PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES TO CADMIUM EXPOSURE IN *FUCUS SERRATUS* (PHAEOPHYCEAE)

by

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A thesis submitted to the University of Plymouth

in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences

Faculty of Sciences

January 2009

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ABSTRACT

Physiological and Biochemical Responses to Cadmium Exposure in *Fucus serratus* (Phaeophyceae)

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Marine macroalgae can accumulate metals from the surrounding waters. But their responses to metals, especially non-essential metals like cadmium, are not well known and require further investigation. Therefore, the effects of cadmium exposure on the physiology and biochemistry of *Fucus serratus* collected from metal-contaminated (Restronguet Point) and clean (Bantham Quay) habitats were investigated.

Although exposed to high concentrations of metal pollution throughout their life cycle, *F. serratus* from Restronguet Point accumulated similar concentrations of total and non-exchangeable cadmium to those of the reference population from Bantham Quay. Total and non-exchangeable contents of cadmium increased with increasing cadmium concentrations and time of exposure, without demonstrating accumulation limits or any visible signs of stress. More than 50% of total cadmium was accumulated intracellularly in both populations and the avoidance and excretion of cadmium were not demonstrated by this research.

Cadmium exposure inhibited the growth of *F. serratus* at 10 μg ~ 10 mg L^{-1} and relative growth rates decreased significantly with increasing cadmium exposure. Cadmium treatment increased the contents of some photosynthetic pigments (chlorophyll *a* and *c* and fucoxanthin), as well as the activities of antioxidant enzymes (catalase, ascorbate peroxidase and glutathione reductase), and the concentrations of glutathione and phytochelatin. However, most parameters of chlorophyll *a* fluorescence did not respond to either cadmium treatment or to the different concentrations used. This suggests that *F. serratus* may have utilised the energy from photosynthesis on preventing cadmium damaging to photosynthetic apparatus at the expense of reduced growth.

This is the first report of phytochelatin production by *F. serratus* under the stress of copper and the wide range of cadmium. Both increased cadmium concentration and a prolonged exposure time produced higher values of phytochelatin, longer chain lengths (PC_{2-5}) and higher contents of glutathione. The copper treatment also induced phytochelatin in the alga, however it produced weaker induction than the treatment of cadmium. The two populations showed different responses to combined metal treatments (cadmium + copper).

Although similar cadmium concentrations were accumulated, the Restronguet Point population showed less diminution and faster recovery of growth, higher levels of chlorophyll *a*, chlorophyll *c* and fucoxanthin, higher activities of catalase and glutathione reductase, and a more rapid biosynthesis of phytochelatin. However, oxidative damage in membrane lipids was higher in the Restronguet Point population and antioxidant activities measured by CUPRAC test and DPPH radical scavenging ability test were higher in the Bantham Quay population.

Therefore *F. serratus* demonstrates strong cadmium tolerance to cadmium exposure resulting from the production of antioxidative enzymes, glutathione and phytochelatin. The tolerant population possessed more efficient abilities to defend against cadmium stress, however the reference population also showed different protective strategies.

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ACKNOWLEGEMENTS

I would like to express my profound gratitude to many people who have helped me for this long work. First of all, I would like to give special thanks to my supervisors Dr Maria Donkin and Dr Murray Brown who gave me this interesting topic and warm guides. And I am also grateful to Dr Barbara Pawlik-Skowrońska in Poland. It was a great chance for me to work with her and her research team. I would also like to thank Dr Stuart Lane and Professor Lena Kautsky for their review and valuable comments for a better thesis.

I owed so much to all of my friends in Plant Physiology and Ecotoxicology groups: Chris and Libby Coleman, Angela and Andrew Harrop, Dr Rima Rahah Nasser and her family, Leyla Shams, Dr Tean Mitchell, Chih Ming Hsu, Yanan Di, and all friendly colleagues and technicians. I would also like to thank Owen Smith for proofreading of parts of this thesis, and it was really helpful. Everyone I met in Poland, Dr Tadeusz Skowroński, Magdalena Trzcinska, Dr Renata Kalinowska, Dr Jacek Pirszel and other technicians, was so kind and friendly, and I didn't feel myself as a stranger at all.

I cannot express my gratitude enough to Professor Gi-Tae Yeo and his lovely wife, Professor Taejun Han, Hyang Ok and Anna Carr, and all Korean friends I met in Plymouth for their love and warm care. I am also very appreciative of Professor Ki Wan Nam, Professor Young Sik Kim, Professor Han Gil Choi, and my previous professors and colleagues in Pukyong National University for their unique support.

For this academic journey, Overseas Scholarship program of Korean Government supported me and gave a good chance to meet all of my scholarship colleagues. Also the British Council and the Polish Committee for Scientific Research supported this work in the frame of British-Polish Research Partnership Programme (WAR/341/254).

Most of all, I would like to thank my parents, my brother and his family, and my friends in Korea, without whom I may not be here. They always gave me unsparing love and support. I will not forget any piece of their love and encouragement.

AUTHOR'S DECLARATION

At no 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.

This study was financed with the aid of the Korean Government's Overseas Scholarship.

Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for experiment and consultation purposes and papers are in the process of preparation for publication.

Presentation and Conferences Attended:

Plymouth (2006). 54th Annual Meeting of British Phycological Society.

Hong Kong (2007). 5th International Conference on Marine Pollution and Ecotoxicology. Poster presentation: Phytochelatin and glutathione production in *Fucus serratus* (Phaeophyceae) on exposure to cadmium. The best student poster presentation prize reward.

Plymouth (2007). Marine Institute conference: "Understanding marine systems". Poster presentation: Phytochelatin and glutathione production in *Fucus serratus* (Phaeophyceae) on exposure to wide range of cadmium.

Bristol (2008). 56th Annual Meeting of British Phycological Society. Poster presentation: Phytochelatin and glutathione production in *Fucus serratus* (Phaeophyceae) on exposure to elevated cadmium concentrations.

External Contacts (visiting research):

23rd May ~ 12th June 2005. Centre for Ecological Research in Lublin, Poland.

Phytochelatin production in marine macroalgae (green, red and brown algae).

14th Oct. ~ 6th Nov. 2006. Centre for Ecological Research in Lublin, Poland.

Phytochelatin/GSH analysis in marine macroalgae (*Fucus serratus*).

10th Apr. ~ 17th Apr. 2007. Centre for Ecological Research in Lublin, Poland.

Discussion on publication.

Word count of main body of thesis: 51,454

Signed Soon Jeong Lee.

Date 29/05/09

Chapter 1. General introduction

1. 1. Trace metals as pollutants in the marine environment

Environmental contamination by metals is not a recent subject threatening wildlife and humans. It has been widespread since the late 19th century due in the main to anthropogenic sources, such as mining activities and smelting ores, industrial effluents and wastes, burning fossil fuels, agricultural fungicide or fertilizer runoff, urban runoff, sewage treatment plants, domestic waste dumps, etc. (Lobban and Harrison, 1994, MacFarlane and Burchett, 2001, Pinto et al., 2003). The worldwide mine production of Cd, Cu, Pb and Hg is extensive and the impact of heavy metals on people's daily life is considerable (Kennish, 1996). Although metal pollution used to be considered a regional problem, it is now becoming a global matter of concern (Mallick and Mohn, 2003).

Although metals are known to be common pollutants in aquatic environments as well as terrestrial and atmospheric environments, they are normally found at low concentrations in oceanic surface waters (Pinto et al., 2003). Metal levels are usually much higher in coastal waters due to river runoff, sewage outlet, and the release of industrial effluents, while those by atmospheric transport and upwelling are lower in oceanic surface waters (Pinto et al., 2003). Therefore exposure to low concentrations of metals for long periods is described as chronic pollution whereas short exposures to extreme levels of metals are described as acute pollution. In contrast to most other pollutants, metals are among the most common non-biodegradable and persistent pollutants in the environment (MacFarlane and Burchett, 2001, Mallick and Mohn, 2003). Therefore, a number of metals, particularly those non-essentials to plant and animal metabolism, can affect aquatic organisms even in low concentrations (Baker, 1981, MacFarlane and Burchett, 2001). In addition, the eutrophication of available

nutrients from anthropogenic sources often occurs simultaneously in coastal areas. Since accumulation of metals by algae can be dramatically increased by nutrient availability (particularly nitrogen), metal pollution by river runoff, agricultural runoff or industrial effluents increases the entry of metals into the food web (Pinto et al., 2003).

A wide range of environmental factors are associated with the biological availability of trace metals in water to aquatic organisms. Among these factors are chemical speciation of the metal, presence and concentration of other metals and anions, water and sediment pH, salinity, water temperature, light intensity, organic chelators, particles and complexing agents, oxygen levels and thereby the redox potential (Förstner and Wittmann, 1979, Brinkhuis et al., 1980, Wahbeh, 1984, Kautsky, 1998, Ralph and Burchett, 1998, Greger, 1999).

1. 2. Cadmium

Cd is a well-known pollutant and a non-essential trace element for plants and animals with no known homeostasis mechanism in organs and tissues (Nriagu, 1988, Myklebust and Pedersen, 1999).

1. 2. 1. Distribution and concentrations of Cd

In most unpolluted natural waters which are not exposed to metal elements, such low levels of Cd are reported that they were generally below the standard of allowable concentration of Cd (10 ppb) set by the World Health Organisation for drinking water (National Research Council Canada, 1979). Cd concentration varies with nutrient level and location of the water column (Ray and McLeese, 1987, Lobban and Harrison, 1994). Higher values of Cd can be found in coastal waters near industrial districts or in water

circulation-limited areas and the values can reach to several μg L^{-1} (Ray and McLeese, 1987, Lobban and Harrison, 1994). In uncontaminated soils, the levels of Cd are commonly below 0.5 ppm, however levels can reach up to 3 ppm depending on the geological background (Schachtschabel et al., 1984, Hagemeyer, 1999). Native Cd has not been reported yet and the metal is mostly found with native Zn with 250 of the Zn / Cd ratio in terrestrial rocks (National Research Council Canada, 1979). Most of Cd has been won from Zn deposits, such as limestones, dolomites, skarn, tuff, basalt and other volcanic rocks (National Research Council Canada, 1979). Two oxidation states of Cd have been found in nature, the metallic state and the Cd^{2+} state. There are eight stable isotopes that have been reported with conventional abundances as follows: ^{106}Cd (1.21%), ^{108}Cd (0.88%), ^{110}Cd (12.39%), ^{111}Cd (12.75%), ^{112}Cd (24.07%), ^{113}Cd (12.26%), ^{114}Cd (28.86%), ^{116}Cd (7.58%) (National Research Council Canada, 1979).

Much higher levels of Cd are accumulated by physiochemical processes in the tissues of marine organisms when compared with the levels in the surrounding environment. Ray and McLeese (1987) mentioned three possible sources of Cd for marine organisms: (1) in solution or in colloidal suspension; (2) in food or in suspended particulate matter; and (3) in bottom sediment. In case of marine macro- and microalgae, unlike filter feeding invertebrates, metal elements in the water may be the only source of Cd supply. Reported bio-concentration factors of Cd are various depending on the materials: plankton 10^4; seaweed $10^2 \sim 10^3$; mollusc $10^3 \sim 10^4$; crustacean 10^3; and fish 10^2, respectively (Preston, 1973). Bryan (1976) determined the Cd concentrations in marine organisms as follows: phytoplankton 2; seaweed 0.5; zooplankton (copepods) 4; bivalve molluscs 2; oysters 10; gastropod molluscs 6; decapods crustaceans 1; and fish 0.2 μg g^{-1} dry weight, respectively. These values can vary with habitats or areas (Adriano, 1986). Knauer and Martin (1973) reported $<1 \sim 7$ μg Cd g^{-1} dry weight in phytoplankton in Monterey Bay, California.

1. 2. 1. 1. Concentrations of Cd in seaweed

Cd has been shown to be easily adsorbed by marine algae and have $10^2 \sim 10^3$ of bio-concentration factors by algae (Preston, 1973, National Research Council Canada, 1979). While the local aqueous Cd levels ranged from <0.01 to 0.30 ppb and from <0.01 to 85 ppb, accumulated Cd levels in algae were much higher than levels in the water: 0.7 ~ 13 ppm in *Enteromorpha* sp.; 1.8 ~ 12.5 ppm in *Fucus vesiculosus*; 1.0 ~ 11.5 ppm in *Ascophyllum nodosum*; 2.3 ~ 13 ppm in *F. serratus*; and 0.9 ~ 20 ppm in *Chorda filum* (Stenner and Nickless, 1974). *Porphyra* and *Fucus* were reported to accumulate 660 and 2700 times of Cd over water levels (Preston et al., 1972) and *F. vesiculosus* accumulated 2.6×10^4 times over the water level (Morris and Bale, 1975). However the concentration factor was independent of the Cd level in the water and irreversible (National Research Council Canada, 1979).

1. 2. 2. Chemical form of Cd in marine environment

Cd in the three phases of the marine environment (i. e. water, particulate matter and sediment) may be in a balance with one another (Ray and McLeese, 1987). Cd is in solution as free hydrated ions or complexed with labile / non-labile chemical forms, both inorganic and organic. In seawater, Cd stays mainly in the ionic forms (more than 80%) and most often in a variety of chloride complexes ($CdCl^+$) (Ray and McLeese, 1987). In particulate matters, relatively much lower levels of Cd are present. On the other hand, Cd and most of other trace metals in the sea are mainly stored within sediments which may then affect the dissolved metal concentrations in waters (Ray and McLeese, 1987).

1. 2. 3. Environmental factors affecting bioaccumulation of Cd

The accumulation of Cd by marine organisms and the degree and nature of Cd complexation rely on a wide range of physiochemical factors, such as salinity, temperature, pH, season, light, nutrient concentration, redox potential, sorption, chelation, complexation, precipitation, hydrolysis and the interactions of those factors (Ray and McLeese, 1987, Topcuoğlu et al., 2003). Here, some of these factors are discussed.

1. 2. 3. 1. Salinity

Salinity shows considerable variability, especially in estuarine and coastal environments. There have been some reports of increased uptake rate of Cd coincident with reduction of salinity (Coombs, 1979, Ray and McLeese, 1987). Several causes of a salinity effect were postulated: (1) Increased oxygen consumption by organisms may enhance metal uptake (Bass, 1977, Taylor, 1977); (2) The lower the salinity, the higher total amount of biologically available Cd^{2+} (Mantoura et al., 1978, Engel et al., 1981); and (3) A higher level of Ca at higher salinities diminishes uptake of Cd (Wright, 1977a, Wright, 1977b).

1. 2. 3. 2. Temperature

Increasing temperature results in increase of bioaccumulation of Cd, which is possibly related to the stimulation of metabolic activity by higher temperatures. Significantly higher accumulation of Cd has been analysed in algae *Stichococcus bacillaris* Naegli, *Vaucheria compacta* (F.S. Collins) F.S. Collins ex W.R. Taylor and *V. debaryana* Woronin, clams *Mya arenaria* Linnaeus, *Mulinia lateralis* Say and *Nucula*

proxima Say, oysters *Crassostrea virginica* Gmelin and *Saccostrea echinata* Quoy and Gaimard, crab *Callinectes sapidus* M. J. Rathbun, prawn *Leander adspersus* Rathke, shrimp *Lysmata seticaudata* Risso and coelenterate hydrozona *Laomedea loveni* Allman, except mussel *Mytilus edulis* Linnaeus, with increased temperature (Jackim et al., 1977, Theede et al., 1977, Denton and Burdon-Jones, 1981, Hung, 1982, Skowroński, 1986, Ray and McLeese, 1987, Skowroński et al., 1998).

1. 2. 3. 3. pH

pH in the surrounding water may have an indirect effect on bioaccumulation of Cd since this factor is closely related to the migration of Cd. While Cd has a high mobility in acidic waters, the element is largely adsorbed or precipitated by sediments or organic / inorganic colloids in neutral and alkaline waters (National Research Council Canada, 1979).

1. 2. 3. 4. Interactive effect

The accumulation of trace metals including Cd is not often explained as a simple factor and a number of environmental parameters are often closely related to each other. Life stage (such as gametogenesis and age), seasonal variation, etc. can produce combined factors and several different outcomes can be expected. Coombs (1979) reported an additive effect of salinity and temperature on accumulation of Cd by marine organisms.

1. 2. 4. Toxicity of Cd

Lobban and Harrison (1994) reported the general order of metal toxicity to algae as Hg > Cu > Cd > Ag > Pb > Zn. This order can show wide variations dependent on a

number of factors, such as the algal species, experimental conditions, ionic speciation, chemical interactions and algal mobility (Lobban and Harrison, 1994, Ralph and Burchett, 1998). Indeed Lyngby and Brix (1982) published a slightly different order of metals with seagrass *Zostera marina*, Hg > Cu > Cd > Zn > Cr > Pb and Kennish (1992) postulated similar list of metals with marine flora and fauna, Hg > Cd > Cu > Zn > Ni > Pb > Cr > Al > Co. Cd is one of the most toxic trace metals in all of these orders and has a number of biochemical effects, mostly adverse to aquatic organisms.

1. 2. 5. Phytochelatin (PC) and metallothionein (MT) activity against Cd toxicity

Some living organisms have specific cellular mechanisms detoxifying metal stress. Chelation of metal elements by high-affinity ligands is one of the key mechanisms for metal detoxification and tolerance (Hall, 2002). Amino acids, organic acids, and peptides are the potential ligands and phytochelatins (PCs) and metallothioneins (MTs) are the cysteine-rich, metal-binding peptides (Rauser, 1999, Clemens, 2001, Hall, 2002). Although both are low-molecular weight cytoplasmic peptides, their synthetic processes are different. MTs are gene-encoded proteins and are found in animals, plants, algae and fungi (Rauser, 1990). On the other hand, PCs are enzymatically synthesized peptides in higher plants, algae, fungi and lichens (Rauser, 1990, Pawlik-Skowrońska et al., 2007). PC synthase is known to be activated in the presence of metal ions (Cobbett, 2000) and has been reported to be hypersensitive to Cd among many potential PC inducers in many reports (Grill et al., 1985, Ahner and Morel, 1995, Cobbett and Goldsbrough, 2002, Inouhe, 2005).

1. 2. 6. Production of reactive oxygen species (ROS) and antioxidative enzymes against Cd stress

Cd-induced antioxidative mechanism is not yet well understood (Lee and Shin, 2003) since Cd has a different oxidative process from transition metals like Cu and Fe (Robinson, 1989). However toxicological research has suggested that Cd could induce oxidative stress in different tissues of both animals and some algae (Reed and Gadd, 1990, Okamoto et al., 1996). Nevertheless, there is much less information about Cd-induced oxidative stress and the defence systems in marine algae (Lee and Shin, 2003).

Most research has been focused on microscopic algae. Cd increased superoxide dismutase (SOD), ascorbate peroxidase (APX) and β-carotene levels, increased oxidation of protein and lipids, and decreased GSH levels in the marine dinoflagellate *Gonyaulax polyedra*, which may be due to Cd-induced reactive oxygen species (ROS) metabolism (Okamoto et al., 2001b, Okamoto and Colepicolo, 2001). The marine microalga *Nannochloropsis oculata* showed enhanced lipid peroxidation and H_2O_2 content, and increased activity of APX, glutathione peroxidase (GPX) and catalase (CAT) after Cd exposure (Lee and Shin, 2003). In the prasinophyte *Tetraselmis gracilis*, increased SOD activity by generating ROS was reported (Okamoto et al., 1996). Moreover, the red macroalga *Gracilaria tenuistipitata* showed increased lipid peroxidation, oxidation of protein and activity of CAT and SOD (Collén et al., 2003). All of these results indicate that Cd can induce oxidative stress in which ROS production may be involved.

Antioxidative responses varied according to the status of Cd stress. Significant increases in SOD and APX activities and high GSH content were shown in *Gonyaulax polyedra* under chronic stress; however no significant change in APX, a slight increase in SOD, increased oxidation of proteins and lipids, and a reduced GSH pool were detected under acute stress (Okamoto et al., 2001b). Therefore a higher antioxidative

capacity was run in cells under chronic stress and hyperoxidative cellular status was attacking cells under acute stress (Okamoto et al., 2001b). GSH and free cysteine levels were increased according to Cd dose and time of exposure in *Euglena gracilis* (Coppellotti, 1989), indicating Cd toxicity depends on various factors such as Cd concentration, period of exposure, and the test organism (Okamoto et al., 1996).

1. 2. 7. Bioavailability of Cd

Although Cd has no known metabolic function in organisms, Cd^{2+} is readily taken up by plants and algae (Pinto et al., 2003). In contrast to cupric ions, Cd cannot supply $^{\cdot}OH$ in the Fenton reaction (Pinto et al., 2003, Halliwell and Gutteridge, 2007). However, Cd increased the growth of the diatom *Thalassiosira weissflogii* in Zn-deficient culture and the alga showed 90% of their maximum growth rate with Cd-replete condition (Price and Morel, 1990). Price and Morel (1990) proposed that the depleted Zn was replaced by Cd and the substitution was done in one or more metalloenzymes such as carbonic anhydrase based on the similar size distribution of Cd and Zn in cytoplasmic proteins. Zn depletion reduced the activity of carbonic anhydrase, which inhibited the growth of the diatom due to the reduced bicarbonate uptake (Morel et al., 1994). Although Cd replacement restored part of carbonic anhydrase activity in Zn-limited cultures (Morel et al., 1994) and Cd shifted with the band of carbonic anhydrase in gel electrophoresis (Lee et al., 1995), it was not clear if Cd merely substituted for Zn in the enzyme (Küpper and Kroneck, 2005). Later, the protein size of Cd-carbonic anhydrase of *T. weissflogii* was analysed and it revealed a much larger structure (43 kD) than Zn-carbonic anhydrase (27 kD) (Lane and Morel, 2000). In addition, the fact that TWCA1, the diatom's major intracellular Zn-requiring isoform of carbonic anhydrase, remained low when Cd replaced Zn but the growth rate increased

showed Cd-carbonic anhydrase is indeed specifically synthesized as a Cd enzyme (Lane and Morel, 2000, Küpper and Kroneck, 2005).

Another substitution by Cd is related to the Mg^{2+} in chlorophyll molecules. Some toxic metal elements (such as Cu, Zn, Hg and Cd) can replace the Mg ion during metal stress. Since metal-chlorophylls are not suitable for photosynthesis, these new compounds can produce much lower fluorescence quantum yield and energy transfer from the antenna pigment complexes to the reaction centres (Küpper et al., 1996, Küpper et al., 1998). Therefore the formation of metal-chlorophylls due to metal contamination can finally cause a fatal collapse of photosynthesis (Küpper et al., 1996).

1. 3. Responses of aquatic organisms to metal pollution

For several decades the toxicity of metals on plant and animal metabolic processes has been one of main topics in biology (Fernandes and Henriques, 1991, Küpper et al., 1998). Simpson (1981) and Myklebust and Pedersen (1999) presented heavy metals as one of the worst forms of pollution in the marine environment. Marine organisms can accumulate metals to levels several fold higher than found in the water, which becomes more serious in higher trophic levels of the food chain due to biological magnification (Lobban and Harrison, 1994).

Some of trace metals, such as Cu, Fe, Mn, Zn, and Mg are vital for the metabolic processes of plants while other metals like Cd, Pb, Ag and Hg do not have any known biochemical functions (Lyngby and Brix, 1982, Ralph and Burchett, 1998). Although essential metals are required for the physiology of organisms, both essential and non-essential metals can be toxic if accumulated in excess (Vallee and Ulmer, 1972, Van Assche and Clijsters, 1990).

Accumulation of metals by aquatic organisms is normally related to the metal concentrations in the water, which shows initial rapid uptake followed by equilibrium (Ralph and Burchett, 1998). A generalised model of metal uptake for macrophytes has been proposed: exchange adsorption by passive uptake, diffusion across the plasmalemma into the protoplast, and active accumulation into the cell (Malea, 1994). This whole process of metal uptake by seagrasses and seaweeds is known to be influenced by the levels of biologically available metals in the water rather than by the metal loads in the sediment (Ralph and Burchett, 1998). Although, as a result of complexation and precipitation, there are much higher loads of metals in the sediment, correlation between accumulation by organisms and levels in the sediment was often limited (Luoma et al., 1982, Ralph and Burchett, 1998). Luoma et al. (1982) reported that concentrations of Cu, As, Pb, Zn and Ag in *Fucus vesiculosus* have close mutual relationship with the total levels of these metals in sediment while concentrations of Cd, Co, Hg, Fe and Mn in *Fucus* lack any correlation with the levels of these metals in surface sediment.

Aquatic organisms may be expected to undergo various different reactions to metal stress because different species have different characteristics and different detoxifying mechanisms (Payne and Price, 1999). To date, many researchers have reported that toxic levels of metals affect the cellular and biochemical processes of aquatic algae, such as photosynthesis, nucleic acid production, protein and lipid biosynthesis, uptake of inorganic nutrients, and nitrogen fixation (Boyle, 1984, Pinto et al., 2003, García-Ríos et al., 2007). Stunted growth, chlorosis, necrosis and discoloration of thalli are the first symptoms of phytotoxicity (Van Assche and Clijsters, 1990). Inhibition of photosynthesis, reduction in pigment concentrations, inhibition of nitrate uptake and the production of nitrate reductase, and the loss of other cations (such

as K^+) are some of the harmful effects of metals, which may result in the previously mentioned symptoms (Rijstenbil et al., 1994, Gledhill et al., 1997).

Metal toxicity may be caused by the binding of metals to sulphydryl groups in proteins or the displacement of essential elements (such as Mg^{2+}) (Van Assche and Clijsters, 1990, Hall, 2002). Accumulated metals can disrupt electron transport in photosystem II (PS II), ruin development of the gametophyte or disturb the allocation of proteins, lipids, sterols, sterol esters, and free fatty acids (Smith et al., 1986, Anderson et al., 1990, Gledhill et al., 1997). Another adverse effect is that of stimulating the formation of free radicals and oxidative stress. Both an increase in the cellular levels of ROS and a reduction in cellular antioxidative ability can be expected by metal stress (Sies, 1999, Pinto et al., 2003).

Therefore aquatic organisms, especially marine macroalgae as used in this study, have unique mechanisms of defence against metal toxicity. A series of mechanisms enabling metal toxicity tolerance have been elucidated to date, namely detoxifying antioxidants (such as GSH, ascorbic acid, tocopherols, polyphenol, MT and PC), antioxidative enzymes (such as CAT, SOD, glutathione reductase (GR), APX and GPX), exudation of metal ions and intracellular compartmentation of metal ions.

1. 4. Marine macroalgae as bioindicators

Because metal levels in seawater are very low and variable and levels in sediments are altered by many factors, such as pH, grain size, organic matter content, and oxidation-reduction potential, concentrations of metals or changes in their availability in the environment have been frequently monitored by measuring metal levels in marine organisms (Kautsky, 1998, Topcuoğlu et al., 2003). Therefore research

on organisms which are suitable for monitoring the surrounding environment has been developed. These organisms are known as biological indicators (bioindicators), bioaccumulative indicators, biomonitors or sentinel organisms (Phillips, 1994), and represent one or more properties of the ecosystem to which they belong (Ferrat et al., 2003). They accumulate information on variations in the environment and provide data which would be unobtainable by chemical analyses (Lyngby, 1990).

Metal ions in the environment cannot be destroyed by microbial processes but can be modified, immobilized or detoxified by following methods: biosorption, bioaccumulation, reduction, solubilisation, precipitation, and methylation (Gavrilescu, 2004). Biosorption (= adsorption) is defined as the passive uptake of metals to cell walls or extracellular polymers, such as polysaccharides, proteins and nucleic acids. Bioaccumulation is defined as the energy-requiring uptake of metal cations, which comprises a two step process: passive binding to the cell wall and energy-consuming transfer associated with Mg and K transport (Gavrilescu, 2004).

Three different strategies of metal uptake by plants were proposed by Baker (1981). The first category is an excluder which takes up low levels of metals at quite high external metal concentrations. Certain kinds of barriers or protective mechanisms operate (such as exudation of metal complexing ligands) in these plants to prevent uptake of metals. However, under extremely high metal concentrations, this protection collapses and massive uptake occurs. The second group of plants, which accumulates metals even at very low external concentrations, is known as an accumulator. Some mechanisms of detoxification in tissues may allow the plants to accumulate metals without serious problems. Nevertheless, under extremely high external metal concentrations, they cannot increase the uptake due to competition of metal ions at the cell wall. The final type of plant uptake is found in plants which increase their uptake linearly with increasing external metal concentrations. They are known as indicators

owing to the linear relationships between their metal uptake and the external metal concentrations.

Benthic marine macroalgae have been used to evaluate metal pollution in the environment since they meet many of the requirements of bioindicators. Many algal species are easy to collect and to identify taxonomically. They are widespread geographically, available year-round and are tolerant of changes in salinity and turbidity (Phillips, 1994). They are considered to be useful biomonitors since the metal contents in the algal tissues are directly linked to the dissolved metal concentrations in the surrounding water (Bryan, 1983). Since algae do not have a dietary route for metal uptake and do not use particulate metals, these organisms exhibit only dissolved metals from solutions (Luoma et al., 1982, Bryan et al., 1985). They can accumulate levels of metals in the tissues several thousand times higher than those of seawater (concentration factor of algae: 10^3~10^5), providing an indication of metal levels in the water (Förstner and Wittmann, 1983, Bryan and Langston, 1992, Phillips, 1994, Muse et al., 2006). Macroalgae have been regarded as better indicators than phytoplankton since they are sessile and have longer life spans (Bryan, 1971, Förstner and Wittmann, 1983, Topcuoğlu et al., 2003). The longer life span allows for a longer period of metal exposure (Förstner and Wittmann, 1983) and, for that reason, the older part of *Fucus vesiculosus* accumulates considerably higher metal concentrations than the area around the growing point (Bryan, 1971). Therefore macroalgae can be useful bioindicators in the aquatic environments since they provide a regular and consistent record of changes in the metal values of tissues (Fuge and James, 1974).

Among algal divisions, brown or green macroalgal species have been used most frequently (Phillips, 1994, Topcuoğlu et al., 2003, Hédouin et al., 2008). Most research on green algae was performed with *Ulva* spp. (including former *Enteromorpha*) (Say et al., 1990, Rijstenbil et al., 1998a, Locatelli, 1999, Topcuoğlu et al., 2003, Han and Choi,

2005). Red algae and seagrasses were also employed as bioindicators but less frequently (Phillips, 1994, Costanzo et al., 2000, Ferrat et al., 2003). Brown algae have been used as bioindicators of metal pollution in the costal environment since the early seventies (Burrows, 1971, Bryan, 1983, Hédouin et al., 2008). Brown algae are particularly effective and reliable at removing, for example, Cu^{2+}, Cd^{2+}, Pb^{2+}, and Zn^{2+}, due to their abundant extracellular polymers and their high metal tolerance when compared with the many types of biosorbents (for example bacteria, fungi and yeasts) (Davis et al., 2003). Their strong adsorption capacity (biosorption with polysaccharides such as alginates) and absorption (bioaccumulation with polyphenols and PC) make them useful bioindicators since concentrations of metal are high enough to detect and stable (Phillips, 1994, Hédouin et al., 2008).

1. 5. *Fucus serratus* L.

