds (See Chapter 5 for further details). With increasing Pt concentrations, available binding sites on the cell surface are continuously loaded with Pt, but the complexation rate of Pt carriers (for transferring Pt into the cell) remains slow and the rate is not proportional to the amount of Pt at the surface. More Pt was internalized ($p < 0.05$) when PGE were added individually (33% - 48% for +TM, and 21% - 32% for -TM) than when added as a combined PGE solution (17% - 31% for +TM and 7% - 18% for -TM). It is assumed that when Pt is individually added to solution, there are more free metal

binding sites on the algal surface. These sites will rapidly be occupied with metals which react faster toward the algal surface (especially Pd) when these metals are also present in the solution. Therefore, when there are no faster competing metals, all binding sites of the metal carriers would be occupied by Pt and this metal is consequently transferred into the cell resulting in an increase in Pt internalization..

Table 4.2. Correlation coefficients between percentage of internalized metal and total metal accumulated by *C. stigmatophora* exposed to various concentrations of Rh, Pd and Pt for 24 h

<i>Experiment</i>		<i>r</i>	
		<i>+TM</i>	<i>-TM</i>
A	Rh	0.761	0.081
	Pd	0.842	-0.663
	Pt	-0.952	-0.976
B	Rh	0.665	0.482
	Pd	0.831	0.815
	Pt	-0.783	-0.828
C	Rh	-0.207	—
	Pd	-0.426	—
	Pt	-0.802	—

4.3.4. Adsorption Isotherms of Platinum Group Elements

Isotherms defining Rh, Pd and Pt adsorption on the cell surface of *C. stigmatophora*, measured over a period of 24 h and determined from the EDTA washes of the microalga, are shown in Figure 4.4. Isotherms are frequently used to describe adsorption behaviour. Metals generally associate with phytoplankton cells in relation with Freundlich adsorption isotherms (Fisher, 1986; Gonzalez-Davila, 1995; Mahan et al., 1989) and this is also the

case for PGE. Exposure of algal cells to various concentrations of PGE showed a proportional relationship between aqueous PGE and its adsorption by the algal surface, which could be mostly defined by a Freundlich-type equation (Equation 4.1) (Schwarzenbach et al., 1993).

$$C_s = K_F \cdot C_w^n \quad (4.1)$$

where C_s is metal concentration adsorbed to the algal cell surface ($\mu\text{g g}^{-1}$), C_w is dissolved metal concentration ($\mu\text{g L}^{-1}$), K_F is the Freundlich constant (L g^{-1}) and n is a measure of the nonlinearity related to the adsorption mechanism. Constants, derived from linear regression analysis of the logged datasets, are presented in Table 4.3. According to the results, at lower concentrations of PGE (Experiments A and B), algal and aqueous concentrations of Rh, Pd and Pt are non-linearly related, although the extent of non-linearity is rather different amongst the three metals and is not particularly great (i.e. n is close to unit value implying a single type of reaction throughout the metal concentration range). As shown in Figure 4.4, the isotherms for PGE adsorption in +TM cultures are slightly convex, i.e. they follow the first type of Freundlich isotherm relationship (where $n < 1$), which indicates that the extent of surface adsorption in +TM gradually diminishes with increasing concentration of metal. In other words, increasing concentration of PGE makes it more difficult for *C. stigmatophora* cells to adsorb additional metal ions. Adsorption of Pt in –TM cultures, both as combined (Experiment A) and individual (Experiment B) metal solution, as well as Pd in –TM cultures in Experiment A, showed evidence of a synergistic effect since n is slightly greater than unit value (Pérez-Rama et al., 2010). Thus, under these conditions, Pt ions (and Pd ions in Experiment A) are favourably adsorbed by *C. stigmatophora* cells when there is no essential trace metals in the solution to compete with

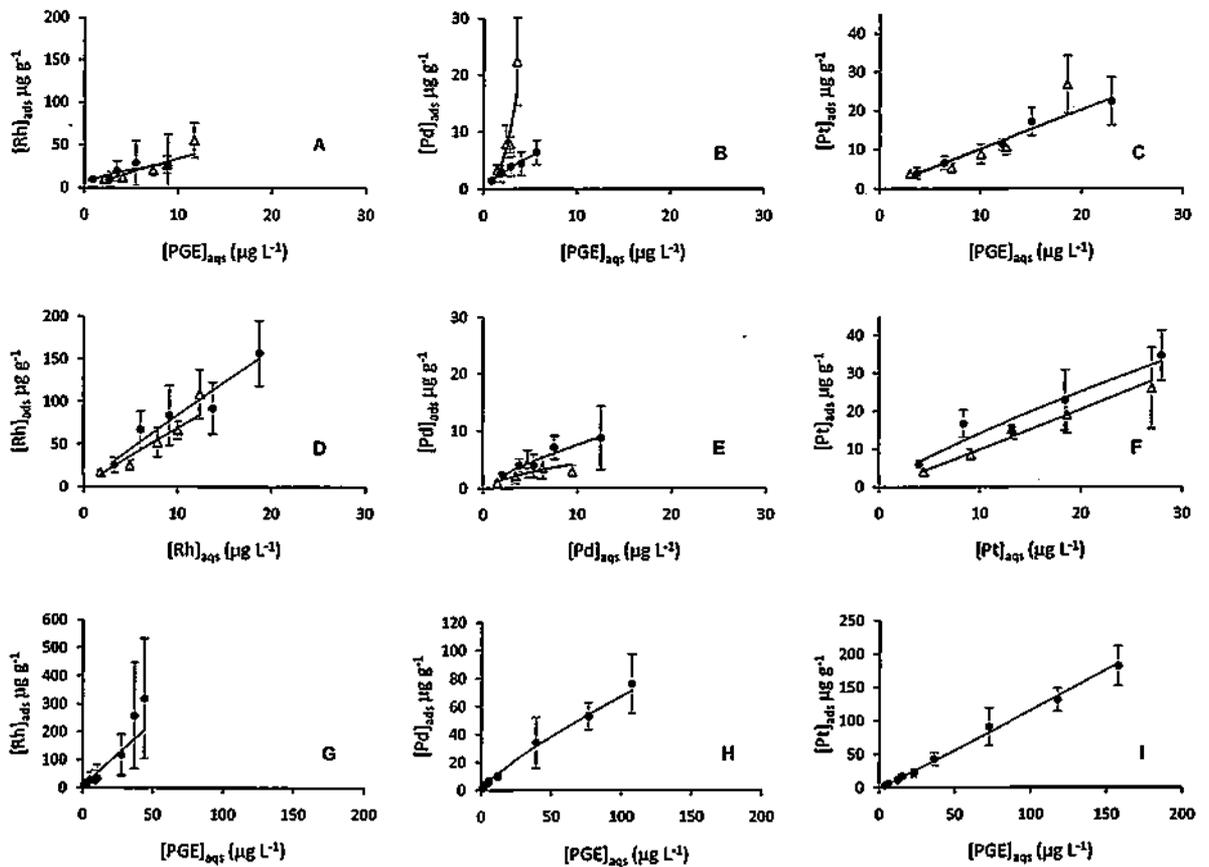


Figure 4.4. Isotherms defining the adsorption of various concentrations of PGE to the cell surface of *C. stigmatophora* exposed to 5 – 30 $\mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a combined solution (A – C), 5 – 30 $\mu\text{g L}^{-1}$ of each of Rh, Pd and Pt individually (D – F) and 5 – 200 $\mu\text{g L}^{-1}$ of each metal in a combined solution (G – H) after 24 h under culture conditions detailed in Table 4.1. Solid lines are best-fits to the data according to the Freundlich isotherm (Eq. 4.1) and defined by the constants given in Table 4.3. (●) represents data for +TM cultures, (Δ) represents data for –TM cultures. pH alterations in each experiment is detailed in Appendix 2.1. Error bars denote the standard deviations about the mean of four independent measurements.

Pt. Presuming that accumulation of Pt, as a potentially toxic metal, by *C. stigmatophora* is carried out through the transport system and binding sites of nutrient metals (Sunda, 2000; Sunda & Huntsman, 1998), other essential trace metals (i.e. Fe, Mn, Zn, Cu, Co and Mo) in +TM cultures would bind to the functional groups on the algal cell surface due to the competition for binding sites and due to the slower reaction kinetic of Pt complexation, whereas in –TM cultures, where there is no competitive metal (apart from Pd and Rh),

more Pt complexation to the binding sites occurs. However, due to the slow rate of Pt complexation the saturation of functional groups with metal will be achieved at a later stage.

A comparison of K_F values as quantitative indicators of sorption capacity (Bayramoglu et al., 2006; Zhou et al., 1998) amongst the cultures and the three metals revealed that surface adsorption of Rh depends on the presence of other metals in the solution, since its adsorption capacity increased in the presence of other essential metals. Reasons for this are unclear but it is possible that the presence of the nutrient metals induces receptors that are specific to or favourable for Rh binding at the algal surface. Platinum adsorption did not differ between the +TM and -TM cultures in all experiments. However, in +TM cultures when individually exposed to algae, its K_F value was two fold greater than other cultures

Table 4.3. Results of adsorption isotherms for Rh, Pd and Pt accumulated by *C. stigmatophora* derived from Freundlich equation (Eq. 4.1, See Figure 4.4)

Experiments		$K_F (L g^{-1})$		n		R^2	
		+TM	-TM	+TM	-TM	+TM	-TM
A	Rh	9.3	4.2	0.507	0.916	0.725	0.850
	Pd	1.7	1.1	0.753	2.179	0.989	0.854
	Pt	1.1	1.0	0.980	1.010	0.990	0.861
B *	Rh	9.8	8.1	0.932	0.932	0.925	0.903
	Pd	1.3	0.9	0.778	0.671	0.936	0.676
	Pt	2.1	0.9	0.832	1.051	0.903	0.986
C	Rh	5.8	—	0.942	—	0.894	—
	Pd	1.6	—	0.818	—	0.996	—
	Pt	1.0	—	1.044	—	0.997	—

* Samples analysed with a different model of ICP-MS (See Section 4.2)

indicating that the competitive effect of Rh and Pd with Pt to occupy the surface binding sites is more considerable than with other essential trace metals. Therefore, a greater amount of Pt adsorbed on the surface of *C. stigmatophora* when no Rh or Pd is present.

4.3.5. Total Accumulation Isotherms of Platinum Group Elements

Accumulation of metal by algae is known to involve three processes (Miao et al., 2005): i) diffusion of the metal from the solution to the biological surface, ii) binding site complexation at the cell surface, and iii) internalization of the metal into the cytosol. Surface binding is an important step on which isotherms are based on. The adsorption isotherms for Rh, Pd and Pt were explained in the previous section. However, since metal internalization is a significant part of algal-metal interactions, total accumulation isotherms have been devised to define the overall PGE accumulation by the microalgae.

Figure 4.5 shows isotherms defining total Rh, Pd and Pt accumulation by *C. stigmatophora* measured over a period of 24 h. Similar to surface adsorption, total accumulation of PGE showed a proportional relationship with its dissolved concentration and was mostly defined by a Freundlich type equation. Constants, derived from the regression analysis of the logged data sets are presented in Table 4.4. As seen in Figure 4.5, the dissolved and algal concentrations of the three metals are non-linearly related, although the extent of non-linearity is rather different among the metals and treatments. The isotherm for Rh and Pt accumulation follows the first type of Freundlich isotherm relationship with n values just below unit value. For Pd, however, the isotherm was more concave which follows the third type of relationship in the Freundlich isotherm equation ($n > 1$), an effect accompanied by

the high tendency of this metal to be internalized. In Experiments A and B (metal concentrations of $5 - 30 \mu\text{g L}^{-1}$), by transferring the surfaced adsorbed Pd ions into the cytosol, the binding sites which become free after internalizing Pd will adsorb more Pd ions and subsequently transfer them into the cell. Therefore, accumulated concentrations of Pd by algal cells increase and result in concavature in the isotherms.

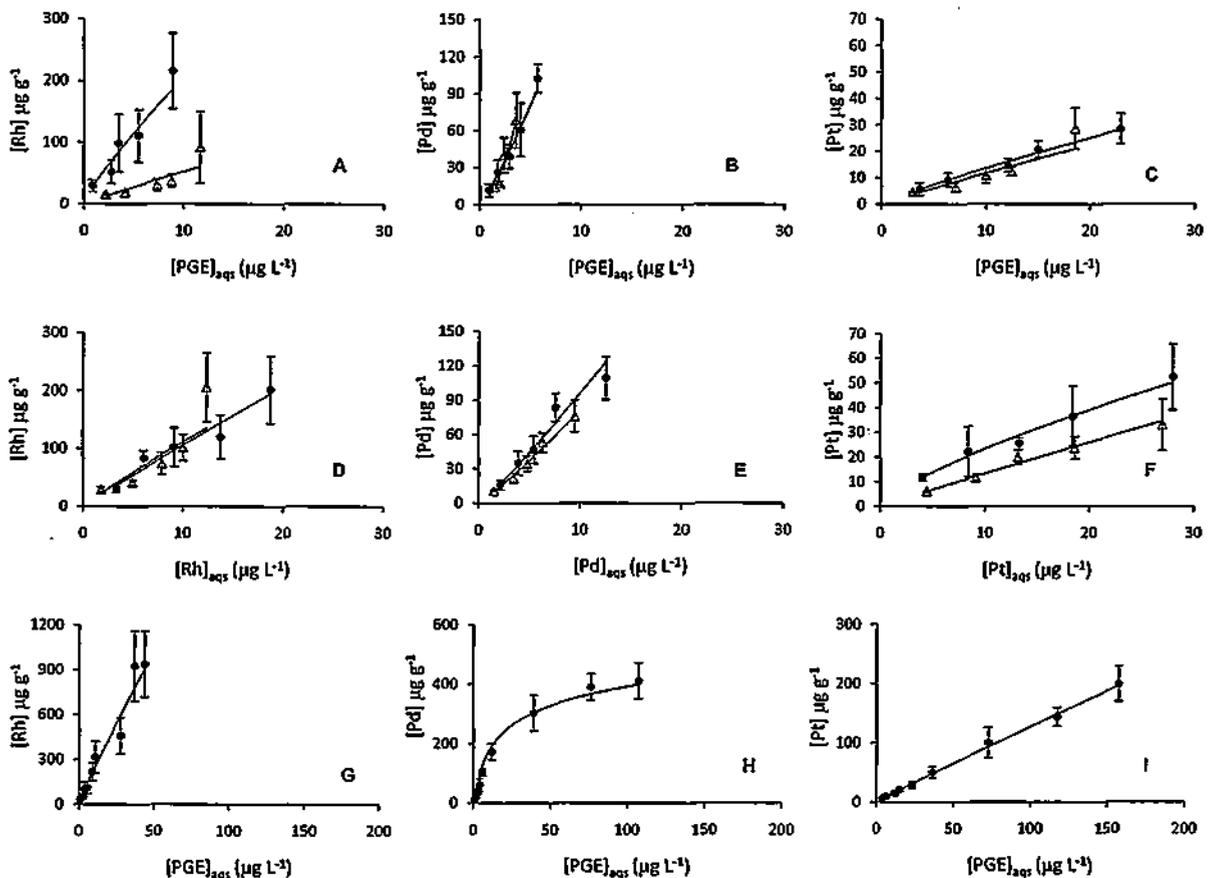


Figure 4.5. Isotherms defining the accumulation of different concentrations of PGE to *C. stigmatophora* exposed to $5 - 30 \mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a combined solution (A - C), $5 - 30 \mu\text{g L}^{-1}$ of each of Rh, Pd and Pt individually (D - F) and $5 - 200 \mu\text{g L}^{-1}$ of each metal in a combined solution (G - I) after 24 h under culture conditions detailed in Table 4.1. Solid lines are best-fits to the data according to the Freundlich isotherm (Eq. 4.1) and defined by the constants given in Table 4.2. (●) represents data for +TM cultures, (Δ) represents data for -TM cultures. pH alterations in each experiment is detailed in Appendix 2.1. Error bars denote the standard deviations about the mean of four independent measurements.

Table 4.4. Results of accumulation isotherms for Rh, Pd and Pt by *C. stigmatophora* derived from Freundlich equation (Eq. 4.1, See Figure 4.5)

Experiments		$K_F (L g^{-1})$		n		R^2	
		+TM	-TM	+TM	-TM	+TM	-TM
A	Rh	29.3	5.9	0.847	0.944	0.933	0.819
	Pd	12.5	6.3	1.159	1.871	0.991	0.928
	Pt	1.8	1.3	0.886	0.949	0.992	0.871
B *	Rh	11.1	13.2	0.976	0.923	0.937	0.829
	Pd	7.2	6.3	1.122	1.102	0.978	0.987
	Pt	4.1	1.5	0.748	0.963	0.981	0.987
C	Rh	27.7	—	0.923	—	0.972	—
	Pd	**	—	**	—	**	—
	Pt	1.5	—	0.963	—	0.997	—

* Samples analysed with a different model of ICP-MS (See Section 4.2)

** See text for details

At higher concentrations of metal ($50 - 200 \mu g L^{-1}$), the accumulated Pd concentrations by algae did not proportionally increase by enhancing its concentration in solution and considerable deviation from the linear relationship was observed. This indicates that Pd ions become bound first to the highest-affinity surface ligands which can internalize Pd into the cytoplasm and subsequently to those of lesser activity. An increase in the dissolved concentration of Pd increased the accumulation capacity until the limit was reached. Such a value represents the maximum Pd accumulation capacity by *C. stigmatophora* when all active sites have been occupied by the metal. This implies that Pd accumulation by algae is carried out through specific functional groups or binding sites on the algal cell membrane and once the binding sites are saturated, due to the increasing dissolved concentration of Pd in solution, no more Pd can be accumulated and the bound versus aqueous Pd ratio

declines. Therefore the isotherm would have a hyperbolic shape which follows the Langmuir Equation (Equation 4.2) (Schwarzenbach et al., 1993).

$$C_s = \frac{C_s \text{max} \cdot K_L \cdot C_w}{1 + K_L \cdot C_w} \quad (4.2)$$

where $C_s \text{max}$ is the maximum metal concentration accumulated by the alga or metal concentration accumulated at saturation ($\mu\text{g g}^{-1}$) and K_L is the Langmuir constant (L g^{-1}).

Rearranging Eq. 4.2 gives:

$$\frac{C_w}{C_s} = \frac{C_w}{C_s \text{max}} + \frac{1}{K_L \cdot C_s \text{max}} \quad (4.3)$$

which is the linearised form of the isotherm (Lee et al., 2009).

Table 4.5 shows the values of parameters calculated from the linear form of the Langmuir equation. Plotting C_w / C_s versus dissolved Pd concentration (Figure 4.6) yields a slope =

$\left(\frac{1}{C_s \text{max}}\right)$ and an intercept = $\left(\frac{1}{K_L C_s \text{max}}\right)$ which can be used for calculating the Langmuir

constant and saturation concentration of Pd by *C. stigmatophora* as shown in Table 4.5.

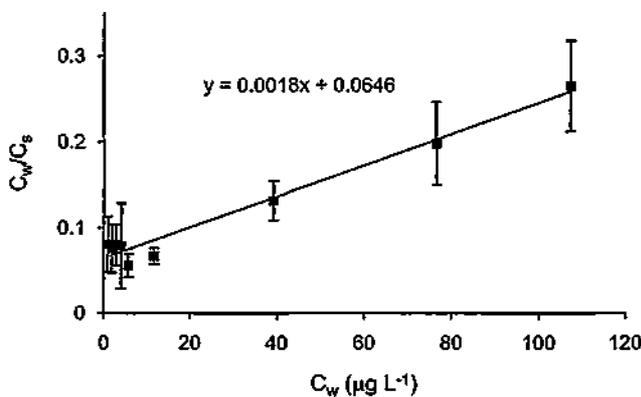


Figure 4.6. Langmuir linear regression for Pd on *C. stigmatophora* exposed to 5 to 200 $\mu\text{g L}^{-1}$ of Pd in a combined solution of PGE for 24 h under culture conditions detailed in Table 4.1. Error bars denote the standard deviations about the mean of four independent measurements.

Table 4.5. Results of sorption isotherms of Pd accumulated by *C. stigmatophora* derived from Langmuir linear regression equation (See Figures 4.6)

	$C_{s,max}$ ($\mu\text{g g}^{-1}$)	K_L (L g^{-1})	R^2
Pd	550	28	0.971

The straight line indicates there is a single type of sorption site responsible for binding of Pd over the range of studied concentrations and for this reason, Pd accumulation would be restricted at higher concentrations when all of the specialized functional groups for Pd accumulation on the cell surface are occupied and no more metal is able to bind. According to the results, the saturation Pd concentration, $C_{s,max}$, of *C. stigmatophora* is estimated at $550 \mu\text{g L}^{-1}$ under the experimental conditions employed. The saturation of binding sites might possibly happen for Rh and Pt as well since their algal and aqueous concentrations are not linearly related ($n < 1$). However this might not be achieved within the concentration range of metal applied in this study, possibly because of kinetic constraints on their coordination reactions.