Among about 1500 species of brown algae (Phaeophyceae), *Fucus vesiculosus* and *F. serratus* are probably the best known British seaweeds (Knight and Parke, 1950). The genus *Fucus* has been reported in a wide range of intertidal rocky shores in North East Atlantic coasts (Williams, 1996, Malm et al., 2001, Cairrão et al., 2004). While patches of *F. vesiculosus* are distributed in the mid-intertidal zone, patches of *F. serratus* are commonly found in the lower intertidal zone (Knight and Parke, 1950, Lewis, 1964, Malm and Kautsky, 2003). *F. serratus* has fronds with serrated edges and dichotomous branching thalli (Fig. 1. 1 and 1. 2). These robust thalli are known to grow to a maximum of about one meter in length, and average elongation rate of thalli from the Devon coast was 0.49 cm per week in a range of 0.31 ~ 0.71 cm per week (Knight and Parke, 1950). *F. serratus* is a perennial alga and is known to live normally 3 years

(Rees, 1932). However it can live the fourth and even fifth year in very sheltered areas although the ones in very exposed areas barely live more than 2 years (Knight and Parke, 1950). Unlike *F. vesiculosus* which achieve the maximum weight in the third year, *F. serratus* may gain the more weight in the fourth year (Knight and Parke, 1950).

Fucoid algae have been used as one of the supreme bioassay materials owing to their ease of culture, fast growth and relatively quick responses to stress sources (Bond et al., 1999, Braithwaite and Fletcher, 2005). Some authors have used fucoid seaweeds as bioindicators of metals in inshore waters (Nickless et al., 1972, Preston et al., 1972, Bryan and Hummerstone, 1973, Luoma et al., 1982, Forsberg et al., 1988, Rönnberg et al., 1990, Cairrão et al., 2004, Stengel et al., 2004).

F. serratus, a dioeious species, is actively reproductive throughout the year although the peak of reproduction is commonly in the autumn and winter (Knight and Parke, 1950, Quatrano, 1980). It has diplontic life cycle, diploid thalli and haploid gametes (Quatrano, 1980). Its sexual reproduction, external oogamous fertilization, is common to certain angiosperims (Quatrano, 1980). Reproductive structures on the receptacles (i.e. conceptacles, ca. 0.5 cm) secrete ripe gametes via the ostiole.

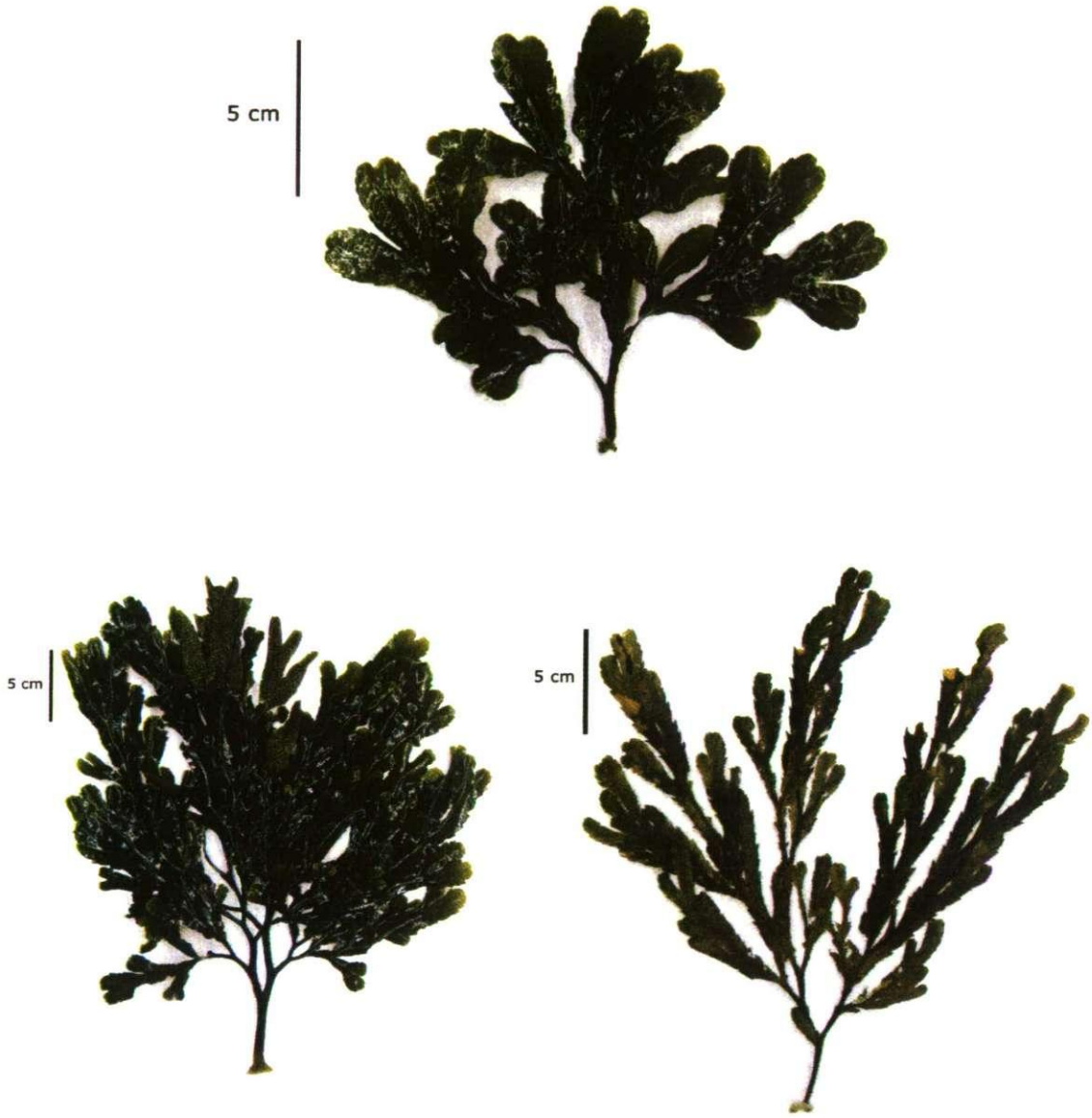

Fig. 1. 1. *Fucus serratus* L. Upper, late first year; lower, left, second year with receptacles; lower, right, third or fourth year.

Fig. 1. 2. *Fucus serratus* from the Avon Estuary. Upper, natural population patches on the rocks; lower, receptacle possessing conceptacles for reproduction.

The big immotile egg (ca. 75 μm) is fertilised by a small motile sperm which has lateral biflagellate (ca. 5 μm) (Quatrano, 1980). For over one hundred years, zygotes of the Fucales have been utilized for studies of polarity, polar axis determination, cell-wall assembly of the wall-less egg (Quatrano, 1974, Quatrano and Stevens, 1976, Nielsen et al., 2003a) since the polar development and embryogenesis of *Fucus* is similar to those of other plants, including angiosperms (Quatrano, 1980).

1. 6. Object of this study

The use of algae, particularly marine macroalgae, to indicate and monitor environmental pollution is no longer new. The increasing use of algae as bioindicators as well as bioremediation agents has been shown on many occasions. The genus *Fucus* is composed of vigorous marine macroalgae found in metal-polluted areas and they have frequently been selected for many experimental studies. However, most research has focused on Cu and Zn as metal sources and *F. vesiculosus* as the algal species. Therefore the physiological and biochemical responses of *F. serratus* to heavy metal pollution have been based on understanding gained from research into *F. vesiculosus* or other brown algae rather than *F. serratus* itself. In addition, the effects of Cd have received less attention due to its non-essential role in plant physiology and its lower toxicity compared with Cu or Hg. Since each metal element and algal species has unique physiological, biochemical and geochemical characteristics, specific and detailed information for each algal and metal species is very important.

In this work, *F. serratus* from two different areas with completely different histories of metal contamination were investigated for photosynthetic performance,

antioxidative reactions and detoxification of Cd. In previous research (Nielsen et al., 2003b, Collén and Davison, 1999a, Collén and Davison, 1999c), stress-tolerant populations have been generally shown to possess more efficient mechanisms for defence against metal stress therefore comparisons between the two populations were addressed in each chapter.

The bioaccumulation of Cd by *F. serratus* was measured as well as growth rate, chlorophyll *a* fluorescence parameters and contents of photosynthetic pigments. Total and non-exchangeable concentrations of Cd taken by *F. serratus* and the efficiency of the alga as a bioindicator were described. The effects of Cd as a toxic metal element on photosynthesis were evaluated with various chlorophyll *a* fluorescence parameters and levels of chlorophyll *a* and *c* and two accessory pigments were measured.

The expression of oxidative stress by *F. serratus* was determined. Lipid peroxidation, CUPRAC, DPPH free radical scavenging capacity and three antioxidative enzymes were estimated after Cd exposure.

Detoxification of Cd stress closely related to the synthesis of PC and the precursor, GSH was addressed in Chapter 5. Comparison between two populations and compound data with accumulated contents of Cd explained these different strategies against Cd stress.

Finally, in Chapter 6, results from each chapter were discussed as a whole process of physiological and biochemical responses of *F. serratus* exposed to Cd.

Chapter 2. General material and methods

2. 1. Description of collecting sites

Fucus serratus was collected from two sites in South West England with different history of exposure to metal contamination. As a reference population, *F. serratus* from Bantham Quay (BQ; 50°16'70''N; 3°51'05''W), at the mouth of the river Avon, was selected (Fig. 2. 1 and 2. 2). The Avon Estuary in South Devon has little or no industry, very limited domestic sewage input (Bamber and Depledge, 1997), and there has been no mineral exploitation (Grant et al., 1989, Bryan and Langston, 1992, Bamber and Depledge, 1997). Bryan and Langston (1992) regarded the Avon Estuary as one of the least polluted coastal areas in the UK in which 18 $\mu g\ g^{-1}$ of total Cu was measured in sediment (Table 2. 1). Diverse algal species were found in this area (Fig. 2. 3).

Restronguet Point (RP; 50°11'60''N; 5°4'10''W) is at the entrance to Restronguet Creek, a branch of the Fal Estuary, in South West Cornwall (Fig. 2. 1 and 2. 4). Restronguet Creek is the most metal-contaminated area in the Fal Estuary (Pirrie et al., 2003). This estuary has received long-term contamination as a result of polymetallic mining activity; it is also an important marine habitat for invertebrates, fish and algae (Pirrie et al., 2003). Research has shown that the flora and fauna inhabiting this polluted estuary have evolved to have resistance mechanisms to metals such as Cu and Zn: e.g. annelida *Nereis diversicolor* (Bryan and Hummerstone, 1971, Bryan and Hummerstone, 1973, Bryan, 1974) and *Nephtys hombergi* (Bryan, 1976), brown algae *Fucus vesiculosus* (Bryan and Gibbs, 1983) and *Fucus serratus* (Nielsen, 2002, Nielsen et al., 2003b), and mollusca *Scrobicularia plana* (Bryan, 1976). Restronguet Creek receives

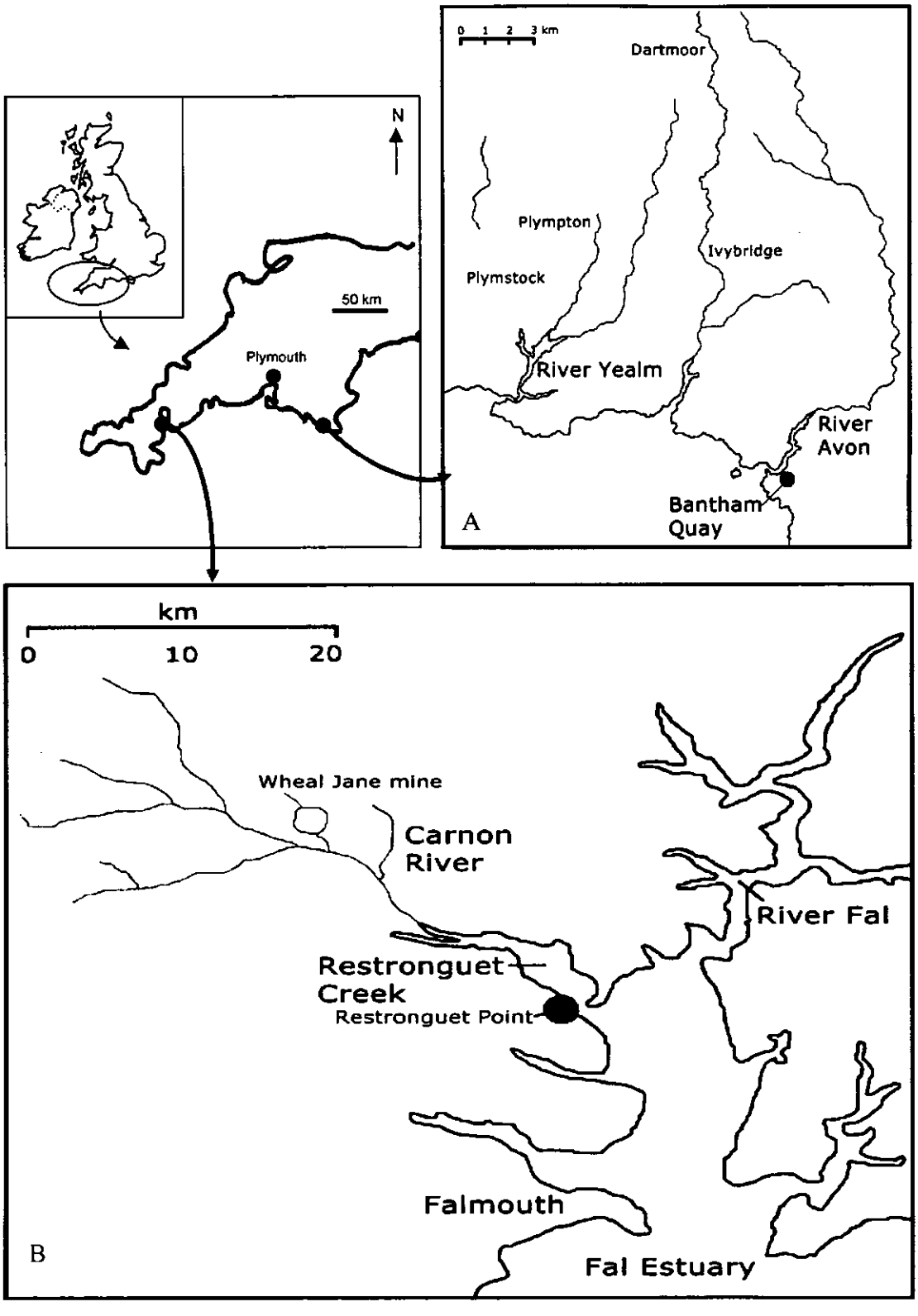

Fig. 2. 1. Collecting sites for *Fucus serratus*, A, Bantham Quay; B, Restronguet Point, South West England.

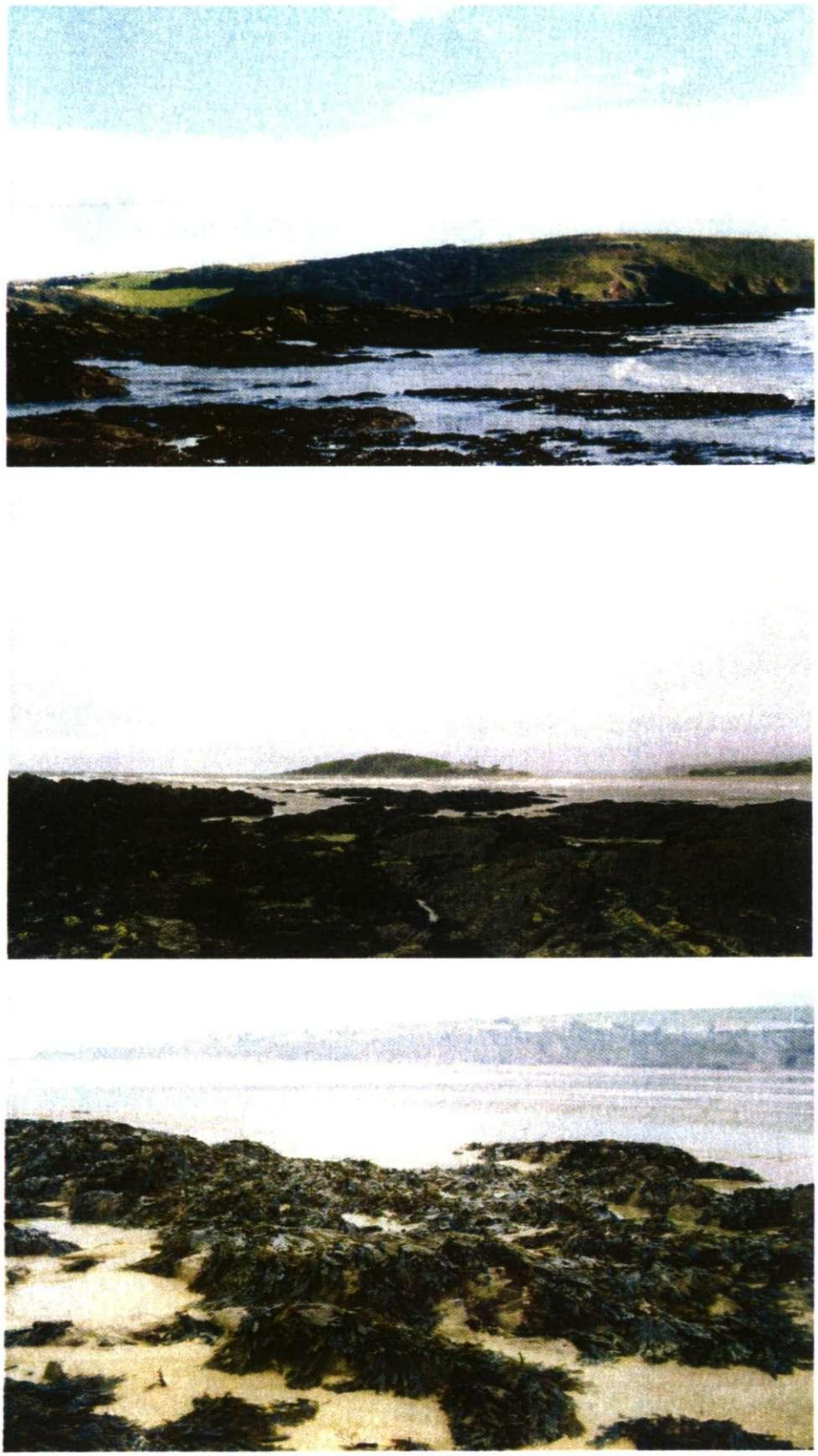

Fig. 2. 2. Bantham Quay, the mouth of the Avon Estuary, South Devon, England.

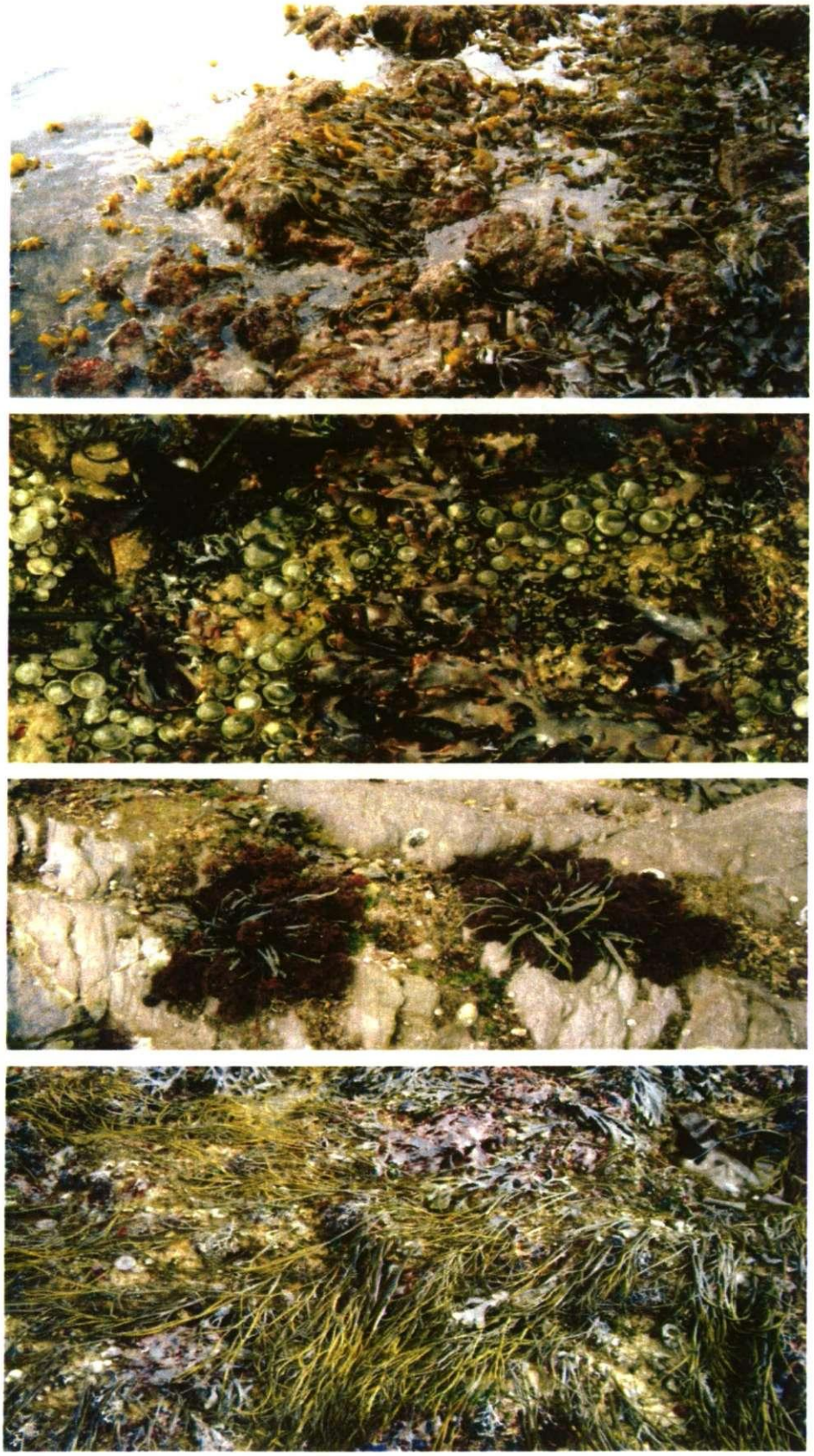

Fig. 2. 3. Diverse benthic flora of Bantham Quay. Emergence of various species in red, green and brown algae can be identified.

Table 2. 1. Concentrations of total metals in sediments and water column

Metals	Water (μg L^{-1})		Sediment (μg g^{-1} D.W.)					
	Restronguet Creek	Carnon River	Avon Estuary		Restronguet Creek			Fal Estuary
Ag		0.45		0.06	3.76			
As		233		13.0	1740		983 - 2803	1467 – 3939
Cd	<0.1 - 38	24.8		0.08	1.53			2.35 – 3.76
Co		70		10	21			
Cr				28	32			
Cu	2 – 176	593	19	18	2398	3000	1172 - 5073	1591 – 4507
Fe		5024	19400	18361	49071			51228 – 95418
Hg				0.12	0.46			
Mn	3 - 1513	1777		326	485			439 – 710
Ni	1 – 18	107		23	58			
Pb	<2 – 4	40		68	341		217 - 570	
Sn				3.9	55.9		752 ~ 3400	
Zn	7 - 22460	12470	98	82	2821	2500	950 ~ 6600	2332 - 5809
Reference	Bryan and Gibbs (1985)	Bryan et al. (1987)		Bryan and Langston (1992)		Bryan et al. (1987)	Pirrie et al. (2003)	Langston et al. (2003)

acidic (pH 3.8 ~ 4) and metal-enriched water (such as Fe, Zn, Mn and Cu) from the Carnon River due to various mining and smelting works and from the erosion of spoil heaps (Dines, 1969, Bryan and Gibbs, 1983, Perryman, 1996). The history of mining in Cornwall dates back to the early Bronze Age, with fluvial tin (Sn) deposits being the main target (Gerrard, 2000). Hard rock mining for Sn, Cu and a range of other metals was performed from the 13th century (Gerrard, 2000). Production of Cu and Sn reached at its peak in the 1860s and 1890s respectively and up to 50% of the world's mineral supply of Cu, Sn, As, Pb and Zn came from more than 1000 mines in this region (Dines, 1969, Burt, 1998). However output decreased rapidly during the 20th century and the last Sn mine (Wheal Jane, Carnon Valley) closed in 1991 (Somerfield et al., 1994). In January 1992, sudden discharge of 50 million litres of acidic (pH 3.1), metal-laden mine water, the so called 'Wheal Jane incident', happened (Somerfield et al., 1994, Banks et al., 1997, Younger, 2002, Pirrie et al., 2003). While there was visual evidence of contamination by metals, biological studies with benthic invertebrate community did not show significant impacts due to limited mixing of water column, large dispersal seawards and metal-tolerance of the species (Bryan and Gibbs, 1983, Somerfield et al., 1994, Millward and Grant, 2000, Pirrie et al., 2003). Nevertheless, in excess of £20 million was spent for remediation for contaminated water system after this accident (Younger, 2002). Although all metal extract has now ceased, drainage from the old mine adits and erosion of the spoil heaps by water continues, resulting in very high concentrations of metals in the sediments and waters in the Fal Estuary (Bryan and Gibbs, 1983, Pirrie et al., 2003). Among them, Zn, Mn, Cu and Cd remain in solution while As, Fe and Pb form insoluble compounds associated with the sediment (Bryan and Gibbs, 1983). Bryan and Langston (1992) and Bryan and Gibbs (1983) reported 200 nM of total Cu and over 153 μM of Zn in the water of Restronguet Creek and Pirrie et

Fig. 2. 4. Restronguet Point, the Fal Estuary, south west Cornwall, England.

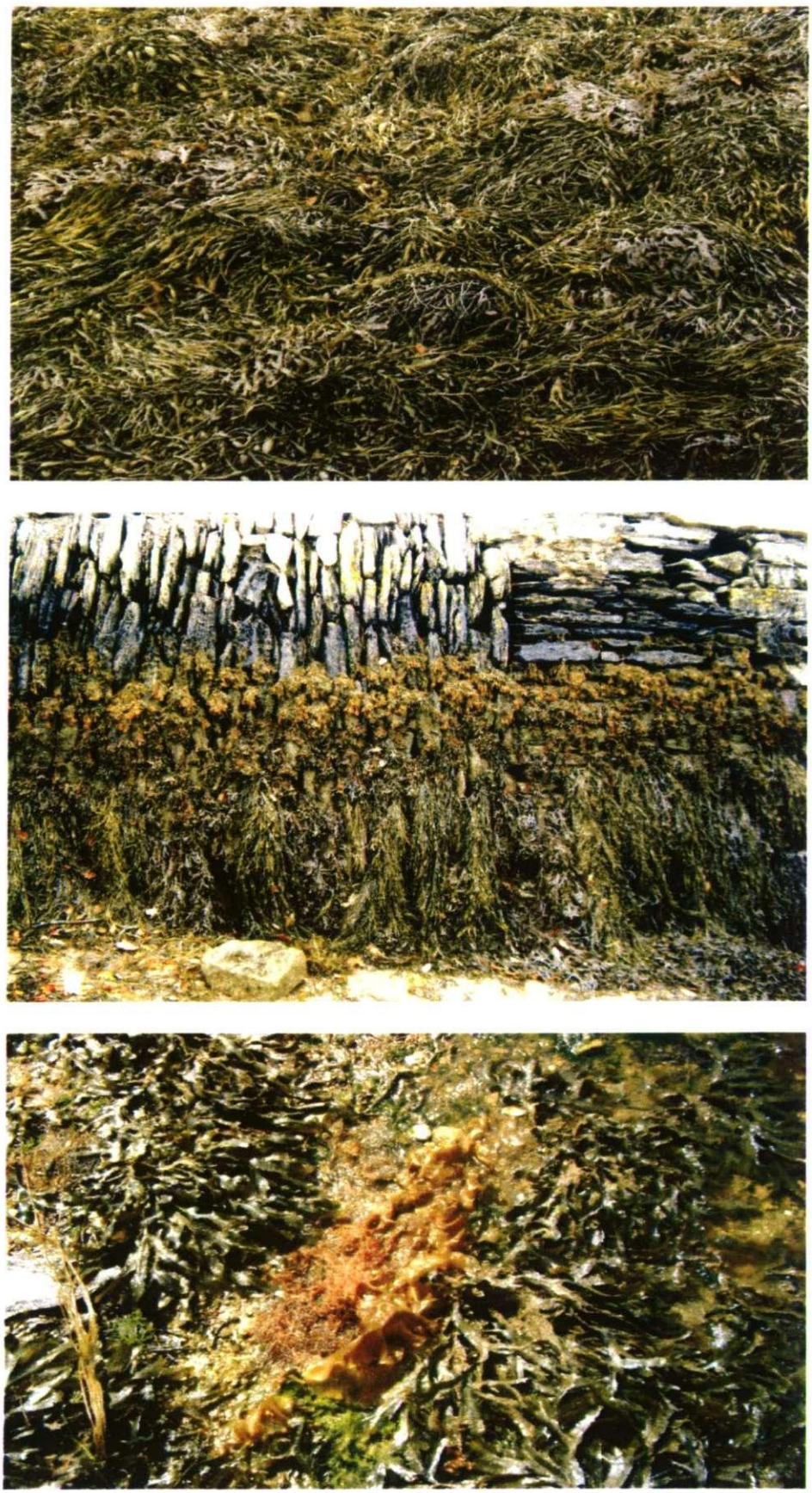

Fig. 2. 5. Relatively limited benthic flora of Restronguet Point. *Ascophyllum nodosum*, *Fucus vesiculosus* and *F. serratus* occupy most of the area.

al. (2003) published 2803 ppm As, 5073 ppm Cu, 3400 ppm Sn, 902 ppm Pb and 6600 ppm Zn in surficial sediments from the Fal Estuary. Compared with the BQ area, the seaweed flora is far less diverse with a few brown algae (*Ascophyllum nodosum*, *Fucus vesiculosus* and *F. serratus*) dominating in this contaminated area (Fig. 2. 5). Compared with those in BQ, frequencies of occurrence of red and green algae were relatively lower and only a few (*Ulva*, *Solieria* and *Rhizoclonium*) were found in RP.

Therefore, Restronguet Creek is an ideal site to study the chronically metal-exposed population of *F. serratus* and to compare the effects and responses with that of a population from the reference site, Bantham Quay, the Avon Estuary.

2. 2. Experimental algae

Vegetative thalli of *Fucus serratus* were collected at spring tide from October 2006 to November 2007. Approximately 1 year-old non-reproductive algae were selected from two sites in South West England with different levels of metal contamination. Algae were transported to the laboratory within 2 hr in the natural seawater from their local habitats.

In the laboratory, materials were rinsed three times in filtered seawater and all visible epiphytes and sediment were removed by brushing. Healthy apical tips (c. 400 mg) were cut from fronds, placed in acid-washed 2 L plastic tanks containing aerated filtered seawater obtained from their natural habitats and maintained for 1 ~ 5 days to recover from cutting and any trauma caused by acclimatisation to laboratory conditions.

2. 3. Culture conditions

Culture conditions were maintained for up to 14 d at 15 °C, 250 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR) and 12 : 12 h light : dark cycle. Light was controlled by cool white fluorescent lamps with a time controller. 100 mL plastic containers were used for the algal culture. Culture media were mixed and aerated by means of a shaker (60 rpm) rather than filtered air injection to avoid contamination by air (Fig. 2. 6). The location of each culture container was changed every 48 hr to reduce any light gradient effect and eliminate any differences in the shakers.

2. 4. Culture medium, Aquil

For experimentation, seaweeds were cultured under the same conditions as described above but in the chemically defined seawater medium, Aquil (Price et al., 1988/89, Gledhill et al., 1997). This artificial medium composition was initially devised for study of trace metals in planktonic algae (Morel et al., 1979) and has been successfully applied for culture of brown macroalgae (Bond et al., 1999, Gledhill et al., 1999, Nielsen, 2002). For the medium, Milli-Q water (Milli-Q water system ZFMQ 230 04, Millipore Corporation, France) and high quality reagent salts (analytical grade) were always used since salts and water are known as the most frequent sources of major trace metal contamination (Morel et al., 1979). To avoid adsorption onto the wall of the medium container and culture vessels or tanks, high density polyethylene (HDPE) polymer or polycarbonate polytetra-fluoroethylene (Teflon) were always used. To minimize metallic contamination, all equipments such as flasks, tanks and pipette tips were washed with acid (5% HCl) for at least 48 hr (Nriagu, 1988), rinsed 2 times in

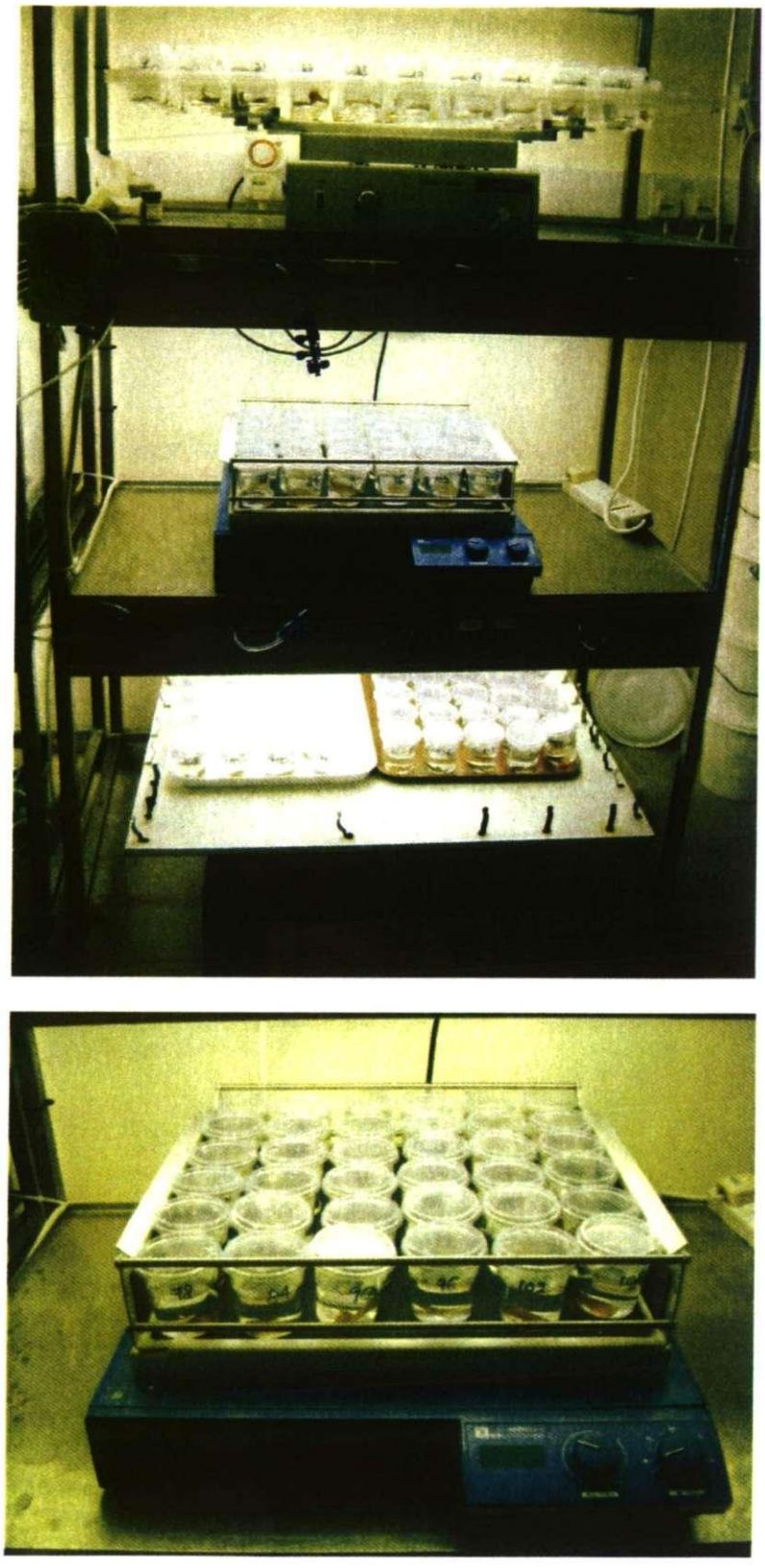

Fig. 2. 6. Culture apparatus in temperature-controlled room.

Distilled water, subsequently rinsed once in ultrapure water and dried in a laminar flow hood.

Chemical composition of Aquil is shown in Table 2. 2. Artificial chelating agents, EDTA, were not added since EDTA is known to prevent metals from being adsorbed by cell wall of algae (Simkiss and Taylor, 1995, Ma et al., 2003). Medium was prepared in three parts, synthetic ocean water (SOW), nutrients and trace metals. For SOW, salts were weighed and kept in a dry place as a powder mixture and anhydrous and hydrated salts were dissolved separately for complete mixing (Harrison et al., 1980, Price et al., 1988/89). Major and minor nutrients of phosphate, nitrate, silicate, etc. were made from the individual stocks (×1000) and then diluted to their final concentrations. Trace metals were also prepared as stock solutions in advance and reagent grade chemicals were dissolved in 0.01 M HCl to reduce impurities. Acid-washed materials (flasks, stoppers, containers, etc.) and Milli-Q water were prepared as described above. The pH was set to 8.2 as the media were prepared. Medium was prepared at least 24 hr before the lab experiment for ionic stabilisation.

2. 5. Metal concentration in medium

For metal exposure, stocks of 2 mM cadmium sulphate hydrate ($3CdSO_4 \cdot 8H_2O$) and 2 mM pentahydrated cupric sulphate ($CuSO_4 \cdot 5H_2O$) were prepared in 0.01 M HCl. Concentrations of Cd between 0 and 10 mg L^{-1} were added for the elevated Cd treatments and those between 0 and 1000 μg Cd L^{-1} were added for lower Cd

Table 2. 2. Composition of Aquil, modified from Morel et al. (1979) and Nielsen (2002)

Constituent	Amount added g L^{-1} Milli-Q water	Concentration in stock (M)	Final concentration in Aquil (M)
Synthetic Ocean Water (SOW)			
NaCl	24.03		4.20×10^{-1}
Na_2SO_4	4.09		2.88×10^{-2}
KBr	0.10		8.40×10^{-4}
KCl	0.70		9.39×10^{-3}
H_3BO_3	0.03		4.85×10^{-4}
$NaHCO_3$	0.20		2.38×10^{-3}
All anhydrated salts were completely dissolved before adding the hydrated salts.			
$CaCl_2 \cdot 6H_2O$	1.51		1.05×10^{-2}
$MgCl_2 \cdot 6H_2O$	11.10		5.46×10^{-2}
Nutrients			
$SrCl_2 \cdot 6H_2O$	1.70	6.38×10^{-2}	6.38×10^{-5}
NaF	0.30	7.14×10^{-2}	7.14×10^{-5}
KI	0.04	2.40×10^{-4}	2.40×10^{-7}
$NaNO_3$	1.70	2.00×10^{-2}	2.00×10^{-5}
NaH_2PO_4	0.276	2.00×10^{-3}	2.00×10^{-6}
Each stock was made individually and 1 mL L^{-1} was added to SOW.			
Trace metals			
Solution 1	g L^{-1} 0.01 M HCl		
$FeCl_3 \cdot 6H_2O$	0.122	4.51×10^{-4}	4.51×10^{-7}
$CuSO_4 \cdot 5H_2O$	0.249	9.97×10^{-4}	9.97×10^{-10}
Solution 2A	g 100 mL^{-1} 0.01 M HCl		
$Na_2MoO_4 \cdot 2H_2O$	2.42	1.00×10^{-1}	1.00×10^{-7}
$CoCl_2 \cdot 6H_2O$	0.595	2.50×10^{-2}	2.50×10^{-8}
Solution 2B	g 100 mL^{-1} 0.01 M HCl		
$ZnSO_4 \cdot 7H_2O$	0.115	4.00×10^{-3}	4.00×10^{-9}
$MnCl_2 \cdot 4H_2O$	0.460	2.30×10^{-2}	2.30×10^{-8}
Solution 2A and 2B at 1 mL L^{-1} were added to each to solution 1. Then solution 1 was added at 1 mL L^{-1} to SOW.			

Treatments, since the previous ranges of Cd were extraordinarily higher than contents of Cd in natural environment. Physiological responses of *F. serratus* to Cd exposure were determined in material exposed to Cd for different time periods: 6 hr, 12 hr, 24 hr, 7 d and 14 d for elevated Cd treatment, and 24 hr, 96 hr and 7 d for lower Cd treatment. The medium was exchanged every 48 h to ensure that the seaweeds did not become nutrient limited.

2. 6. Relative growth rate (RGR)

Relative growth rate (RGR) of fronds was calculated using the following equation based on fresh weight (FW, g). Pieces of *Fucus* thalli washed with filtered seawater to remove mud/sand and epiphytes were blotted dry and weighted. Rates were expressed as percentage (Hunt, 1982).

$$RGR(\%d^{-1}) = \frac{(\ln(m_f) - \ln(m_i))}{t} \times 100$$

where 'm_f' and 'm_i' are masses of the frond (g FW) on the final/initial day of measurement and 't' is time in days/hours between measurements.

2. 7. Statistical analyses

Data were analysed using the statistical package SPSS version 16.0 for Windows (SPSS Inc.). Before all parametric tests, the data were tested for homogeneity of variance and normality (Sokal and Rohlf, 1995). Multivariate test of General Linear Model (GLM) was used for analyzing the effects of locality, metal concentration,

exposure time and their interactions, and Tukey HSD was used for the *Post Hoc* multiple comparisons. When additional analyses were required, One-way ANOVA was performed to check for differences, especially within a population or under certain treatments. In all analyses, differences were considered to be significant at a probability of 5% ($p < 0.05$). Number of replicates in each experiment varied between 3 and 10.

Chapter 3. Bioaccumulation, growth and photosynthetic performances of *Fucus serratus* in response to cadmium stress

3. 1. Introduction

The toxic effects of metals on plants and algae, and the ways by which photosynthetic organisms respond depend on, for example, the metal species, the concentration of metal, length of exposure, and the developmental stage of the organism (Joshi and Mohanty, 2004). Typically, the effects on growth and photosynthesis have been studied to assess the toxicity of metal exposure. This is the approach adopted here and reported in the current chapter. To investigate the effects of Cd on different components of the photosynthetic apparatus, the technique of chlorophyll (Chl) *a* fluorescence has been used. In addition, the accumulation of Cd by the seaweed has been determined, allowing the inter-relationship between bioaccumulation and toxic effect to be discussed.

3. 1. 1. Photosynthetic responses of algae to metal stress

Metals are known to be crucial inhibitors of photosynthesis in higher plants, seagrasses and algae (Clijsters and Van Assche, 1985, Prasad and Strzałka, 1999, MacFarlane and Burchett, 2001). They have been shown to reduce photosynthesis (Rijstenbil et al., 1994, Ralph and Burchett, 1998, Xia et al., 2004), inhibit electron transport in photosystem II (PS II) (Shioi et al., 1978, Yruela et al., 2000), influence activity of PS I and PS II (Joshi and Mohanty, 2004, Kalaji and Loboda, 2007), inhibit photophosphorylation (Clijsters and Van Assche, 1985, Macinnis-Ng and Ralph, 2002), and decrease concentrations of pigments which may be the result of metal-induced inhibition of pigment biosynthesis (especially Chl *a*) (Rijstenbil et al., 1994, Xia et al.,

2004). However, the results of various studies would suggest that the main target of some metal ions (e.g. Cd^{2+}) is on CO_2 fixation as a consequence of the inhibition of Calvin-Benson cycle enzymes (e.g. ribulose-1,5-bisphosphate carboxylase/oxygenase, Rubisco) (Clijsters and Van Assche, 1985, Van Assche and Clijsters, 1990, Krupa and Baszynski, 1995, Joshi and Mohanty, 2004). Cd-related symptoms include the disintegration of chloroplast membrane ultrastructure (i.e. disorganization of the lamellar structure, mainly grana stacks) (Baszyński et al., 1980) and inhibition of protein synthesis (Krupa and Baszynski, 1995, Joshi and Mohanty, 2004).

PS II is regarded as one of the most sensitive components of the photosynthetic apparatus to metal exposure, especially within algae (Mallick and Mohn, 2003). Cd, and several other metals including Pb, Ag, and Zn, are known to inhibit electron flow at the water-splitting site of PS II (see review by Mallick and Mohn, 2003), whereas metals such as Hg, Ni and Cr reportedly cause inactivation of the PS II reaction centre directly and Cu affects more than one site in electron transport chain of the N_2-fixing cyanobacterium, *Anabaena doliolum* (Rai et al., 1995).

Some metals (e.g. Cu, Zn, Cd and Hg) can substitute for the magnesium ion (Mg^{2+}) at the reaction centre of Chl PS II, inhibiting the light harvesting processes of photosynthesis resulting in the decay (bleach) of Chls and ultimately the cessation of photosynthetic activities (Küpper et al., 1996, Küpper et al., 1998, Prasad and Strzałka, 1999, MacFarlane and Burchett, 2001, Küpper et al., 2007). These metal-Chl compounds are thermally unstable and have much lower fluorescence quantum yields than Mg-Chl compounds; therefore, further transfer of energy from the antenna pigment complexes to reaction centres is inhibited (Watanabe et al., 1985, Küpper et al., 1998). Effects on Rubisco are possibly related to the substitution of Mg^{2+} in the ternary enzyme-CO_2-$metal^{2+}$ complex (Clijsters and Van Assche, 1985, Stiborova et al., 1986). The metal-induced indirect effects on primary photochemistry are known to result from

a lower consumption of ATP and NADPH and from a higher thylakoid proton-gradient due to a lower photochemical yield (Krupa et al., 1992, Krupa et al., 1993).

Despite the extensive literature detailing the inhibitory effects of a range of metals on various components of the photosynthetic apparatus in algae and plants, there have been far fewer studies on how Cd affects the photosynthetic machinery of marine macroalgae. Cd is found to disturb the uptake of $^{14}CO_2$ and reduce pigment contents but the harmful effects on marine algae are relatively less studied than other metal ions (such as Cu and Zn) (see review by Rai et al., 1981).

3. 1. 1. 1. Photosynthetic pigments and metal stress

Exposure to some metals (e.g. Cu, Zn, Cd, Hg, and Pb) is known to affect the concentration of photosynthetic pigments (e.g. Chl *a*, Chl *b* and carotenoids) by inhibiting biosynthesis and/or degrading the pigments already synthesised (Clijsters and Van Assche, 1985, Kastori et al., 1998, Prasad and Strzałka, 1999, Macinnis-Ng and Ralph, 2002). For example, enzymes involved in Chl biosynthesis, including δ-aminolevulinic acid (ALA)-dehydratase (EC 4.2.1.24) (Van Assche and Clijsters, 1990) and protochlorophyllide reductase (De Filippis and Pallaghy, 1994), can be inhibited and iron (Fe) depletion or substitution of the central Mg^{2+} can limit light harvesting and result in the decay of Chl molecules (Prasad and Strzałka, 1999). Lipid peroxidation related to changes in membrane permeability and chloroplast ultrastructure by metal stress can decline pigment levels due to the increases in peroxidise activity and the depletion of other antioxidants such as the carotenoids (Dietz et al., 1999). Therefore these increases of peroxides and decreases of photosynthetic pigments may describe the potential phytotoxicity in certain environmental conditions (e.g. metal contaminated estuary) (MacFarlane and Burchett, 2001).

Baker and Walker (1990) reported that the decrease of pigment content is directly related to the reduction in photosynthetic activity and carbon fixation, and, consequently, can be possibly related to all structures and functions at the whole plant level.

3. 1. 1. 2. Effects of Cd on photosynthesis

Though the effects of Cd on the physiology and biochemistry of algae has been reported much less than those of Cu and other metals because of its relative rareness (Lobban and Harrison, 1994), it was reported that Cd uptake has effects on growth, pigment contents, and carbon assimilation in *Ulva lactuca* and *Laminaria saccharina* (Markham et al., 1980a, Markham et al., 1980b). Moreover, Cd inhibits protein synthesis of *L. saccharina* leading to enzyme deficiencies and a series of secondary effects (Kremer and Markham, 1982). Cd is also known to affect Chl biosynthesis, disappearance of granal stacks, PS I and II, degradation of thylakoid acyl lipids, release of some polypeptides associated with the oxygen evolving complex (OEC), disorganisation of OEC and light-harvesting complex II (LHC II) antenna system. Direct effect of Cd on the structure, composition and functionality of PS II has been investigated on thylakoid membranes although Cd exposure is also related to the substitution of Mn bound to OEC, inhibition of some enzymes of the CO_2 assimilation pathway, inhibition of Rubisco activity by decrease of protein biosynthesis or by –SH bond destabilisation, and decreases of the stomata density and conductance in higher plants (see review by Joshi and Mohanty, 2004, Krupa et al., 1993).

Primary photochemistry (F_v / F_m) is known to be relatively stable or there is little change with Cd exposure (Greger and Ögren, 1991, Krupa et al., 1992) whether the thylakoids are exposed to Cd *in situ* or *in vitro* (Baszyński et al., 1980, Krupa et al.,

1987). Cd was reported to increase NPQ slightly (Krupa et al., 1993) but qP was unchanged. Increased NPQ indicates a higher dissipation of energy without fluorescence (Krupa et al., 1993, Skorzynska-Polit and Baszynski, 1997) and stable qP indicates a normal rate of photosynthetic electron transport (Joshi and Mohanty, 2004). Since extremely high concentrations of Cd can decrease F_v / F_m (greater than 50 mg Cd L^{-1} depending on the algal species), the inhibition effect on the photosynthetic electron transport of PS II by Cd stress is regarded as an indirect response to a suppressed Calvin-Benson cycle (Krupa et al., 1993).

3. 1. 2. Chlorophyll *a* fluorescence

Light energy in Chl of plant tissue is known to be used or exhausted in three different ways. Some is used for photosynthetic activity (photochemistry), some is dissipated as heat and the remainder, approximately 1 ~ 2% of total light absorbed by photosynthetic pigments, is re-emitted as light. This re-emitted light from Chl *a* molecules in PS II can be measured by a fluorometer as Chl *a* fluorescence (Maxwell and Johnson, 2000). This can provide information on changes in the efficiency of photochemistry and dissipation as heat since these three processes are inter-related. Moreover Chl *a* fluorescence can be used to elucidate the potential of plants to resist environmental stresses (commonly abiotic stresses) and to determine the damaging effects of such stresses to the photosynthetic apparatus (Maxwell and Johnson, 2000, Sarkar et al., 2004, Strasser et al., 2004).

Fig. 3. 1 shows a typical pattern of Chl *a* fluorescence quenching after tissue has been dark adapted (Table 3. 1 for further information and definition of parameters). Kautsky and co-workers (1960) observed that on transferring dark-adapted plant leaves

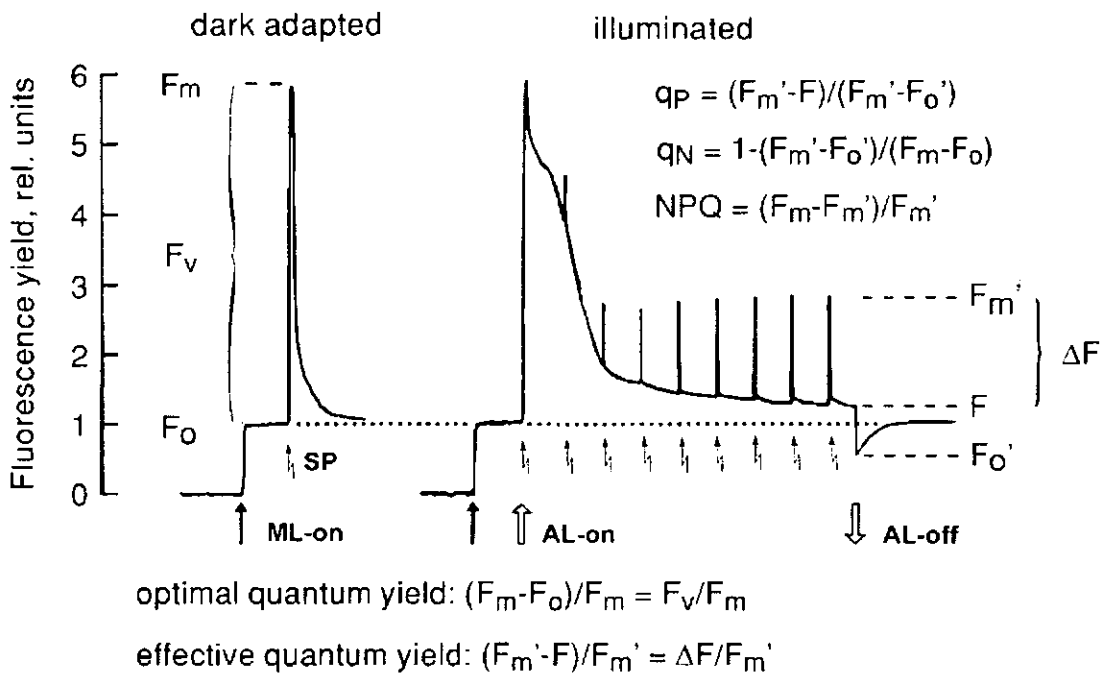

Fig. 3. 1. Typical fluorescence quenching diagram. For further information and definition of parameters, see the text and Table 3. 1. Reproduced after Schreiber et al. (1998).

Table 3. 1. Chlorophyll *a* fluorescence parameters used in this study

Parameter	Measurement	Derivation
Extracted and technical fluorescence parameters		
F_0	Minimal fluorescence level from dark-adapted thalli (t = 50 μs)	
F_0'	Minimal fluorescence level when the thallus is exposed to actinic light and all PS II centres are open	
F_{100}	Fluorescence intensity at 100 μs	
F_{300}	Fluorescence intensity at 300 μs	
F_J	Fluorescence intensity at J-step (at 2 ms)	
F_I	Fluorescence intensity at I-step (at 30 ms)	
F_m	Maximal fluorescence level from dark-adapted thalli	
F_m'	Maximal fluorescence level from thalli in light	
F_v	The dark-adapted variable fluorescence	$= F_m - F_0$
F_v / F_0	The potential activity of PS II	$= (F_m - F_0) / F_0$
F_s	Steady state fluorescence yield	
T_{Fm}	The time needed to reach F_m	
Area	Area above fluorescence curve between F_0 and F_m	$= \int_0^{T_{F_{max}}} (F_m - F_s)dt$
Quantum efficiencies or flux ratios		
qP	The coefficient of photochemical quenching	$= (F_m' - F_s) / (F_m' - F_0')$
NPQ	Non-photochemical quenching	$= (F_m - F_m') / F_m'$
F_v / F_m	The maximum quantum efficiency of PS II	$= (F_m - F_0) / F_m$ $= \Phi_{PSII} / qP$
Φ_{PSII} (=$\Delta F/F_m'$)	Quantum efficiency of PS II, actual quantum yield of PS II	$= (F_m' - F_s) / F_m'$

Table 3. 1. Continues.

Parameter	Measurement	Derivation
Phenomenological fluxes or phenomenological activities		
ABS / CS	Effective antenna size of a cross section (CS). Expresses the total number of photons absorbed by Chl molecules per CS	$\approx F_0$
TR_0 / CS	Maximal trapping rate of PSII. Describes the maximal rate by which an excitation is trapped by the CS (at t = 0)	$= \varphi_{P0}$ (ABS / CS_0)
ET_0 / CS	Electron transport flux per CS (at t = 0)	$= \varphi_{P0} \cdot \psi_o \cdot$ (ABS / CS_0)
DI_0 / CS	Effective dissipation per CS (at t = 0)	= (ABS/CS) – (TR_0/CS)
Density of reaction centres		
RC / CS_0	Gives the number proportional to the active reaction centres to the cross-section of the measured sample (t = 0)	$= \varphi_{P0}$ / (V_J / M_0) F_0
RC / CS_m	Gives the number proportional to the active reaction centres to the cross-section of the measured sample (t = m)	$= \varphi_{P0}$ / (V_J / M_0) F_m
Performance index or vitality index		
PI_{ABS}	Performance index, which explains photosynthetic efficiency	= (RC/ABS)[φ_{P0}/(1- φ_{P0})][Ψ_0/(1- Ψ_0)]

* Note: $\varphi_{Po} = TR_0 / ABS = 1 - (F_0 / F_m)$
$\psi_o \equiv ET_0/TR_0 = 1 - V_J$
$V_J = (F_{2ms} - F_0) / (F_m - F_0)$
$M_0 = TR_0 / RC - ET_0 / RC$
PFD (photon flux density) is absorbed light (μmol photon m^{-2} s^{-1}) (measured using an integrating sphere).
0.5 is a factor that accounts for the partitioning of energy between PS II and PS I.

into the light there is a rapid rise in fluorescence emission termed the Kautsky effect. Any additional electrons are not accepted by PS II (particularly plastoquinone A, Q_A) until the previously accepted one is passed to a subsequent electron carrier (plastoquinone B, Q_B). This 'close' state of the reaction centre causes reduction of the efficiency of photochemistry and increment of the yield of Chl *a* fluorescence (Maxwell and Johnson, 2000). However this rapid increase of fluorescence is usually followed by the fall termed fluorescence quenching. This phenomenon was explained as (1) the increased rate of electrons transported from PS II (photochemical quenching) and (2) the increased efficiency of energy converted to heat (non-photochemical quenching, NPQ).

Electron transport in plant thylakoids can be inhibited by metal ions either by (1) preventing electron flow from H_2O to $NADP^+$/methyl-viologen or (2) disturbing light trapping complex and membrane structure (Tripathy and Mohanty, 1980, Joshi and Mohanty, 2004).

3. 1. 2. 1. Photochemical processes

Photochemical processes can be measured by the following three parameters: Φ_{PSII}, the efficiency of PS II photochemistry; qP, photochemical quenching; F_v / F_m, the maximum quantum efficiency of PS II in dark adapted tissue (Maxwell and Johnson, 2000).

$\Phi_{PSII} = (F_m' - F_s) / F_m'$

$qP = (F_m' - F_s) / (F_m' - F_o')$

$F_v / F_m = (F_m - F_o) / F_m$

Φ_{PSII}, sometimes expressed in a different way as $\Delta F / F_m'$, represents the proportion of absorbed light energy by Chl in PS II, which evaluates the rate of linear electron

transport and consequently overall photosynthesis (Maxwell and Johnson, 2000). Photochemical quenching (qP) describes the proportion of 'opened' reaction centres in PS II. Some researchers use an alternative term, 1 – qP (excitation pressure) which gives the proportion of 'closed' reaction centres in PS II (Maxwell et al., 1994). Changes in qP are related to the closed reaction centres saturated by light. F_v / F_m, the intrinsic efficiency of PS II, is one of the most widely used parameters and provides information on the maximum quantum efficiency when all PS II centres were open. Changes in F_v / F_m are related to changes in the efficiency of non-photochemical quenching. A value of about 0.83 is considered optimal for most plant species (Björkman and Demmig, 1987, Johnson et al., 1993) and lower values indicate a stress exposed state, particularly photoinhibition (Maxwell and Johnson, 2000).

3. 1. 2. 2. Non-photochemical processes

NPQ and qN determine changes of heat dissipation related to the dark-adapted state. They may increase as a consequence of processes that protect the plant from light-induced damage or of the damage itself (Maxwell and Johnson, 2000). NPQ commonly varies in the range of 0.5 ~ 3.5 at saturating light although the values can be widely altered depending on plant species, physiological and environmental state of the plants (0 ~ inifinity).

$NPQ = (F_m - F_m') / (F_m - F_0')$

where F_0 and F_m are minimal and maximal fluorescence levels in dark-adapted thalli and F_0' and F_m' are minimal and maximal fluorescence levels from thalli in light.

Another parameter used for quantifying non-photochemical quenching is qN, varying 0 ~ 1 (Van Kooten and Snell, 1990); however, this term is no longer widely used and was not measured in the present study. Neither parameter is recommended for

direct comparisons between plants with different histories or plants of different species which may have distinctly different values of F_v / F_m (Maxwell and Johnson, 2000).

3. 1. 2. 3. O-J-I-P test

The JIP-test analysis is a relatively new technique for assessing and understanding the stress response of plants by analysing the polyphasic rise of the Chl *a* fluorescence transient (OJIP phases, Fig. 3. 2) (Strasser and Strasser, 1995, Tsimilli-Michael et al., 1995, Tsimilli-Michael et al., 1996, Strasser et al., 2004). Fig. 3. 2 shows a typical Chl *a* fluorescence transient curve which is plotted on a logarithmic time scale. The Chl *a* fluorescence transient of dark-adapted plant rises from F_0 to F_p (= F_m) by a red saturating light and on a logarithmic time scale the transient presents fast polyphasic behaviour (Strasser et al., 2000). F_0 represents fluorescence at 50 μs when all reaction centres of PS II are open and F_p reveals fluorescence under saturating excitation light when all reaction centres of PS II are closed. A fluorometer with high time resolution (10 μs), such as the Handy PEA (Hansatech, England), can measure fluorescence values at 50 μs (F_0, step O), 100 μs (F_{100}), 300 s (F_{300}), 2 ms (Step J), 30 ms (Step I) and maximal (F_m, step P) (Strasser et al., 1995, Bussotti et al., 2007).

This new tool is relatively easy and fast to use, providing repeatable and robust data (Reddy and Strasser, 2000, Bussotti et al., 2007). The transient curve is derived from the physiological state of the plant or alga when exposed to different environmental condition. Therefore, the JIP-test can help the understanding of the 'Structure-Function' relationship of photosynthetic organisms (Reddy and Strasser, 2000).