The Langmuir fit is considered to be evidence that biosorption of Pd stops at one monolayer, consistent with specific biosorption onto functional binding sites. The values of K_F for lower concentration of Pd ($5 - 30 \mu\text{g L}^{-1}$) also did not show a significant difference ($p < 0.05$) between different experiments of combined and individual PGE solutions, +TM and -TM cultures (Table 4.3). This may support the assumption of equally available adsorption sites, monolayer surface coverage, and no interaction between adsorbed Pd ions.

Comparison between the adsorption isotherms and total accumulation isotherms indicates

the difference between metal intensity and capacity for PGE adsorption and PGE internalization. The adsorption of metals to the surface of algal cells is considered to be via two kinds of sites (Galceran et al., 2006), one of which is physiologically active (i.e. adsorption followed by internalization) and the other is not active (i.e. adsorption only). The capacity of each group of sites differs with metal species. The results of this study revealed among the three metals, the most significant difference was observed for Pd which showed a 5.5 to 7.5 fold larger K_F for its total accumulation relative to its surface adsorption (Table 4.3 and 4.4). This indicates that Pd has a greater affinity toward active binding sites on the surface. However, the lowest difference was observed for Pt which did not exhibit great internalization possibly due to its slow coordination kinetics.

4.4. Summary

Chlorella stigmatophora cultures were exposed to various concentrations of Rh, Pd and Pt as an individual or combined stock solution to investigate the isotherms defining PGE adsorption and accumulation by the microalgae. Summarizing the results of this study, the following conclusions can be drawn:

- Consistent with previous results, uptake of PGE by the algae followed the order Rh > Pd >> Pt.
- PGE surface adsorption as well as Rh and Pt total accumulation by *C. stigmatophora* followed the 1st type of Freundlich isotherm relationship, indicating that increasing concentration of adsorbed PGE makes further adsorption more difficult for algal cell. Pd total accumulation showed the 3rd type of relationship;

however, at greater concentrations, Pd followed the Langmuir isotherm which evidence that Pd accumulation is through specific functional binding sites and stops at one monolayer.

- Removal of individually added Rh and Pd by algae was lower than their removal from a combined PGE solution, while Pt removal did not compete with other metals, presumably due to its slow rate of exchange with surface sites.
- Palladium showed the greatest tendency to be internalized by the algal cells (up to 90%) and the extent and trend of its internalisation was not dependent on the presence of Rh and Pt; clearly, Pd is more reactive towards algal cells compared with Rh and Pt. On the other hand, Rh and Pt internalisation was dependent on the presence of the other PGE.

CHAPTER 5

Accumulation Kinetics of Platinum Group Elements by

Chlorella stigmatophora

Accumulation Kinetics of Platinum Group Elements by *Chlorella stigmatophora*

5.1. Introduction

Metal accumulation by biological materials causes changes in cellular physiology, depending on the extent of metal being adsorbed on the surface or bioaccumulated inside the cell. Alterations in the environmental concentrations of metals may cause the variation in the extracellular metal and consequently in the total concentration. Extracellular metal adsorption is a reversible process (Gonzalez-Davila, 1995; Sunda, 2000) and therefore it tends to reflect the environmental situation immediately before the collection of samples. Intracellular metal is not affected as greatly as extracellular metal content, and due to its slower reaction speed is more representative of the average conditions of the environment (Fernández et al., 2006). Since the measurement of cellular metal content at different stages is useful in biomonitoring studies, it is essential to understand the kinetics of metal accumulation in different stages of cell life. The kinetic data can then be fitted to suitable models, and could be used for calculating the values of maximum accumulation or accumulation rate.

The removal kinetics of metal ions by biological materials, and in particular by microalgae, have been widely studied (Fernández et al., 2006; Fraile et al., 2005; Quigg et al., 2006; Santana-Casiano et al., 1995; Schmitt et al., 2001; Tuzun et al., 2005; Yan & Pan, 2002; Yu & Wang, 2004). However, although PGE emissions to the environment have recently

become a concern, there are not many studies about their environmental behaviour (Sures & Zimmermann, 2007; Turner et al., 2007; Zimmermann et al., 2005) and nothing is known about the kinetics of their accumulation by microalgae. Therefore the present chapter describes a study into the kinetics of uptake and internalisation of the PGE by *C. stigmatophora* under otherwise identical experimental conditions to those employed in Chapters 3 and 4.

5.2. Methodology

Chlorella stigmatophora cells in their mid-exponential growth phase were centrifuged at 4000 rpm for 10 min and the pellets were resuspended separately in both +TM and -TM cultures as explained in Chapter Two (Section 2.4). Experiments were carried out in triplicate by transferring 1.5 L of algae into 2 L narrow mouth polycarbonate bottles (Nalgene, Nalge Nunc International) for each cultures of +TM and -TM. All bottles were spiked with 20 $\mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a mixed PGE stock solution and incubated under the conditions detailed in Table 5.1. Cultures of algae with no added PGE were set for each of +TM and -TM cultures as controls. The bottles were continuously aerated using a Pasteur pipette vertically placed in each bottle, connected to an air pump via polyethylene airline tubing (as described in Chapter Two, Section 2.3). Following the addition of metal into the cultures, phytoplankton cells were filtered at time 0 h, 0.5 h and every 12 h thereafter up to 156 h and processed for PGE analysis as explained in Chapter Two (Section 2.6). Samples were stored in a cold room at 4°C for further analysis by ICP-

Table 5.1. Incubation conditions of *C. stigmatophora* cultures exposed to $20 \mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a combined stock solution for 7 days. (+TM: cultures included nutrient trace metals and EDTA; -TM: cultures with no added nutrient trace metals and EDTA)

<i>culture</i>	<i>Light</i> ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	<i>Temperature</i> ($^{\circ}\text{C}$)	<i>salinity</i>	<i>pH</i> *	<i>Cell concentration</i> ($\sim \text{mL}^{-1}$)	<i>No. of replicates</i>
+TM	56	16 \pm 1	34.9	7.55	1,840,000	3
-TM	56	16 \pm 1	33.8	7.50	1,950,000	3

* Values are the initial records of pH at time 0 h.

MS to determine the intra-cellular, extra-cellular and aqueous PGE concentrations. Limits of detection of ICP-MS based of unspiked matrix are detailed in Appendix 1.

The pH was recorded initially and before each filtration throughout the experiment and adjusted by adding microlitre volumes of 1M HCl or 1M NaOH as required. The alteration of pH during the experiment is shown in Figure 5.1 and detailed in Appendix 2.1. The dry biomass of algal cells (mg L^{-1}) was calculated using the weight of filtered algae at internal filtrations and are shown in Figure 5.1. The algal weight calculated in this way was an estimation of biomass based on the weight of 15 ml filtered algal solution at each stage and was not calculated from the solution cell concentration.

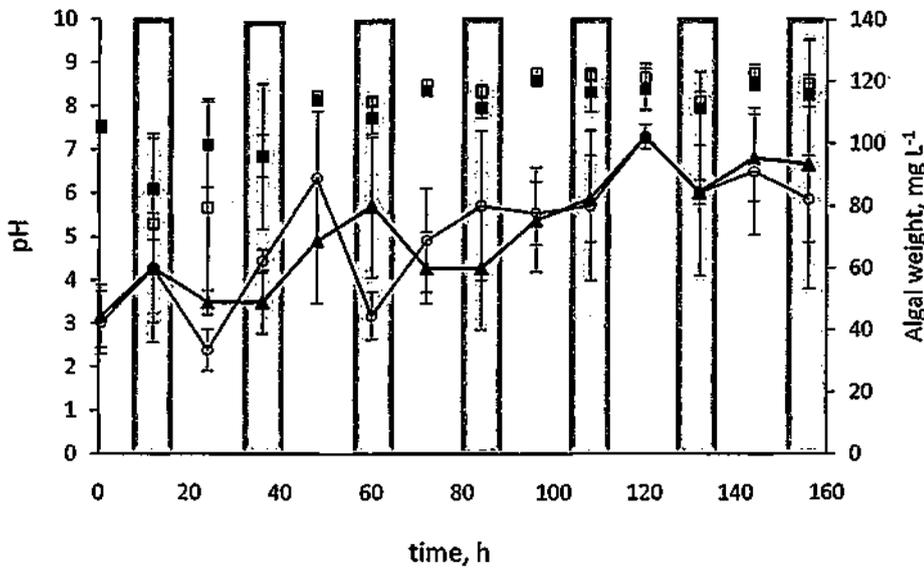


Figure 5.1. Alterations of pH and algal growth in *C. stigmatophora* cultures exposed to 20 mg L⁻¹ of PGE for 7 days under the light regime of 14 h light : 10 h dark (shaded columns). (■) and (□) represents pH values for +TM and -TM cultures, respectively. (▲) and (○) represents algal weight in +TM and -TM cultures, respectively. Error bars denote the standard deviations about the mean of three independent measurements.

5.3. Results and Discussions

5.3.1. Recovery of PGE

Recovery of Rh, Pd and Pt over seven days of exposure of *C. stigmatophora* to the PGE solution was calculated as the ratio of PGE mass balance to the initial metal concentration and the results are presented as percentages in Figure 5.2. A time dependent decrease in the available concentration of Rh, Pd and Pt was observed in all culture groups after the addition of the PGE at the beginning of the experiment. The results revealed that at early stages of exposure, Pt was the most available metal in the solution and above 90% of it was recovered in the cultures while shortly after metal addition (0.5 h) to the solution, 25% and

30% of Rh and Pd were lost, respectively, most likely due to the adsorption to the container walls.

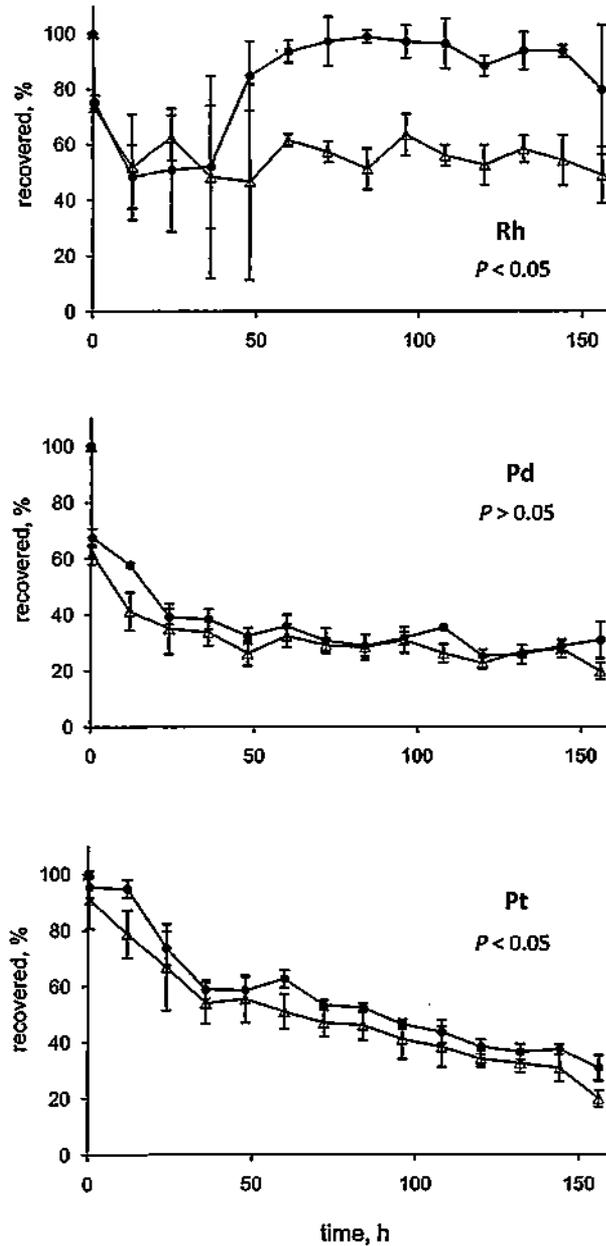


Figure 5.2. Recovery of $20 \mu\text{g L}^{-1}$ of Rh, Pd and Pt in the presence of *C. stigmatophora* as a function of time. (●) represents data for +TM cultures, (Δ) represents data for -TM cultures. Initial algal cell concentrations are detailed in Table 5.1. Error bars denote the standard deviations about the mean of three independent measurements. *P* values indicate the significant and / or non-significant differences of the data between +TM and -TM cultures.

The results of PGE recovery within the first 24 h was consistent with other results previously explained in Chapters 3 and 4. However the extent of recovery altered as the reaction progressed. The loss of Pd (up to 40%) occurred rapidly within 24 h, where after remained constant between 20% and 30% with not a significant difference between +TM and -TM cultures. This was due possibly to the saturation of contact surfaces of the container by Pd which resulted in equilibrium between Pd concentration on surface and in the solution. The recovery of Pt in both types of cultures gradually declined from ~ 90% to ~ 20% in the final day of exposure. It did not equilibrate even at the end of exposure time and there is a possibility of further reduction in case the exposure carried on. As was stated in previous experiments and other studies, Pt appears less reactive toward surfaces and is kinetically inert relative to Pd and Rh. Therefore although its early stage loss is not significant, it becomes significant with increasing time. The increasing extent of loss is likely due to the adsorption of the metal ion to the container walls. Precipitation of sparingly soluble Pt species cannot be ruled out, but solids were not observed to the naked eye or microscopically throughout the experiments.

The initial loss of Rh to about 50% was also rapid within the first 12 h and did not change considerably afterwards in -TM cultures, while in +TM it recovered back to about 95% after 40 h and remained relatively constant towards the end of the experiment.

5.3.2. Removal of PGE by *C. stigmatophora* over Time

Exposure of *C. stigmatophora* to 20 $\mu\text{g L}^{-1}$ of Rh, Pd and Pt in a mixed stock solution showed that PGE removal by phytoplankton cells from seawater was time dependent and

occurred as a biphasic process for Rh and Pd (Fig. 5.3). An initial phase, in which metal was removed by *C. stigmatophora* within a short period of time (about 24 – 48 h for Rh and 12 h for Pd), was followed by a second slower phase in which further metal was accumulated by the algae at a slower rate until equilibrium was achieved. The instantaneous reaction rates, λ ($\mu\text{g g}^{-1} \text{min}^{-1}$), of PGE towards algal cells were determined as the slope of the line between the origin and total cellular concentration of PGE after 30 min. The reaction rate of Pd was 2 and 3 folds greater than that of Rh and Pt in +TM and –TM cultures, respectively, and consequently the ratio of Pd removal from the solution was 3 to 4 times greater than that of the other metals (Table 5.2). Although the instantaneous removal of Rh and Pt was not significantly different ($p < 0.05$) within the first 30 min of exposure, the removal of Pt by algae, however, showed a systematic linear trend afterwards and the percentage of Pt removal continuously increased towards the end of the exposure. The extent of overall metal removal by *C. stigmatophora* at the end of the experiment was

Table 5.2. The half time removal (50% removal time, $t_{1/2}$), and instantaneous reaction data of Rh, Pd and Pt in response to exposure to *C. stigmatophora*

metal	culture	Instantaneous Reaction (within 0.5 h)		$t_{1/2}$, h
		Removal, %	λ , $\mu\text{g g}^{-1} \text{min}^{-1}$	
Rh	+TM	7.8	0.85	36
	-TM	7.1	0.75	39
Pd	+TM	17.7	1.85	4
	-TM	26.2	2.64	1
Pt	+TM	6.1	0.91	91
	-TM	5.8	0.84	91

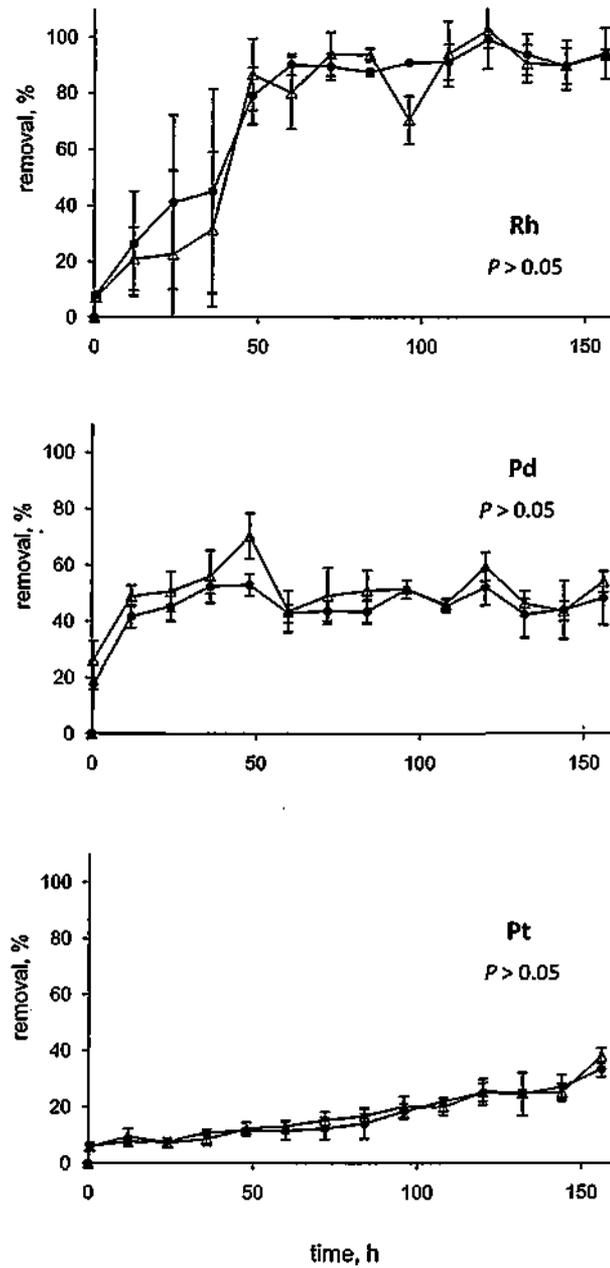


Figure 5.3. Percentage of Rh, Pd and Pt removed from the aqueous phase by *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a mixed stock solution for 7 days under culture conditions detailed in Table 5.1. (●) represents data for +TM cultures and (Δ) represents data for -TM cultures. Error bars denote the standard deviations about the mean of three independent measurements. P values indicate the non-significant differences of the data between +TM and -TM cultures.

Rh > Pd > Pt, consistent with previous results and similar to that found for PGE removal by the marine macroalga *Ulva lactuca* exposed to $10 \mu\text{g L}^{-1}$ of each of Rh, Pd and Pt for 100 h (Turner et al., 2007). Removal of Pd was considerably faster than that of Rh and especially Pt, an effect that has also been reported by other authors for *U. lactuca* (Cosden et al., 2003; Turner et al., 2006).

The 50% removal time, $t_{1/2}$, defined as the time required for removal of 50% of the total metal accumulated by the algae at equilibrium, is also shown in Table 5.2. Metal concentration at equilibrium was considered as the endpoint which appeared to be equilibrium for Rh and Pd but not for Pt which exhibited continuous uptake throughout. For a given metal, $t_{1/2}$ is similar between +TM and -TM treatments. The difference in the kinetic behaviour between Pt and Pd is consistent with the relatively slow rate of exchange of ligands coordinated to Pt relative to ligand exchange rates for Pd (Cosden et al., 2003). Since Pt is much slower to react with ligands and its rate of removal from solution by the algae in the present experiments is controlled by reactions with surface sites on the algae, these observations are expected. Other studies have also examined removal of PGE by organisms over time and overall removal trends for Pt and Pd are similar to those in this study.

The time dependent accumulation of PGE by *C. stigmatophora* is also shown in terms of the accumulation factor (AF), defined as the ratio of cellular to aqueous concentration of PGE. Here, uptake is normalised to dry weight of algae, which changed throughout the time course of the experiment (Figure 5.4). Consistent with previous results, Rh showed the greatest AF among the three metals (about $30 \times 10^4 \text{ L kg}^{-1}$ at maximum AF) and Pt presented the lowest, which was 100 times lower than Rh. The AF of Rh and Pt increased

over time. Despite the large variation of data, the mean overall AF of Rh remained relatively constant after 50 h, while the AF for Pt continuously increased. The steady increase of AF for Pt throughout the exposure time is due to the slow reaction kinetics of Pt in binding with the cell surface which consequently results in a gradual increase in removal of metal by the microalga. The greatest AF values for Pd were observed between 24 and 36 h (AF = 22 – 25 L kg⁻¹) which thereafter decreased to about 10 L kg⁻¹ and remained relatively constant towards the end of the exposure period. The reduction in AF may be attributed to the reduction of intra-cellular Pd concentration which is assumed to take place through metal effluxing.

By increasing the exposure time and consequently increasing the biomass of algae in seawater (as a result of growth), a reduction in AF for all metals may be expected as a result of the particle concentration effect (or biomass) on PGE accumulation (see Chapter 3, Section 3.3.2.2). However, the results indicated that the PGE accumulation process is time dependent and that time is more important than the concentration effect in the present experiments. This means that although higher concentrations of algal cells resulted in an increasing amount of exudates in the solution, there was adequate time for ligand exchange between PGE and the algal cell surface to proceed.