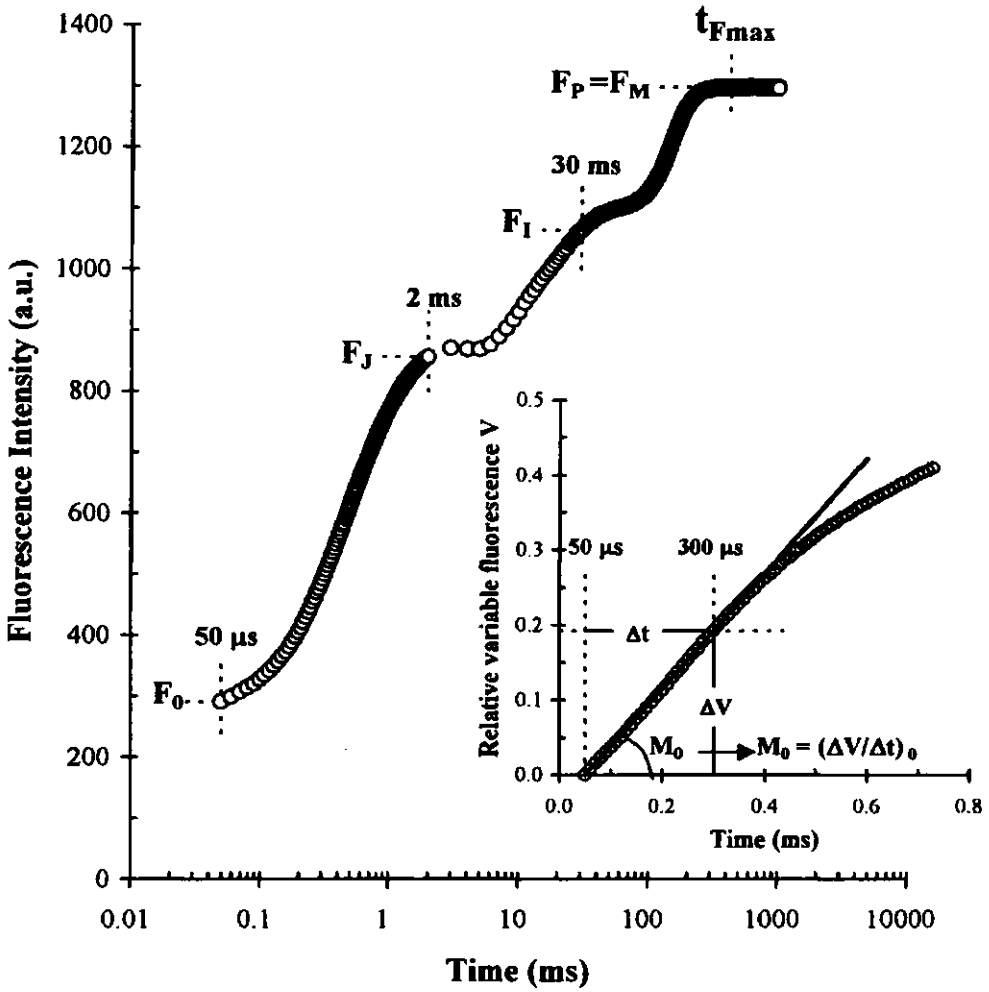

Fig. 3. 2. Typical Chl *a* fluorescence transient O-J-I-P curve, plotted on a logarithmic timescale from 50 µs to 1 s. Each point indicates the fluorescence intensity at the time: the fluorescence intensity F_0 (at 50 µs); the fluorescence intensities F_J (at 2 ms) and F_I (at 30 ms); the maximal fluorescence intensity $F_P = F_m$ (at T_{Fm}). The inserted graph represents the relative variable fluorescence on a linear timescale, from 50 µs to 0.8 ms. Reproduced after Tsimilli-Michael et al. (2000).

The JIP-test was developed from the theory of energy flow in thylakoid membranes (Strasser et al., 2004, Romanowska-Duda et al., 2005, Kalaji and Loboda, 2007). The theory of equilibrium between the inflow and outflow of energy for photosynthetic pigments can provide information on the probability of the fate of the absorbed energy (Kalaji and Loboda, 2007). These indices calculated by the JIP-test are termed 'specific' and 'phenomenological' parameters and include absorption (ABS), trapping (TR), electron transport (ETR) per reaction centre (RC) or measured area of sample (cross section, CS) and performance index (PI) (Kalaji and Loboda, 2007).

3. 1. 2. 4. Applications of Chl *a* fluorescence and fluometry

Chl *a* fluorescence has been applied extensively to studies in agronomy, forestry, marine environment, ecotoxicology, plant physiology and plant breeding (Ciscato et al., 1999, Chaerle and Van Der Straeten, 2000, Strasser et al., 2000, DeEll and Toivonen, 2003, Christen et al., 2007). This analytical tool has been employed to understand the structure and function of the chloroplasts, and biotic and abiotic factors affecting photosynthetic yield: e.g. drought (Haupt-Herting and Fock, 2000, Bukhov and Carpentier, 2004), light intensity (Haldimann et al., 1996, Bruce and Vasil'ev, 2004), nutrient deficiency (Morales et al., 2000), temperature (Fracheboud et al., 1999), herbicides or air pollutants toxicity (Pfündel, 2003, Popovic et al., 2003, Dewez et al., 2008), and herbivores (Zangerl et al., 2002, Tang et al., 2006). Moreover, this measurement has been applied to measure algal biomass (Mallick and Mohn, 2003) and physiological responses of plants (Joshi and Mohanty, 2004) and to monitor environmental pollution especially based on metal ion toxicity in plants (Sgardelis et al., 1994). From the 1990s research into Chl *a* fluorescence transients has been used to assess toxic metal stress on photosynthetic responses of seagrasses and algae (Ralph and

Burchett, 1998). Varying results have been reported due to the capacity to de-activate metal ions, the chemical composition and physiological state of the algae, species specificity, ecotypes and exposed algal stage of life history (Boyle, 1984, Strömgren, 1980, Davies, 1976, Jensen et al., 1974, Strömgren, 1979).

The recent frequent use of this technique was made possible by the introduction of Chl fluorometers, such as the pulse amplitude modulation (PAM) fluorometry or the non modulated fluorimeter (plant efficiency analysis, PEA). They make it possible to measure the fluorescence yield and to investigate fluorescence quenching processes (Mallick and Mohn, 2003). These instruments have been evaluated as sensitive and non-invasive for studying the photosynthetic efficiency of plants especially for the responses to metal stresses (Küpper et al., 1996, Küpper et al., 1998, Macinnis-Ng and Ralph, 2002) since the extraction of photosynthetic pigments is not required (Küpper et al., 1996, Küpper et al., 1998).

3. 1. 3. Purpose of this study

Brown algae are known to be very resistant to abiotic environmental stresses. Various approaches to measure physiological responses of *Fucus serratus* and *F. vesiculosus* to cupric ion have been reported: growth, respiration, Chl fluorescence, oxygen evolution, Chl *a* content, Cu accumulation, early development, osmoregulation, inheritance of the tolerance, etc. However, our knowledge on responses of *F. serratus* to Cd exposure is yet limited. Therefore, *Fucus serratus*, collected from both the contaminated and the clean sites in South West England, was exposed to Cd under controlled laboratory conditions and the effects of Cd on accumulation, growth and photosynthesis were investigated.

3. 2. Materials and methods

3. 2. 1. Plant material and culture condition

Fucus serratus was prepared as described in Chapter 2.

3. 2. 2. Culture medium, Aquil, and Cd concentration

The medium and metal concentrations were prepared as described in Chapter 2.

3. 2. 3. Determination of total and non-exchangeable accumulation of Cd

Cultured algae (ca. 100 mg fresh weight) were prepared in two different ways for 1) total (non-exchangeable + exchangeable) and 2) internal (non-exchangeable) metal/metalloid contents. To determine the total concentration of metals/metalloids in the samples, *Fucus* thalli were washed three times with ultrapure water and blotted dry with filter paper. To discriminate between extracellular sorption and intracellular uptake of metals/metalloids, duplicate seaweed samples, from the same thalli for total contents determination, were subjected to sequential chemical treatment (EDTA and ultrapure water treatment) prior to digestion by nitric acid (HNO_3) (Vasconcelos and Leal, 2001). Materials were washed with 5 mM EDTA three times (10 minutes each), rinsed twice with ultrapure water and blotted dry with filter paper.

Washed and dried materials were frozen at -20°C overnight and freeze-dried (Super Modulyo freeze-drier; Girovac, United Kingdom). After weighing (mg), the

freeze-dried samples were placed in Teflon vessels with 3 mL 70% nitric acid and digested in a microwave (CEM-2000; CEM Microwave Technology, United Kingdom) at 2 kW for 30 min. Digests were then transferred to borosilicate volumetric flasks and diluted to a volume of 25 mL with ultrapure water ready for analyses. Cd, Cu, Pb, Zn and As contents were determined by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS, PlasmaQuad PQ2+ Turbo, Thermo Elemental, Winsford, Cheshire, UK). Metal/metalloid standards were made using certified standard solutions (Merck, UK), acidified to the same pH as the samples with HNO_3. Results (total and non-exchangeable concentrations of metals/metalloids) are expressed as means ± standard deviations of three replicates.

3. 2. 4. Physiological responses of *Fucus serratus* to the presence of Cd

To observe the effect of Cd on the physiology of *F. serratus*, growth and photosynthetic performances were monitored for different time periods at a range of external concentrations. Algae were exposed to either 0 ~ 10 mg Cd L^{-1} for 6 h, 12 h, 24 h, 7 d and 14 d or 0 ~ 1000 μg Cd L^{-1} for 24 hr, 96 hr and 7 d.

3. 2. 4. 1. Relative growth rate (RGR)

Algal growth rates were determined as RGR based on fresh biomass as described in Chapter 2.

3. 2. 4. 2. Chlorophyll *a* fluorescence for measuring photosynthetic performance

Changes in fluorescence emission were reported to be related to changes in photosynthetic rate (Baker and Oxborough, 2004). Chl *a* fluorescence was measured using a Handy PEA (Handy-Plant Efficiency Analyser, Hansatech Instruments LTD,

King's Lynn, Norfolk, U.K.) for algae exposed to 0 ~ 10 mg Cd L^{-1} and a pulse amplitude modulated fluorescence monitoring system (PAM FMS, Hansatech Instruments LTD, Norfolk, U.K.) for algae exposed to 0 ~ 1000 μg Cd L^{-1}. The measured parameters used in this study are given in Table 3. 1. For some parameters, e.g. F_0, F_m, F_v, F_v / F_0 and F_v / F_m, thalli were dark adapted for ca. 20 minutes prior to taking measurements.

3. 2. 4. 3. Photosynthetic pigment analysis

Determination of photosynthetic pigment of *F. serratus* followed the protocols outlined by Evans (1988) (Fig. 3. 3). Because of their photosynthetic activities under the light condition and unstable characters to air (oxidation), analysis of pigments was carried out under minimal exposure to air and light. During analyses, a thick black cotton cloth was used to keep them under dark (at least dim) conditions. All solutions were kept in brown glass bottles and stored in the fridge. Freshly opened methanol and acetone (analytical grade) were used.

After harvesting, Cd exposed samples (0.06 ~ 0.2 g FW) were rinsed under running tap water to remove any remaining Aquil medium and Cd, flushed with liquid nitrogen immediately and stored at -20° in plastic tubes covered by □luminium foil until they were analysed.

Seaweed samples were ground to a powder with a pestle and mortar using liquid nitrogen on ice and then extracted in dimethyl sulfoxide (DMSO) three times for a total of 25 min (final volume 1 mL). At each step the mixtures were well shaken, and then centrifuged to collect the extracts. The concentrations of pigments in the extract were

determined spectrophotometrically (Unicam Heλios β UV-VIS spectrophotometer, Spectronic Unicam, Cambridge, U.K.) using the following formulae (a) (Evans, 1988).

(a) DMSO Extract

$$[Chl\ a] = A_{665} / 72.8$$

$$[Chl\ c] = (A_{631} + A_{582} - 0.297\ A_{665}) / 61.8$$

$$[Fx] = (A_{480} - 0.722\ (A_{631} + A_{582} - 0.297\ A_{665}) - 0.049\ A_{665}) / 130$$

where Chl *a*, chlorophyll *a*; Chl *c*, chlorophyll *c*; Fx, fucoxanthin.

To ensure complete recovery of pigments, the seaweed samples were further extracted in acetone. The remaining samples were extracted in acetone four to five times for 20 ~ 30 minutes (total acetone volume 2.4 ~ 3 mL). After this treatment, *Fucus* samples were white and acetone extracts were clear. The acetone extracts were combined, and 1 mL hexane and 1 mL distilled water were added to it and then gently swirled.

The upper hexane phase and the lower aqueous phase were separated with a pipette. The hexane phase was washed with an equal volume of 80% methanol (2 ~ 3 times) until the washing was colourless and the methanol washing was added to the acetone extract. The combined reagent was measured spectrophotometrically with a glass cuvette using the following formulae (b) (Evans, 1988).

(b) Acetone : Methanol : Water extract

$$[Chl\ a] = A_{664} / 73.6$$

$$[Chl\ c] = (A_{631} + A_{581} - 0.300\ A_{664}) / 62.2$$

$$[Fx] = (A_{470} - 1.239\ (A_{631} + A_{581} - 0.300\ A_{664}) - 0.0275\ A_{664}) / 141$$

Next, the previously separated hexane phase was diluted with fresh acetone (10 mL) and measured spectrophotometrically using a glass cuvette (formulae (c)) (Evans, 1988).

(c) Acetone : Hexane Extracts

$$[Chl\ a] = A_{661} / 83.3\ (or\ A_{615} / 15.4)$$

$$[\beta\text{-}carotene] = (A_{480} - 0.033\ A_{661}) / 193$$

Each value represents a concentration of pigment in each different volume of reagent. All the values were combined together after conversion to the same unit, which shows total pigment contents in *F. serratus* thalli.

3. 2. 5. Statistical tests

Statistical tests were performed as described in Chapter 2 using SPSS (for windows, version 16.0, SPSS Inc.). Three replicates were used for determination of metal concentrations and photosynthetic pigments and six ~ ten replicates were used for statistical analyses of photosynthetic performances and growth.

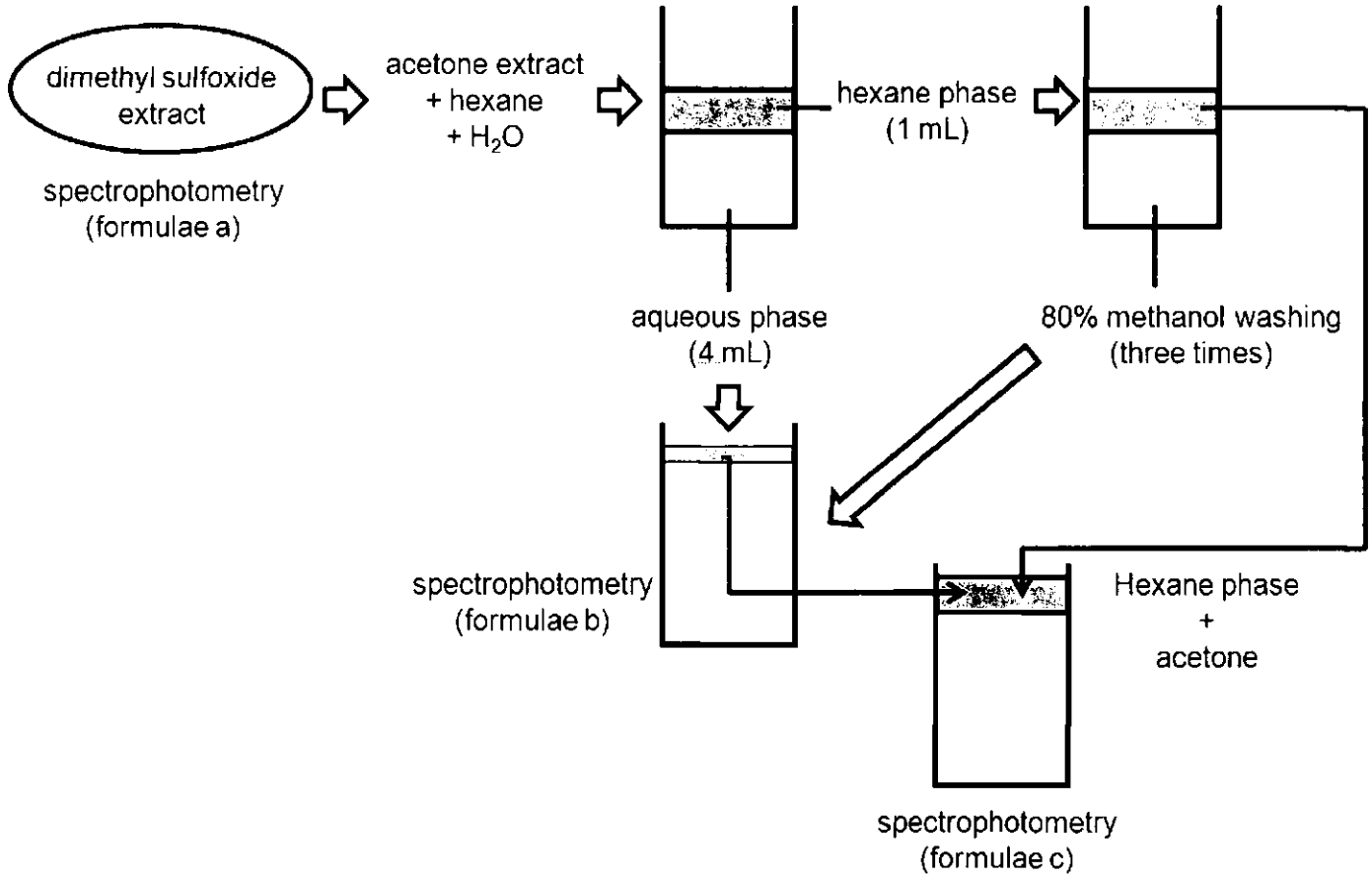

Fig. 3. 3. Protocol for pigment extraction and separation. Modified from Evans (1988).

3. 3. Results

3. 3. 1. Metal concentrations in natural thalli of *Fucus serratus*

The non-exchangeable and total concentrations of metals in *F. serratus* collected from Restronguet Point (RP) and Bantham Quay (BQ) are tabulated in Table 3. 2. The seaweed samples collected from RP had significantly ($p < 0.001$) higher concentrations of Cu, Zn and Pb than those from BQ, whereas the concentration of Cd in samples from the two sites was the same ($p > 0.05$).

Most of the accumulated metals were found in the non-exchangeable fraction, although the proportions varied between sites (Table 3. 2). Approximately 74.7% of Cd, 87.9% of Zn, 100% of Cu and 60.9 % of Pb were found intracellularly in samples from RP while 49.3% of Cd, 68.4% of Zn, 86.0% of Cu and 75.9% of Pb were found within cells in BQ samples.

3. 3. 2. Metal concentrations of *Fucus serratus* exposed to higher and extended Cd stress

Accumulated metal contents were analysed with *F. serratus* exposed to 0 ~ 10 mg Cd L^{-1} for 7 and 14 d (Table 3. 3 ~ 3. 4, Fig. 3. 4). Description of metal contents is focused on Cd concentration, since Cu, Zn and Pb were not additionally supplied except for essential levels in Aquil media (Table 2. 1).

In both populations, total Cd contents increased with increasing Cd concentrations in the medium ($p < 0.0001$) as well as with time of Cd exposure ($p =$

0.012) (Table 3. 3, Fig. 3. 4). No significant difference was discovered between total Cd contents of both populations ($p > 0.05$). Above 50% of the total Cd was found in the non-exchangeable fraction within the cells in both populations. The highest ratio between non-exchangeable and total Cd concentration was in cells of the control treatments and the percentages decreased with increasing Cd contents in the media as well as exposure time.

Non-exchangeable Cd fraction showed the same pattern to total Cd contents. Non-exchangeable Cd concentrations were enhanced by increasing Cd concentrations in the medium ($p < 0.0001$) as well as time of exposure ($p = 0.005$) regardless of the population (Table 3. 4). Both the polluted and the reference populations showed the similar non-exchangeable Cd concentrations at 7 and 14 d ($p > 0.05$) (Fig. 3. 4).

Levels of other measured metals (Cu, Zn and Pb) were significantly higher at RP than BQ (Table 3. 3 ~ 4). Changes in total and non-exchangeable concentrations of metals did not show a pattern of increase/decrease with Cd concentration or time of exposure.

Table 3. 2. The concentrations (μmol g^{-1} DW) of non-exchangeable and total metals in *Fucus serratus* collected from Restronguet Point and Bantham Quay in May 2007. Data are expressed as means ± S. D. (n = 3). RP, Restronguet Point; BQ, Bantham Quay.

	Location	Cu (μmol g^{-1} DW)	Zn (μmol g^{-1} DW)	Pb (μmol g^{-1} DW)	Cd (μmol g^{-1} DW)	Total metal ions (μmol g^{-1} DW)
Non-exchangeable	RP	0.9143 ± 0.1232	3.1070 ± 0.2399	0.0042 ± 0.0002	0.0049 ± 0.0010	4.3424 ± 0.3157
	BQ	0.0413 ± 0.0166	0.2907 ± 0.1245	0.0022 ± 0.0015	0.0034 ± 0.0004	0.3376 ± 0.1430
Total	RP	1.2236 ± 0.0739	3.5336 ± 0.4897	0.0069 ± 0.0009	0.0062 ± 0.0009	4.4583 ± 0.6140
	BQ	0.0480 ± 0.0033	0.4252 ± 0.0464	0.0029 ± 0.0002	0.0069 ± 0.0002	0.4830 ± 0.0501

Table 3. 3. The concentrations (μmol g^{-1} DW) of total metals in *Fucus serratus* after cadmium exposure for 7 and 14 d. Data are expressed as means ± S. D. (n = 3). RP, Restronguet Point; BQ, Bantham Quay.

Location	Time	Cd (mg L^{-1})	Cu (μmol g^{-1} DW)	Zn (μmol g^{-1} DW)	Pb (μmol g^{-1} DW)	Total metal ions (μmol g^{-1} DW)
RP	7 d	0	0.94 ± 0.13	4.24 ± 0.41	0.011 ± 0.006	5.191 ± 0.546
		1	0.94 ± 0.16	4.16 ± 1.33	0.011 ± 0.008	5.111 ± 1.498
		5	0.98 ± 0.17	3.78 ± 1.10	0.007 ± 0.005	4.767 ± 1.275
		10	0.63 ± 0.24	3.75 ± 1.31	0.007 ± 0.006	4.387 ± 1.556
	14 d	0	0.58 ± 0.35	3.37 ± 1.63	0.011 ± 0.008	3.961 ± 1.988
		1	0.71 ± 0.14	4.21 ± 1.15	0.011 ± 0.003	4.931 ± 1.293
		5	0.64 ± 0.37	3.71 ± 1.88	0.008 ± 0.008	4.358 ± 2.258
		10	0.71 ± 0.08	3.79 ± 0.30	0.011 ± 0.002	4.511 ± 0.382
BQ	7 d	0	0.12 ± 0.01	0.59 ± 0.04	0.006 ± 0.001	0.716 ± 0.051
		1	0.13 ± 0.04	0.59 ± 0.20	0.005 ± 0.001	0.725 ± 0.241
		5	0.19 ± 0.09	0.64 ± 0.51	0.006 ± 0.003	0.836 ± 0.603
		10	0.10 ± 0.06	0.47 ± 0.04	0.006 ± 0.005	0.576 ± 0.105
	14 d	0	0.13 ± 0.04	0.78 ± 0.15	0.003 ± 0.000	0.913 ± 0.190
		1	0.09 ± 0.00	0.61 ± 0.29	0.005 ± 0.004	0.705 ± 0.294
		5	0.11 ± 0.02	0.44 ± 0.04	0.004 ± 0.002	0.554 ± 0.062
		10	0.09 ± 0.00	0.62 ± 0.16	0.006 ± 0.003	0.716 ± 0.163

Table 3. 4. The concentrations (μmol g^{-1} DW) of non-exchangeable metals in *Fucus serratus* after cadmium exposure for 7 and 14 d. Data are expressed as means ± S. D. (n = 3). N.D. represents not detected. RP, Restronguet Point; BQ, Bantham Quay.

Location	Time	Cd (mg L^{-1})	Cu (μmol g^{-1} DW)	Zn (μmol g^{-1} DW)	Pb (μmol g^{-1} DW)	Total metal ions (μmol g^{-1} DW)
RP	7 d	0	0.45 ± 0.03	3.67 ± 1.30	0.006 ± 0.003	4.126 ± 1.333
		1	0.65 ± 0.28	3.92 ± 1.06	0.008 ± 0.005	4.578 ± 1.345
		5	0.64 ± 0.19	1.94 ± 1.40	0.004 ± 0.007	2.584 ± 1.597
		10	0.57 ± 0.07	2.46 ± 0.53	N.D.	3.030 ± 0.600
	14 d	0	0.45 ± 0.12	2.53 ± 0.52	0.001 ± 0.003	2.981 ± 0.643
		1	0.65 ± 0.14	3.49 ± 0.75	0.003 ± 0.003	4.143 ± 0.863
		5	0.53 ± 0.16	3.24 ± 1.14	0.007 ± 0.006	3.777 ± 1.306
		10	0.60 ± 0.16	3.37 ± 0.71	0.002 ± 0.002	3.972 ± 0.872
BQ	7 d	0	0.05 ± 0.01	0.49 ± 0.09	0.001 ± 0.001	0.541 ± 0.101
		1	0.04 ± 0.03	0.41 ± 0.08	0.003 ± 0.002	0.453 ± 0.112
		5	0.07 ± 0.01	0.51 ± 0.09	0.012 ± 0.016	0.592 ± 0.116
		10	0.08 ± 0.02	0.44 ± 0.10	0.002 ± 0.001	0.522 ± 0.031
	14 d	0	0.07 ± 0.01	0.75 ± 0.10	0.003 ± 0.000	0.148 ± 0.020
		1	0.08 ± 0.02	0.36 ± 0.04	0.002 ± 0.001	0.442 ± 0.061
		5	0.08 ± 0.01	0.30 ± 0.02	0.004 ± 0.002	0.384 ± 0.032
		10	0.08 ± 0.02	0.33 ± 0.06	0.003 ± 0.002	0.413 ± 0.082

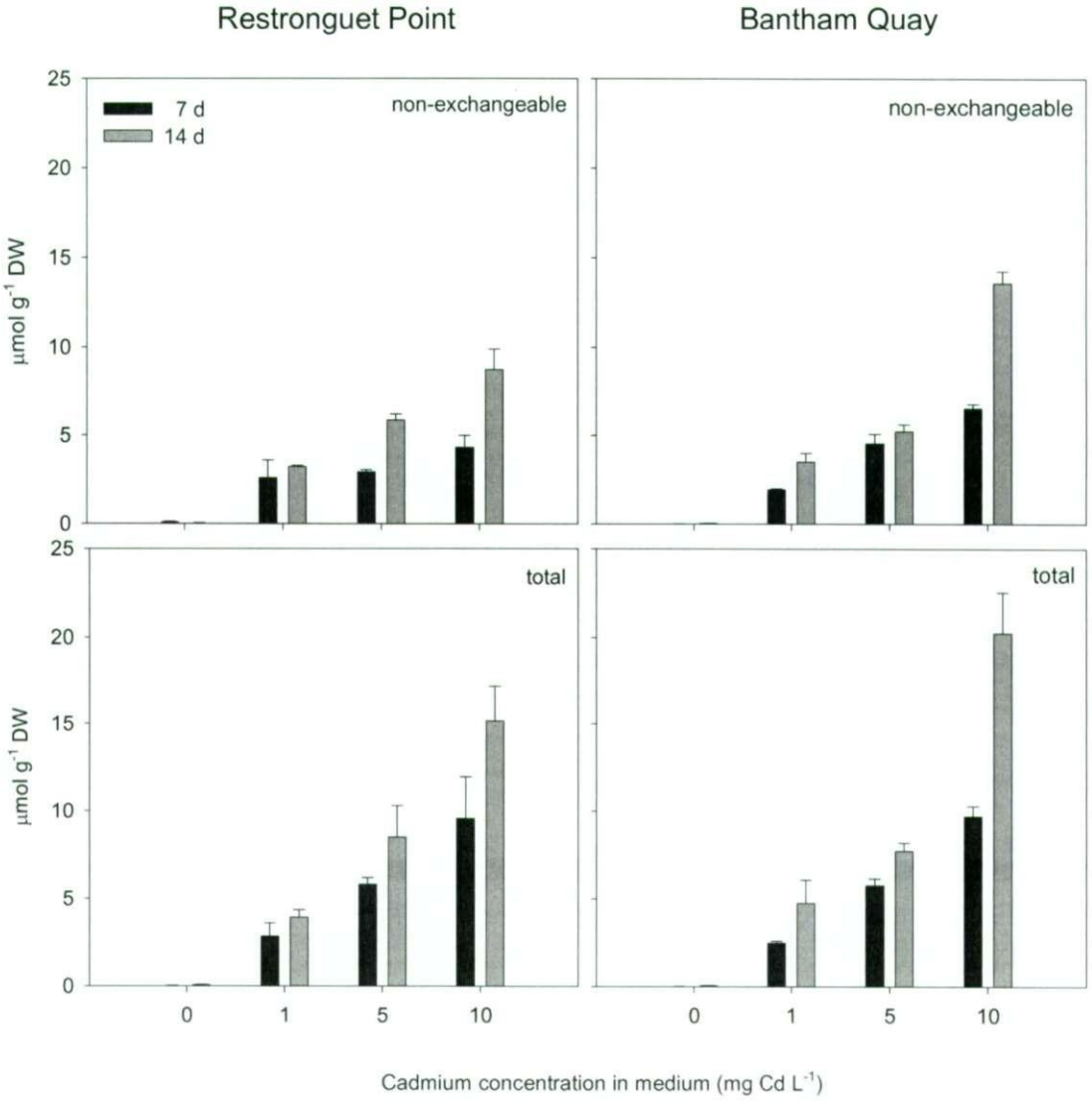

Fig. 3. 4. Total and non-exchangeable cadmium concentrations in *Fucus serratus* from Restronguet Point and Bantham Quay exposed to a range of cadmium concentrations for 7 days and 14 days. Values are means and standard deviations (n = 3).

3. 3. 3. Metal concentrations of *Fucus serratus* exposed to lower and shorter Cd stress

Accumulated metal concentrations of *F. serratus* which was exposed to lower Cd doses (0 ~ 1000 μg Cd L^{-1}) for shorter period (24 hr, 96 hr and 7 d) were tabulated in Tables 3. 5 ~ 3. 8 and Fig. 3. 5. In this experiment likewise, only changes in Cd levels were focused since other metals were kept at the levels in the medium. Mean concentrations of total and non-exchangeable Cd of both populations significantly increased with time of Cd exposure ($p = 0.015$ for total; $p = 0.017$ for non-exchangeable) and Cd concentration in the medium ($p < 0.0001$ for both non-exchangeable and total concentrations, Fig. 3. 5). However locality did not affect total Cd concentration. Both the contaminated and the reference populations revealed similar levels of total Cd concentrations at each 24 hr, 96 hr and 7 d ($p > 0.05$). Difference by locality was shown with non-exchangeable Cd concentration at 24 hr. Internal Cd contents of the RP population exposed to Cd for 24 hr were significantly lower than the BQ population. However, except this 24 hr treatment, above 50% of Cd was accumulated intracellularly in both populations.

The RP population accumulated significantly higher levels of Cu, Zn and Pb (Table 3. 5 ~ 3. 8). Total and non-exchangeable concentrations of these three metals were significantly higher at the RP population. Total and non-exchangeable concentrations of As were not different from each population. In the mean time, changes of Cu, Zn, Pb and As were not related to the Cd concentration or culture period (time of Cd exposure).

Table 3. 5. The concentrations (μmol g^{-1} DW) of total metals in *Fucus serratus* from Restronguet Point after cadmium exposure for 24 hr, 96 hr and 7 d. Data are expressed as means ± S.D. (n = 3). RP, Restronguet Point.

Location	Time	Cd (μg L^{-1})	Cu (μmol g^{-1} DW)	Zn (μmol g^{-1} DW)	Pb (μmol g^{-1} DW)	As (μmol g^{-1} DW)	Total metal/metalloids (μmol g^{-1} DW)
RP	24 hr	0	0.94 ± 0.12	3.64 ± 0.46	0.029 ± 0.019	0.29 ± 0.07	5.160 ± 0.669
		10	1.46 ± 0.21	3.79 ± 1.16	0.026 ± 0.004	0.35 ± 0.06	5.626 ± 1.434
		100	0.99 ± 0.33	4.14 ± 0.56	0.029 ± 0.025	0.29 ± 0.05	5.449 ± 0.965
		1000	1.38 ± 0.10	3.34 ± 0.48	0.015 ± 0.004	0.37 ± 0.02	5.105 ± 0.604
	96 hr	0	1.10 ± 0.14	8.65 ± 0.86	0.022 ± 0.003	0.27 ± 0.03	10.042 ± 1.033
		10	1.15 ± 0.09	2.60 ± 0.25	0.014 ± 0.005	0.31 ± 0.06	4.074 ± 0.405
		100	1.00 ± 0.10	5.39 ± 0.59	0.016 ± 0.003	0.29 ± 0.07	6.696 ± 0.763
		1000	0.81 ± 0.08	5.12 ± 2.09	0.038 ± 0.007	0.33 ± 0.05	6.298 ± 2.227
	7 d	0	0.92 ± 0.11	3.56 ± 0.42	0.013 ± 0.012	0.39 ± 0.05	4.883 ± 0.592
		10	1.21 ± 0.32	4.71 ± 1.01	0.023 ± 0.009	0.37 ± 0.04	6.313 ± 1.379
		100	1.14 ± 0.09	4.17 ± 0.37	0.016 ± 0.003	0.31 ± 0.05	5.636 ± 0.513
		1000	0.79 ± 0.23	4.35 ± 0.20	0.015 ± 0.004	0.34 ± 0.02	5.495 ± 0.454

Table 3. 6. The concentrations (μmol g^{-1} DW) of total metals in *Fucus serratus* from Bantham Quay after cadmium exposure for 24 hr, 96 hr and 7 d. Data are expressed as means ± S.D. (n = 3). BQ, Bantham Quay.

Location	Time	Cd (μg L^{-1})	Cu (μmol g^{-1} DW)	Zn (μmol g^{-1} DW)	Pb (μmol g^{-1} DW)	As (μmol g^{-1} DW)	Total metal/metalloids (μmol g^{-1} DW)
BQ	24 hr	0	0.46 ± 0.13	0.79 ± 0.12	0.014 ± 0.008	0.32 ± 0.05	1.584 ± 0.308
		10	0.40 ± 0.05	0.65 ± 0.04	0.008 ± 0.001	0.23 ± 0.08	1.288 ± 0.171
		100	0.54 ± 0.08	1.16 ± 0.29	0.013 ± 0.003	0.34 ± 0.04	2.053 ± 0.413
		1000	0.35 ± 0.11	0.74 ± 0.19	0.013 ± 0.005	0.25 ± 0.03	1.353 ± 0.335
	96 hr	0	0.42 ± 0.15	0.91 ± 0.30	0.011 ± 0.006	0.32 ± 0.03	1.661 ± 0.486
		10	0.49 ± 0.12	0.89 ± 0.35	0.013 ± 0.004	0.28 ± 0.10	1.673 ± 0.574
		100	0.59 ± 0.10	1.35 ± 0.23	0.043 ± 0.007	0.37 ± 0.05	2.353 ± 0.387
		1000	0.52 ± 0.15	0.96 ± 0.20	0.013 ± 0.009	0.34 ± 0.06	2.323 ± 0.419
	7 d	0	0.37 ± 0.03	1.25 ± 0.34	0.008 ± 0.002	0.32 ± 0.07	1.948 ± 0.442
		10	0.53 ± 0.16	2.22 ± 0.92	0.017 ± 0.005	0.42 ± 0.07	3.187 ± 1.155
		100	0.37 ± 0.15	0.85 ± 0.03	0.009 ± 0.002	0.37 ± 0.04	1.599 ± 0.222
		1000	0.54 ± 0.26	0.95 ± 0.38	0.014 ± 0.003	0.29 ± 0.05	1.794 ± 0.693

Table 3. 7. The concentrations (μmol g^{-1} DW) of non-exchangeable metals in *Fucus serratus* from Restronguet Point after cadmium exposure for 24 hr, 96 hr and 7 d. Data are expressed as means ± S.D. (n = 3). RP, Restronguet Point.

Location	Time	Cd (μg L^{-1})	Cu (μmol g^{-1} DW)	Zn (μmol g^{-1} DW)	Pb (μmol g^{-1} DW)	As (μmol g^{-1} DW)	Total metal/metalloids (μmol g^{-1} DW)
RP	24 hr	0	0.77 ± 0.09	1.11 ± 0.31	0.021 ± 0.019	0.26 ± 0.08	2.161 ± 0.499
		10	0.83 ± 0.17	1.91 ± 0.08	0.012 ± 0.002	0.34 ± 0.04	3.092 ± 0.292
		100	0.95 ± 0.20	1.77 ± 1.10	0.012 ± 0.004	0.26 ± 0.10	2.992 ± 1.404
		1000	0.72 ± 0.08	2.11 ± 1.39	0.014 ± 0.000	0.25 ± 0.02	3.094 ± 1.490
	96 hr	0	0.95 ± 0.22	1.57 ± 0.64	0.021 ± 0.011	0.19 ± 0.02	2.560 ± 0.891
		10	0.66 ± 0.05	1.98 ± 0.46	0.014 ± 0.001	0.19 ± 0.01	2.844 ± 0.521
		100	0.85 ± 0.19	1.75 ± 0.26	0.014 ± 0.003	0.18 ± 0.04	2.794 ± 0.493
		1000	0.66 ± 0.09	1.44 ± 0.13	0.022 ± 0.012	0.17 ± 0.02	2.292 ± 0.252
	7 d	0	0.79 ± 0.08	2.15 ± 0.54	0.013 ± 0.002	0.06 ± 0.00	3.013 ± 0.622
		10	0.87 ± 0.09	2.99 ± 1.57	0.016 ± 0.004	0.10 ± 0.02	3.886 ± 1.684
		100	0.80 ± 0.05	1.60 ± 0.20	0.013 ± 0.003	0.10 ± 0.01	2.423 ± 0.263
		1000	0.76 ± 0.04	1.57 ± 0.44	0.011 ± 0.007	0.09 ± 0.02	2.431 ± 0.507

Table 3. 8. The concentrations (μmol g^{-1} DW) of non-exchangeable metals in *Fucus serratus* from Bantham Quay after cadmium exposure for 24 hr, 96 hr and 7 d. Data are expressed as means ± S.D. (n = 3). BQ, Bantham Quay.

Location	Time	Cd (μg L^{-1})	Cu (μmol g^{-1} DW)	Zn (μmol g^{-1} DW)	Pb (μmol g^{-1} DW)	As (μmol g^{-1} DW)	Total metal/metalloids (μmol g^{-1} DW)
BQ	24 hr	0	0.27 ± 0.00	0.68 ± 0.13	0.009 ± 0.006	0.25 ± 0.09	1.209 ± 0.226
		10	0.27 ± 0.01	0.53 ± 0.04	0.007 ± 0.002	0.18 ± 0.03	0.987 ± 0.082
		100	0.29 ± 0.05	0.70 ± 0.12	0.008 ± 0.002	0.33 ± 0.03	1.328 ± 0.202
		1000	0.25 ± 0.02	0.61 ± 0.15	0.007 ± 0.002	0.18 ± 0.07	1.047 ± 0.242
	96 hr	0	0.29 ± 0.04	0.63 ± 0.23	0.011 ± 0.002	0.12 ± 0.04	1.051 ± 0.312
		10	0.23 ± 0.05	0.67 ± 0.16	0.008 ± 0.000	0.15 ± 0.02	1.058 ± 0.230
		100	0.33 ± 0.06	0.76 ± 0.14	0.007 ± 0.002	0.13 ± 0.02	1.227 ± 0.222
		1000	0.32 ± 0.08	0.89 ± 0.22	0.010 ± 0.001	0.10 ± 0.02	1.320 ± 0.321
	7 d	0	0.34 ± 0.03	0.67 ± 0.08	0.008 ± 0.001	0.07 ± 0.01	1.088 ± 0.121
		10	0.29 ± 0.04	0.49 ± 0.11	0.007 ± 0.003	0.06 ± 0.01	0.847 ± 0.163
		100	0.30 ± 0.04	0.58 ± 0.19	0.008 ± 0.000	0.06 ± 0.02	0.948 ± 0.250
		1000	0.31 ± 0.08	0.79 ± 0.07	0.011 ± 0.003	0.08 ± 0.04	1.191 ± 0.193

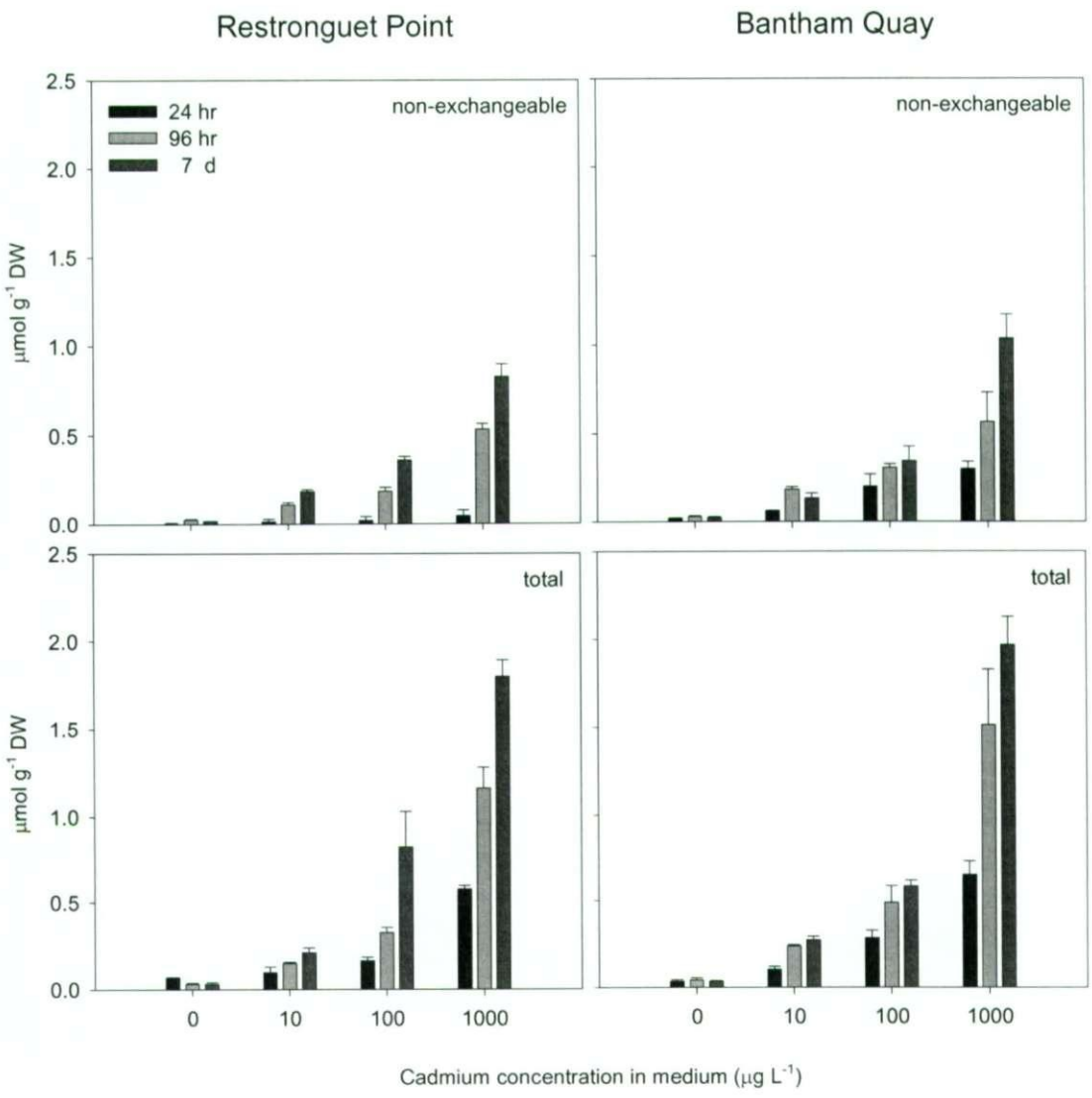

Fig. 3. 5. Non-exchangeable and total cadmium concentrations in *Fucus serratus* from Restronguet Point and Bantham Quay exposed to cadmium for 24 hr, 96 hr and 7 days. Values are means and standard deviations (n = 3).

3. 3. 4. Relative growth rate (RGR)

The effect of high concentrations of Cd (1 ~ 10 mg L^{-1}) on RGR of *Fucus serratus* was measured on the basis of weight change for 7 ~ 14 d exposure (Fig. 3. 6). In the RP population, RGRs of *F. serratus* decreased for the first 7 d in all treatments except 0 μg Cd L^{-1}. The higher concentrations showed the more decrease, however RGRs were not significantly different between 5 and 10 mg Cd L^{-1} for 7 and 14 d ($p > 0.05$). After 14 d of Cd exposure, recovery of weight was shown as increased RGRs in RP (Fig. 3. 6). 5 and 10 mg Cd L^{-1} exposure increased RGRs of *F. serratus* after 14 d even though they were lower than 0 mg L^{-1}. 1 mg L^{-1} showed the latest recovery of growth in RP.

The BQ population also showed drastic decrease in RGRs for the first 7 d. RGRs of BQ have also decreased with increasing Cd concentrations however 1 mg L^{-1} showed the similar decrease to 10 mg L^{-1} ($p > 0.05$). After 14 d, RGRs were still lower than 0 except for 0 mg L^{-1}. All Cd treatments above 1 mg L^{-1} demonstrated similar RGRs in BQ ($p > 0.05$). For 7 and 14 d, all RGRs values from Cd-exposed materials were significantly lower than RGRs of the RP population ($p < 0.0001$).

F. serratus was cultivated with Cd in the second experiment, however this time lower concentrations of Cd and shorter exposure were used, i.e. 0 ~ 1000 μg Cd L^{-1} and 24 hr, 96 hr and 7 d (Fig. 3. 7). RGRs decreased with increasing time of exposure and Cd treatment in RP, however the effect of concentration was not clear at 24 hr (Fig. 3. 7). 1000 μg L^{-1} showed the lowest growth after 96 hr and 10 μg L^{-1} showed the highest RGR after 7 d.

RGRs in BQ have also reduced with increasing time of Cd exposure and Cd treatment (Fig. 3. 7). After 24 hr, RGRs decreased very significantly with increasing Cd concentrations ($p < 0.0001$). However this significance disappeared at 96 hr and values of Cd-exposed materials were very similar at 96 hr ($p > 0.05$). The effect of increasing

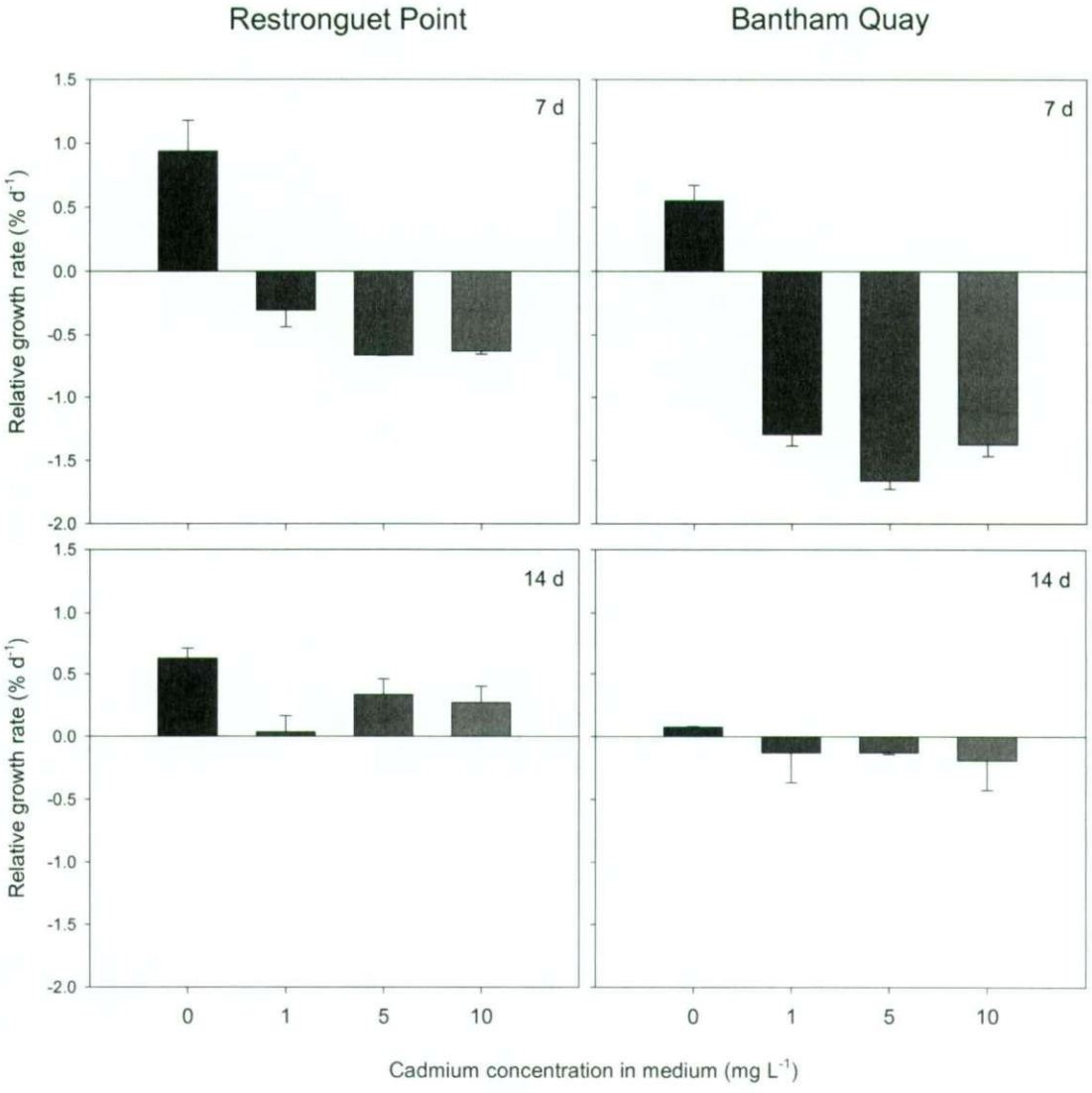

Fig. 3. 6. Relative growth rates of *Fucus serratus* from Restronguet Point and Bantham Quay exposed to cadmium for 7 and 14 days. Values were expressed by means and standard deviations (n = 3).

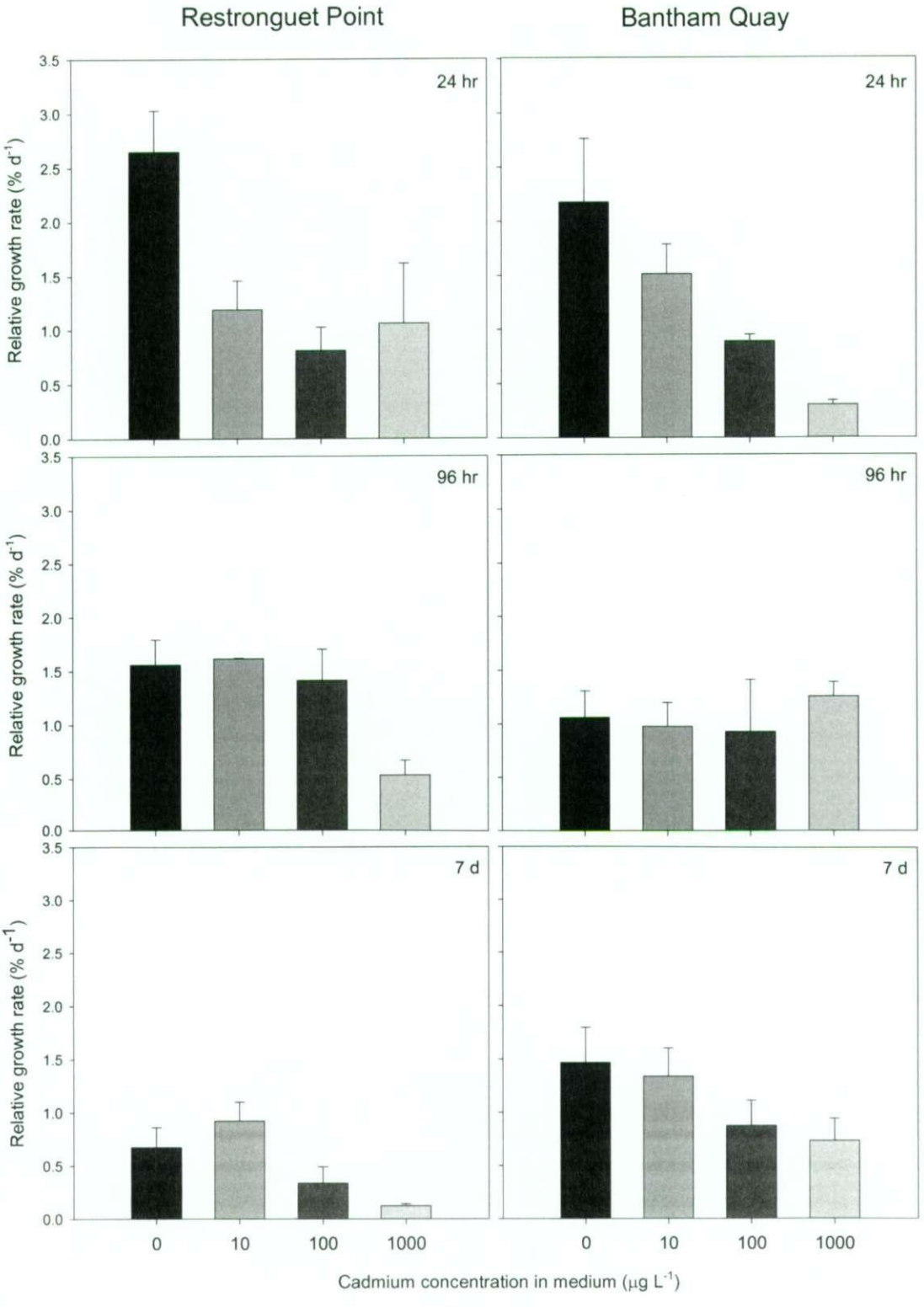

Fig. 3. 7. Relative growth rates of *Fucus serratus* from Restronguet Point and Bantham Quay exposed to cadmium for 24 hr, 96 hr and 7 d. Values are means and standard deviations (n = 3).

Cd concentration on RGRs have reappeared at 7 d ($p < 0.05$), and RGRs in BQ were higher than in RP.

3. 3. 5. Chlorophyll *a* fluorescence parameters

F_0 (minimal fluorescence level from dark-adapted thalli)

Minimum fluorescence of dark-adapted material (F_0) of *F. serratus* was measured in 0 ~ 10 mg Cd L^{-1} for 7 and 14 d (Fig. 3. 8). In RP, F_0 levels including the control significantly increased with time of exposure ($p = 0.001$). The difference between 7 and 14 d exposures were more apparent at 5 and 10 mg Cd L^{-1} than the lower concentrations ($p = 0.001$). After 14 d, effects of Cd treatment (1 ~ 10 mg L^{-1}) did not show a pattern of increase/decrease related to the concentration ($p > 0.05$).

In BQ, F_0 values were not affected either by Cd treatment (0 ~ 10 mg L^{-1}) or by culture period ($p > 0.05$, respectively). All *Fucus* materials including the control showed the lower F_0 levels at 14 d than the levels at 7 d, but no statistical significance was estimated because of the wide variations.

At 7 d, both populations showed similar values of F_0. However, at 14 d, the RP population had significantly higher levels than the BQ population ($p < 0.0001$).

For evaluating shorter exposure, 0 ~ 10 mg L^{-1} treatment was determined for 6, 12 and 24 hr (Fig. 3. 9). According to GLM, algae from RP showed significant difference by Cd concentrations ($p = 0.004$) but not by time of exposure ($p > 0.05$). However, the significance was not related to the increasing Cd concentration ($0 = 5 \leq 1 = 10$ mg L^{-1}). However, neither time of exposure nor Cd concentration affected F_0 levels in the BQ population ($p > 0.05$, respectively).

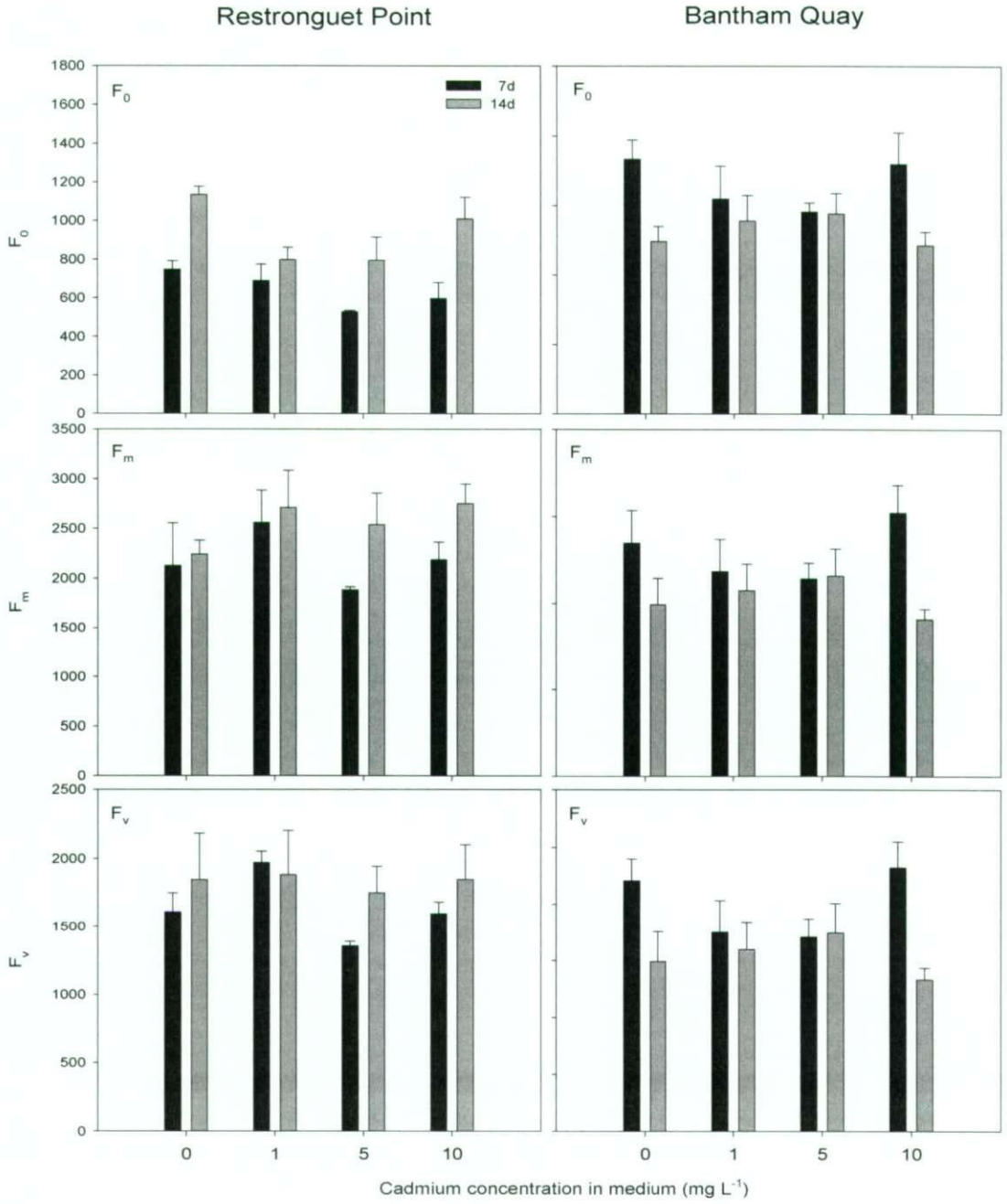

Fig. 3. 8. Changes of Chl *a* fluorescence parameters in *Fucus serratus* exposed to cadmium (0 ~ 10 mg Cd L^{-1}) for 7 and 14 d. Restronguet Point is a polluted site and Bantham Quay is a reference site. F_0, minimal fluorescence level from dark-adapted thalli (t = 50 μs); F_m, maximal fluorescence level from dark-adapted thalli; F_v, the dark-adapted variable fluorescence. Values were expressed by means and standard deviation (n = 8).