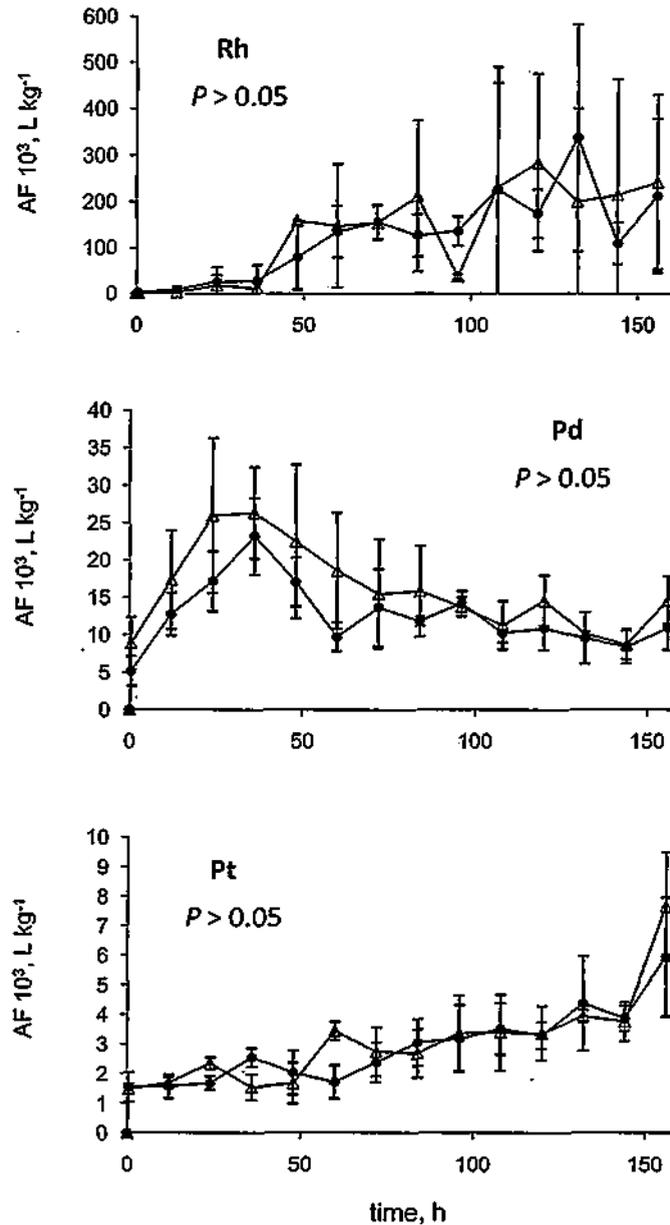


Figure 5.4. Uptake of Rh, Pd and Pt by *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of mixed solution of PGE for 7 days, shown in terms of accumulation factor – AF, as function of time. Initial algal cell concentrations are detailed in Table 5.1. (●) represents data for +TM cultures and (Δ) represents data for –TM cultures. Error bars denote the standard deviations about the mean of three independent measurements. P values indicate non-significant differences between the data between +TM and –TM cultures.

5.3.3. Intra- vs Extracellular PGE Accumulation

The extracellular or adsorbed concentration (i.e. metal removed from the cell surface by EDTA washing) and intracellular concentration (i.e. cellular metal remaining after the EDTA wash) of PGE per weight unit of *C. stigmatophora* is shown in Figure 5.5. Cell surface adsorption took place at the greatest rate in the initial 30 min of exposure which was 20 – 25 $\mu\text{g g}^{-1}$ of algae (slightly above 25 $\mu\text{g g}^{-1}$ for Pt). Considering the differences in kinetic behaviour of Rh, Pd and Pt, the similar extents of extracellular concentrations of the three metals suggests that cell surface accumulation of PGE is the result of non-specific binding to a variety of chemical functional groups on cell surfaces which is believed to be a rapid process, occurring in the first few minutes or hours of exposure depending on the metal species involved (Quigg et al., 2006; Ruangsomboon & Wongrat, 2006). At later stages, the rates of adsorption slowed down to various extents for the three metals until quasi equilibrium was achieved or a period of relaxation occurred.

The results also showed that intracellular accumulation of PGE was not a single-phase process and was metal dependent. Intracellular accumulation of PGE by *C. stigmatophora* cells takes place more slowly relative to surface adsorption and may be controlled by the diffusion process through the cell wall or regulated by intracellular metabolic processes (Santana-Casiano et al., 1995). After 24 h Pd was the most internalized metal ($\sim 80 \mu\text{g g}^{-1}$) and consistent with previous findings, Pt showed the lowest degree of internalisation. However, by increasing the exposure time, intracellular Pd reduced and reached to minimum concentration (20 – 30 $\mu\text{g g}^{-1}$) after 100 h. Internalised Rh also peaked after 70 – 80 h in +TM and 60 h in –TM cultures and a gradual reduction occurred afterwards, although the extent of this decline was not as low as that for Pd.

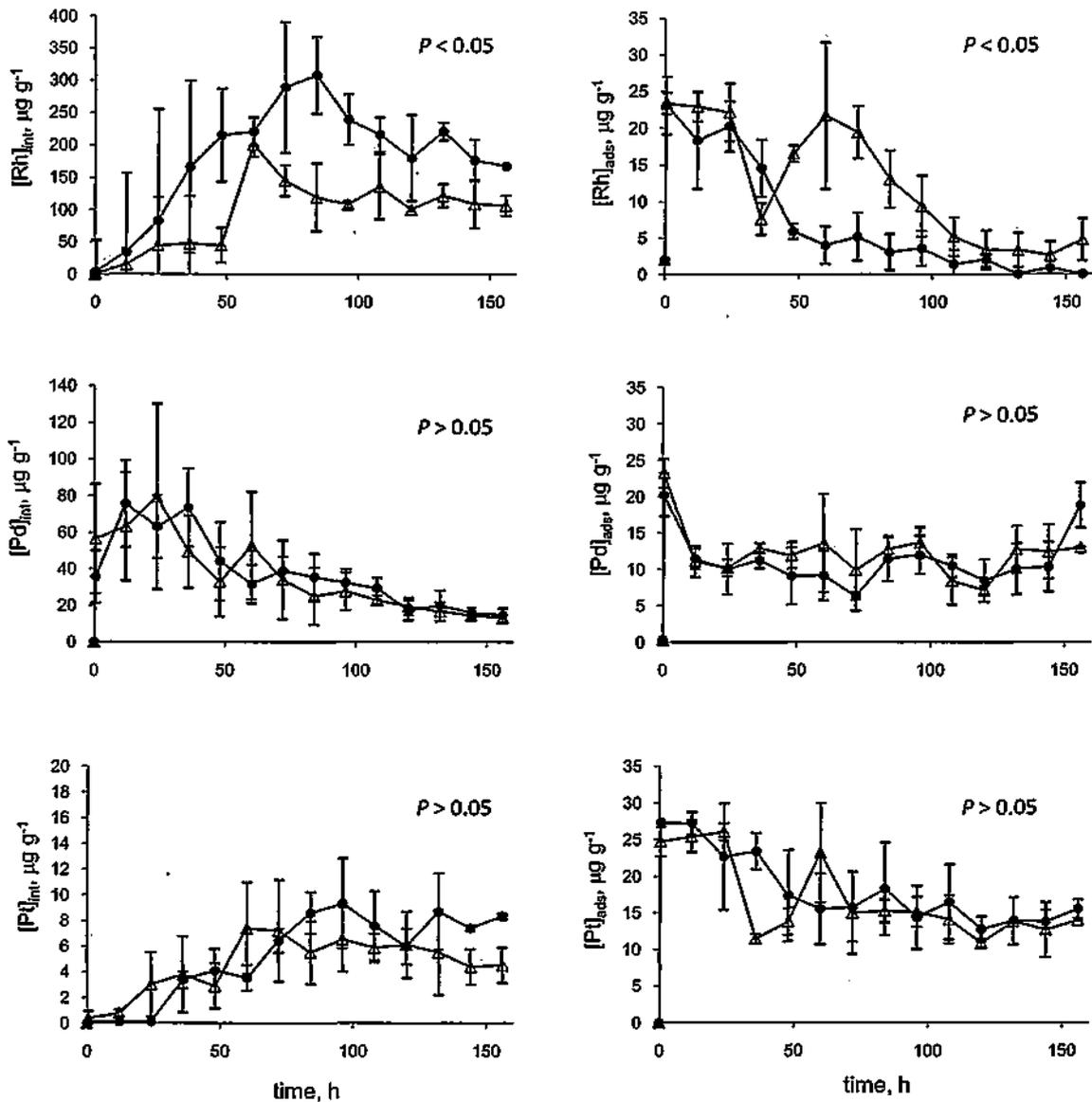


Figure 5.5. Accumulation of Rh, Pd and Pt by *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of PGE solution for 7 days, shown in terms of internalised metal concentration per unit of algal weight (left) and cell surfaced adsorbed metal concentration per unit of algal weight (right), as function of time. Initial cell concentrations of algae are detailed in Table 5.1. (●) represents data for +TM cultures and (Δ) represents data for -TM cultures. Error bars denote the standard deviations about the mean of three independent measurements. *P* values indicate the significant and / or non-significant differences of the data between +TM and -TM cultures.

The increase in the exposure time allows exhaustion of intracellular metal to occur (Gonzalez-Davila, 1995). Microalgae often possess efflux systems for toxic metals, as observed for Cd in diatoms (Levy et al., 2008) and Cu in *Chlorella vulgaris* (Worms et al., 2006). Some diatoms have been shown to exclude metal-complexes; for example, *Thalassiosira weissflogii* is able to export Cd-phytochelatin chelates (Sunda & Huntsman, 1998). Efflux systems are induced by high intracellular metal concentrations, and decrease toxicity by minimizing toxic metal concentrations. Assuming PGE are potentially toxic metals, increasing their intracellular concentrations will induce the efflux system of algal cells and as a consequence the intracellular concentrations of PGE will decrease. This reduction takes place rapidly for more reactive species like Pd, and as observed in Figure 5.5, for Pt no reduction in intracellular concentration occurred within the time frame of the experiment because of its relatively slow reaction kinetics. Although its concentration more or less equilibrated after 60 h, it is predicted that by maintaining Pt in contact with the algae for longer periods of time, an intracellular reduction would occur through some detoxification process as with Pd.

For a clearer understanding of cellular PGE variation over time, the results are also presented as the percentage of internalised metal relative to total metal accumulated by the algae (Figure 5.6). The greatest extent of Pd internalisation (86%) occurs in the early stage of exposure, followed by increasing its extra cellular concentration by time (indicating the efflux or metal exhaustion of Pd by cells which may involve cellular mechanisms as explained above), confirming the high affinity of Pd to complex and react with binding sites. On the other hand, Pt gradually internalized and barely exceeded 40% of the total accumulated Pt. Almost 99% of total Rh was found to be intracellularly located after 36 and 60 h in the +TM and -TM cultures, respectively, and remained constant with no

significant differences between the +TM and -TM cultures, although in -TM more surface adsorption and less internalisation of Rh took place compared with the +TM treatment. No significant differences were observed for the accumulation and internalization of Pd and Pt between the +TM and -TM cultures.

Compared with Pt, accumulation of Pd by the macroalgae *U. lactuca* (Cosden et al., 2003) and microalgae *C. vulgaris* and yeast *S. cerevisiae* (Godlewska-Zylkiewicz, 2003) was reported to be faster and greater in extent. The removal of Pd by the zebra mussel, *Dreissena polymorpha*, was reported to attain equilibrium one day after exposure (Sures & Zimmermann, 2007); in a separate study in which there was a continuous supply of metal (twice weekly) Pt equilibration by this species was attained after 7 weeks (Singer et al., 2005).

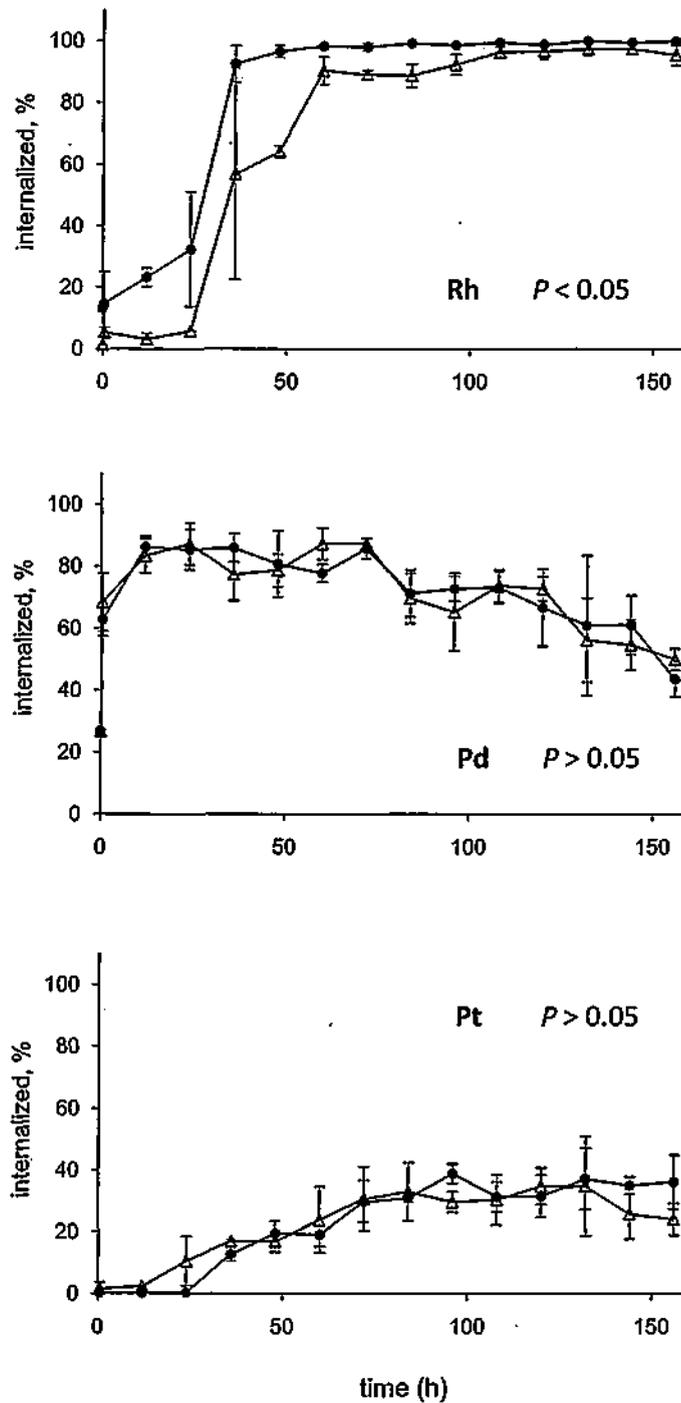


Figure 5.6. Percentage of internalized PGE by *C. stigmatophora* exposed to $20 \mu\text{g L}^{-1}$ of each of Rh, Pd and Pt in a mixed stock solution for 7 days under culture conditions detailed in Table 5.1. (●) represents data for +TM cultures and (Δ) represents data for -TM cultures. Error bars denote the standard deviations about the mean of three independent measurements. P values indicate the significant and / or non-significant difference of the data between +TM and -TM cultures.

5.3.4. Mechanisms of PGE Accumulation Kinetics

The multi-phasic uptake kinetics of PGE may indicate the existence of parallel uptake mechanisms which occur on the surface of the cell and is metal species dependent. The accumulation of some metals (e.g. Cr, Se and Fe) by certain microalgae over time has shown a linear trend (Chen et al., 2003; Wang & Dei, 2001b) indicating one type of reaction is involved in the uptake, at least under the experimental conditions applied in those experiments. However, most investigations carried out on the kinetics of metal accumulation by microalgae exhibit bi- or multi-phasic uptake (Garnham et al., 1992; Gonzalez-Davila, 1995; Lee et al., 2009; Levy et al., 2008; Quigg et al., 2006; Vasconcelos & Leal, 2001; Yan & Pan, 2002). The results of the present study suggest that when a single, initial concentration of PGE is added to an algal culture solution, uptake of metal into algal cells is at least a two phase process. The first phase of accumulation is fast and metabolism-independent (Garnham et al., 1992; Gonzalez-Davila, 1995) indicating this process to be passive. In this stage, fast PGE adsorption to the binding sites on the cell surface occurs at both non-active sites and metabolically active sites at which Rh, Pd and Pt may subsequently enter the cell through them. In the second phase, which is considered as an energy-dependent process (Garnham et al., 1992) and happens at a slower rate, internalization of PGE into the algal cells takes place via ion pores, channels or transporters in the algal cell membrane (Levy et al., 2008). In this stage, surface adsorption slows down because initially a large number of surface binding sites may be available for adsorption and after some time the remaining sites may be difficult to be occupied (Melčáková & Růžovič, 2010). Although surface adsorption of metals slows down, for fast reacting species (such as Pd in this study) the ratio of internalised to surface adsorbed metal reduces over time. This could be due to a detoxification response of the algae to the

increasing concentration of potentially toxic species of metal (Levy et al., 2008; Wells et al., 1995; Yan & Pan, 2002). As a defence mechanism algae can detoxify metal ions by producing cell exudates which can bind to internalised metal. Detoxification may also occur for Rh and Pt, but since Pd complexation takes place faster, the exhaustion of only this metal from the algal cells was seen within the applied exposure period. The same process may be observed for Rh and Pt when the alga is exposed to metal for a longer period of time.

5.4. Summary

The accumulation kinetics of $20 \mu\text{g L}^{-1}$ of Rh, Pd and Pt by *C. stigmatophora* was studied under controlled experimental conditions. The recovery, removal and cellular concentration of metals over seven days of exposure were analyzed. The overall outcome of this experiment indicated that:

- Platinum exhibited greatest recovery initially, but progressively more was lost over the time and by the end of exposure more Pt was lost than Pd and Rh. This occurs mainly because Pt is a slow reactive species, and its reaction with surfaces take place longer compare with Rh and Pd.
- 90% of Rh was removed by the algal cells over the reaction time, while removal of Pt did not exceed 40% which is due to its slow reaction kinetics. Pd removal rapidly occurred within 12 h and did not change appreciably afterwards. This might be explained by the nature of the specific binding sites which can be saturated after a certain period of time.

- Accumulation of Rh, Pd and Pt was a biphasic, time-dependent process; a fast and non-specific surface adsorption was followed by a slow specific internalization.
- Adsorption of PGE to the algal cell surface was a non-specific, fast process initially. Due to the saturation of the binding sites along with the action of internalisation, surface adsorption decreased over time with the fastest rate for Pd and the slowest for Pt.
- Internalization of Pd took place very rapidly due to its fast reaction kinetics and began to efflux after 80 h which is attributed to the defence mechanism of alga to detoxifying metal from the cells. Rh and Pt internalization occurred more slowly and while almost all of the accumulated Rh internalized into the algal cell, only 40% of Pt was found intracellularly.

CHAPTER 6

Growth Inhibitory Effects of Platinum Group Elements on *Chlorella stigmatophora*

Growth Inhibitory Effects of Platinum Group Elements on *Chlorella stigmatophora*

6.1. Introduction

Increasing quantities of platinum group elements are entering the environment from automobiles (via catalytic converters) and hospital wastes, yet little information is known about their toxicity to terrestrial and marine biota. For example, Battke et al. (2008) showed that Pd concentrations above 100 mg kg^{-1} of road side particles could reduce the length of barley leaves by up to 13%. PGE strongly induced heat shock protein (hsp70) in zebra mussels *Dreissena polymorpha* exposed to $500 \text{ } \mu\text{g L}^{-1}$ of Rh, Pd and Pt for 10 weeks (Singer et al., 2005). The 50% lethal concentration of Pt for the freshwater crustacean *Asellus aquaticus* was found to be $100 \text{ } \mu\text{g L}^{-1}$ after 4 days of exposure to metal (Rauch & Morrison, 1999).

The inhibitory effects of metals on phytoplankton growth have been frequently reported. Because of their key position as primary producers in aquatic systems, and as target of metal pollution, microalgae are considered to be sensitive indicators of environmental change and therefore are important species for the regulatory assessment of metals (Capelo et al., 1993; Goudey, 1987; Levy et al., 2007). Therefore, this chapter aims to investigate the toxicity and inhibitory effects of Rh, Pd and Pt on the growth of the marine microalga *C. stigmatophora* by investigating the two important factors of pigment content as well as the specific growth rate of algae (as the measure of the growth) after exposure to various concentrations of PGE. The results from this toxicity test will provide important

information for assessing the potential toxicological impact of platinum group elements both individually and in combination on this important group of aquatic organisms.