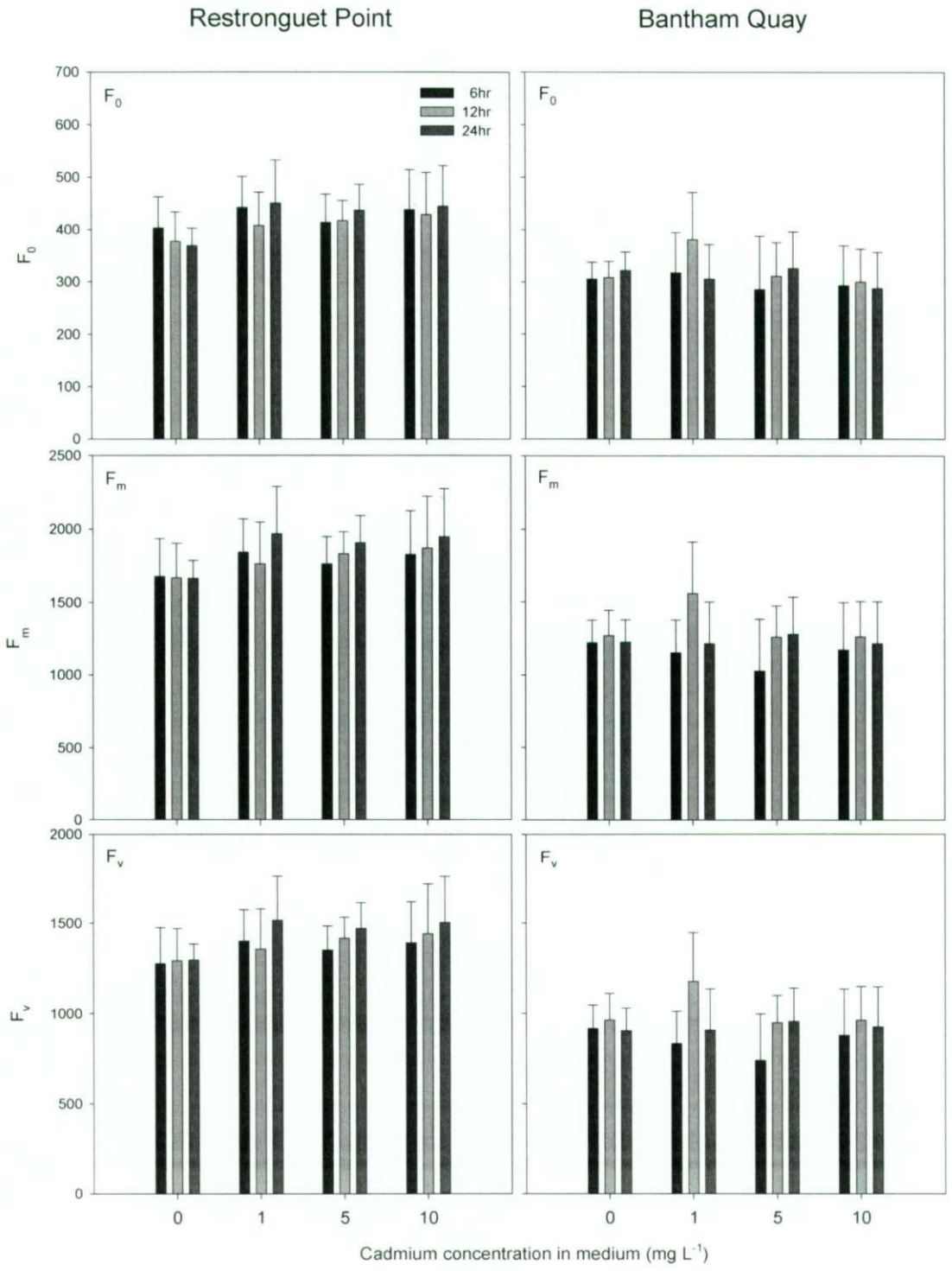

Fig. 3. 9. Changes of Chl *a* fluorescence parameters in *Fucus serratus* exposed to cadmium (0 ~ 10 mg Cd L^{-1}) for 6, 12 and 24 hr. Restronguet Point is a polluted site and Bantham Quay is a reference site. For the definition of parameters, see Fig. 3. 6. Values were expressed by means and standard deviation (n = 10).

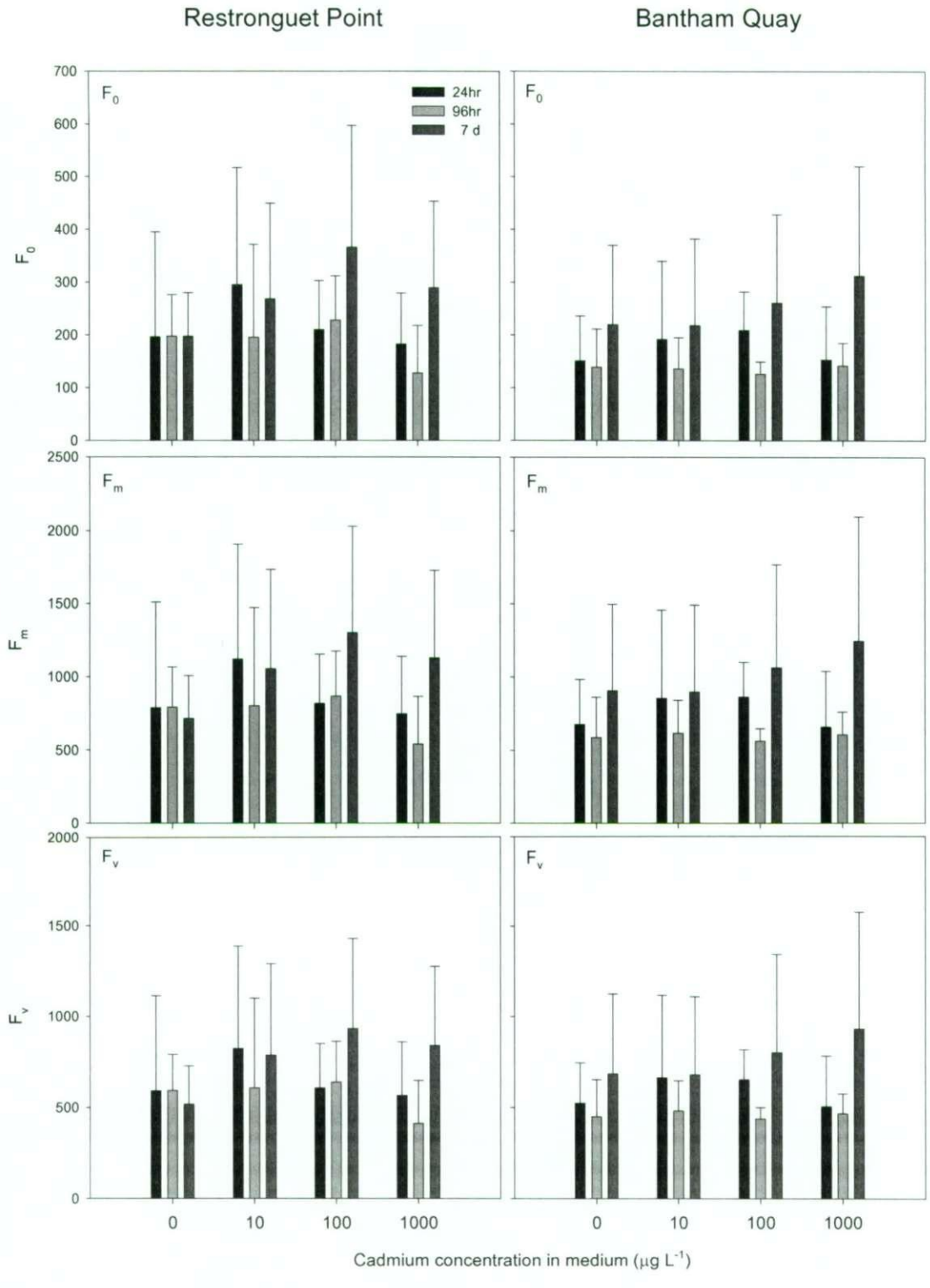

Fig. 3. 10. Changes of Chl a fluorescence parameters in *Fucus serratus* exposed to cadmium (0 ~ 1000 μg Cd L^{-1}) for 24 hr, 96 hr and 7 d. Restronguet Point is a polluted site and Bantham Quay is a reference site. For the definition of parameters, see Fig. 3. 6. Values were expressed by means and standard deviation (n = 6).

Fucus from BQ had significantly lower minimum fluorescence than *Fucus* from RP ($p < 0.0001$). Shorter exposure with 1 ~ 10 mg Cd L^{-1} showed F_0 values approximately half that of the longer exposures (7 and 14 d).

Since 1 ~ 10 mg L^{-1} are extremely high Cd concentrations, which may be impossible to reach in the natural environment, much lower concentrations of Cd (10, 100, and 1000 μg L^{-1}) were applied for 24 hr, 96 hr and 7 d. F_0 values were variable with wide variations in both populations and concentrations of 0 ~ 1000 μg Cd L^{-1} were not found to be related to the changes of F_0 in either RP or BQ populations (Fig. 3. 10). Values in RP were not affected by exposure time ($p > 0.05$), however values of 96 hr in BQ were lower than other exposure ($p = 0.05$). Minimum fluorescence in BQ decreased at 96 hr and recovered at 7 d (96 hr ≤ 24 hr ≤ 7d). Total values of both populations were similar ($p > 0.05$). When compared with extremely high Cd concentrations (1 ~ 10 mg L^{-1}), lower concentrations (10 ~ 1000 μg L^{-1}) had lower F_0 values which were even lower than the values for 1 ~ 10 mg L^{-1} with shorter exposure (up to 24 hr).

F_m (maximal fluorescence level from dark-adapted thalli)

Maximum fluorescence of dark-adapted materials (F_m) after Cd exposure for 7 and 14 d did not differ with Cd concentrations of 0 ~ 10 mg L^{-1} in either population ($p > 0.05$, Fig. 3. 8). While 14 d exposure showed significantly higher F_m than 7 d in RP ($p > 0.005$), especially at 5 ~ 10 mg L^{-1}, there was no difference in BQ ($p > 0.05$). As with F_0, F_m of 0 mg Cd L^{-1} was higher than those of 1 and 5 mg L^{-1} in BQ, however no statistical difference was detected ($p > 0.05$). F_m of *F. serratus* after 7 and 14 d exposure to 0 ~ 10 mg L^{-1} did not show any significant differences between the two populations ($p > 0.05$).

With shorter exposure (6, 12 and 24 hr), 0 ~ 10 mg Cd L^{-1} had significant effect on algae from the polluted site ($p = 0.007$) and 1 and 10 mg L^{-1} increased F_m values

compared to the control (Fig. 3. 9). However, exposure times of 6, 12 and 24 hr did not cause any significant differences at the polluted site ($p > 0.05$). Meanwhile F_m values were not affected by Cd concentration with algae from the control site ($p > 0.05$). 12 hr treatment showed an apparent increase compared with 6 hr in BQ, especially with 1 mg Cd L^{-1}. Algae from RP showed significantly higher F_m values compared to algae from BQ ($p < 0.0001$). Compared with values with longer exposure (Fig. 3. 8), approximately from a half to two thirds of F_m values were measured.

F_m values with lower Cd concentrations (10 ~ 1000 μg L^{-1}) were also measured at 24 hr, 96 hr and 7 d (Fig. 3. 10). As F_0, the RP population had widely varied values and was affected by neither Cd concentration nor exposure time ($p > 0.05$). F_m of BQ population also showed similar pattern to F_m of RP and was not affected by Cd concentration ($p > 0.05$). F_m of BQ significantly decreased at 96 hr ($p = 0.009$) and increased again at 7 d (96 hr ≤ 24 hr ≤ 7 d). Each population showed similar F_m value and no statistical significance was observed ($p > 0.05$). When total value of each population was compared with values for longer or shorter exposure at higher concentration (~ 10 mg Cd L^{-1}), much lower value was evaluated.

F_v (dark-adapted variable fluorescence)

Variable fluorescence of dark-adapted algae between F_m and F_0 ($F_v = F_m - F_0$) was calculated (Figs 3. 8 ~ 10). High Cd concentration (0 ~ 10 mg L^{-1}) did not affect F_v values in either population for 7 and 14 d ($p > 0.05$) (Fig. 3. 8). While there was no difference between time of exposure in BQ ($p > 0.05$), 14 d exposure showed higher F_v levels than 7 d in RP, especially at higher Cd concentrations (5 and 10 mg L^{-1}) ($p = 0.025$).

Although both populations of *F. serratus* showed similar fluorescence values after 7 d exposure to 0 ~ 10 mg Cd L^{-1}, RP showed significantly higher levels after 14 d exposure ($p > 0.05$).

When *F. serratus* was exposed to Cd for less than 24 hr, F_v from the RP population was affected by Cd concentration (0 ~ 10 mg L^{-1}) ($p = 0.011$) (Fig. 3. 9). Cd exposure increased F_v values and the control was statistically different from 1 and 10 mg Cd L^{-1} ($p = 0.039$ and $p = 0.013$, respectively). Exposure of 5 mg L^{-1} was not different from the other treatments ($p > 0.05$).

While time of exposure did not affect F_v of *F. serratus* from RP, algae from BQ were affected very significantly ($p = 0.001$). 12 hr exposure showed the highest values and they were significantly higher than 6 hr exposure ($p = 0.001$). After 24 hr, the values were lower than after 12 hr of exposure and were not different from the other time conditions ($p > 0.05$).

F_v values between the two populations were very distinct and values of RP were significantly higher than those of BQ ($p < 0.0001$). When compared with F_v values for longer exposure (7 and 14 d), the values with shorter exposure (24 hr, 96 hr and 7 d) were lower.

F. serratus exposed to 0 ~ 1000 μg Cd L^{-1} for 24 hr, 96 hr and 7 d showed widely varying F_v values in which Cd concentration was not involved in either population (Fig. 3. 10) ($p > 0.05$). In RP, time of exposure also did not affect F_v. In BQ, however, exposure time was related to the change of F_v ($p = 0.012$). Time of exposure decreased values at 96 hr, but recovered at 7 d (96 hr ≤ 24 hr ≤ 7 d).

Both populations showed similar F_v values and no statistical difference was detected ($p > 0.05$). Total values of F_v with lower Cd concentration showed lower levels than values with 1 ~ 10 mg Cd L^{-1} at both longer and shorter exposure.

F_v / F_m (maximum quantum efficiency of photosystem II)

The maximum quantum efficiency of PS II was evaluated by F_m and F_0 (F_v / F_m = (F_m - F_0) / F_m) (Figs 3. 11 ~ 3. 13). When *F. serratus* was exposed to 0 ~ 10 mg Cd L^{-1} for 7 and 14 d, time of Cd exposure did not affect the efficiency values in either population ($p > 0.05$) (Fig. 3. 11). F_v / F_m increased at 1 and/or 10 mg L^{-1} after 7 d exposure in both populations, however the values were not significant ($p > 0.05$, Fig. 3. 11).

The BQ population presented significantly higher maximum quantum efficiency levels than RP, especially at 14 d ($p = 0.001$).

Shorter times of exposure of 6 to 24 hr with the same Cd concentrations (0 ~ 10 mg L^{-1}) had a significant impact on F_v / F_m values of *F. serratus* from both polluted and clean sites ($p < 0.0001$) (Fig. 3.12). Values after the shortest exposure (6 hr) were significantly lower than the other treatments in both populations ($p < 0.0001$, respectively), and values after 12 and 24 hr were also similar in both populations ($p > 0.05$). While exposure time had significant effect on F_v / F_m in both *Fucus* populations, Cd concentration produced a different effect. Although the RP populations did not have any significant effect ($p > 0.05$), values of the BQ population were changed

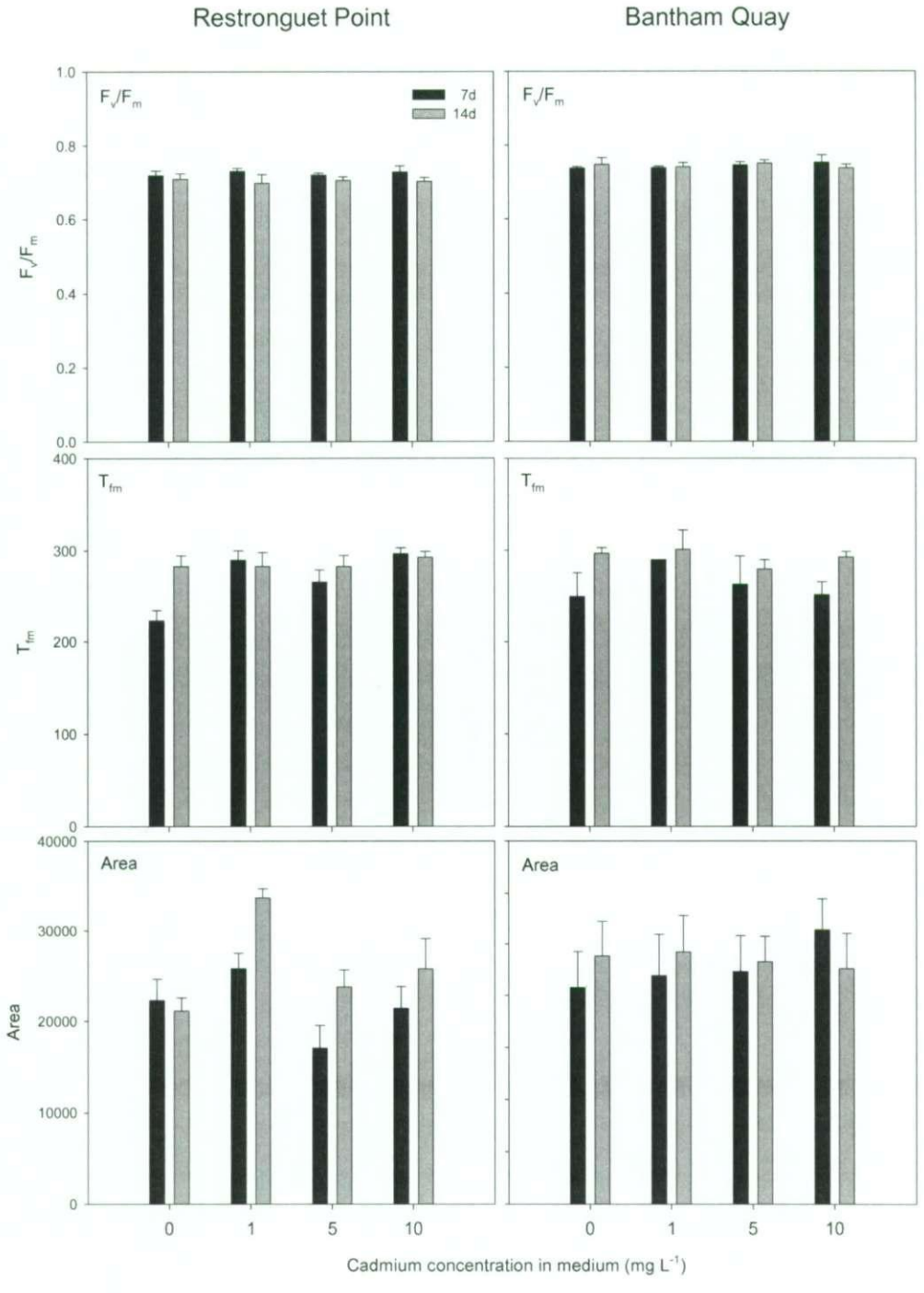

Fig. 3. 11. Changes of Chl *a* fluorescence parameters in *Fucus serratus* exposed to cadmium (0 ~ 10 mg Cd L^{-1}) for 7 and 14 d. Restronguet Point is a polluted site and Bantham Quay is a reference site. F_v/F_m, the maximum quantum efficiency of PS II; T_{fm}, the time needed to reach F_m; Area, area above fluorescence curve between F_0 and F_m. Values were expressed by means and standard deviation (n = 8).

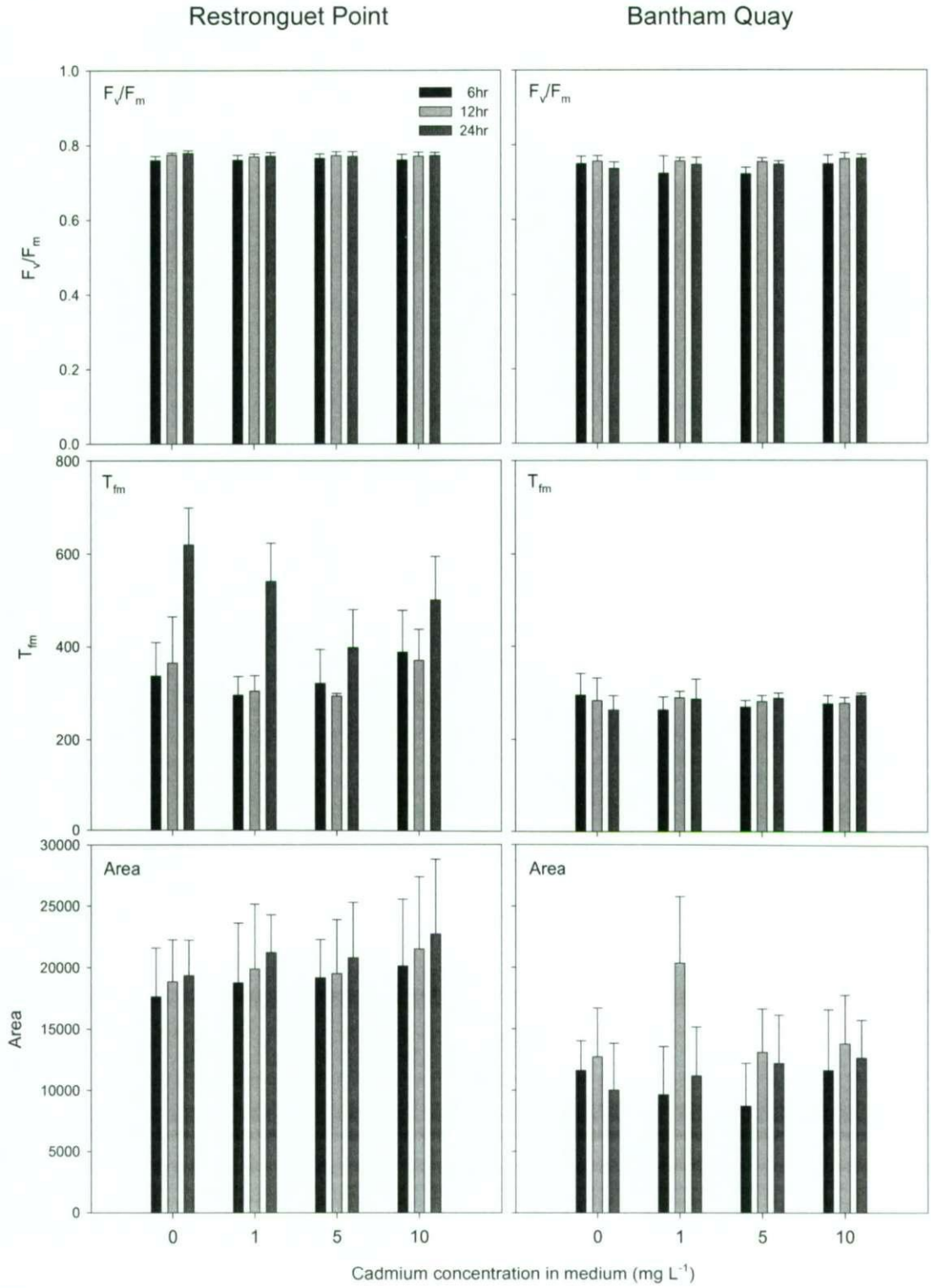

Fig. 3. 12. Changes of Chl *a* fluorescence parameters in *Fucus serratus* exposed to cadmium (0 ~ 10 mg Cd L^{-1}) for 6, 12 and 24 hr. Restronguet Point is a polluted site and Bantham Quay is a reference site. For the definition of parameters, see Fig. 3. 9. Values were expressed by means and standard deviation (n = 8).

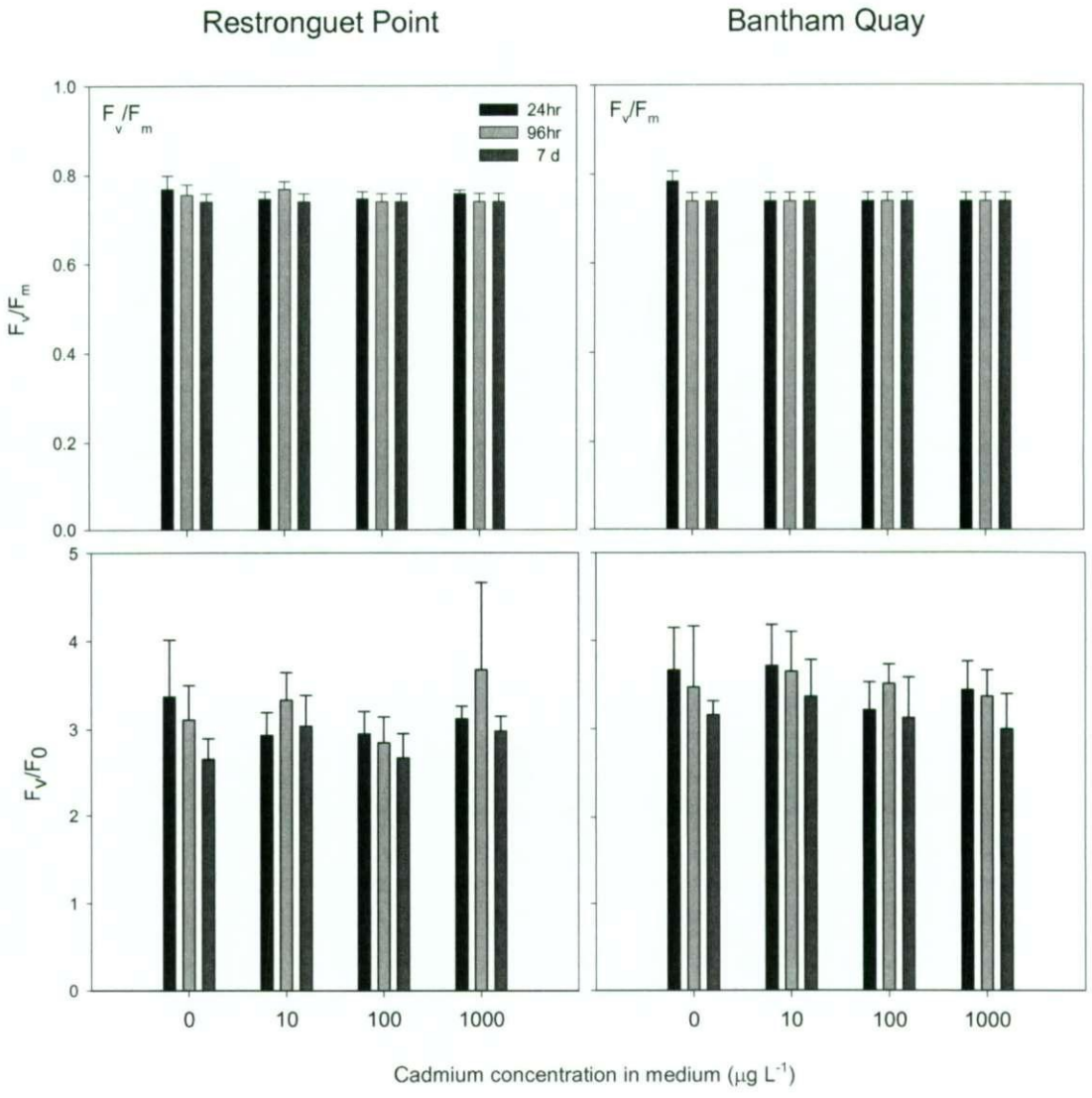

Fig. 3. 13. Changes of Chl a fluorescence parameters in *Fucus serratus* exposed to cadmium (0 ~ 1000 μg Cd L^{-1}) for 24 hr, 96 hr and 7 d. Restronguet Point is a polluted site and Bantham Quay is a reference site. F_v/F_m, the maximum quantum efficiency of PS II; F_v/F_0, the potential activity of PS II. Values were expressed by means and standard deviation (n = 6).

significantly by Cd concentrations ($p = 0.006$). 1 and 5 mg Cd L^{-1} in BQ showed lower values than 0 and 10 mg L^{-1}, especially after 6 hr.

Total values of F_v / F_m were significantly higher at RP than at BQ ($p < 0.0001$). Values with shorter exposure (up to 24hr) were higher than values with longer exposure (7 and 14 d, Fig. 3. 11).

The maximum quantum efficiency was measured with lower Cd concentration of 0 ~ 1000 μg L^{-1} for 24 hr, 96 hr and 7 d (Fig. 3. 13). In RP, Cd concentration affected F_v / F_m values ($p = 0.012$) and 100 μg L^{-1} was significantly lower than 1000 μg L^{-1} ($p = 0.008$). Time of Cd exposure was more significantly related to F_v / F_m values in RP ($p = 0.002$). 7 d exposure produced significantly lower values than 24 hr ($p = 0.035$) and 96 hr ($p = 0.002$), however values remained until 96 hr (24 hr = 96hr, $p > 0.05$).

Meanwhile F_v / F_m values were not affected by Cd concentration in BQ ($p > 0.05$) (Fig. 3. 13). Exposure time was significantly correlated with F_v / F_m in BQ ($p = 0.004$), and 7d exposure showed significantly lower values than 24 hr and 96 hr treatments ($p = 0.010$ and $p = 0.012$, respectively).

Total values from each population were significantly different and values from BQ were higher than values from RP ($p < 0.0001$). F_v / F_m values of 0 ~ 1000 μg Cd L^{-1} were higher than values with long exposure and high Cd concentrations (7 and 14 d, 1 ~ 10 mg L^{-1}) and similar to values with short exposure and high Cd concentrations (6, 12 and 24 hr, 1 ~ 10 mg L^{-1}).

T_{fm} (time needed to reach F_m)

The time needed to reach F_m was measured by millisecond (ms) levels with handy PEA (Figs 3. 11 ~ 3. 12). In RP, Cd treatment significantly affected T_{fm} values at 7 d ($p < 0.0001$) but not at 14 d ($p > 0.05$)(Fig. 3. 11). Cd exposure increased T_{fm} values at 7 d. Time of exposure increased T_{fm} levels and 14 d exposure showed significantly higher values than 7 d treatment ($p = 0.002$).

However, Cd exposure did not induce any significant effect on T_{fm} in BQ at both 7 and 14 d exposure ($p > 0.05$). As the RP population, longer exposure (14 d) increased time to reach F_m ($p = 0.001$).

Total values of each population were similar and no significant difference was detected between them ($p > 0.05$).

When exposed to shorter Cd exposure (6 ~ 24 hr) with same Cd concentrations (0 to 10 gm Cd L^{-1}), T_{fm} of the RP population was significantly affected by exposure time and Cd concentration (Fig. 3. 12). It took much longer time to reach F_m at 24 hr than 6 hr and 12 hr, regardless of Cd concentration ($p < 0.0001$). However 6 hr and 12 hr exposure did not show significant difference ($p > 0.05$). 5 mg Cd L^{-1} showed significantly shorter time to get to maximum fluorescence than 0 and 10 mg L^{-1}. 0 mg L^{-1} also showed significantly high values after 24 hr in RP.

However, the population from BQ was not affected by either exposure time or Cd concentration ($p > 0.05$) (Fig. 3. 11). In addition, values of BQ were significantly lower than values of RP ($p < 0.0001$), indicating it took shorter time to reach F_m in BQ.

Area (area above fluorescence curve between F_0 and F_m)

Area above fluorescence curve between F_0 and F_m was calculated by the Handy PEA automatically (Fig. 3. 11). When *F. serratus* was exposed to high Cd concentrations (0 ~ 10 mg L^{-1}), the area between F_0 and F_m did not show a pattern of change, although there was a significant difference ($p < 0.0001$). 5 mg L^{-1} had the lowest value at 7 d and 1 mg L^{-1} had the highest value at 7 and 14 d in RP. Time factor had a significant effect in RP ($p < 0.0001$). 14 d exposure had higher values than 7 d except the control in RP. While values at RP increased with extended exposure time, Area values at BQ were quite stable with extended exposure time (7 and 14 d) and Cd treatment ($p = 0.05$). Considering the dissimilarity of the two locations, area values were not different ($p > 0.05$).

In the shorter exposure experiment, *Fucus* from RP did not respond significantly until 24 hr with 0 ~ 10 mg Cd L^{-1} ($p > 0.05$, Fig. 3. 12). Mean values of fluorescence curve area increased with extended exposure time and Cd concentration in RP, however statistical significances were not detected ($p > 0.05$). Total area values of BQ were significantly lower than values of RP ($p < 0.0001$). In BQ, 12 hr exposure always showed high values in any Cd concentrations ($p < 0.0001$) and the highest value was measured with 1 mg Cd L^{-1} (Fig. 3. 12). Higher Cd concentration enhanced area values in BQ ($p = 0.042$).