6.2. Methodology

6.2.1. Algal Culture

Chlorella stigmatophora cells in their mid-exponential growth phase, were centrifuged at 4000 rpm for 10 min and the pellets resuspended in +TM cultures as explained in Chapter Two (Section 2.4). Two sets of experiments were performed, covering a range of Rh, Pd and Pt concentrations. Experiments were carried out in quadruplicate by transferring 120 mL of algae into separate 150 mL styrolux (crystal polystyrene) containers. In the first experiment (Experiment A), containers containing algae were spiked with 0 (control), 5, 10, 15, 20, 30, 50, 60, 70, 80, 90, 100, 150 and 200 $\mu\text{g L}^{-1}$ of each of Rh, Pd and Pt as a combined PGE solution. In the second experiment (Experiment B), algae were exposed to the same concentrations of each of Rh, Pd and Pt individually. The containers were incubated for 48 h under the conditions detailed in Table 6.1 and constantly agitated on a Denley orbital mixer and two UNIMAX2010 shakers at 85 rpm. Containers were covered with clear lids to prevent evaporation and introduction of any contamination. The lids were slightly left open for air exchange. The pH was recorded initially and at the time of filtration, as well as at two intervals throughout the exposure. Alterations in pH were adjusted by adding microlitre volumes of 1 M HCl or 1 M NaOH as required.

Table 6.1. Incubation conditions of *C. stigmatophora* exposed to various concentrations of combined and individual solution of Rh, Pd and Pt for 24 h for toxicity studies

Experiment	Light ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	Temperature ($^{\circ}\text{C}$)	salinity	pH (initial)	Cell concentration ($\sim \text{mL}^{-1}$) [*]	No. of replicates
A	65	15 \pm 1	34.1	7.55	1,590,000	4
B	65	15 \pm 1	33.7	7.52	2,860,000	4

* Initial cell concentration

6.2.2. Extraction of Cellular Chlorophyll *a*

The cellular chlorophyll *a* (Chl *a*) contents were measured at 24 h after metal exposure. To this end 15 mL suspensions of algae from each container were vacuum filtered through a glass microfibre filter (MF300, 47 mm diameter, Fisher) and immediately frozen by placing the filters in liquid nitrogen in order to accelerate Chl *a* extraction. As the samples freeze, ice crystals can break the cell membrane and make the extraction more effective (MacIntyre & Cullen, 2005). Each frozen filter paper was placed in a Petri dish and covered with foil to minimize the photodegradation of Chl *a*. To complete the extraction, frozen algae on filter papers were ground in 10 mL of 90% acetone (99+%, GLC, Specified) using a porcelain mortar and pestle. The ground sample were transferred to a polypropylene centrifuge tube (50 mL universal containers, polyethylene cap, Sterilin) and diluted with 10 mL of 90% acetone. The cap was tightly closed to prevent evaporation and the tube covered with foil and placed in a fridge at 4 $^{\circ}\text{C}$. After 24 h, the samples were centrifuged at 5000 rpm for 10 min to remove cellular debris. The supernatant was decanted into 1 cm spectrophotometer glass cuvettes and the absorbance was immediately measured at 664, 647, 630 and 750 nm by spectrophotometry. The entire process from

filtration until reading the absorbance was carried out in the dark (or minimum light) to prevent Chl *a* degradation (MacIntyre & Cullen, 2005). The spectrophotometer was calibrated at each wavelength using a glass spectrophotometer cuvette filled with 90% acetone. Each absorbance at 750 nm (which measures the turbidity of the samples) was subtracted from the corresponding absorbance at other wavelengths. Concentrations of Chl *a* were then calculated using equation 6.1 (Stirling, 1985):

$$\text{Chlorophyll } a, \mu\text{g mL}^{-1} = (11.85 A_{664} - 1.54 A_{647} - 0.08 A_{630}) \cdot \frac{V}{v \cdot L} \quad (6.1)$$

where A_x is absorbance at wavelength x , V (mL) is the final volume of acetone extract, v (mL) is the volume of filtered algae and L (cm) is the path length of the spectrophotometer cuvette.

6.2.3. Measurement of Algal Growth

Algal cell concentration in each container was measured immediately before adding the metals and at 48 h after exposure. Two hundred μL of algal suspension was transferred to vials and diluted with 15 mL of seawater. The cell concentration of each vial was measured at particles size ranges of 3 – 5 μm , 5 – 7 μm and > 7 μm using a Coulter Counter (Z2 Beckman Coulter). Background particle concentrations of seawater for the same particle sizes were also measured and subtracted from the corresponding particle concentrations of algal samples. The growth of algae was calculated using the specific growth rate equation (Fogg & Thake, 1987):

$$\text{Specific Growth Rate, } \mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1} \quad (6.2)$$

where N_1 and N_2 are algal cell number concentrations at times t_1 and t_2 , respectively.

6.2.4. Data Analysis

The most common parameter used in toxicity assays is the EC_{50} , which is expressed as the effective concentration giving 50% reduction in algal growth rate over a known period of time compared to the controls (Sparks, 2000). Calculations of 24 h EC_{50} for Rh, Pd and Pt toxicity, expressed as the 24 h EC_{50} and 48 h EC_{50} , were carried out by two common methods of dose response analysis according to Newman (1995) and as summarised below.

Probit analysis: The portability unit (PU) of the growth reduction percentage was calculated in Excel 2007 and the data were compared with Response Metameters Relationship Table (Newman, 1995) for accuracy. EC_{50} was calculated by a linear regression between PU and log of initial metal concentration when PU is set to 5. This method of data transformation is a classical method for sigmoid response curves.

Arcsine transformation: The percentage of growth reduction was converted to *arcsine* values and then plotted against log of initial metal concentration. EC_{50} was calculated by a linear regression when *arcsine* is set to 45. *Arcsine* transformation moves scattered values toward the centre, giving a linear relationship of the response data.

The lowest observed effect concentration (LOEC), defined as the metal concentration at which the response becomes significantly ($P < 0.05$) lower than the control, and the no observed effect concentration (NOEC), defined as the highest tested metal concentration that yields no significant difference from a control, were estimated by multiple comparison of the data.

Assessment of significant differences among treatments ($P < 0.05$) for the growth rate, cell concentration and chlorophyll *a* content of each culture was analysed using one way ANOVA. *Post hoc* multiple comparison tests were applied to establish where significant differences occurred.

6.3. Results and discussion

6.3.1. Chl *a* Content of Metal-Treated *C. stigmatophora* Cultures

The concentration of Chl *a* of *C. stigmatophora* cultures was measured after 24 h of exposure to individual and combined stock solutions of Rh, Pd and Pt. The Chl *a* concentrations in non-exposed algal cultures (controls) were significantly different in Experiments A and B. The Chl *a* concentration of non-exposed (control) cultures after 24 h for Experiment A was $2.49 \pm 0.4 \mu\text{g mL}^{-1}$ and for Experiment B was $0.97 \pm 0.1 \mu\text{g mL}^{-1}$. The difference is most likely due to the initial algal density of the stock cultures utilized for the two experiments, which were carried out at different times, although the inoculums for both experiments were taken from the exponential growth phase of the stock cultures. Therefore, to simplify the interpretation of the results, data were normalized and presented as the percentage of Chl *a* reduction in each treatment relative to control cultures (Figure 6.1). The results revealed a significant reduction of cellular Chl *a* content with increasing concentration of metal, although Rh, Pd and Pt affected Chl *a* concentrations to different extents. After 24 h, Rh and Pt at their highest concentrations ($200 \mu\text{g L}^{-1}$) reduced the Chl *a* of algae up to 70% and 80% of the control, respectively. The decrease was more moderate in Rh and Pt exposed cultures, whereas $5 - 30 \mu\text{g L}^{-1}$ of Pd and combined PGE

caused a significant reduction of Chl *a* followed by negligible changes at higher concentrations. The sudden decline in Chl *a* content of Pd and PGE exposed cultures indicated that cellular Pd concentration exceeds the threshold of algal capacity (Fisher et al., 1984), suggesting that each cell is capable of accommodating a certain amount of metal before sensitive sites within the cell are affected.

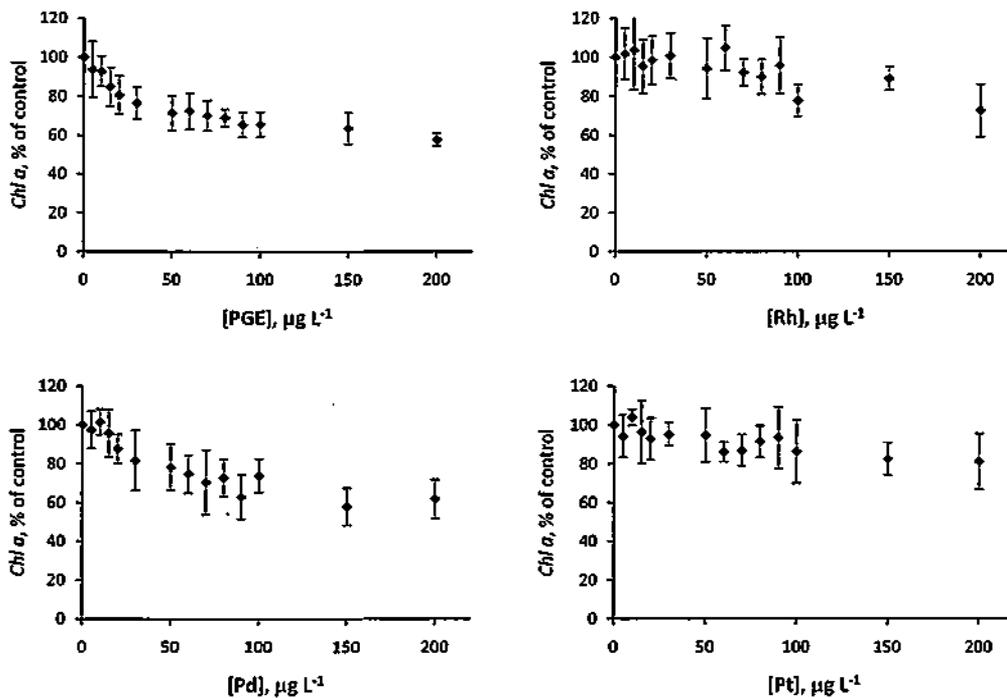


Figure 6.1. Variations in Chl *a* content of *C. stigmatophora* cultures exposed to 5 – 200 $\mu\text{g L}^{-1}$ of Rh, Pd and Pt as combined and individual stock solutions for 24 h, in terms of % relative to control. Error bars denote the standard deviations about the mean of four independent measurements.

In order to evaluate the possible effect of PGE at cellular level, the concentrations of Chl *a* were normalized to the algal biomass (Figure 6.2) and the results indicated a linear relationship between the number of cells and Chl *a* concentrations for Pd and Pt exposed cultures, and a non-linear relationship for Rh exposed cultures with a significant reduction

in cellular Chl content at Rh concentrations of $> 90 \mu\text{g L}^{-1}$. This suggests that Rh may either inhibit the Chl a biosynthesis process or induce its degradation. Since Fe is quantitatively the most important metal involved in algal photosynthesis, it is predicted that the interference of Rh with Fe may cause this effect possibly via binding to biological ligands responsible for Fe accumulation and blocking its reaction sites. However, more investigations regarding the pathways that PGE can affect algal cell Chl a at the molecular level are recommended.

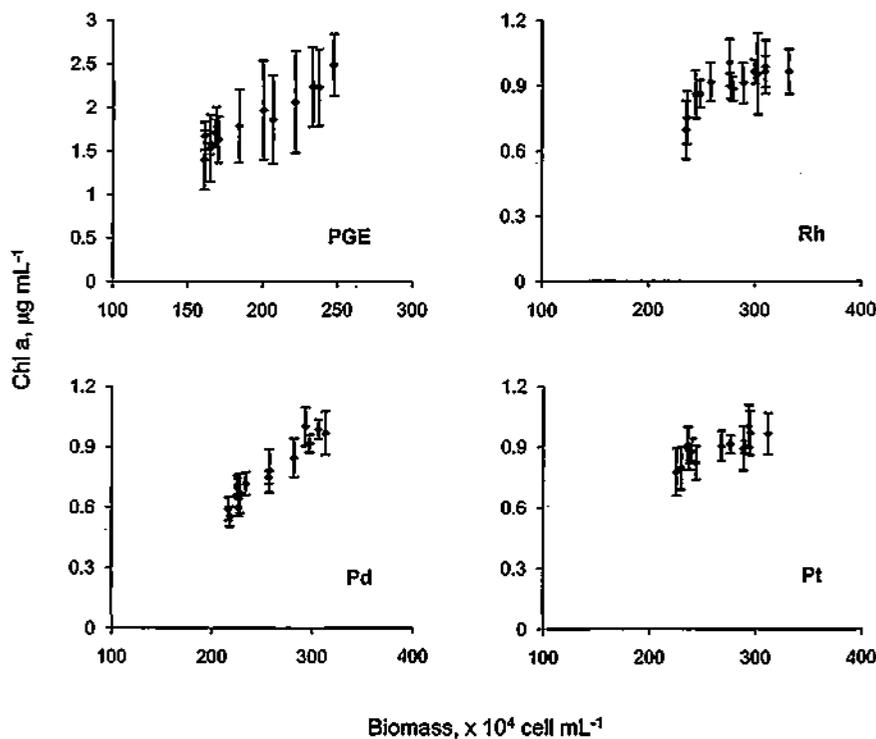


Figure 6.2. Relationship between the concentration of Chl a and the algal biomass obtained from *C. stigmatophora* cultures after 24 h exposure to 5 – 200 $\mu\text{g L}^{-1}$ as combined and individual stock solutions of Rh, Pd and Pt. Algal biomass was calculated using the 48 h specific growth rate. Error bars denote the standard deviations about the mean of four independent measurements.

The reduction of Chl *a* content and other pigments of algal cells is a common symptom of metal toxicity in higher plants, green algae and cyanobacteria (Joshi & Mohanty, 2004). The decrease in Chl can occur due to both the inhibition of the organism's biosynthesis and the induction of its degradation (Molas, 1997). The action of a metal on the photosynthetic system of plants has been studied extensively and various mechanisms are shown to be involved in the inhibition process. Copper interferes with various physiological processes such as inhibiting the ability of thylakoids to undergo light state transition (Joshi & Mohanty, 2004) or interfering with Fe metabolism causing Fe deficiency (Lidon & Henriques, 1993). Mercury inhibits photosynthetic electron transport at various sites (Joshi & Mohanty, 2004) and displaces Mg in Chl, leading to Chl degradation (Patra & Sharma, 2000). Aluminium causes morphological and ultra-structural changes in chloroplasts, and also reduces photosynthetic electron transport, CO₂ fixation and net photosynthesis (Moustakas et al., 1997). There are no studies demonstrating the mechanism(s) by which PGE can inhibit photosynthesis function or reduce Chl *a* content. Odjegba et al. (2007) showed that combined solution of PGE concentration of 1 ppm resulted in a 10% reduction of maximum quantum yield of photosynthetic activity in the lettuce, *Lactuca sativa*. However the cellular mechanism was not investigated.

In the present work, some preliminary studies were carried out with Chl fluorescence to investigate the inhibitory mechanism(s) of PGE at the cellular level. A dual-channel photosynthesis yield analyser Toxy-PAM (WALZ, Heinz Walz GmbH) was used to measure the effective quantum yield of photosystem II (PSII), *Y*, via assessment of Chl fluorescence yield in the algal cells. Any substance which causes a limitation of photosynthetic electron flow will cause a corresponding lowering of *Y*, and Toxy-PAM is designed to carry out *Y* determination with extraordinary accuracy. However, the

preliminary studies specified that measurement of toxicity in microalgal cells within the applied range of PGE concentrations may not be applicable by ToxY-PAM. Because, for accurate measurement, microalgae in the cuvettes must remain suspended, whereas, precipitation and settlement of *C. stigmatophora* cells after 50 min (determined by ToxY-PAM) meant that a clear toxicity response could not be determined within this time scale. The results from this approach are, therefore, not presented here.

The effective concentration of PGE as 24 h EC₅₀ for Chl *a* reduction relative to the control was calculated by applying both *Probit* analysis and *Arcsine* transformation (Figure 6.3). The calculations indicated that the effective concentrations of Rh, Pd and Pt required for a 50% reduction of Chl *a* within 24 h are much higher than the applied experimental concentrations. The EC₂₀, or effective concentration of metal for 20% reduction in Chl *a*, was therefore calculated and the results are shown in Table 6.2. The similarity of the EC₂₀ for Pd and combined PGE (~30 and ~40 µg L⁻¹, respectively) shows that the inhibitory effect of PGE on Chl *a* of *C. stigmatophora* cultures is more likely to be caused by Pd. However the action of Rh and Pt is considered to be additive with respect to the total metal concentrations (PGE), i.e. metal mixtures may behave in a synergistic manner and enhance toxicity (e.g. lower 24 h EC₂₀ in the combined PGE exposure than in the Pd exposure). The toxicity mechanism of Pd on phytoplankton is unclear. However, considering the inhibitory effect of Ni on photosynthetic electron transport (Krupa et al., 1993) and the similar biochemical potentials of Pd and Ni, it is possible that these two metals exert similar inhibitory effects on primary producers.

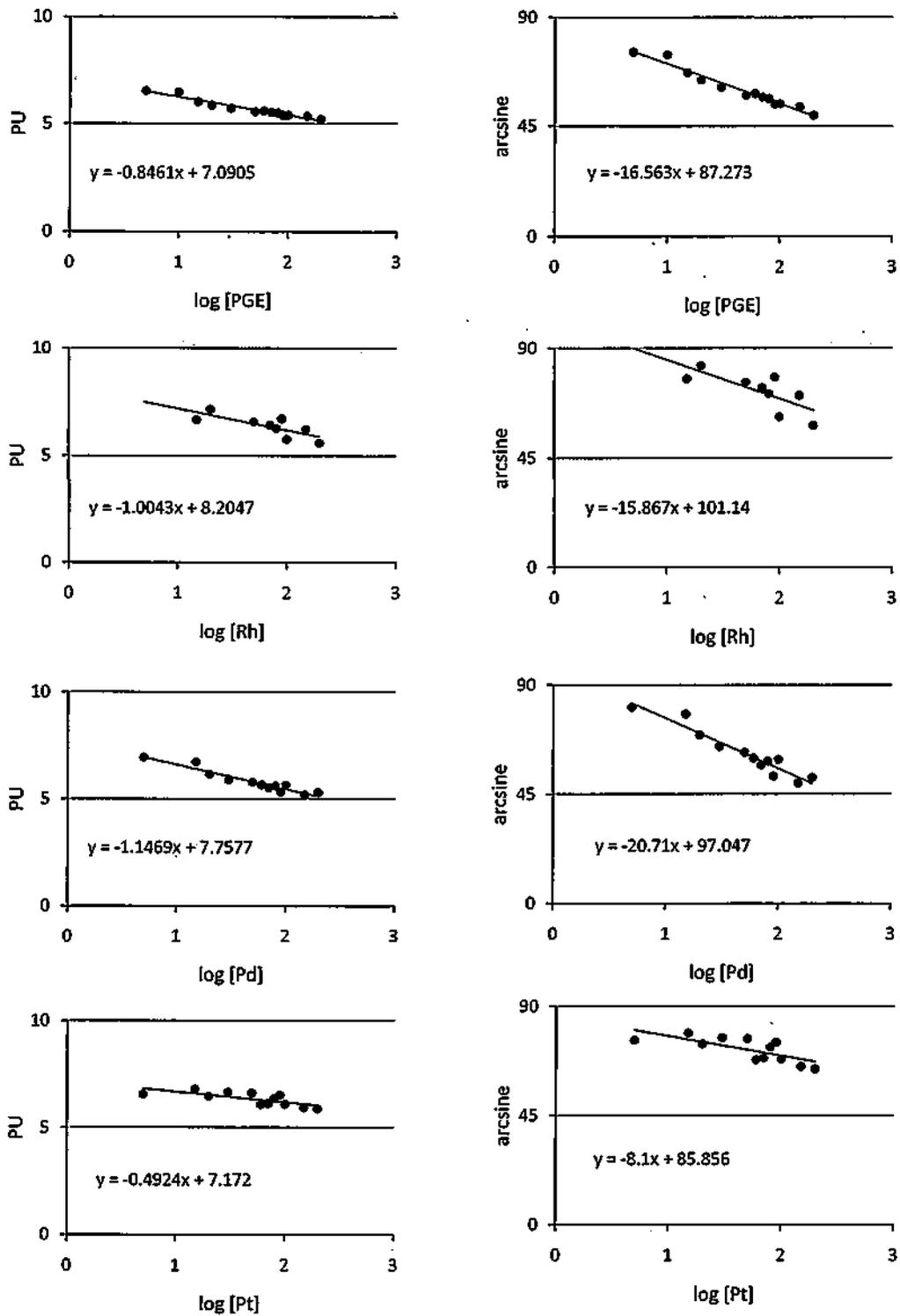


Figure 6.3. Linear regression of dose response equation between log of initial metal concentration ($\mu\text{g L}^{-1}$) and PU (left) or *arcsine* (right) of percentage of Chl *a* reduction in *C. stigmatophora* exposed to 5 – 200 $\mu\text{g L}^{-1}$ of individual and combined solutions of Rh, Pd and Pt for 24 h.

Table 6.2. Effect of Rh, Pd and Pt on the Chl *a* content of *C. stigmatophora* exposed to combined and individual solutions of metals for 24 h (values in parenthesis are concentrations in μM)

Experiment		$EC_{50}, \mu\text{g L}^{-1}$		$EC_{20}, \mu\text{g L}^{-1}$		R^2	
		(μM)		(μM)			
		Probit	Arcsine	Probit	Arcsine	Probit	Arcsine
A	PGE	295	355	30	27	0.950	0.965
B	Rh	1500 (5.7)	3400 (12.9)	225 (0.86)	235 (0.89)	0.600	0.567
B	Pd	255 (1.1)	325 (1.4)	50 (0.22)	42 (0.18)	0.923	0.923
B	Pt	*	*	500 (2.7)	590 (3.0)	0.544	0.545

* The values are above 25 mg L^{-1} .

6.3.2. Algal Specific Growth Rate of Metal-Treated *C. stigmatophora*

Algal growth endpoints are the basis of most chronic algal toxicity studies and are environmentally relevant because changes in population growth may influence species succession and community structure and function (Franklin et al., 2002a). The effect of PGE on the population of *C. stigmatophora* (which is considered as growth index in phytoplankton studies) was investigated in this study and the changes in growth are presented as the effect of metal toxicity. Total cell density of *C. stigmatophora* after 48 h of exposure to $5 - 200 \mu\text{g L}^{-1}$ of metals was calculated (Figure 6.4). A reduction of cell density was seen in all cultures exposed to both individual and combined metal solutions of Rh, Pd and Pt. The extent of reduction in metal concentrations lower than $60 - 70 \mu\text{g L}^{-1}$ varied between the three metals as well as the combined PGE solution. However, at higher

concentrations (above $90 \mu\text{g L}^{-1}$) all metals completely inhibited growth of algal cells (Rh at $> 80 \mu\text{g L}^{-1}$, Pt at $> 70 \mu\text{g L}^{-1}$ and Pd and PGE at $> 60 \mu\text{g L}^{-1}$).

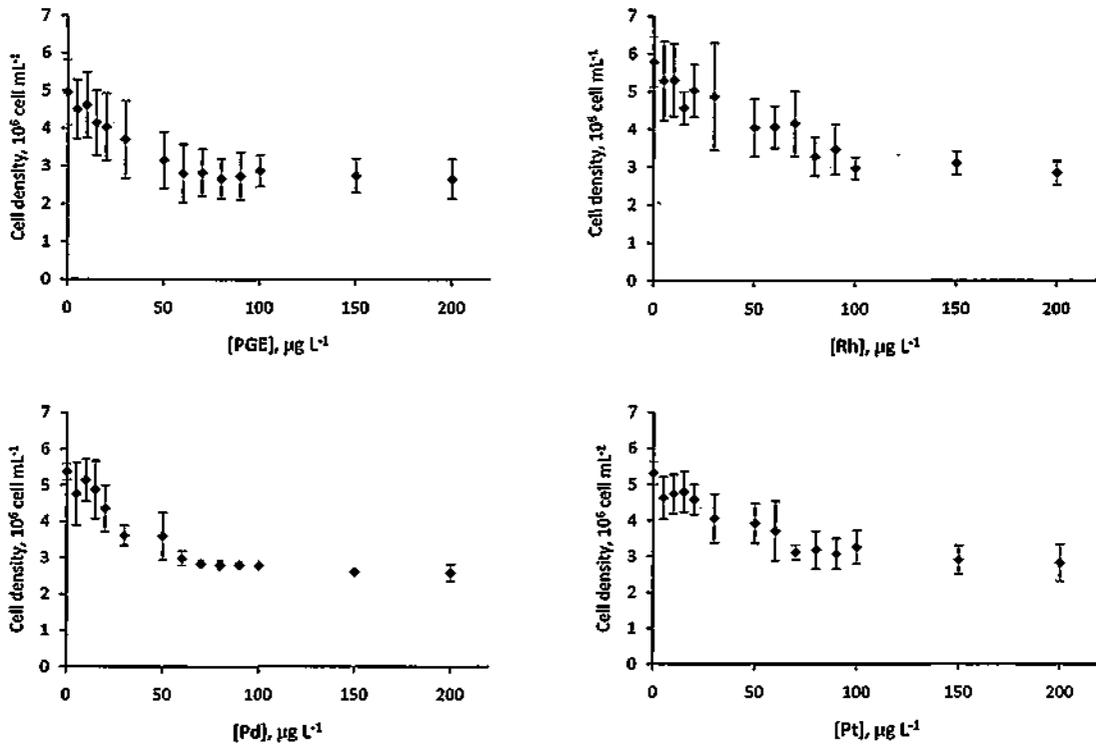


Figure 6.4. Cell concentration of *C. stigmatophora* exposed to 5 – 200 $\mu\text{g L}^{-1}$ of Rh, Pd and Pt as individual and combined stock solutions for 48 h. Error bars denote the standard deviations about the mean of four independent measurements.

The growth rate, μ , observed for non-exposed algae in all experiments was between 0.32 and 0.50 doubling day $^{-1}$, which is just below the maximum μ of *C. stigmatophora* at optimum growth conditions which is reported by Fabregas et al. (1987); i.e. μ_{opt} : 0.51 doubling day $^{-1}$. Among all metals, Pd had the greatest adverse effect on algal growth and at concentrations above $100 \mu\text{g L}^{-1}$ it resulted in a negative μ which is due either to increasing mortality rate of algae relative to cell division or reproduction disorders which results in a

reduction of population. According to the findings of previous chapters, among the three metals Pd exhibits the highest affinity for reacting with algal cell surfaces and consequently internalizing into the cells (see Chapters 3 – 5). Therefore, its effects on cellular functions might be more rapid than those of the other metals. It is known that the metals which have more tendency towards particle surfaces (including microalgae) are more toxic to phytoplankton (Fisher, 1986; Franklin et al., 2000).

The μ of the 48 h metal-treated algal cultures was used to calculate the percentage of growth rate, taking the control sample as 100%. The data were then plotted against the metal concentration (Figure 6.5). A sigmoid plot was obtained for the three metals, which is typical of most aquatic organisms in the presence of a toxicant (Franklin et al., 2000), as a result of a sharp decline in algal μ above a certain threshold metal concentration ($> 10 \mu\text{g L}^{-1}$ PGE, $20 - 30 \mu\text{g L}^{-1}$ Rh and Pt, $15 \mu\text{g L}^{-1}$ Pd).

The lowest applied metal concentration ($5 \mu\text{g L}^{-1}$) was found to be the lowest concentration of metal which significantly inhibited the growth rate of *C. stigmatophora* relative to the control (Table 6.3). Therefore, LOEC values of Rh, Pd and Pt were estimated to be $0 < \text{LOEC} \leq 5 \mu\text{g L}^{-1}$. These values are lower than 48 h LOEC values of Cu ($0.07 \mu\text{M}$), Cd ($0.19 \mu\text{M}$) and Zn ($0.57 \mu\text{M}$) for freshwater *Chlorella* sp. (Franklin et al., 2002b). However, in the mentioned study, the growth rate of algae under incubation conditions of 27°C and $140 \mu\text{mol m}^{-2}\text{s}^{-1}$ was estimated at $1.9 \text{ doubling day}^{-1}$ which is greater than the algal μ in the present study. Algal growth rate is generally inversely related to the cell metal concentration (Sunda, 2000). Thus, under different incubation conditions which result in different growth rates, metal accumulation by algae will vary, which consequently

affects the EC of the toxicant. One should be aware that when comparing the toxicity of metals the precise experimental conditions have to be considered.

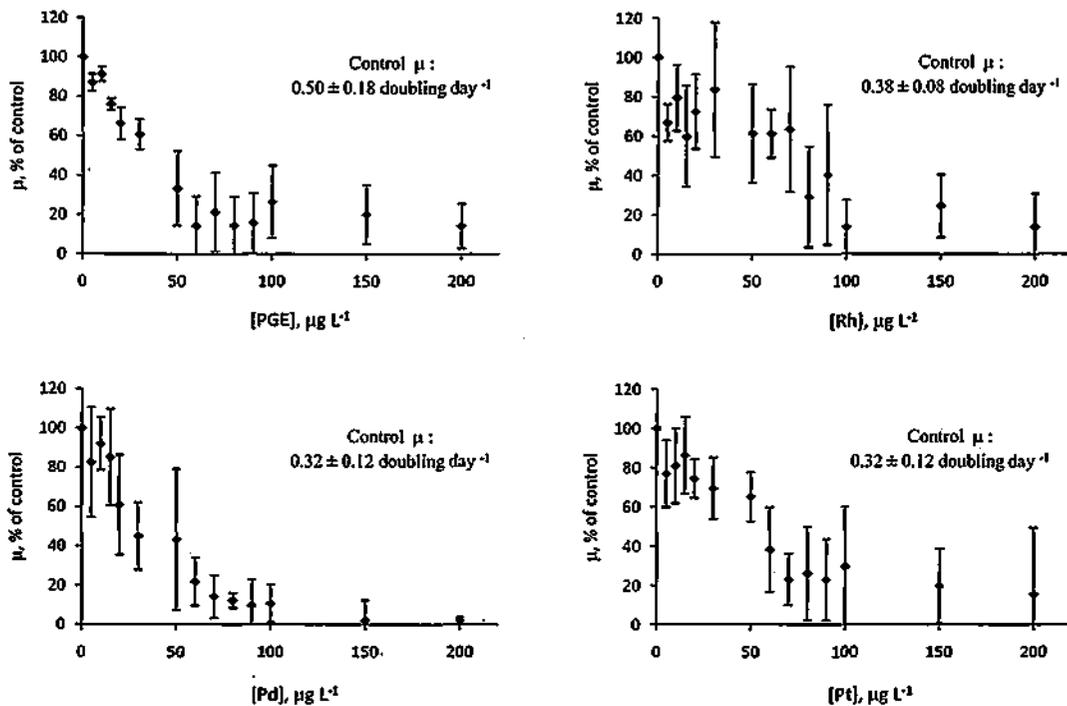


Figure 6.5. Growth rate of *C. stigmatophora* exposed to 5 – 200 $\mu\text{g L}^{-1}$ of Rh, Pd and Pt as individual and combined stock solutions for 48 h, in terms of % relative to control. Error bars denote the standard deviations about the mean of four independent measurements.

The 48 h EC_{50} of individual and combined Rh, Pd and Pt for *C. stigmatophora* growth rate was calculated using regressions obtained from the *Probit* and *arcsine* analyses (Figure 6.6 and detailed in Table 6.3). Among the three metals, Pd showed the highest toxicity of 27 $\mu\text{g L}^{-1}$ for 48 h EC_{50} . Comparison of the EC_{50} results for Chl *a* and μ indicates that PGE may employ different mechanisms for inhibition the different cellular functions. The ability of PGE to form complexes with both organic and inorganic ligands makes them

have the potential not only to disturb cellular equilibrium and replace other essential metals, but also to interact with functional groups of macromolecules, such as proteins, enzymes and DNA/RNA, consequently disrupting a range of cellular processes (Colombo et al., 2008b). Therefore, effective PGE concentrations to inhibit a function depends on the target structure in the algae (e.g. Chl, growth). The toxicity of other metals has also been studied on *Chlorella* sp. The 42 h EC₅₀ of Pb to *C. stigmatophora* was estimated to be 0.034 µM (Nielsen & Clausen, 1990) which is three fold greater than Pd inhibitory effects. However, the toxicity of metals on organisms is species dependent. For instance, the 72 h EC₅₀ of Cu on freshwater *Chlorella* sp. was reported to be 1.5 µg L⁻¹ (Franklin et al., 2000), and 68 µg L⁻¹ and 200 µg L⁻¹ for *C. pyrenoidosa* and *C. lunula*, respectively (Yan & Pan, 2002). Regarding PGE, this is also the case. Although comparison of the results of this research with other studies showed higher toxicity of Pb on *C. stigmatophora*, in metal-treated isopods, *Asellus aquaticus*, PGE demonstrated a clearly stronger *hsp70* (heat shock protein) induction than Cd and Pb (Singer et al., 2005). Therefore, the inhibiting concentrations of metal vary depends on the target species as well as environmental conditions (e.g. pH, light, temperature) which affect growth rate.

Some metals are able to link to cell membranes, thus hindering transport processes through the cell wall (Fraile et al., 2005). They compete with essential metals for active enzyme or membrane protein sites and by reacting with biologically active groups (Keller et al., 1988; Vasconcelos & Leal, 2001). It is recommended that the competitive effect of PGE, in particular Pd, on accumulation of essential trace metals by *C. stigmatophora* to be studied in order to find out whether PGE inhibits the accumulation of biologically essential metals of algae. Moreover, information on the localization of PGE inside the cell would be useful

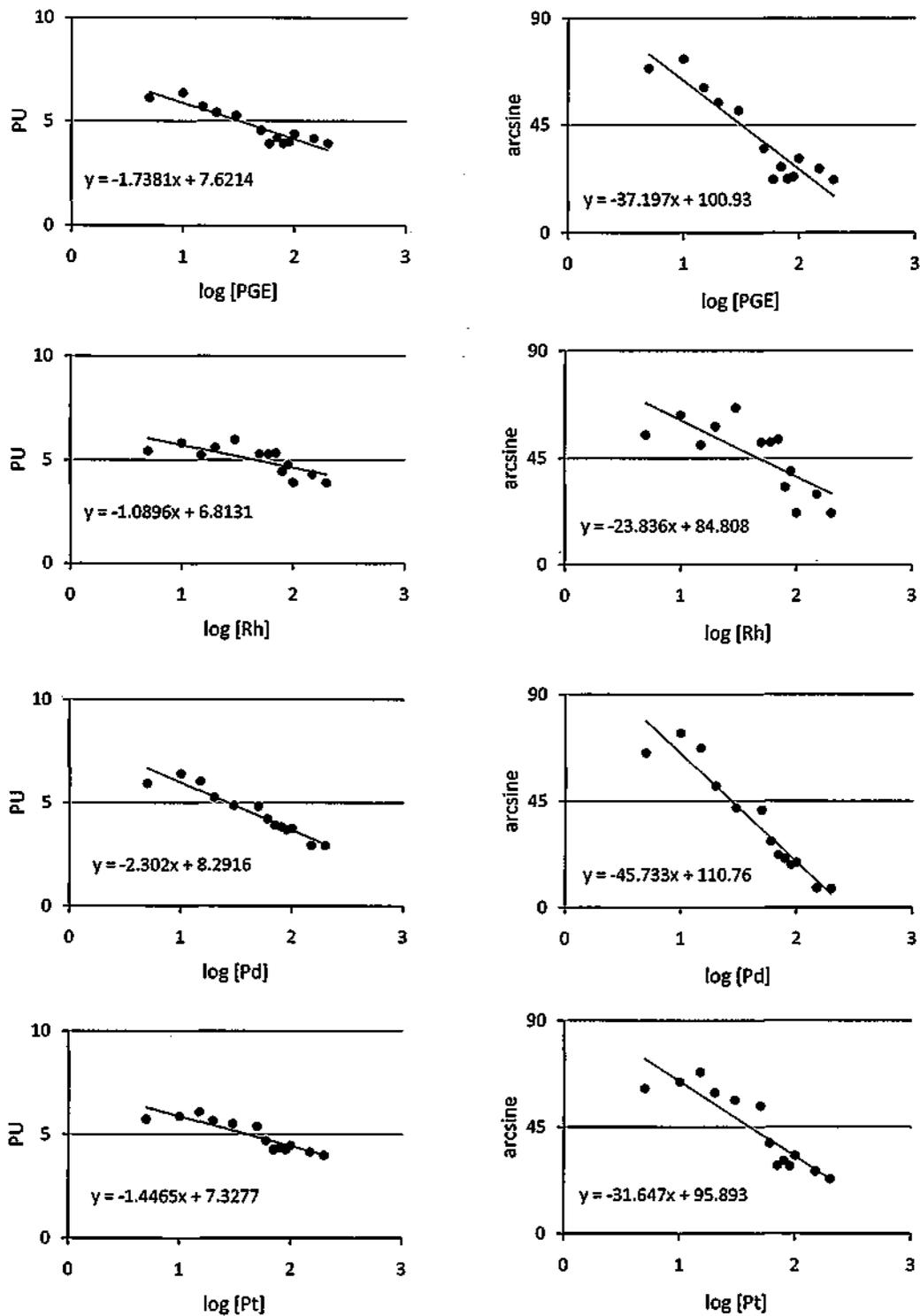


Figure 6.6. Linear regression of dose response equation between log of initial metal concentration ($\mu\text{g L}^{-1}$) and PU (left) and arcsine (right) of percentage of growth rate reduction in *C. stigmatophora* exposed to 5 – 200 $\mu\text{g L}^{-1}$ of individual and combined solutions of Rh, Pd and Pt for 48 h.

for predicting toxicity. Fractionation using ultracentrifugation is recommended for further studies to determine the partitioning of metals within the cell (Levy et al., 2008).

Table 6.3. Effect of Rh, Pd and Pt on the growth rate of *C. stigmatophora* exposed to combined and individual solutions of metals for 48 h (values in parenthesis are concentrations in μM)

Experiment	$EC_{50}, \mu\text{g L}^{-1}$ (μM)		R^2		LOEC, $\mu\text{g L}^{-1}$ (μM)
	Probit	Arcsine	Probit	Arcsine	
A PGE	32	32	0.871	0.875	≤ 5
B Rh	46 (0.18)	47 (0.18)	0.568	0.574	≤ 5 (≤ 0.02)
B Pd	27 (0.12)	27 (0.12)	0.919	0.922	≤ 5 (≤ 0.02)
B Pt	41 (0.21)	41 (0.21)	0.822	0.821	≤ 5 (≤ 0.03)

Extracellular production of organic ligands by phytoplankton may be the most important factor in controlling the effects of biological activity on trace metal-dissolved organic interactions (Levy et al., 2008; Sunda, 2000; Vasconcelos and Leal, 2001). Scanning electron microscopy images prepared from *C. stigmatophora* exposed to 20 and 100 $\mu\text{g L}^{-1}$ of combined PGE solution indicated the cells surrounded by hair-like structures (HLR) which are assumed (pers. comm. Peter Bond, EMC – Plymouth) to be extracellular compounds released by *C. stigmatophora* cells in response to PGE exposure stress (Figure 6.7). HLR were also present in the controls but to a much lesser extent. The exudates are known to be polysaccharides and have been shown to be produced at almost all stages of the microalgae life cycle (Kaplan et al., 1987). However, their production depends on the

physiological state of the cell as well as on environmental factors and the presence of toxic compounds in the medium (Worms et al., 2006).

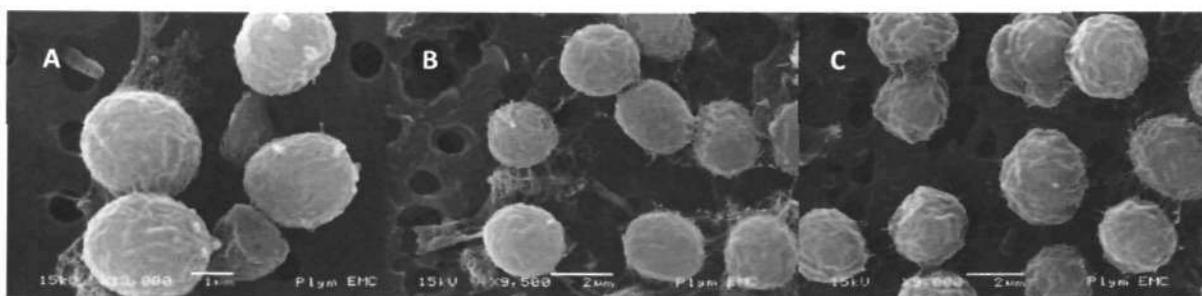


Figure 6.7. Scanning electron microscopy images of *C. stigmatophora* exposed to (A) $0 \mu\text{g L}^{-1}$, (B) $20 \mu\text{g L}^{-1}$ and $100 \mu\text{g L}^{-1}$ of combined solution of PGE for 24 h.

6.3.3. Cell Size Variations in Response to Metal Exposure

Chlorella stigmatophora is a small green microalga with a cell size of $2 - 5 \mu\text{m}$ (Nielsen & Clausen, 1990), although in this study cells smaller and larger than this size range were also found. During previous experiments, it was noticed that some algal cells exposed to certain concentrations of PGE exhibited a larger mean cell diameter than those of controls, and this increase in cell size was observed in all replicate cultures (Figure 6.8). Therefore, a size fraction measurement was carried out on the *C. stigmatophora* cells exposed to $5 - 200 \mu\text{g L}^{-1}$ of individual or combined Rh, Pd and Pt solutions. The results are presented in Figure 6.9 as the percentage of cell sizes smaller than $5 \mu\text{m}$ ($3 - 5 \mu\text{m}$) and larger than $5 \mu\text{m}$. Control cultures had the lowest ratio of $> 5 \mu\text{m}$ cells (with the exception of algal cultures exposed to $15 \mu\text{g L}^{-1}$ of Pd, $p > 0.05$) and the ratio increased with metal concentration, specially at concentrations of $60 - 100 \mu\text{g L}^{-1}$ of Pd and combined PGE solution.