PI_{ABS} (performance index)

The performance Index explaining photosynthetic efficiency was measured using the Handy PEA. For 7 and 14 d PI_{ABS} values of *F. serratus* from RP were not changed by Cd concentrations (0 ~ 10 mg Cd L^{-1}) ($p > 0.05$, Fig. 3. 14), while the values increased with higher Cd concentrations in BQ ($p = 0.04$). PI_{ABS} values were similar at both 7 and 14 d exposure in BQ however the values decreased significantly

with longer exposure time in RP ($p = 0.018$). The differences between populations were significant ($p < 0.0001$) and PI_{ABS} values of RP were much lower than values of BQ, especially at higher and longer Cd treatments.

In both populations the PI_{ABS} values measured with shorter exposure (6 ~ 24 hr) increased with increased Cd concentrations, however statistical differences between them were not present ($p > 0.05$, Fig. 3. 15). Exposure time had a significant effect on PI_{ABS} values in both populations. In RP, 24 hr treatment showed higher values than 6 hr ($p = 0.027$) however the 12 hr treatment did not show differences from either 6 hr or 24 hr ($p > 0.05$). BQ responded more significantly to time of exposure ($p = 0.003$). 12 hr exposure produced much clearly higher values than 6 hr ($p = 0.002$) although the values were not significantly different from values for 24 hr ($p > 0.05$). Total PI_{ABS} values were significantly higher in RP than BQ ($p < 0.0001$).

F_v / F_0 (potential activity of photosystem II)

Proportion of active Chl associated with the reaction centre of PS II ($F_v / F_0 = (F_m - F_0) / F_0$) was calculated after Handy PEA or FMS measurement (Fig. 3. 14 and 3. 15). When *F. serratus* was exposed to 0 ~ 10 mg Cd L^{-1} for 7 and 14 d, F_v / F_0 ratios from RP population increased with increasing Cd concentration (Fig. 3. 14). 10 mg L^{-1} treatment was significantly higher than the control in RP ($p = 0.047$). Values of RP decreased with extended exposure time (7 d > 14 d, $p = 0.013$). F_v / F_0 values from BQ population were affected by neither the Cd treatment nor the time of Cd exposure ($p > 0.05$, Fig. 3. 14). Values representing each population were very significantly different and BQ population showed significantly higher F_v / F_0 ratios than RP population ($p < 0.0001$).

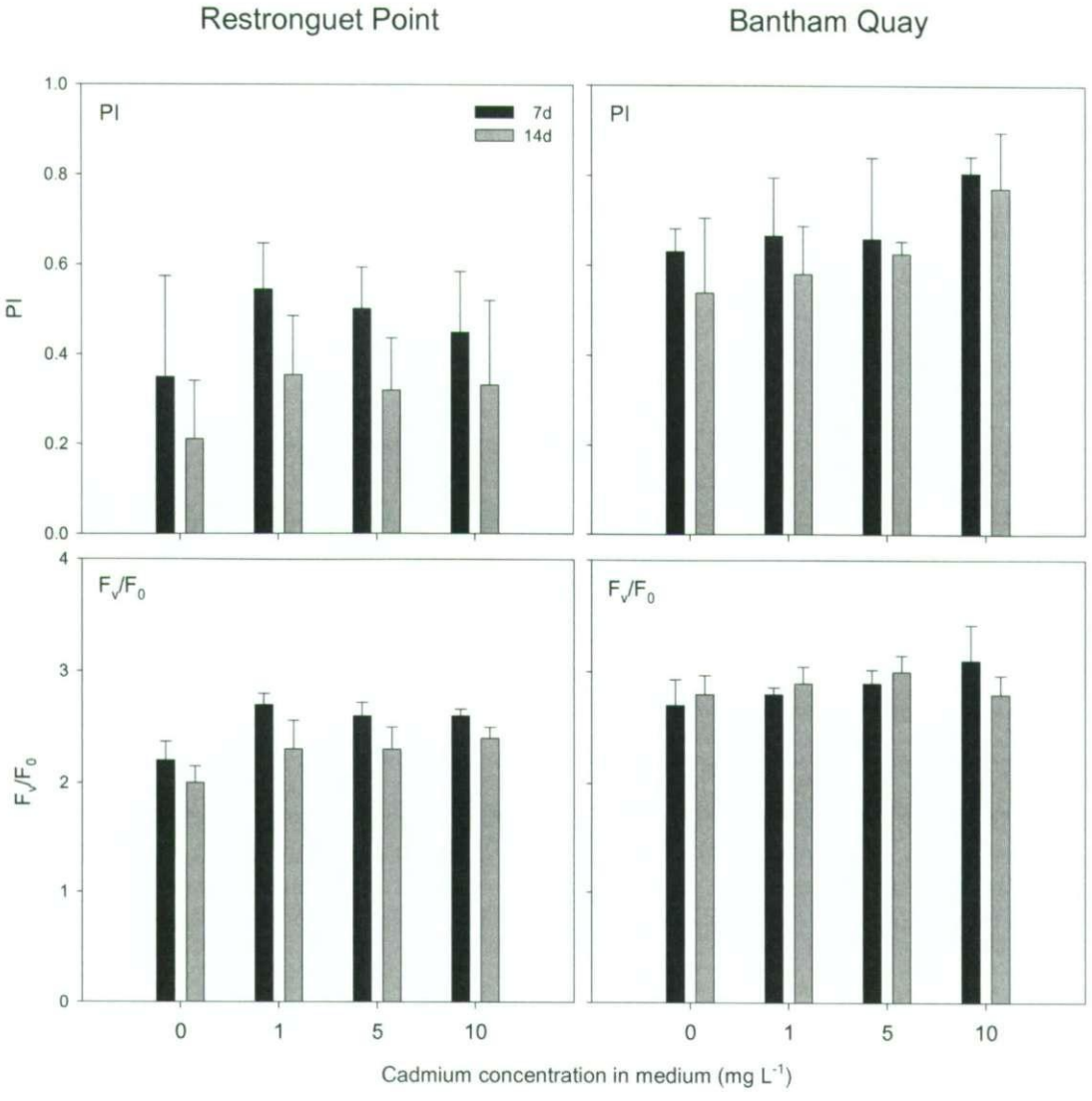

Fig. 3. 14. Changes of Chl *a* fluorescence parameters in *Fucus serratus* exposed to cadmium (0 ~ 10 mg Cd L^{-1}) for 7 and 14 d. Restronguet Point is a polluted site and Bantham Quay is a reference site. PI, performance index; F_v/F_0, the potential activity of PS II. Values were expressed by means and standard deviation (n = 8).

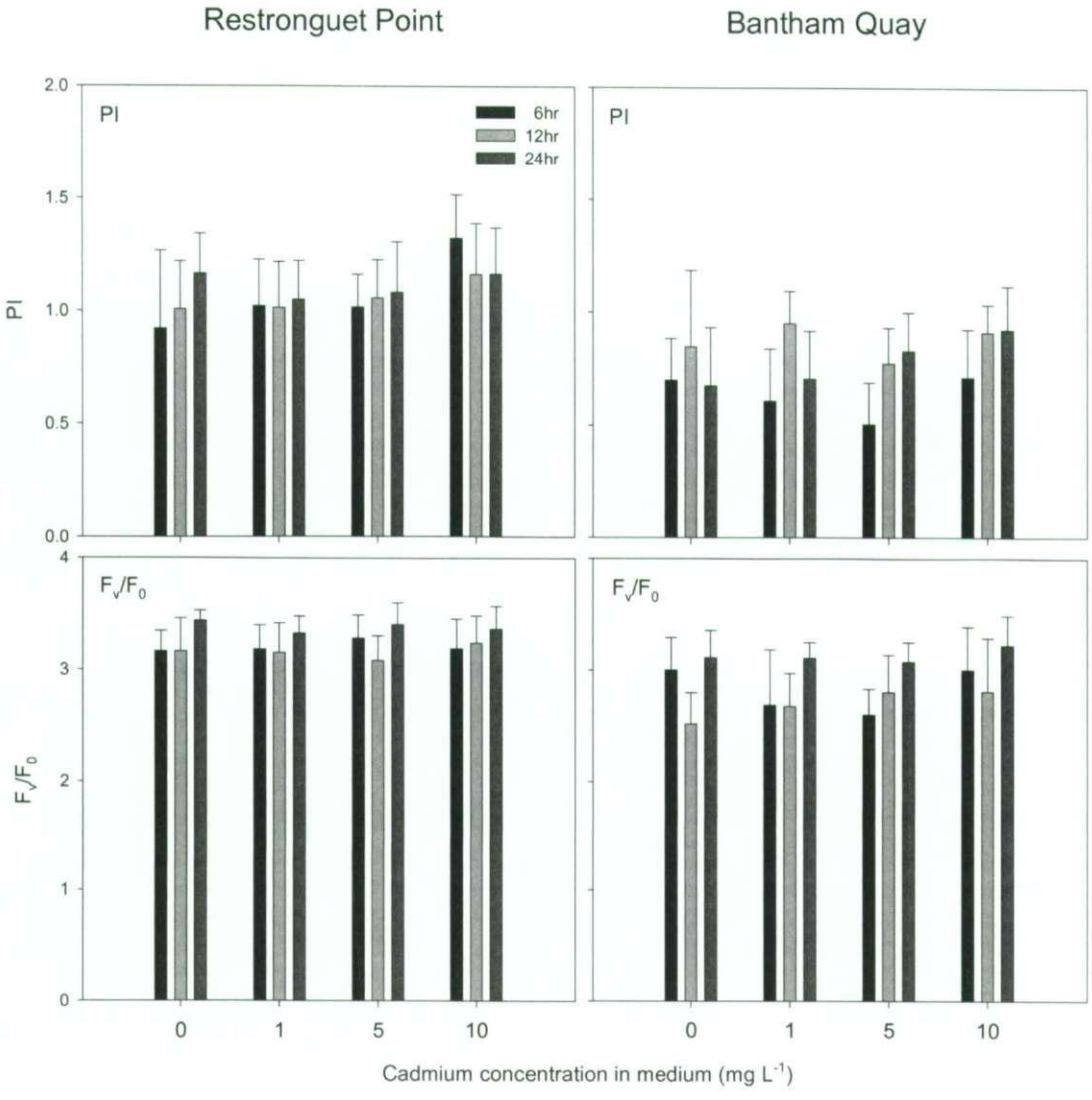

Fig. 3. 15. Changes of Chl *a* fluorescence parameters in *Fucus serratus* exposed to cadmium (0 ~ 10 mg Cd L^{-1}) for 6, 12 and 24 hr. Restronguet Point is a polluted site and Bantham Quay is a reference site. For the definition of parameters, see Fig. 3. 14. Values were expressed by means and standard deviation (n = 8).

Shorter exposure (6, 12 and 24 hr) with 0 ~ 10 mg Cd L^{-1} produced a significant impact on F_v / F_0 ratios (Fig. 3. 15). 12 and 24 hr exposure had much higher ratios of F_v / F_0 than 6 hr exposure in RP ($p < 0.0001$) and values increased with increasing Cd exposure time (6 hr < 12 hr ≤ 24 hr). 12 and 24 hr exposure also gave significantly higher values than 6 hr in BQ ($p < 0.0001$), however 12 hr showed higher values than 24 hr without statistical significance (6 hr < 24 hr ≤ 12 hr). Cd treatment did not have an effect on F_v / F_0 ratios in RP ($p > 0.05$) although 10 mg Cd L^{-1} showed significantly higher values than other concentrations in BQ ($p = 0.001$, $5 \leq 1 \leq 0 < 10$ mg Cd L^{-1}). The total value of the polluted population was much higher than the total value of the reference population ($p < 0.0001$).

Fucus serratus from RP and BQ were exposed to lower Cd concentrations of 0 ~ 1000 μg L^{-1} for 24 hr, 96 hr and 7 d (Fig. 3. 13). Cd concentration had significant effect on F_v / F_0 ratio in RP ($p = 0.03$) however significant difference was present between 100 and 1000 μg Cd L^{-1} only ($p = 0.018$). Ratios of F_v / F_0 were not affected by Cd treatment in BQ population ($p > 0.05$). Length of Cd exposure had a significant effect on F_v / F_0 values of RP ($p = 0.007$), however values were not related to time length. 7 d exposure showed the lowest value and 96 hr showed the highest value in RP. Exposure time had significant impact on F_v / F_0 ratio of BQ ($p = 0.006$) and longer exposure showed lower values (7 d < 96 hr ≤ 24 hr). Total value of RP was very significantly lower than total values of BQ ($p < 0.0001$).

Φ_{PSII} (quantum efficiency of photosystem II)

Quantum efficiency of PS II was estimated by F_m' and F_s ($\Phi_{PSII} = (F_m' - F_s) / F_m'$). Cd treatment and the concentrations did not affect changes of Φ_{PSII} in both

populations ($p > 0.05$, Fig. 3. 16). Meanwhile time of Cd exposure was highly related to quantum efficiency in both populations ($p < 0.0001$). In both populations, values decreased as exposure time increased. 24 hr exposure showed the highest values and was very significantly higher than both 96 hr and 7 d exposures ($p < 0.0001$). Total value of BQ was significantly higher than total value of RP ($p < 0.0001$).

qP (coefficient of photochemical quenching)

The coefficient of photochemical quenching (qP) was measured by FMS for *F. serratus* which was exposed to 0 to 1000 μg Cd L^{-1} for 24 hr, 96 hr and 7 d (Fig. 3. 16). Both populations showed similar photochemical quenching values for up to 7 d ($p > 0.05$). Changes of qP values did not match with changes of Cd concentrations in either populations ($p > 0.05$) however they did match with elapsed time of Cd exposure (Fig. 3. 16, $p < 0.0001$). Time of Cd exposure for RP materials significantly decreased qP values and 24 hr exposure was very significantly higher than 96 hr and 7 d exposure ($p < 0.0001$). Exposure time also decreased qP levels of BQ and values decreased gradually. 24 hr exposure gave significantly higher values than 96 hr ($p < 0.0001$) and 7 d exposure ($p < 0.0001$) and 96 hr exposure again showed produced values than 7 d ($p = 0.012$).

NPQ (non-photochemical quenching)

The non-photochemical quenching (NPQ) was determined by FMS with F_m and F_m' ($NPQ = (F_m - F_m') / F_m'$) and *F. serratus* from RP and BQ was exposed to 0 ~ 1000 μg Cd L^{-1} for 24 hr, 96 hr and 7 d (Fig. 3. 16). No significance was found between polluted and reference populations ($p > 0.05$). 100 μg L^{-1} in RP significantly enhanced

NPQ values and 10 μg L^{-1} showed the lowest values ($p < 0.0001$). Meanwhile Cd treatment in BQ did not give a significant effect on changes of NPQ ($p > 0.05$). 96 hr treatment showed the highest NPQ values and was significantly different from 24 hr and 7 d treatment in RP ($p < 0.0001$). Time of Cd exposure had more clear effect in BQ and longer exposing time produces higher NPQ values (24 hr < 96 hr < 7 d, $p < 0.0001$).

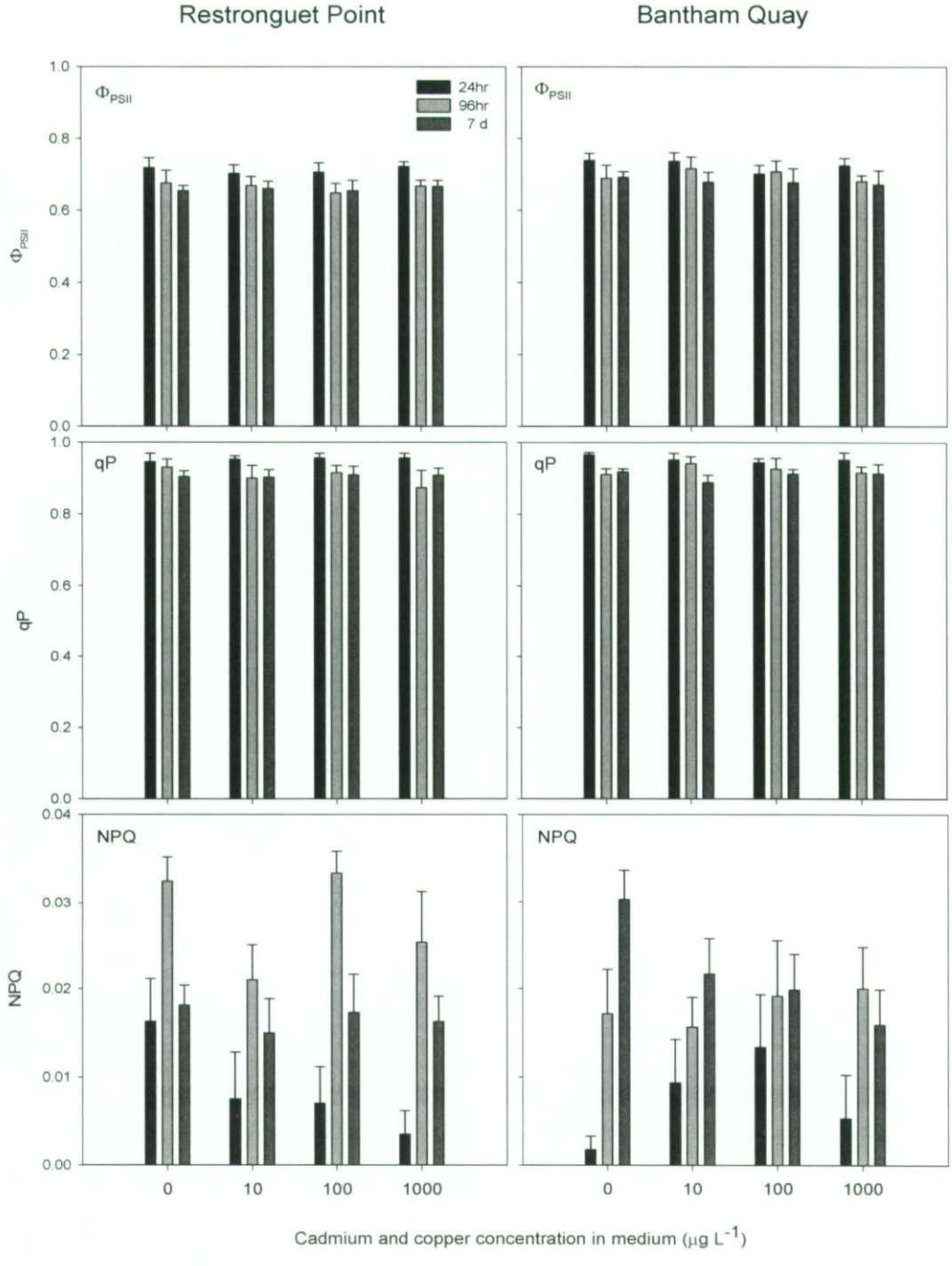

Fig. 3. 16. Changes of Chl a fluorescence parameters in *Fucus serratus* exposed to cadmium (0 ~ 1000 μg Cd L^{-1}) for 24 hr, 96 hr and 7 d. Restronguet Point is a polluted site and Bantham Quay is a reference site. Φ_{PSII}, quantum efficiency of PS II; qP, the coefficient of photochemical quenching; NPQ, non-photochemical quenching. Values were expressed by means and standard deviation (n = 6).

ABS / CS (effective antenna size of a cross section)

Number of absorbed photons by Chl molecules per cross section (ABS / CS) was determined by Handy PEA. ABS / CS was not affected by 0 ~ 10 mg Cd L^{-1} in both populations ($p > 0.05$). Meanwhile the values were not changed by exposure time (7 and 14 d) in BQ, 14 d exposure showed higher ABS / CS than 7 d exposure in RP ($p = 0.001$). In addition, RP population showed higher ABS / CS than BQ population ($p = 0.014$), and the differences were particularly apparent, at 14 d. When the values were compared with the values of each control treatment (0 mg Cd L^{-1} at 7 and 14 d), ABS / CS of RP decreased with increasing Cd concentrations but no differences were found between Cd treatments after 14 d exposure (Fig. 3. 17). Meanwhile, in BQ, ABS / CS of 1 ~ 10 mg Cd L^{-1} was much lower than 0 mg Cd L^{-1} at 7 d and the values increased with Cd concentrations and were higher than the control at 14 d (Fig. 3. 18).

Absorbed photons per CS increased with Cd treatment for shorter exposure (6, 12 and 24 hr, $p = 0.004$). Although ABS / CS was not respond to exposure time of Cd treatment in either populations ($p > 0.05$), RP population showed significantly higher total values than BQ population ($p < 0.0001$). BQ population was not significantly changed by shorter exposure time nor by Cd concentrations (0 ~ 10 mg L^{-1}). When the photon values with Cd exposure were compared with the control, in RP, all Cd treatments showed similar values and increased with time (Fig. 3. 19). However, in BQ, 1 mg L^{-1} showed distinctly higher values, especially at 12 hr and all Cd treatments showed similar values to the control at 24 hr (Fig. 3. 20).

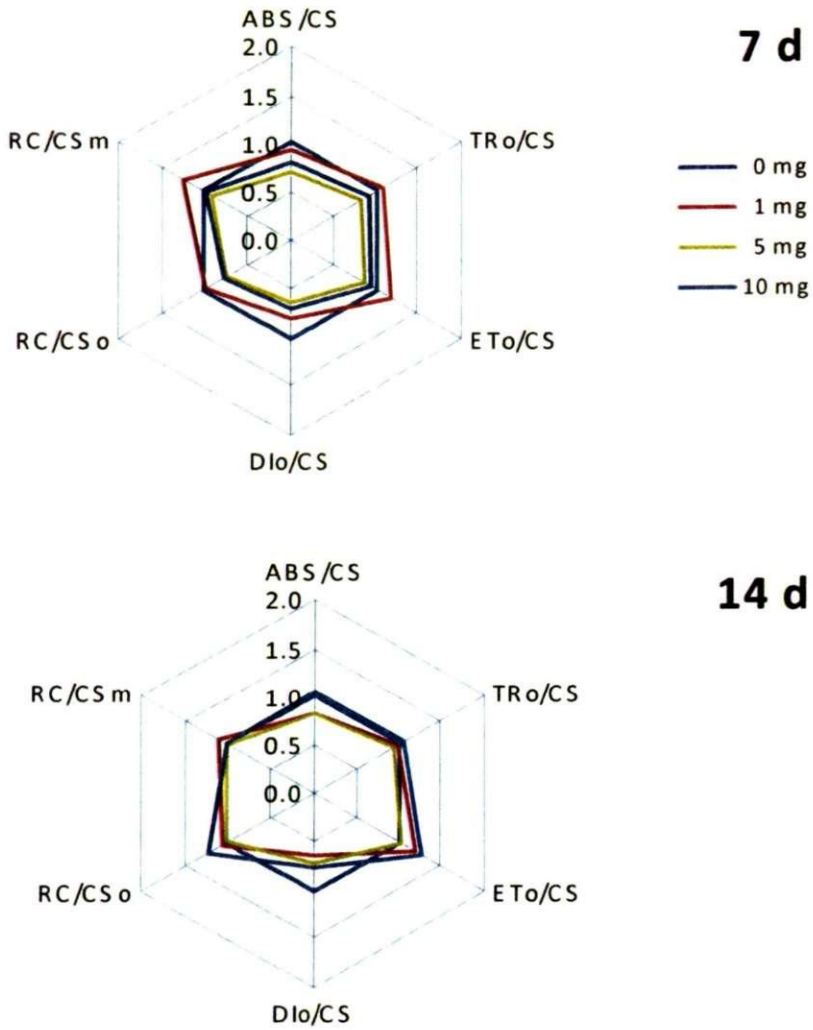

Fig. 3. 17. Influence of cadmium treatment on several selected functional and structural JIP-test parameters plotted relative to the respective controls (set as reference = 1.0). *Fucus serratus* collected from Restronguet Point was exposed to 0 ~ 10 mg Cd L^{-1} for 7 and 14 d. ABS/CS, the total number of photons absorbed by Chl molecules per cross section (CS); TR_0/CS, the maximal rate by which an excitation is trapped by the CS (at t = 0); ET_0/CS, electron transport flux per CS (at t = 0); DI_0/CS, effective dissipation per CS (at t = 0); RC/CS_0, the number proportional to the active reaction centres to the cross-section of the measured sample (t = 0); RC/CS_m, the number proportional to the active reaction centres to the cross-section of the measured sample (t = m).

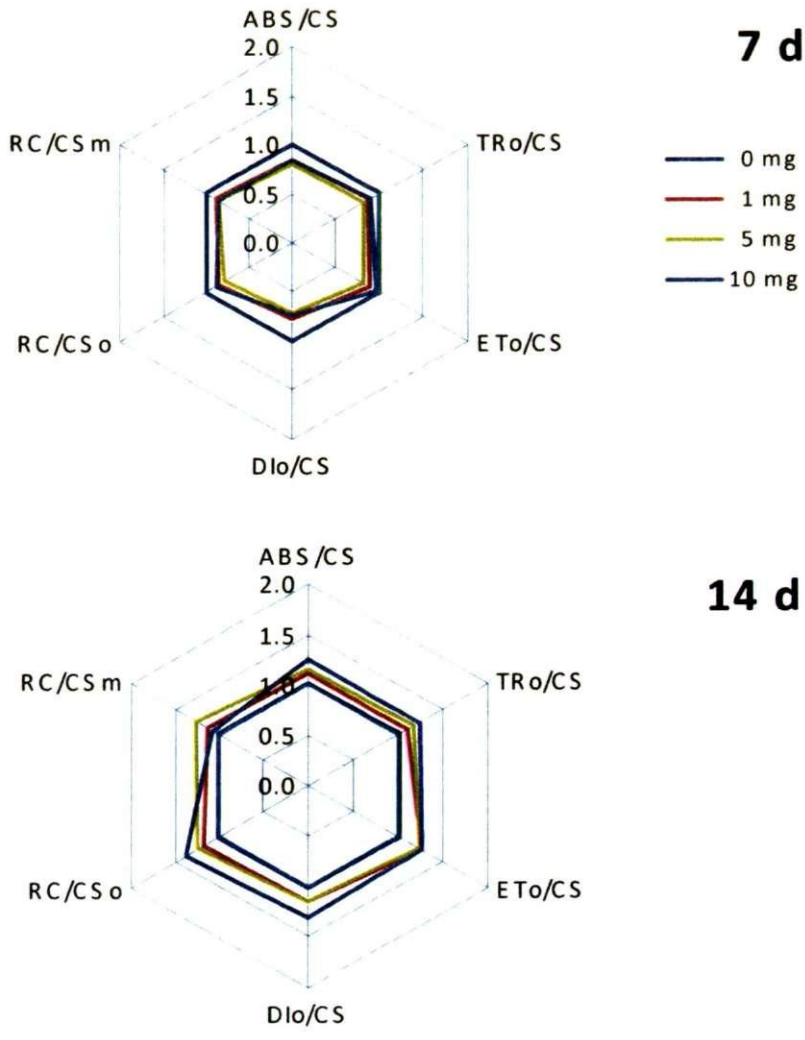

Fig. 3. 18. Influence of cadmium treatment on several selected functional and structural JIP-test parameters plotted relative to the respective controls (set as reference = 1.0). *Fucus serratus* collected from Bantham Quay was exposed to 0 ~ 10 mg Cd L^{-1} for 7 and 14 d. For the definition of abbreviations, see Fig. 3. 17 or text.

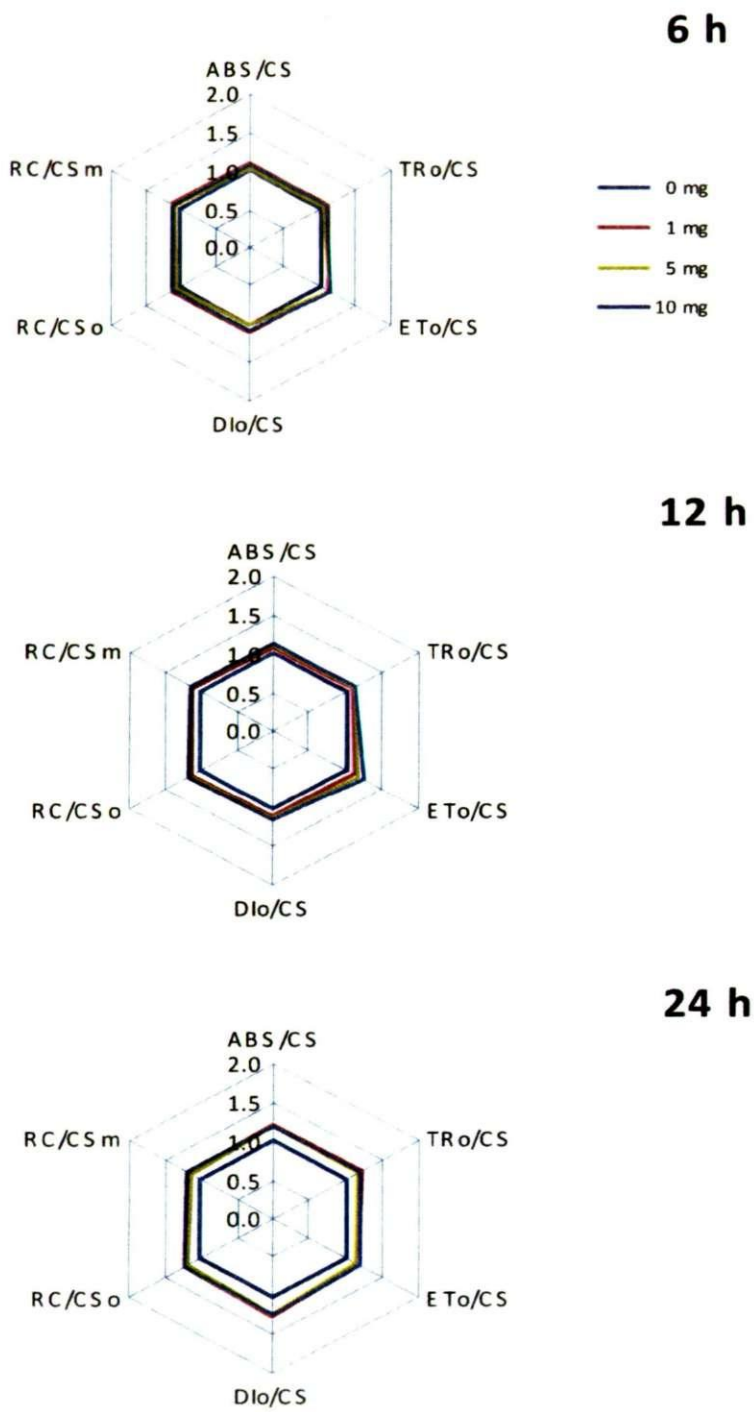

Fig. 3. 19. Influence of cadmium treatment on several selected functional and structural JIP-test parameters plotted relative to the respective controls (set as reference = 1.0). *Fucus serratus* collected from Restronguet Point was exposed to 0 ~ 10 mg Cd L^{-1} for 24 hr, 96 hr and 7 d. For the definition of abbreviations, see Fig. 3. 17 or text.