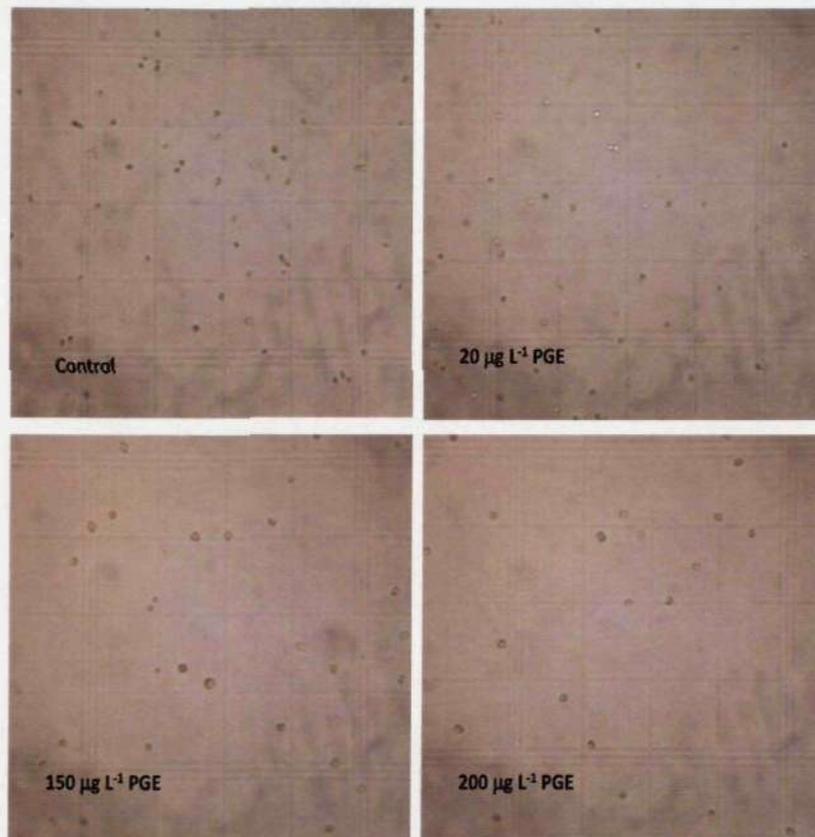


Figure 6.8. Light microscopy images ($\times 40$) of *C. stigmatophora* exposed to various concentrations of combined solution of PGE for 24 h compared with the control ($0 \mu\text{g L}^{-1}$ metal).

Morphological aberrations such as cell enlargement are common indicators of metal toxicity in microalgae (Charles et al., 2002; Starodub et al., 1987) and have also been observed in *Phaeodactylum tricornerutum* exposed to Cu (Cid et al., 1996; Levy et al., 2008), *Scenedesmus quadricornis* exposed to Pb (Starodub et al., 1987) and *Chlorella* sp. exposed to U (Franklin et al., 2000). *C. stigmatophora* cell size has also been shown to increase when exposed to 100 – 1000 nM concentrations of Pb. Various reasons have been put forward to explain changes in the size of cells. UV irradiation is shown to increase cellular protein content which may be associated with the biosynthesis of typical UV stress

proteins (Buma et al., 1996). *P. tricornutum* showed an increase in the number and size of vacuoles in the cells exposed to metal (Levy et al., 2008) which may result in an increase in cell volume. The inability of cells to finish coupling and cell division is known to be another reason for cell size increments (Cid et al., 1996; Starodub et al., 1987). Copper is shown to inhibit mitotic spindle formation in *P. tricornutum* (Levy et al., 2008), thus algal cells continue to photosynthesize whilst the cell division is impaired, leading to swollen and enlarged cells. The increasing permeability to Na^+ in Cu exposed cells is also another reason which is stated by Riisgård et al. (1980) for cell size increase.

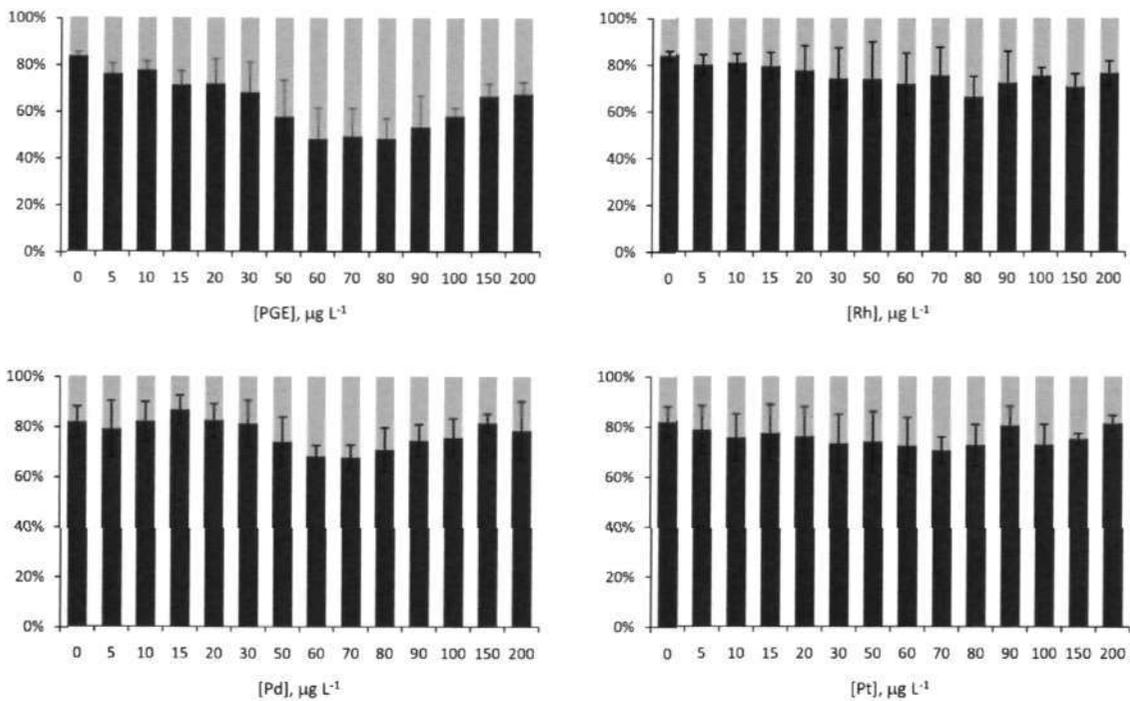


Figure 6.9. Variations of *C. stigmatophora* cell sizes under exposure to 5 – 200 $\mu\text{g L}^{-1}$ of Rh, Pd and Pt as individual and combined stock solutions for 24 h, in terms of % relative to control. Error bars denote the standard deviations about the mean of four independent measurements. Black sections: cell $< 5 \mu\text{m}$, grey sections: cells $> 5 \mu\text{m}$.

In PGE exposed *C. stigmatophora*, the most significant ($p < 0.05$) changes of cell size were observed at 60 – 100 $\mu\text{g L}^{-1}$ in Pd and combined PGE exposed cultures; at higher concentrations, there was a lower ratio of enlarged cells. The possible reason might be that there is a less chance for enlarged cells to survive, especially if the enlargement is due to the cell division disorders. Therefore there is a higher rate of mortality for these affected cells, resulting in a decrease of large cells compared to smaller cells. Reduction of cell membrane elasticity due to denaturation of membrane-bounded proteins by high concentration of metal, as reported for the marine flagellate *Dunaliella tertiolecta* (Riisgård et al., 1980), might be another reason to explain the reduction in the proportion of enlarged cells.

6.4. Summary

- The overall outcome of this study is that, among the PGE, Pd has greater potential to inhibit the physiological functions of *C. stigmatophora* due to its faster kinetics of interaction with the surface of the alga and greater tendency to undergo internalization relative to Rh and Pt. The observed toxicity of the PGE mixture is mainly due to Pd rather than Rh and the kinetically inert Pt.
- Considering the low recovery of Pd (about 50% – 60% from the analysis of the culture medium (see Chapters 3 – 5), the concentration of this metal in solution that effectively inhibits cell growth is likely to be lower than the initial nominal concentrations reported in this chapter. This, therefore, raises concerns about the potentially high toxicity of Pd.

- The Chl *a* content of *C. stigmatophora* cultures decreased by increasing the concentration of combined and individual Rh, Pd and Pt solutions; Pd was the most effective metal to reduce Chl *a* at population level and Rh was the most effective metal to reduce Chl *a* at cellular level due to either inhibiting its biosynthesis or induction of its degradation.
- The 24 h EC₅₀ of all metals for Chl *a* reduction was found to be beyond the metal concentrations applied in the experiment, while the 24 h EC₂₀ of Pd and PGE was estimated to be 30 – 50 µg L⁻¹.
- Increasing concentration of combined and individual Rh, Pd and Pt solutions could considerably reduce the growth of *C. stigmatophora* and at concentrations above 50 µg L⁻¹ (or 100 µg L⁻¹ for Rh) the growth decreased to a minimum rate.
- The 48 h EC₅₀ of metals for growth inhibition was estimated to be between 27 and 47 µg L⁻¹ and the LOEC was found to be ≤ 5 µg L⁻¹.
- Increasing concentrations of Rh, Pd and Pt resulted in an enlargement of cells with more significant effects arising from the combination of PGE; this effect is assumed to be mainly due to cell division disruption caused by the presence of the metals.

CHAPTER 7

General Discussion and Conclusions

General Discussion and Conclusions

7.1. General Discussion

This study is the very first to examine the interaction of an important group of emerging contaminants (namely, PGE), whose concentrations are predicted to increase with increasing use of the catalytic converter and platinum-based chemotherapy drugs (Ek et al., 2004) with marine microalgae. It is also one of a very limited number of studies to investigate the accumulation of PGE by aquatic algae. For example, Cosden et al. (2003) and Turner et al. (2007) studied the accumulation of PGE by the coastal, benthic macroalga, *Ulva lactuca*, while Godlewska-Zylkiewicz (2003) studied the uptake of chloride complexes of Pt and Pd by *Chlorella vulgaris* in order to preconcentrate these metals before analysis by graphite furnace atomic absorption spectrometry. The results of this study are therefore highly significant for an improved understanding of the interactions between primary producers at the base of the food chain and PGE in marine and coastal waters. The findings of this study also have more general implications for our understanding of trace metal biogeochemistry in the marine environment and have practical implications for previous and future studies of metal-algae interactions undertaken empirically under laboratory conditions.

7.1.1. Practical Implications

This study has shown that PGE added to sea water have a significant tendency to adhere to container walls under a variety of experimental conditions; thus, up to 60% of Pd(II), up to

40% of Rh(III) and up to 20% of Pt(IV) were lost in this way. Cobelo-Garcia et al. (2007) suggest that this is due to the adsorption of neutral or charged species to the polymer and, possibly at higher concentrations, precipitation of neutral hydroxides. Because PGE are lost to the container walls, it is important to derive accumulation and kinetic constants from an analysis of both the algae and the water phase. Many other studies derive metal concentrations accumulated by algae from the difference between the added concentration and the amount remaining in solution (Matsunaga et al., 1999; Yan & Pan, 2002). Clearly, the amount of metal taken up by the algae will be overestimated in these cases by an amount dependent on the degree of loss to the containers. With respect to PGE, Cosden et al. (2003) derived first-order kinetic constants for the removal of Pd and Pt from seawater by *U. lactuca* by this approach and their values are therefore subject to uncertainty. Loss of PGE to the containers also means that a proportion of the metals is not available to the algal biomass in toxicity studies. Thus, the concentration of PGE in solution that effectively inhibits algal growth will be lower than the initial (nominal) amount of metal added to the medium. This needs to be considered for the prediction of metal toxicity in the environment. In the present studies, loss to the containers means that PGE (and especially Pd) may be considerably more toxic than the experimental data imply. Moreover, adsorption of PGE to the containers results in a lower dissolved concentration, hence, in a lower concentration of available PGE to algae. According to the data of uptake isotherm studies (Chapter 4), increasing of PGE concentration in water will reduce the AF of metal since it is a function of dissolved concentration of metal. In other words, the amount of PGE accumulated by algae will be greater than if this competition was not present, because more PGE was available to algal cells.

The pH proved difficult to maintain at a near-constant level in the present experiments. The pH usually increases in batch cultures mainly due to the utilization of CO₂ by the algae for photosynthesis. No pH buffer was used in the present study to avoid interference with the growth of algae and also with metal accumulation by algae and with subsequent metal analysis. Rather, acid or base was added dropwise throughout the experiments to correct for any drift in pH. In some cases, mildly acidic conditions occurred when the pH adjustment was not adequate, but in most cases the pH increased, sometimes to about 10. The effects of pH on the protonation and deprotonation of the metal-binding functional groups on the algal surface and on metal speciation in solution were discussed in Chapter 3. With regard to PGE, accumulation by *C. stigmatophora* is relatively insensitive to variations in pH across the range studied, at least compared with other trace metals studies in the literature. Presumably this reflects limited changes to the speciation of the metals across this pH range (although relatively little information exists on the pH-dependent speciation of these metals in seawater). For other trace metals whose speciation is more affected by pH failure to monitor and adjust this parameter in similar experiments may lead to significant errors in estimates of kinetic rate constants of uptake and internalization and of accumulation factors. In many studies involving other metals and microalgae the pH is rarely monitored throughout the experiment and is often reported only at the beginning of the exposure or even before the metals are added. For instance, Quigg et al. (2006) do not report the pH of sea water in copper uptake experiments using a variety of marine phytoplankton, while Vasconcelos and Leal (2001) only report an initial pH of 8.0 when studying Pb, Cd and Cu uptake by *Emiliana huxleyi* over a period of seven days. In equivalent experiments conducted using fresh water algae where pH is either not reported

or monitored (e.g. Lee et al., 2009; Yan & Pan, 2002) the pH drift may be more significant because fresh water has a lower buffering capacity than seawater.

Another variable that had a significant impact on the accumulation of PGE by *C. stigmatophora* was the weight-volume concentration of the algae, or the ratio of algal mass to solution volume. Thus, with decreasing algal concentration, an increase in the accumulation factor (which is weight-normalized) was observed for Rh, Pd and Pt. This effect has been observed with the adsorption of trace metals and other contaminants to sediment and soil suspensions on many occasions (Benoit et al., 1994; Gschwend & Wu, 1985) but not for metal interactions with marine microalgae. Reasons for this phenomenon in the present context are not clear but could be related to an increase in the release of exudates that bind these metals in solution with increasing algal concentration. This effect can be considered as a defence mechanism in the natural environment for algal communities. If this is the case, the effect is not likely to be specific to *C. stigmatophora* nor to the PGE. This means that accumulation factors used for modelling plankton-metal interactions are specific not only to the metal and algal species but also to the precise concentration of algae present (hence the stage of the plankton bloom, time of day, season etc). Moreover, because of the effect an increase in the concentration of algae is not associated with a proportional reduction in the amount of metal remaining in solution. Algae therefore seem to be able to act as a buffer to the amount of metal that can be removed from sea water. Because this effect has not been recognised previously with respect to metals and marine algae, biogeochemical scavenging or uptake models that rely on accumulation factors may require revision or reconsideration (Chen et al., 1996). Clearly, future studies involving metal-algal interactions should incorporate algal concentration as a key variable in their protocols.

7.1.2. Synopsis of PGE Accumulation by and Toxicity to *C. stigmatophora*

This study has shown that the accumulation of PGE by *C. stigmatophora* is a time-dependent process which is influenced by a number of experimental, hence environmental factors. In general, however, Rh is accumulated to the greatest extent by the alga, followed by Pd and then Pt. Reactions kinetics are biphasic with a fast adsorption step followed by a slower internalization phase; Pd is accumulated most rapidly and Pt is accumulated most slowly.

It is difficult to compare the accumulation of the PGE in this study with the accumulation of other trace metals in the marine environment. This is not only because different test species have been employed throughout the literature but because, as discussed above, the precise experimental conditions like pH (and whether it is maintained), time and algal biomass or cell concentration vary among the different studies (e.g. Chen et al., 2003; Wang & Dei, 2001a; Yan & Pan, 2002) and these factors have a significant bearing on the results. Nevertheless, regardless of the precise experimental conditions and species, it appears that the net accumulation of Pt by microalgae is particularly low.

The surface of the alga may be considered as a polyfunctional macromolecule with multiple binding sites, such as carboxylic, sulphhydryl and phosphate groups (Fisher, 1986). The green algal matrix contains complex heteropolysaccharides that may be sulphated. The main acidic groups responsible for metal ion uptake are the carboxyl groups of uronic acids as well as sulphonate groups (Schiewer & Volesky, 2000). Regarding the PGE in this study, it is suggested that the uptake of the Pd(II) ion occurs via carboxylic groups because its accumulation exhibited a moderate reduction with increasing pH, while uptake of Rh(III) and Pt (IV) ions is more likely to occur via phosphate and hydroxyl groups because

their accumulation moderately increased with increasing pH. In addition, it should be noted that Pd and Pt could also be taken up as anionic species like chlorides and hydroxychlorides (such as PdCl_4^{2-} and $\text{PtCl}_5\text{OH}^{2-}$; (Gammons, 1996)) as these forms appear to dominate speciation in seawater and are known to adsorb to dead biomass (Godlewska-Zylkiewicz, 2003). However, because of the negatively charge of most functional groups on the algal surface it is likely that the free ions Rh^{3+} , Pd^{2+} and Pt^{4+} have the greatest affinity for the surface of marine microalgae among the species present. The overall accumulations of the PGE are therefore a combination of both their affinity for the algae and relative abundance of the reactive species. In addition for Pt, and as discussed below and elsewhere, accumulation is also affected by its slow reaction kinetics.

Internalization is the key step in the overall accumulation and consequently the toxicity process. The plasma membrane is biologically active and often able to control the magnitude of the metal internalization fluxes. Due to the overall hydrophobic nature of the biological membrane (Worms et al., 2006), only neutral or non-polar molecules cross into the cytosol by passive diffusion. The majority of environmentally relevant metal species, including those of the PGE, are hydrophilic and their transport through the biological membrane is likely to be carried out by specific proteins. Rhodium and Pd were internalized to greater extents than Pt, and Pd was more toxic than Rh and Pt as evaluated by growth rate and pigment content. This suggests that Pd is able to both cross the membrane effectively, perhaps because its ionic radius or coordination geometry is similar to that of an essential metal ion, and interact with intracellular macromolecules strongly and rapidly.

7.1.3. Environmental Implications

The fate of PGE in the coastal and marine environments will to a large extent depend on their interaction with suspended particles of both lithogenic and biogenic origin. Results of studies into the uptake of PGE by sediment particles suspended in sea water are both qualitatively and quantitatively consistent with the results reported in this study. For example, Turner (2007) determined sediment-water partition coefficients of Rh, Pd(II) and Pt(IV) of about 6000, 1400 and 200 ml g⁻¹ respectively for estuarine sediment suspended in coastal sea water, similar to AF values established in the present study under otherwise similar experimental conditions. Turner and Wu (2007) found an inverse relationship between the partition coefficient of each PGE and estuarine sediment particle concentration, qualitatively similar to the dependence of AF on biomass concentration found in this work and as discussed above.

Both suspended sediment and microalgae act as filters for contaminants fluxing across the coastal zone and in open sea water. Removal of PGE by primary producers will affect both the transport and fate of these metals and their interaction with the food chain. Very little information exists on the trophic transfer of PGE in the marine environment. Mulholland and Turner (2010) studied the uptake of PGE by the snail, *Littorina littorea*, both directly (from water) and when fed with the macroalga, *Ulva lactuca*, that had been pre-contaminated with PGE. It was shown that the gastropod accumulated Rh and Pd more effectively from the diet than from the aqueous phase. For Pt overall accumulation was lower and the aqueous phase appeared to be more important than the diet. The direct accumulation of Pd by the snail was attributed to the exceptional affinity of the metal for low molecular weight cysteine-rich proteins, and its indirect accumulation from the diet

was attributed to its affinity for the algal surface and its tendency to be internalized. In contrast to these observations, equilibrium chemistry predicts that Pt should exhibit similar accumulation and trophic transfer to Pd. However, discrepancies between their behaviours are due to the much slower coordination reactions of Pt. It is predicted that such effects and fractionation will occur with organisms grazing on marine microalgae like *C. stigmatophora*.

The difference in kinetics of coordination between Pd and Pt will have important implications for the biogeochemistries of these metals. As mentioned above, Pd will interact with biological surfaces more rapidly than Pt and accumulate and transfer in the food chain to greater extents. This means that Pd is likely to accumulate in immobile substrates like estuarine sediments and sessile flora like macroalgae to a greater extent than Pt (Cosden et al., 2003). Yang (1989) noted that Pd:Pt ratios in various species of marine macroalgae were greater than in ambient sea water, consistent with this assertion. Although environmental concentrations of PGE are relatively low (on the order of ng g^{-1} in marine macroalgae and pg L^{-1} in sea water; (Lee, 1983; Yang, 1989)), levels are increasing in concert with the growing demand for the catalytic converter and platinum-based chemotherapy drugs (Ek et al., 2004). In the field, macroalgae may serve as useful biomonitors of PGE contamination arising from these sources and because of their similar equilibrium chemistries but different kinetic behaviours, Pd may serve as an indicator of immediate contamination and Pt as an indicator of more historic inputs.

Because Pd reacts more rapidly than Pt, the geochemistries of these metals are also likely to be different in the open ocean. Thus, Pd is predicted to behave in a more nutrient-like way and concentrations are expected to exhibit depletion at the ocean surface and

enrichment at depth as sink and gradually decay. The only vertical oceanic profiles for Pd are reported by Lee (1983) for the Pacific Ocean and show an increase in dissolved concentrations with increasing depth in parallel with increasing concentrations of the nutrients silicate and phosphate. In contrast, because Pt only interacts slowly with growing algae and other natural solids, it is predicted to exhibit more conservative (unreactive) behaviour with depth, or even surface enrichment and depth depletion as the biological means of transferring the metal to depth are kinetically constrained. A vertical profile of Pt reported by Colodner et al. (1993) for the Pacific Ocean reveals a uniform concentration in support of this assertion.

7.2 Future Research

This study has identified areas for future research both generally for trace metal-algal interactions and more specifically with regard to the PGE in the marine environment. Regarding the former, it is important that future studies monitor and check for drifts in reaction-controlling variable like pH, especially in replicated batch type cultures of the kind undertaken here. Studies should also investigate and quantify the effect of algal biomass on metal accumulation as this has highly important implications for metal removal in coastal and oceanic seawater and for improved estimates of metal fluxes and residence times in the oceans. Regarding the PGE, a better understanding of the biogeochemistries of these metals in the marine environment requires a better understanding of their speciation. Thus, the thermodynamic database for these elements requires improvement and expansion. In particular, little is known about their binding with organic ligands except that

Pd has an exceptional affinity for nitrogen-bearing acidic functional groups (Li & Byrne, 1990).

The trophic transfer of PGE from primary producers requires further investigation. For example, the accumulation and transfer by filter-feeding invertebrates like *Mytilus edulis* (e.g. Allison et al., 1998; Gagnon & Fisher, 1997) would be a logical extension of the experiments undertaken in this work. A better understanding of the mechanisms by which the metals are taken up and internalized by microalgae should also involve parallel experiments employing dead biomass under otherwise identical experimental conditions. The role of exudates generated by algae on the behaviour and binding of PGE is also required for a better understanding of many of the processes described above as these exudates have been implicated in many cases regarding toxicity (or detoxification), bioavailability and accumulation.

Other areas for future work include conducting parallel experiments in the presence of contemporary catalytic converter particulates rather than metals added in aqueous form, thereby replicating the availability of PGE directly entering seawater from airborne vehicular emissions. It is important to note that the relative abundances of PGE in catalytic converters has shifted recently with technological advances in the catalytic converter industry. At present, the current trend in autocatalyst technology is to substitute Pt and Rh with Pd (Battke et al., 2008; Colombo et al., 2008a), the metal that is the most reactive kinetically in seawater and that is also the most toxic to at least the test species of alga in the present work. It is also worth noting that Pd is being considered in cytotoxic, antitumour agents to replace current platinum-based drugs (Tusek-Bozic et al., 2008).

Among the PGE, therefore, Pd should perhaps be considered more urgently for future studies.

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APPENDICES

Appendix 1.

Limits of detection of Rh, Pd and Pt, $\mu\text{g L}^{-1}$ in unspiked acidified seawater, 5 mM EDTA and diluted HNO_3 , analysed by ICP-MS

<i>Experiemnt (Methodology Section)</i>		<i>HNO3</i>	<i>EDTA</i>	<i>Acidified Sea Water</i>
pH (Section 3.2.1)	Rh	0.009	0.009	0.284
	Pd	0.033	0.027	0.233
	Pt	0.140	0.056	0.438
Algal Concentration (Section 3.2.2)	Rh	0.303	0.020	0.109
	Pd	0.166	0.149	0.294
	Pt	0.197	0.141	0.243
Fe (Section 3.2.3)	Rh	0.303	0.020	0.109
	Pd	0.166	0.149	0.294
	Pt	0.197	0.141	0.243
Trace metal (Section 3.2.4)	Rh	0.231	0.034	0.358
	Pd	0.222	0.152	0.516
	Pt	0.083	0.071	0.127
Isotherm combined PGE (Section 4.2)	Rh	0.231	0.034	0.358
	Pd	0.222	0.152	0.516
	Pt	0.083	0.071	0.127
Isotherm Individual (Section 4.2)	Rh	0.000	0.001	0.010
	Pd	0.009	0.003	0.058
	Pt	0.002	0.002	0.001
Kinetics (Section 5.2)	Rh	0.048	0.020	0.265
	Pd	0.127	0.346	0.159
	Pt	0.091	0.078	0.169

Appendix 2

Appendix 2.1. Records of the pH of the cultures for each experiment (Data are presented in the attached CD).

Appendix 2.2. PGE concentrations in control samples (obtained by ICP-MS analysis) for each experiment (Data are presented in the attached CD).

Appendix 3

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Uptake of platinum group elements by the marine macroalga, *Ulva lactuca*

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Abstract

Uptake of environmentally relevant platinum group elements (PGE) by the marine macroalga, *Ulva lactuca*, has been studied. Removal of nM concentrations of Rh(III), Pd(II) and Pt(IV) added to filtered sea water appeared to proceed via pseudo-first-order kinetics, with respective forward rate constants of either 0.0039 or 0.0042 h⁻¹, 0.0058 or 0.0096 h⁻¹ and 0.0017 or 0.0032 h⁻¹, depending on whether an irreversible or reversible reaction was invoked. The (quasi-) equilibrium distribution coefficients, derived from linear fits to uptake (sorption) isotherms, were about 1400, 900 and 350 mL g⁻¹ on a dry mass basis for Rh, Pd and Pt, respectively. With increasing sea water pH, over the range 7.9 to 8.4, uptake of Rh by *Ulva* increased considerably, whereas a small increase in Pt removal was observed; in contrast, uptake of Pd exhibited no clear dependence on pH. The percentage of metal taken up that was internalised within cells, evaluated by washing selected algal samples in 3 mM EDTA, was about 40% for Rh, 80% for Pd and 95% for Pt. Results of this study were interpreted in terms of what is known about the aqueous speciation of PGE in sea water. Thus, Rh exists as cationic hydrated chloride complexes which are readily adsorbed at the algal surface. Palladium has an exceptional affinity for organic ligands, and uptake (and internalisation) appears to be governed by competition for Pd²⁺ from aqueous and algal binding sites. Platinum (IV) exists predominantly as a series of (mainly) negatively charged chloride and mixed hydroxychloride complexes that have little propensity to interact with the algal surface; however, its high degree of internalisation requires at least some interaction with specific and perhaps physiologically active sites.

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Keywords: Platinum group elements; *Ulva lactuca*; Uptake; Adsorption; Speciation

1. Introduction

The environmental behaviour of the platinum group elements (PGE), and in particular Rh, Pd and Pt, has received increasing attention over the past decade, largely because of their incorporation in and gradual emission from the catalytic converter of motor vehicles (Rao and Reddi, 2000; Ek et al., 2004). PGE are dispersed as

microparticulates that subsequently undergo partial solubilisation in the environment (Lustig et al., 1996). Although present concentrations in aquatic systems are typically in the pM range (Ravindra et al., 2004), PGE levels are predicted to rise in proportion to their increasing demand (Ek et al., 2004; Zereini et al., 2007).

By spiking sediment-water suspensions with PGE at elevated and readily detectable concentrations, we have developed a preliminary, empirical understanding of the geochemical behaviour and partitioning of these elements in the aquatic environment. Specifically, we have gained

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an insight into (i) the adsorption kinetics of PGE in river water and the dependence of reaction kinetics on aqueous metal pre-equilibration with natural organic ligands and on selective chemical extraction of principal sorbent phases (hydrous metal oxides and organic matter) (Turner et al., 2006); (ii) the effects of salinity (and ionic components thereof) on the adsorption of PGE to estuarine particles; and (iii) the conditions that lead to PGE coagulation and flocculation in fresh and saline waters (Turner, 2007).

In the present paper, this information base is complemented by a study into the uptake of PGE by a marine macroalga. Given the abundance of algae in sea water, it has been proposed that they serve as a surrogate for a generalised particulate organic phase, and that metal–algal interactions form the basis of first-order oceanic scavenging models (Stanley and Byrne, 1990). Moreover, because of their surface binding characteristics, algae are suitable biomonitors of trace metals in the marine environment (Brown et al., 1999; Haritonidis and Malea, 1999) and are also potential biosorbents for the water treatment industry (Zeroual et al., 2003; Sheng et al., 2004). The green alga, *Ulva lactuca*, is particularly useful in these respects because of its wide distribution and relatively simple structure (Ho, 1990); *U. lactuca* has a sheet-like thallus which is two cells thick, resulting in a relatively high surface area of structurally uniform and physiologically active cells. Some information exists on the short-term (~3 h) uptake kinetics of Pd and Pt by this macroalga (Cosden et al., 2003), albeit using very high (μM) metal concentrations. Here, we examine the longer term uptake of nM concentrations Rh, Pd and Pt, along with the effects of important environmental variables on this uptake (specifically, pH and the ratio of algal mass to solution volume). We also discriminate intra- and extracellular metal concentrations following washing of the alga in the complexing agent, EDTA.

2. Materials and methods

Before being used for sampling, sample processing or sample analysis, all plastic-ware was soaked in 5% HCl for at least 24 h, rinsed twice with Millipore Milli-Q water and, where appropriate, stored in zip-lock plastic bags. All reagents were purchased from either BDH/Merck or Fisher Scientific and were of analytical grade or equivalent. Sea water (salinity ~33) used in the experiments had been collected from Plymouth Sound, SW England, at high water, and was supplied to the laboratory via polymer piping and after filtration through a 5- μm pore-size extruded carbon filter (KX Industries)

from a darkened, fibreglass-lined storage tank at the university.

2.1. Sampling and sample processing

Samples of *U. lactuca* were collected at low water from rock pools at Wembury, UK (50°18'59"N; 4°05'01"W), on various occasions during May and June, 2006. Samples were transported in zip-locked plastic bags containing local sea water back to the laboratory. Here, all visible epiphytes and sedimentary material were removed by washing in Milli-Q water. Algal material was maintained in 10 L plastic tanks containing aerated sea water at 13 °C and under 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic active radiation (PAR) on a 16:8 light/dark cycle for a period of one week. Discs of 26 mm diameter (surface area = 530 mm^2) were cut, as required, from the central portions of thalli using a sharpened end of a polyethylene cylinder. Discs were then returned to sea water for a further 24 h before being used in the experiments.

2.2. Experimental

The general approach for the metal uptake experiments is described below. A multiple-container protocol (Harrison and Druehl, 1982), whereby each container is sampled only once, was favoured in order that (i) the algal mass to solution volume was reasonably consistent throughout each experiment, and (ii) both phases (sea water and algae) could be analysed. Thus, 100 mL aliquots of sea water were transferred to 150 mL polyethylene terephthalate beakers. One millilitre of mixed PGE solution, comprising 1 mg L^{-1} each of Pd(II), Pt(IV) and Rh(III) and obtained by serial dilution of individual 10,000 ppm plasma emission standards (originally in 1.2 M HCl) in Milli-Q water, was added to each beaker. Metal addition resulted in a transient (<30 min) reduction in the pH of sea water of less than 1 unit. The resulting PGE concentrations, nominally 10 $\mu\text{g L}^{-1}$ each, or approximately 100 nM of Rh and Pd and 50 nM of Pt, are significantly greater than those encountered in the environment (including sea water and, on a mass basis, green macroalgae; Yang, 1989; Bertine et al., 1993). Critically, however, PGE concentrations are well below the concentrations of complexants in sea water, including chloride and dissolved organic matter (about 270 μM as DOC in the laboratory-supplied sea water), and it is reasonable to assume, therefore, that a good approximation of natural aqueous speciation is attained under the experimental conditions. A single disc of *U. lactuca* was added to each beaker

using plastic tweezers. Beakers were then covered with clear Perspex to prevent evaporation of sea water and gently agitated on a Denley orbital mixer at 100 rpm at 13 °C and under the light conditions specified above for about 100 h. The pH of the sea water was measured at the beginning and end of the incubation period using a calibrated Hanna electrode. Subsequently, 1 mL aliquots of water were pipetted into individual 15 mL polypropylene conical tubes, each containing 9 mL of 1 M HCl, ready for analysis. Discs were retrieved using tweezers and were gently shaken to remove excess sea water before being placed in individual plastic specimen bags and frozen at -25 °C.

The effects of pH on metal uptake were examined by addition of microlitre quantities of either 1 M HNO₃ or 1 M NaOH to individual beakers. The initial pH in these experiments ranged from about 6 to 9, but after 100 h "equilibrium" values ranged from 7.9 to 8.4. The effects of macroalgal surface area (or the ratio of algal mass to solution volume) were examined by varying the number of *U. lactuca* discs (between 1 and 6) in 100 mL of sea water. Sorption isotherms were undertaken by varying the concentration of PGE (between 5 and 30 µg L⁻¹) in different beakers, and the kinetics of metal uptake were examined by incubating different beakers, containing a single disc and 10 µg L⁻¹ of PGE, for different periods of time up to about 100 h. Controls for each set of reaction conditions comprised sea water containing PGE but no algae, and sea water containing algae but no PGE. All experimental treatments and controls were carried out in triplicate.

2.3. Algal digestion

Frozen algal samples were freeze-dried (Super Modulyo freeze-drier; Girovac, UK) for 48 h and then weighed. For complete digestion, dried discs were placed in Teflon vessels containing 2 mL of concentrated HNO₃ and microwave digested at 2 kW for 30 min. Digests plus Milli-Q water rinsings were then transferred to 10 mL borosilicate volumetric flasks and diluted to mark ready for analysis. Selected discs were also subjected to sequential chemical treatment in ethylenediamine tetra-acetic acid (EDTA) and HNO₃ in order to discriminate extracellular sorption and intracellular uptake of PGE (Vasconcelos and Leal, 2001). Thus, replicate discs were first placed into individual beakers containing 30 mL of 3 mM EDTA in 0.6 M NaCl for 15 min and then into individual Teflon vessels containing HNO₃ as above. Sorbed metal and internalised metal were discriminated from analysis of nitric acid digests with and without pre-washing in EDTA.

2.4. Metal analysis

Concentrations of Rh (as ¹⁰³Rh), Pd (as ¹⁰⁸Pd) and Pt (as ¹⁹⁵Pt) in algal digests and diluted, acidified sea water samples were determined by inductively coupled plasma-mass spectrometry (ICP-MS) using a Thermo Elemental PlasmaQuad PQ2+ with a Meinhard dissolved solids nebuliser. The instrument was calibrated over the range 0 to 40 µg L⁻¹ with mixed standards prepared by dilution of individual plasma emission standards in 0.3 M HNO₃. All standards and samples were spiked with ¹¹⁵In and ¹⁹³Ir to a concentration of 100 µg L⁻¹ in order to normalise signals for instrument drift and variations in plasma conditions. Limits of detection, defined as three standard deviations of measured concentrations in replicate samples (*n*=6) of unspiked sea water, were 0.05 µg L⁻¹ (0.5 nM), 0.04 µg L⁻¹ (0.4 nM) and 0.02 µg L⁻¹ (0.1 nM) for Rh, Pd and Pt, respectively. Molecular ion interferences were detected in the diluted sea water and digested algal matrices without added PGE and were corrected for in the samples by subtracting the apparent concentrations in the appropriate controls. Experimental precision was generally better than 15% for all metals in diluted aqueous samples; poorer precision for metals in algal digests (up to 30%) at least partly reflects the variability of algal mass in replicate experiments.

3. Results and discussion

3.1. Reaction conditions

Despite the use of uniformly cut *Ulva* discs throughout all experiments, dry disc mass exhibited considerable variation after 100 h incubation (mean ± SD = 19.3 ± 3.7 mg; *n*=92). Over the period of the

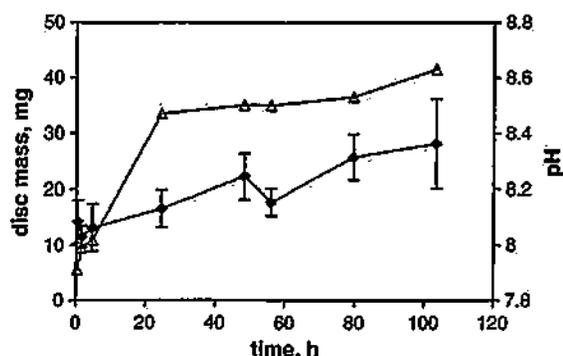


Fig. 1. *U. lactuca* dry disc mass (◆) and sea water pH (△) as a function of incubation time in batch experiments conducted at 13 °C. Error bars (mass only) denote the standard deviation about the mean of three independent measurements.

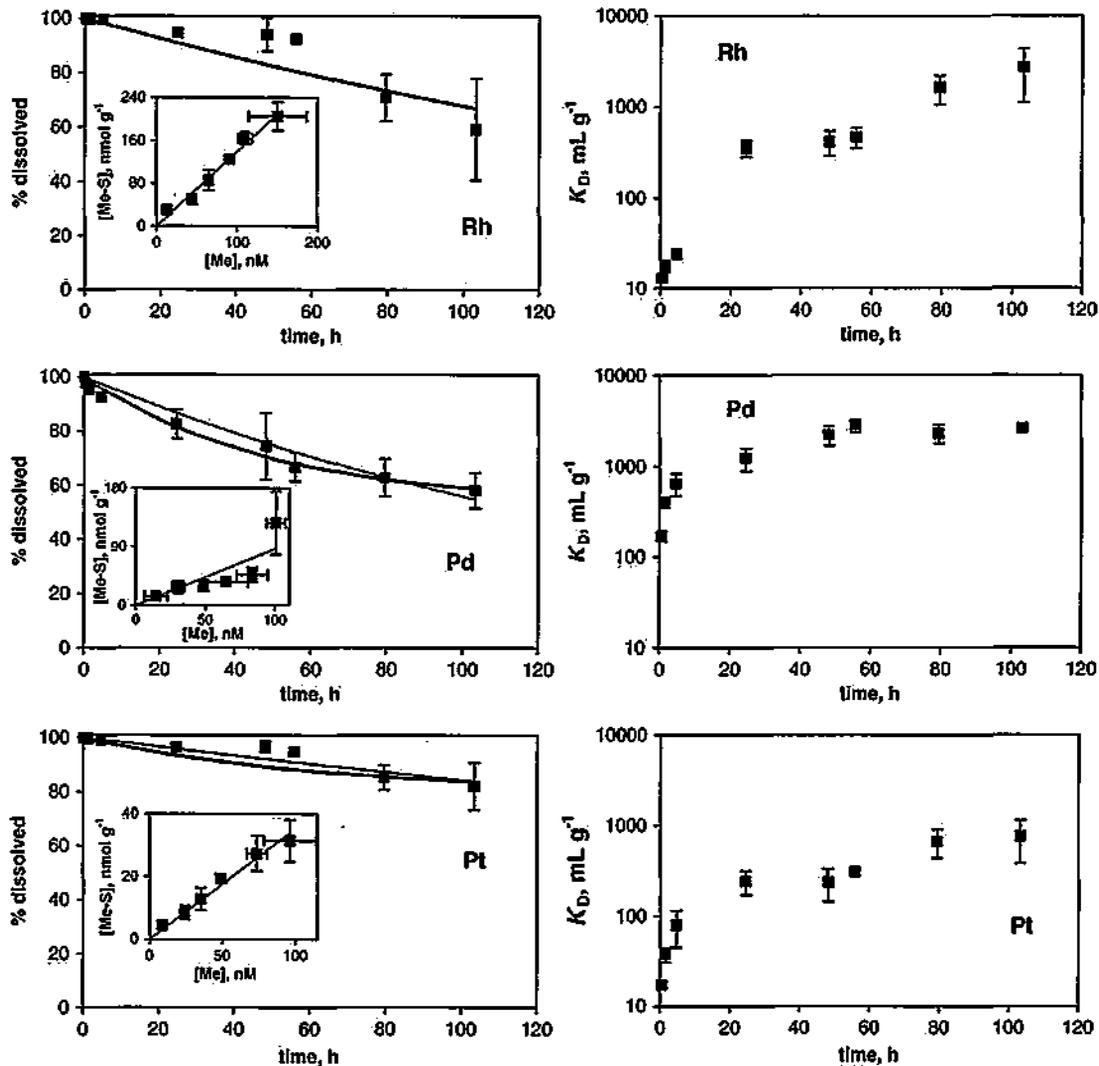


Fig. 2. Time-dependent uptake of 10 ppb of PGE (about 100 nM of Rh and Pd and 50 nM of Pt) by *U. lactuca* in batch experiments conducted at 13 °C. Left-hand panels denote the percentage of metal remaining in sea water and accompanying fits according to first-order reversible and irreversible reactions (thick and thin lines, respectively) using the constants given in Table 1. Right-hand panels denote the corresponding partitioning of metal between *Ulva* and sea water. Inset to left-hand panels are sorption isotherms determined after 100 h incubation; parameters defining equations of best-fit to the isotherms are given in Table 1. In all cases, error bars denote the standard deviation about the mean of three independent experimental determinations.

timed experiment, a net increase in disc mass was observed (Fig. 1). The equation of best fit, derived from linear regression analysis, indicates an original average mass of 12.7 mg and a growth rate of about 0.15 mg h⁻¹. Despite some gaseous exchange between the head space of the beaker and the ambient atmosphere, sea water pH also increased from about 7.9 in the original water sample to around 8.6 after 100 h incubation (Fig. 1).

3.2. Uptake of PGE by *Ulva*

Control experiments conducted in the absence of alga revealed that about 30% of Rh and Pd and up to about

20% of Pt were lost from the aqueous phase over the period of incubation, presumably due to adsorption to the container surfaces. Although container adsorption is unlikely to be as significant in the presence of alga (a competing surface), it is important to consider both aqueous and algal-bound metal concentrations when expressing and interpreting the experimental results. Accordingly, Fig. 2 depicts the time-dependent uptake of PGE in terms of both the percentage of metal remaining in the dissolved phase, derived from the aqueous concentration relative to the total analytical concentration (aqueous plus w/v algal-bound concentrations), and a distribution coefficient, K_D (mL g⁻¹),

representing the w/w concentration of metal in alga relative to its concentration in solution. By expressing particulate concentrations on a weight basis, the latter representation also normalises measurements for variations in disc mass, hence number of binding sites. The results indicate continuous removal of PGE throughout, although there is evidence that Pd and Pt approach equilibrium towards the end of the experiments, and that the extent of metal removal by *Ulva* at the end of the time course is Rh>Pd>Pt.

Shown as inset in Fig. 2 are isotherms defining the (quasi-) equilibrium sorption of PGE to *Ulva*, measured over a period of about 100 h and at a pH of around 8.4. Sorption constants, derived from regression analysis of each data set, are shown in Table 1. For Rh and Pt, particulate (w/w) and aqueous concentrations are linearly related, implying a single type of reaction throughout the metal concentration range studied. For Pd, however, although a linear sorption constant is presented in Table 1, the isotherm is more convex (at least with the exception of the final data point). This suggests that there are a series of independent or successive reactions taking place at the algal surface, and that reaction kinetics are unlikely to be a simple function of metal concentration.

3.3. Modelling PGE uptake kinetics

The kinetics of PGE uptake by *U. lactuca* were modelled from the time-dependent distributions of percentage dissolved metal using simple pseudo-first-order reactions. Assuming that dissolved metal is consumed by *U. lactuca*, a unidirectional, irreversible reaction is appropriate:



whose rate equation is

$$\frac{d[\text{Me}]}{dt} = -k_1 [\text{Me}]_t \quad (2)$$

where Me is aqueous metal, S represents binding sites on the *Ulva* surface, Me-S is algal-bound metal, $[\text{Me}]_t$

is the percentage of aqueous metal at time t and k_1 is the first-order forward rate constant for the reaction. Estimates of k_1 were derived from the integrated form of Eq. (2); specifically, the gradient of $\ln([\text{Me}]_0/[\text{Me}]_t)$ versus time in each case, where $[\text{Me}]_0$ denotes the percentage of aqueous metal at the beginning of the experiment (=100%). The results, shown in Table 1 along with the reaction half-lives ($t_{1/2}=0.693/k_1$), were used to model the metal profiles, as annotated on Fig. 2.

Alternatively, assuming that equilibrium is eventually achieved between aqueous and bound metal, a pseudo-first-order reversible reaction is more appropriate (Ciffroy et al., 2001; Martino et al., 2003):



where k_{-1} is the reverse (desorption) rate constant. The rate equation for this reaction is as follows:

$$\frac{d[\text{Me}]}{dt} = -k_1 [\text{Me}]_t + k_{-1} [\text{Me-S}]_t \quad (4)$$

where $[\text{Me-S}]_t$ represents the percentage of algal-bound metal at time t . Given that a reduction in aqueous metal concentration is directly compensated by an increase in w/w algal-bound metal concentration (that is, $[\text{Me-S}]_t = [\text{Me}]_0 - [\text{Me}]_t$), integration of Eq. (4) yields:

$$[\text{Me}]_t - [\text{Me}]_e = ([\text{Me}]_0 - [\text{Me}]_e) \exp[-(k_1 + k_{-1})t] \quad (5)$$

where $[\text{Me}]_e$ is the percentage of dissolved metal at equilibrium. Since, at equilibrium, $d[\text{Me}]/dt=0$, we may define an equilibrium constant, K , and rewrite Eq. (4) as follows:

$$K = \frac{k_1}{k_{-1}} = \frac{[\text{Me}]_0 - [\text{Me}]_e}{[\text{Me}]_e} \quad (6)$$

Combining Eqs. (5) and (6) then yields:

$$k_1 t = \left(\frac{[\text{Me}]_0 - [\text{Me}]_e}{[\text{Me}]_0} \right) \ln \left(\frac{[\text{Me}]_0 - [\text{Me}]_e}{[\text{Me}]_t - [\text{Me}]_e} \right) \quad (7)$$

The gradient of a plot of the right-hand side of Eq. (7) versus time represents an estimate of the first-

Table 1
Results of first-order kinetic analysis of PGE uptake data (see text for definition of reactants and constants)

Metal	Me + S $\xrightarrow{k_1}$ Me-S (Eq. (1))			Me + S $\xrightleftharpoons[k_{-1}]{k_1}$ Me-S (Eq. (3))				[Me-S] (w/w) vs [Me]		
	k_1 , h ⁻¹	r^2	$t_{1/2}$, h	$[\text{Me}]_e$, %	k_1 , h ⁻¹	k_{-1} , h ⁻¹	r^2	t_{resp} , h	$(K_D)_e$, mL g ⁻¹	r^2
Rh	0.0039	0.813	178	20	0.0042	0.0011	0.801	187	1400	0.982
Pd	0.0058	0.952	119	53	0.0096	0.0108	0.984	49.0	870	0.667
Pt	0.0017	0.880	408	80	0.0032	0.0128	0.773	62.5	352	0.975

The regression coefficient defines the goodness of fit in the derivation of k_1 in each case ($n=8$). Also shown is the quasi-equilibrium distribution coefficient, $(K_D)_e$, derived from linear regression analysis of the isothermic data ($n=6$).

order forward rate constant for the reaction; an estimate of the reverse reaction constant is then obtained from Eq. (6). Results of such analysis of the PGE data are presented in Table 1, and model predictions, generated by incorporating the constants into Eq. (5), are annotated on Fig. 2. For Pd and Pt, the equilibrium metal concentration was based on or was close to the final data point in the time course. Since Rh did not appear to approach equilibrium throughout the timed experiments, an extrapolated value was derived iteratively to yield the best “eye-fit” to the timed data. Also given in Table 1 are estimates of the system response time, τ_{resp} , defined as the reciprocal of the summed rate constants (Millward et al., 1992):

$$\tau_{\text{resp}} = (k_1 + k_{-1})^{-1} \quad (8)$$

and representing the time required for the system to reach 63% of the new equilibrium.

For Rh and Pt, fits to the data, or at least derivation of the forward rate constant, were better for the irreversible reaction than the reversible one. An irreversible reaction is often invoked to define the kinetics of metal uptake by macroalgae (Vasconcelos and Leal, 2001; Cosden et al., 2003), but ultimately this requires total consumption of metal. Given the reversible nature of the interactions between metals and other solids (Koeppenkastrup and de Carlo, 1993; Ciffroy et al., 2001; Gee and Bruland, 2002; Martino et al., 2003), including interactions between PGE and estuarine sediment (Turner et al., 2006), and that metal–algal interactions involve some degree of ion exchange (Crist et al., 1994), we suspect that equilibrium is ultimately achieved but that better data fitting using the reversible reaction is hampered by the difficulty in estimating an equilibrium concentration of metal. The dispersion of metal data (especially Rh) towards the end of the timed experiments likely reflects accompanying variations in other reaction-controlling variables, and in particular pH.

3.4. Effects of pH on PGE uptake

The effects of pH on metal uptake by *U. lactuca* were examined in experiments in which acid or base was added to a series of samples. The results of these experiments are shown in terms of the percentage of metal remaining in the aqueous phase, as defined above, versus “equilibrium” pH (recorded at the end of the incubation period) in Fig. 3. Uptake of Pd by *Ulva* displays no clear or systematic dependence on pH across the range studied (7.9–8.4), while Pt exhibits a small increase in uptake with increasing pH. In contrast,

uptake of Rh is enhanced considerably with increasing pH. For comparison, the percentage of aqueous Rh measured in the timed experiment is also shown as a function of the corresponding pH. The two Rh distributions are qualitatively similar, with the timed data shifted rightwards. Clearly, therefore, the uptake of Rh over the time course is affected to a significant extent by the accompanying evolution of pH.

The pH dependencies of PGE uptake are consistent with equivalent results obtained in sediment–sea water suspensions (Turner, 2007) and may be understood by considering what (relatively little) is known about the aqueous speciation of these elements in sea water. Thus, given the net negative charge of the algal surface (Xue et al., 1988; Crist et al., 1994), uptake of cationic forms of Rh chlorides (e.g. $[\text{RhCl}(\text{H}_2\text{O})_5]^{2+}$; $[\text{RhCl}_2(\text{H}_2\text{O})_4]^+$; Baes and Mesmer, 1976) is predicted to be highly sensitive to pH. That said, it must also be appreciated that Rh may be subject to precipitation or co-precipitation with alkali and trace metal hydroxides above a pH of about 8 (Bertine

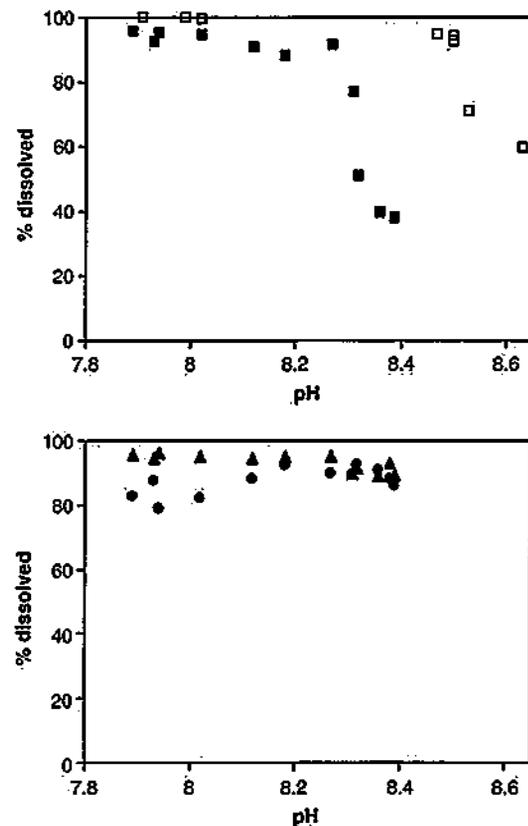


Fig. 3. Uptake of 10 ppb of Rh (■), Pd (●) and Pt (▲) by *U. lactuca* at 13 °C, shown in terms of the percentage of metal remaining in solution as a function of pH (varied by addition of acid or base to sea water). Uptake of Rh during the timed experiment is also shown as a function of the corresponding pH (□).

et al., 1996; Cobelo-Garcia et al., 2007). Inorganic speciation of Pd in sea water is predicted to comprise of PdCl_4^{2-} and $\text{PdCl}_3\text{OH}^{2-}$ (Cosden et al., 2003). However, given that Pd has an exceptional affinity for natural polyelectrolytes (Larrivee et al., 2003) and specific ligands (Stypinski-Mis and Anderegg, 2000), its uptake is likely to be controlled by the competition for Pd^{2+} from aqueous ligands (including *Ulva* exudates) and functional groups of sulphated polysaccharides at the algal surface. Accordingly, the precise pH distribution for this metal reflects the characteristics (e.g. dissociation and complexation constants) of aqueous and particulate binding sites. With respect to Pt(IV), its inorganic speciation in sea water appears to be dominated by PtCl_6^{2-} and $\text{PtCl}_5\text{OH}^{2-}$ (Gammons, 1996); organic complexes are predicted to be important but their formation is kinetically hindered (Cosden et al., 2003). Evidently, complexation of Pt(IV) at the organic-rich, algal surface is also kinetically constrained (see Table 1). While a predominance of the former, inorganic aqueous species may account for the relatively low affinity of Pt for the algal surface, the existence of some cationic forms of lower ligand number are also required for the overall pH dependence of its uptake.

3.5. Effects of *Ulva* concentration on PGE uptake

An additional, potentially confounding variable in the experiments is the mass or surface area of *U. lactuca*, hence the concentration of metal binding sites. Where data are expressed as percentage aqueous metal, algal mass is an inherent variable. While variation in mass is normalised when data are represented in terms of K_D , adsorption experiments and field studies employing other natural particulate phases (including phytoplankton) and a variety of chemicals often report an inverse dependence of this parameter on the ratio of particle mass to solution volume, or a "particle concentration effect" (Delbeke et al., 1995; Faye and Diamond, 1996; Lindström, 2001; Upadhyay et al., 2002). The influence of algal mass on metal uptake was examined in experiments in which the number of discs was varied under otherwise identical conditions, and the results are shown in Fig. 4. Accounting for the variability of replicate measurements, uptake of Pt appears to be insensitive to the concentration (or biomass) of *Ulva*, but uptake of Rh increases and Pd diminishes with increasing algal concentration. With respect to Rh, the results may, in part, be related to a small increase in pH that accompanied increasing algal concentration. It is also possible that precipitation of Rh, mentioned above, is catalysed by a moderate increase in surface area of solid.

Regarding Pd, observations are equivalent to a particle concentration effect. However, explanations for the effect observed in sediment or phytoplankton suspensions and pertaining to inter-particle collisions or the presence of a third, colloidal phase (Schrapp and Opperhuizen, 1992; Benoit, 1995) are clearly inappropriate in the present case. More likely, the observations are related to the saturation or availability of aqueous or particulate binding sites since the sorption isotherm for Pd was non-linear, or to the release of algal exudates that act to stabilise Pd in the aqueous phase. Regardless of the precise cause of the effect, its magnitude is sufficient to exert an influence on the results of the timed experiment for this metal since a doubling of algal mass was observed over the 100-h incubation period (Fig. 1).

3.6. Sorption versus internalisation

Table 2 discriminates metal taken up by *U. lactuca* that is, by operational definition, adsorbed and internalised. Clear differences in the extents of metal internalisation are evident, and there was an inverse relationship between percentage of metal taken up by *Ulva* and the percentage thereof that is bound intracellularly. Internalisation does not appear to be some simple function of reaction rate and was not distinguishable from adsorption as a separate reaction in the timed experiment (Fig. 2).

The rationale for the present evaluation of internalisation is that the product of the stability constant of the metal–EDTA complex and the free ligand concentration exceeds the product of the constant defining metal complexation at the algal surface and the concentration

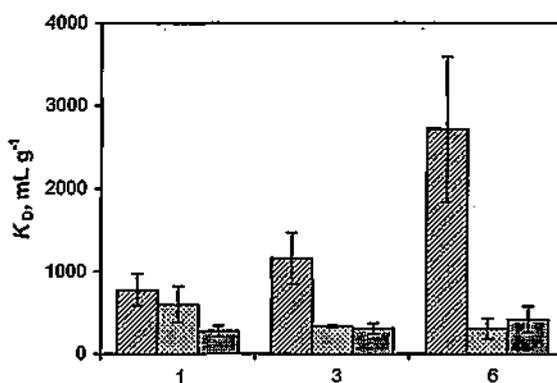


Fig. 4. Distribution coefficients defining the partitioning of 10 ppb of Rh (hatched), Pd (stippled) and Pt (shaded) between *U. lactuca* and sea water (at 13 °C) as a function of number of algal discs incubated. Error bars denote the standard deviation about the mean of three independent measurements.

Table 2

Concentrations of PGE taken up by *U. lactuca* after 100 h incubation, determined after nitric acid digestion with and without pre-washing in EDTA

Metal	Total concentration, nmol g ⁻¹	Concentration after EDTA wash, nmol g ⁻¹	% Internalised	% Adsorbed
Rh	50.7±9.7	19.3±4.1	38.1	61.9
Pd	28.3±9.4	22.8±4.5	80.6	19.4
Pt	8.6±2.2	8.1±2.5	94.2	5.8

Error bars denote the standard deviation about the mean of three independent determinations. Also shown are the internalised and adsorbed fractions of each metal, calculated from the mean concentrations with and without EDTA washing.

of surface binding sites (Hassler et al., 2004). An additional assumption is that the rate of metal desorption (or dissociation) from the algal surface is greater than the rate of metal internalisation. The stability constant of the Pd-EDTA complex ($\log K \sim 26$; Nowack and Sigg, 1996) greatly exceeds equivalent values for other divalent transition metals for which the technique has been validated ($\log K$ typically 15–18). Thus, assuming that Pd complexes are not kinetically inert (Cosden et al., 2003), we surmise that our estimate of intracellular Pd is reasonably accurate. Reliable stability constants for Rh and Pt(IV) complexes with EDTA do not appear to exist, and our assessments of internalisation of these metals may be more indicative than absolute. Specifically, we suspect that internalisation of Pt may have been over-estimated because of its slow kinetics of complexation and dissociation (Cosden et al., 2003).

Since relatively large anionic species or hydrophilic organic complexes are unlikely to contribute to the intracellular pool (Lee et al., 2004; Slaveykova and Wilkinson, 2002), internalisation of PGE by *U. lactuca* likely proceeds via the transport of simple cationic species through the cation channels. As a precursor, this process may involve adsorption of cations to specific, physiologically active sites (Slaveykova and Wilkinson, 2002; Galceran et al., 2004). These sites are likely to be occupied at relatively low metal concentrations and by metals having an affinity for specific ligands, perhaps explaining why Pd (and possibly Pt) is preferentially internalised over Rh. This assertion is qualitatively consistent with the convexity of the sorption isotherm observed for Pd (Fig. 1). We note that a similarly high degree of Ni internalisation (about 80%) has been observed in the green alga, *Chlorella kesslerii* (Hassler et al., 2004). Significantly, Ni and Pd have similar ionic potentials, and common biogeochemical pathways in sea water have previously been reported (Lee, 1983).

3.7. General discussion

This study has provided a valuable insight into the nature of PGE uptake by an important coastal macroalga and the factors controlling this uptake. The broad findings are consistent with those of an earlier study focussing on the interactions between PGE and suspended sediment particles in sea water (Turner, 2007). This is, perhaps, unsurprising since the surfaces of both algae and sediment are characterised by a heterogeneous distribution of a multitude of organic binding sites (Xue et al., 1988; Byrne and Kim, 1990). In view of the limited thermodynamic data for PGE, the pH dependencies of their uptake are particularly informative in providing further empirical evidence for the likely aqueous speciation of PGE in the marine environment. The contrasting speciation of PGE that is evident explains, at least partly, the relative magnitude of their interactions with *U. lactuca* and their extents of internalisation. Overall, however, the magnitudes of these interactions are small compared with those of other, more widely studied trace metals. For example, bioconcentration factors upwards of 10⁴ mL g⁻¹ have been reported in kinetic experiments using radiolabelled Cd and Zn (Wang and Dei, 1999), and factors in the range of 10³ to 10⁴ mL g⁻¹ have been calculated for a suite of metals (including Cr, Cu and Pb) measured *in situ* (Conti and Cecchetti, 2003). Although, on this basis, PGE may be regarded as relatively soluble constituents, the high degree of internalisation displayed by Pd (and possibly Pt) suggests that there is potential for these metals to be biotransformed or to exert toxic effects. With respect to the latter, preliminary experiments have indicated a reduction in photosynthetic activity in *U. lactuca* at PGE concentrations (in combination) below a few tens of nM.

The findings of this work also have more general implications for the study of metal uptake by macroalgae. Thus, firstly, careful attention should be paid to the evolution of pH during the incubation period, especially in batch-type experiments and even over relatively short (hourly) timescales. We note that pH is often reported at the beginning of such experiments or in the original medium but is rarely monitored throughout (e.g. Boisson et al., 1997; Lee and Wang, 2001). Chemical buffering of sea water would appear to be a logical means of countering this problem, but commonly employed reagents have been shown to affect metal uptake and the release of complexing algal exudates (Vasconcelos and Leal, 2002). Secondly, based on our experimental results in the presence of varying biomass, we predict that distribution coefficients (or bioconcentration factors) for many other trace metals are likely to display some dependence on the ratio of algal mass to solution

volume. As such, therefore, concentration factors should be reconsidered as being conditional to the experimental conditions employed, and it is recommended that caution be exercised when using these parameters to predict metal concentrations in the field (Wang and Dei, 1999; Muse et al., 2006).

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