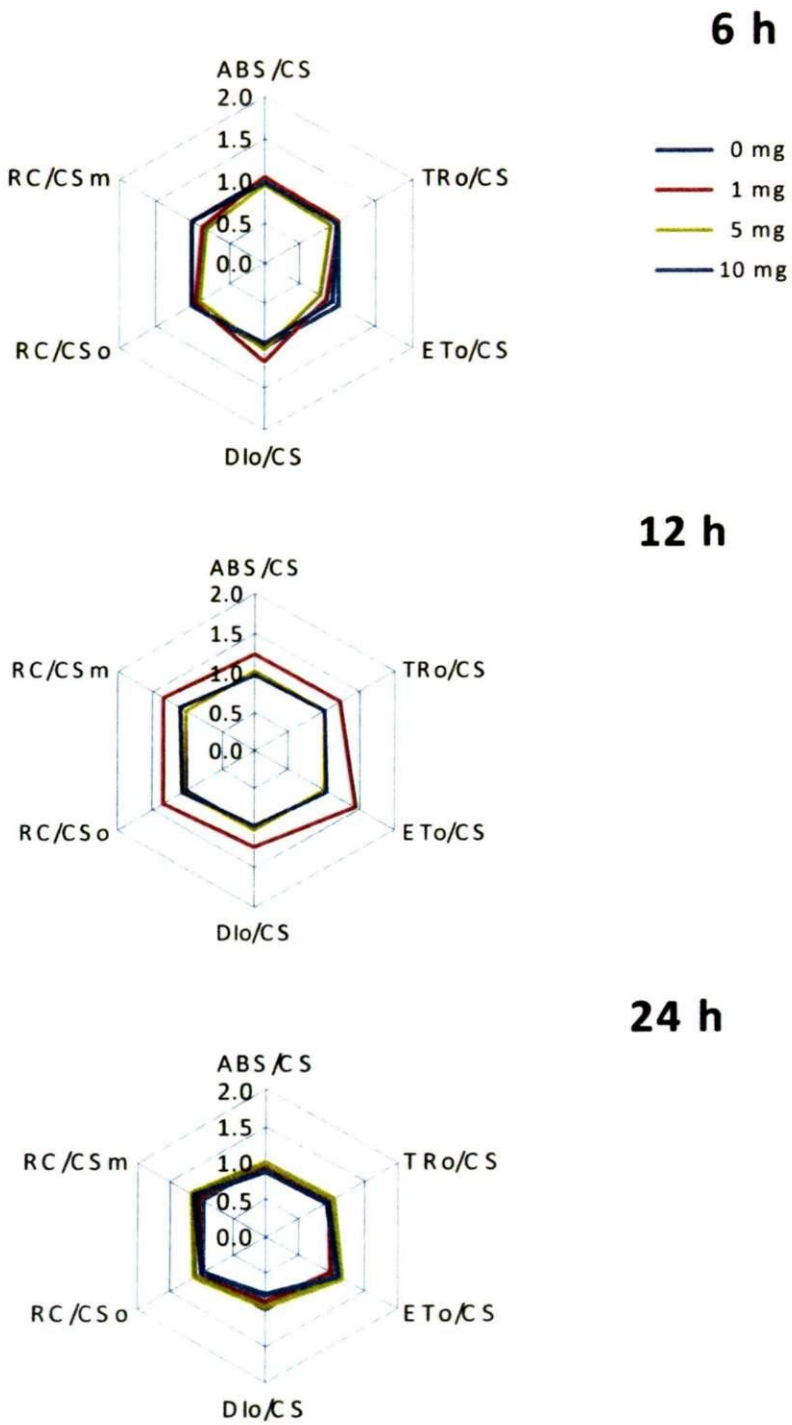

Fig. 3. 20. Influence of cadmium treatment on several selected functional and structural JIP-test parameters plotted relative to the respective controls (set as reference = 1.0). *Fucus serratus* collected from Bantham Quay was exposed to 0 ~ 10 mg Cd L^{-1} for 24 hr, 96 hr and 7 d. For the definition of abbreviations, see Fig. 3. 17 or text.

TR_0 / CS (maximal trapping rate of photosystem II)

Maximal rate of trapped excitation by the CS at t = 0 (TR_0 / CS) was quantified by Handy PEA. Like ABS / CS, TR_0 / CS was not developed with 0 ~ 10 mg Cd L^{-1} in either population for 7 and 14 d. There were no significant differences between the exposure times (7 and 14 d) in BQ, however, in RP, 14 d showed higher TR_0 / CS than 7 d. The RP population showed slightly higher TR_0 / CS than the BQ population ($p = 0.041$), especially after 14 d exposure. When factors were plotted as spider web with values compared with each control treatment, TR_0 / CS at concentrations of 5 and 10 mg Cd L^{-1} was lower than 0 and 1 mg L^{-1} at 7 d. However all Cd treated materials showed similar values after 14 d exposure in RP (Fig. 3. 17). In BQ, values of TR_0 / CS were much lower than the control at 7 d. However values were higher at 14 d and the higher Cd treatment showed higher trapped energy (Fig. 3. 18).

Shorter exposure of 6, 12 and 24 hr did not affect TR_0 / CS levels in either RP or BQ with 0 ~ 10 mg Cd L^{-1} ($p > 0.05$). However the Cd treatment showed different effects on each population. In RP, TR_0 / CS values increased by Cd treatments ($p = 0.004$), especially by 1 and 10 mg L^{-1}, and 5 mg L^{-1} showed similar levels to all other Cd treatments ($p > 0.05$). In BQ, values of trapped excitation were not different by Cd treatments ($p > 0.05$). Total values of TR_0 / CS from RP population were two times higher than values from BQ for 24 hr ($p < 0.0001$). When values were compared with the control, trapped excitation by the CS increased with exposure time in RP (Fig. 3. 19). However the changes were not clear in BQ population and only 1 mg L^{-1} showed significant increase after 12 hr exposure (Fig. 3. 20).

ET_0 / CS (electron transport flux per cross section)

Electron transport flux per CS was measured by Handy PEA at t = 0 (ET_0 / CS). Neither Cd doses from 0 to 10 mg Cd L^{-1} nor Cd exposure time (7 and 14 d) changed electron transport flux in either population ($p > 0.05$). Differences between the two populations were also not detected ($p > 0.05$). The values compared to each control were calculated and described in Figs 3. 17 and 3. 18. Only 1 mg Cd L^{-1} was higher at 7 d, however all Cd treatments (1 ~ 10 mg L^{-1}) raised the values at 14 d in RP (Fig. 3. 17). Meanwhile, in BQ, Cd exposed materials had lower ET_0 / CS than the control at 7 d, although the values increased and were higher at 14 d (Fig. 3. 18). There was no statistical significance between the two locations from which the algae were collected ($p > 0.05$).

Exposure time and Cd concentration had different effects on RP and BQ with shorter exposures (6, 12, 24 hr). Length of Cd exposure had no effect on electron transport flux in RP ($p > 0.05$) but had an effect in BQ ($p = 0.001$). 12 hr exposure showed the highest ET_0 / CS in BQ ($p = 0.001$), especially with 1 mg Cd L^{-1}, and 6 and 24 hr exposure showed similar levels ($p > 0.05$). Meanwhile, although the RP population was not changed by exposure time, this population responded to Cd exposure ($p = 0.006$). ET_0 / CS increased with Cd concentrations and 5 and 10 mg L^{-1} treatment showed significant differences to the control ($p = 0.026$, $p = 0.006$ respectively). 1 mg L^{-1} exposure was not different from the other Cd concentrations ($p > 0.05$). However Cd concentration did not affect ET_0 / CS in BQ population and all Cd concentrations showed similar values for 6, 12 and 24 hr ($p > 0.05$). The total values of ET_0 / CS were significantly higher at RP than the values at BQ ($p < 0.0001$). When each value was compared with the control of each exposure time, ET_0 / CS in RP increased with Cd concentration at 6 hr, however the difference was not clear at 24 hr (Fig. 3. 19). In BQ, Cd treatments values decreased at 6 hr and increased again at 12 and 24 hr except in the case of 1 mg L^{-1}. The 1 mg L^{-1} treatment showed significantly higher ET_0 /

CS after 12 hr and decreased again at 24 hr showing lower values than 5 and 10 mg L^{-1} treatments (Fig. 3. 20).

DI_0 / CS (effective dissipation per cross section)

Effective dissipation per CS at t = 0 (DI_0 / CS) was estimated by Handy PEA. In both populations, all Cd treated algae showed lower values than 0 mg Cd L^{-1} at 7 d, however the differences were not significant after 14 d. For the first 7 d of exposure, two populations showed similar values, however mean values of RP were much higher than those of BQ at 14 d ($p = 0.010$). Compared with each control, 1 ~ 10 mg L^{-1} of Cd treatments reduced the values after 7 and 14 d of exposure in RP and the decreased rates were lower at 14 d than at 7 d (Fig. 3. 17). In BQ, unlike RP, DI_0 / CS was much lower than the control at 7 d however the values were boosted with increased Cd treatment at 14 d (Fig. 3. 18). The values from each population showed significant differences ($p = 0.01$).

DI_0 / CS was not affected by shorter exposure (6 ~ 24 hr) with 0 ~ 10 mg Cd L^{-1} in either population ($p > 0.05$). However Cd exposure had significant effect on DI_0 / CS in both populations. Cd treatment (1 ~ 10 mg L^{-1}) increased DI_0 / CS in RP and 1 and 10 mg L^{-1} had significantly higher values than 0 mg L^{-1} ($p = 0.013$, $p = 0.010$ respectively). In BQ, only a slight difference was found between 1 mg L^{-1} and 10 mg L^{-1} ($p = 0.043$). The total values of DI_0 / CS in RP were significantly higher than the values in BQ with shorter exposure time ($p < 0.0001$). Each value was compared with each control (Fig. 3. 19 and 3. 20). The values of DI_0 / CS increased in RP with exposure time (Fig. 3. 19). Cd treatment enhanced higher dissipation in RP, however differences between Cd concentrations were not significant. In BQ, only 1 mg Cd L^{-1} showed a clear increase at 6 and 12 hr and the other treatments were similar to the control. After 24 hr, all

treatments showed similar values except 10 mg L^{-1} which was lower than the control (Fig. 3. 20).

RC / CS_0

Proportions of the active reaction centres to the CS of the measured sample at t = 0 (RC / CS_0) was measured by Handy PEA. For the first 7 d RC / CS_0 decreased with Cd treatment without statistical significance ($p > 0.05$), however, the values were very similar at all Cd concentrations in both populations ($p > 0.05$). Neither exposure time nor metal-exposure history (location factor) showed significant differences ($p > 0.05$). Compared with each control value, in RP, 5 and 10 mg Cd L^{-1} showed lower, but not significant values at 7 d, while the values were not different from the control at 14 d (Fig. 3. 17). In BQ, the values were much lower than the control after 7 d of Cd exposure however were enhanced at 14 d (Fig. 3. 18).

When *Fucus* was given shorter exposure times (6 ~ 24 hr) with same Cd concentrations (0 ~ 10 mg L^{-1}), active reaction centres to the CS at t = 0 in both populations responded to the exposure time. In RP, time of Cd exposure increased reaction centre values, with the differences between 24 hr exposure and 6 hr exposure being particularly noticeable ($p = 0.036$). In the case of BQ, 12 hr treatment was higher than the other two exposure time ($p = 0.036$). With Cd concentration of 0 to 10 mg L^{-1}, the two populations showed a different response. In RP RC / CS_0 values increased with Cd exposure ($p = 0.003$) and all Cd treatments (1 to 10 mg L^{-1}) gave significantly higher values when compared with 0 mg L^{-1}. The BQ population did not respond significantly to Cd treatment ($p > 0.05$). Total RC / CS_0 values were significantly higher in RP than BQ ($p < 0.0001$). When each value was compared with each control, Cd exposure increased the reaction centre proportions from 6 hr in RP (Fig. 3. 19) however the

values were similar to each other irrespective of Cd concentration (1 to 10 mg L^{-1}). In BQ, RC / CS_0 values were similar to the control at 6 hr (Fig. 3. 20). At 12 hr exposure, only 1 mg L^{-1} showed much higher reaction centre values and, at 24 hr, only 5 mg L^{-1} showed slightly higher levels.

RC / CS_m

The number proportional to the active reaction centres to the CS at t = m (RC / CS_m) was also determined using the Handy PEA. RC / CS_m was affected neither by Cd treatment (0 ~ 10 mg Cd L^{-1}) nor by exposure time (7 and 14 d). No local difference was determined. When the values were compared with each control, only 1 mg Cd L^{-1} showed increased values at 7 and 14 d in RP (Fig. 3. 17). In BQ, Cd treatment diminished RC / CS_m at 7 d however all values were enhanced at 14 d (Fig. 3. 18) .

Fucus responded significantly to shorter exposure times with 0 to 10 mg L^{-1} Cd in both populations. In RP, RC / CS_m was similar until 12 hr, however significantly increased values were measured at 24 hr ($p = 0.006$). In BQ, the response was faster than RP and the only significant differences were detected were between 6 and 12 hr exposure times ($p = 0.002$). Like RC / CS_0, only the RP population showed significant response to Cd concentration ($p = 0.012$). Cd treatment increased RC / CS_m in RP, while Cd exposure did not affect the reaction centre values in BQ. 1 and 10 mg L^{-1} showed higher RC / CS_m values than the control ($p = 0.045$, $p = 0.016$ respectively) and 5 mg L^{-1} was not different from the other treatments ($p > 0.05$). The total values at t = m was very significantly higher at RP than BQ ($p < 0.0001$). When each value was compared with each control, in RP, Cd treatment (1 to 10 mg L^{-1}) enhanced values from 6 hr, however differences among exposure time or Cd concentrations were not clear (Fig. 3. 19). In BQ, 1 and 5 mg L^{-1} slightly decreased values at 6 hr, however they recovered by

12 hr, except 1 mg L^{-1} (Fig. 3. 20). Only 1 mg L^{-1} showed significantly higher value at 12 hr.

JIP-curve

Changes of Chl *a* fluorescence transients were plotted on a logarithmic timescale from 50 μs to 1 s (Figs 3. 21 and 3.22). After 7 d exposure in RP, 5 mg L^{-1} showed an apparent decrease and 10 mg L^{-1} had similar pattern and values to those of the control (Fig. 3. 21). 1 mg Cd L^{-1} treatment achieved a higher curve than the other treatments. However, after 14 d exposure in RP, every curve showed higher values than 7 d exposure and all Cd treatments had apparently higher F_p (= F_m) levels than the control. Meanwhile, in BQ, the control of 7 d exposure had much higher values whereas the other treatments were relatively similar to one another. In the 14 d exposure, the control of BQ decreased and showed the lower F_I and F_P values than the other Cd treatments (Fig. 3. 21).

When *F. serratus* was exposed to Cd (0 ~ 10 mg L^{-1}) for 6 and 12 hr, the Chl *a* fluorescence transient curves were not changed and any significant differences among the treatments were not discovered in RP (Fig. 3. 22). After 24 hr of Cd exposure, Cd treated materials showed slightly higher values than the control of RP. BQ populations always showed lower curves than the RP population for 6 to 24 hr (Fig. 3. 22). After 6 hr exposure 10 mg L^{-1} showed different F_P levels and after 12 hr exposure 1 mg L^{-1} had different F_P levels in BQ. However after 24 hr exposure, all materials showed very similar patterns.

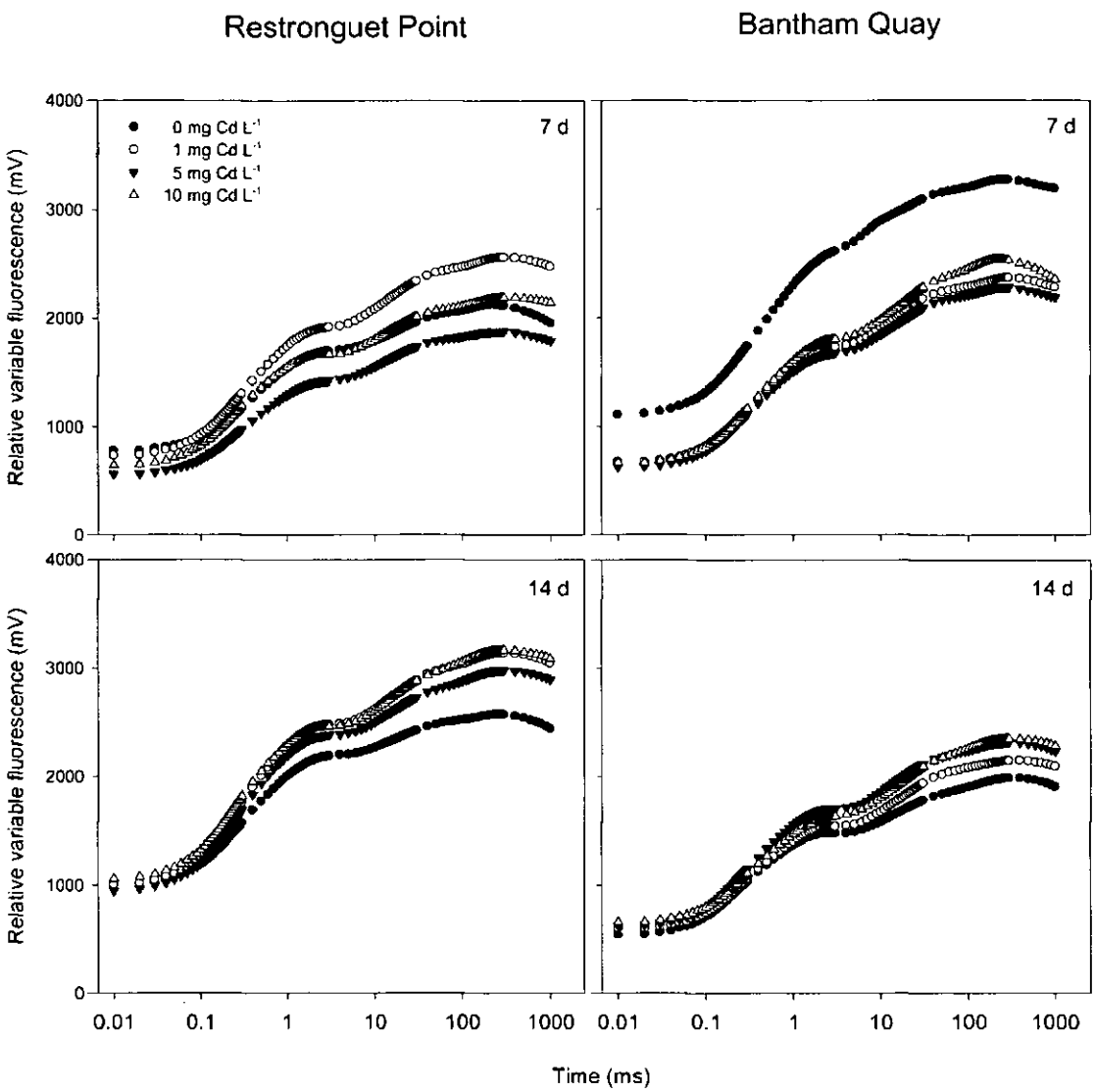

Fig. 3. 21. The change of rapid polyphasic kinetics of chlorophyll *a* fluorescence transients plotted on a logarithmic timescale when *Fucus serratus* was exposed to 0 ~ 10 mg Cd L^{-1} for 7 and 14 d. Values are expressed by mean (n = 8).

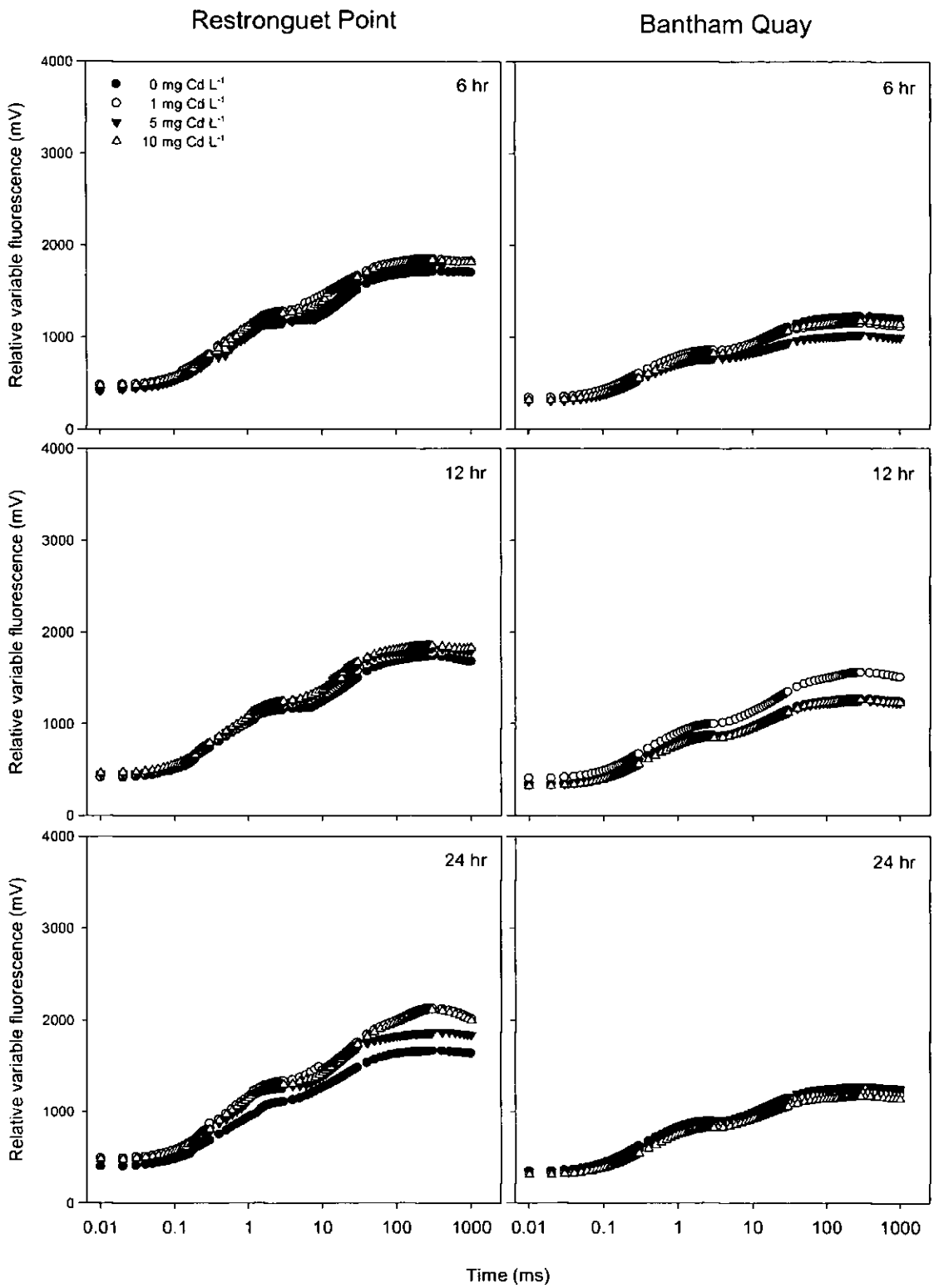

Fig. 3. 22. The change of rapid polyphasic kinetics of chlorophyll *a* fluorescence transients plotted on a logarithmic timescale when *Fucus serratus* was exposed to 0 ~ 10 mg Cd L^{-1} for 24 hr, 96 hr and 7 d. Values are expressed by mean (n = 10).

3. 3. 6. Pigment

Changes in contents of some photosynthetic pigments were evaluated in *F. serratus* which was exposed to 0 ~ 1000 μg Cd L^{-1} for 24 hr, 96 hr and 7 d (Fig. 3. 23 and 3.24). Chl *a*, Chl *c*, Fx and β-carotene here are well-known photosynthetic pigments of marine brown algae.

3. 3. 6. 1. Chlorophyll *a* (Chl *a*)

Cd exposure significantly increased the contents of one of the main photosynthetic pigments, Chl *a*, in RP (Fig. 3. 23). Extended Cd exposure (time of exposure and concentration) increased Chl *a* values, however no statistical significance was estimated between 10 ~ 1000 μg L^{-1} ($p > 0.05$).

In BQ, the effect of exposure time and Cd concentration was not significant ($p > 0.05$) (Fig. 3. 23). Chl *a* in BQ decreased significantly only at 1000 μg Cd L^{-1} exposure for 24 hr and 96 hr, however the value increased again at 7 d exposure.

The polluted population showed very significantly higher values than the values from the reference population ($p < 0.0001$).

3. 3. 6. 2. Chlorophyll *c* (Chl *c*)

Another main photosynthetic pigment of brown algae, Chl *c* was also presented significantly higher levels in the polluted population, RP ($p < 0.0001$, Fig. 3. 23). Like Chl *a* in RP population, Chl *c* also increased with Cd treatment in RP, however the effect of exposure time on Chl *c* contents was not statistically significant between 10 ~ 1000 μg L^{-1} ($p > 0.05$). Meanwhile Cd concentrations in RP showed clear effects and

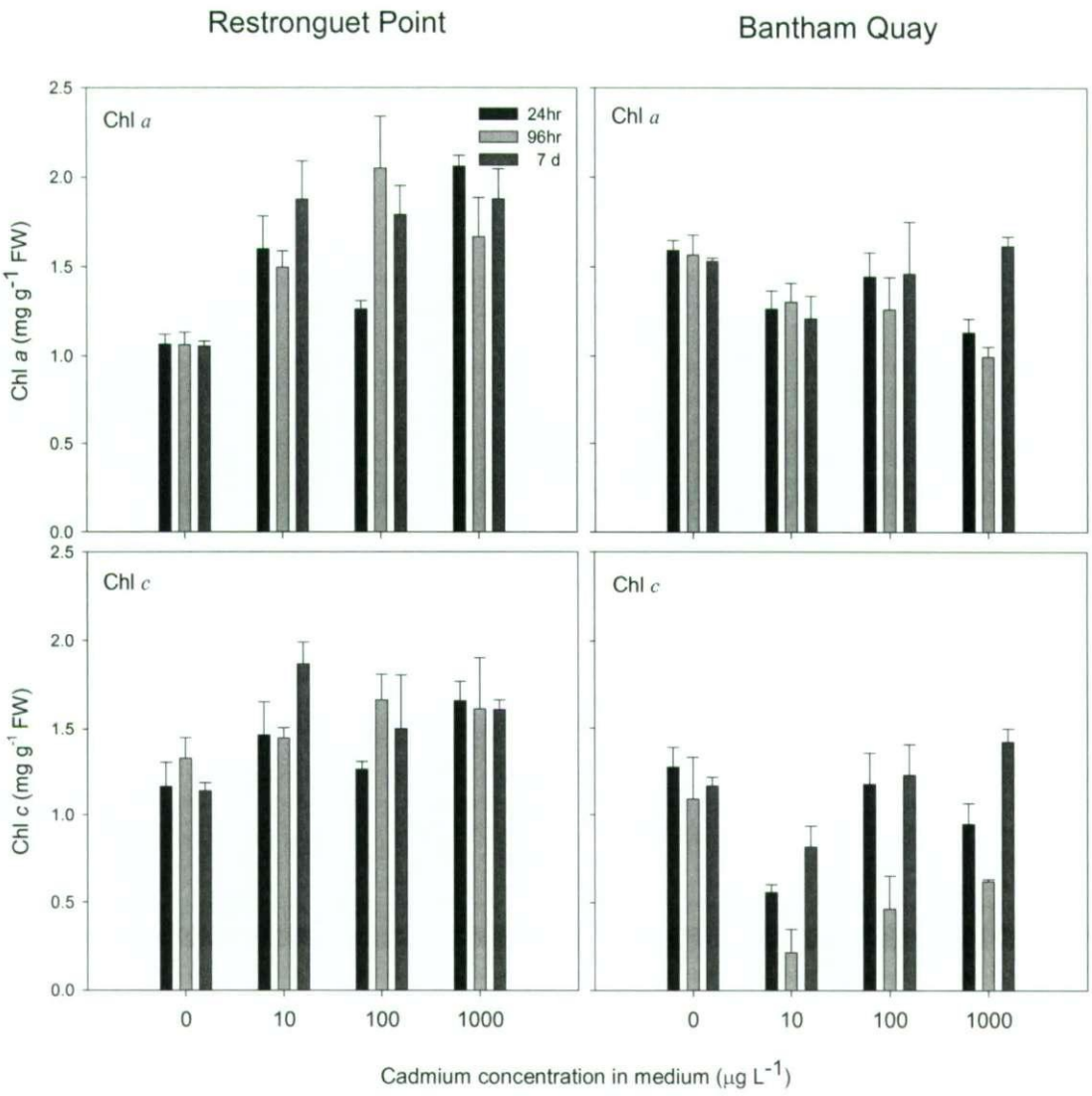

Fig. 3. 23. Chlorophyll *a* (Chl *a*) and *c* (Chl *c*) contents changes in *Fucus serratus* exposed to cadmium for 24 hr, 96 hr and 7 d. Restronguet Point is the metal-polluted site and Bantham Quay is the reference site in South West England. Values are means and standard deviations (n = 3).

100 and 1000 μg Cd L^{-1} significantly increased Chl *c* values compared to lower concentrations ($0 \leq 10 \leq 100 = 1000$, $p = 0.005$).

Chl *c* contents in BQ showed unexplained pattern except the 7 d treatment (Fig. 3. 23). 96 hr exposure showed significantly low values in 10 ~ 1000 μg Cd L^{-1} ($p < 0.0001$) and values of 24 hr and 7 d were higher in order (96 hr < 24 hr < 7 d). The effect of Cd concentration was also clear ($p < 0.0001$) and a concentration of 10 μg Cd L^{-1} was significantly low ($p < 0.0001$).

3. 3. 6. 3. Fucoxanthin (Fx)

Contents of Fx were measured with *F. serratus* exposed to 0 ~ 1000 μg Cd L^{-1} for 24 hr, 96 hr and 7 d (Fig. 3. 24). Both populations showed significant effects by both time of Cd exposure and Cd concentrations ($p < 0.0001$, respectively). In RP, Cd treatment increased the Fx content and all Cd treated materials presented significantly higher values than 0 μg Cd L^{-1}. Cd treatment and longer exposure induced higher Fx contents, but there was no difference between Cd concentrations (10 ~ 1000 μg L^{-1}).

In BQ, Cd treatment decreased the contents of Fx in *F. serratus*. Cd treatment and time of exposure affected the Fx contents, but there was no significance between Cd concentrations (10 ~ 1000 μg L^{-1}). 96 hr exposure produced the lowest values followed by 7 d and 24 hr ($p < 0.0001$). Cd treatment reduced Fx content in BQ until 96 hr ($p < 0.0001$) and levels of Fx significantly decreased with increasing Cd concentrations in the media ($p < 0.0001$, Fig. 3. 24). At 7 d, Fx contents increased again at 100 and 1000 μg Cd L^{-1} and showed similar values to 10 μg Cd L^{-1}.

Without Cd treatment, the BQ population possessed higher values of Fx than the RP population. However, after Cd treatment, the polluted population possessed very significantly higher contents of Fx than the reference population ($p < 0.0001$).

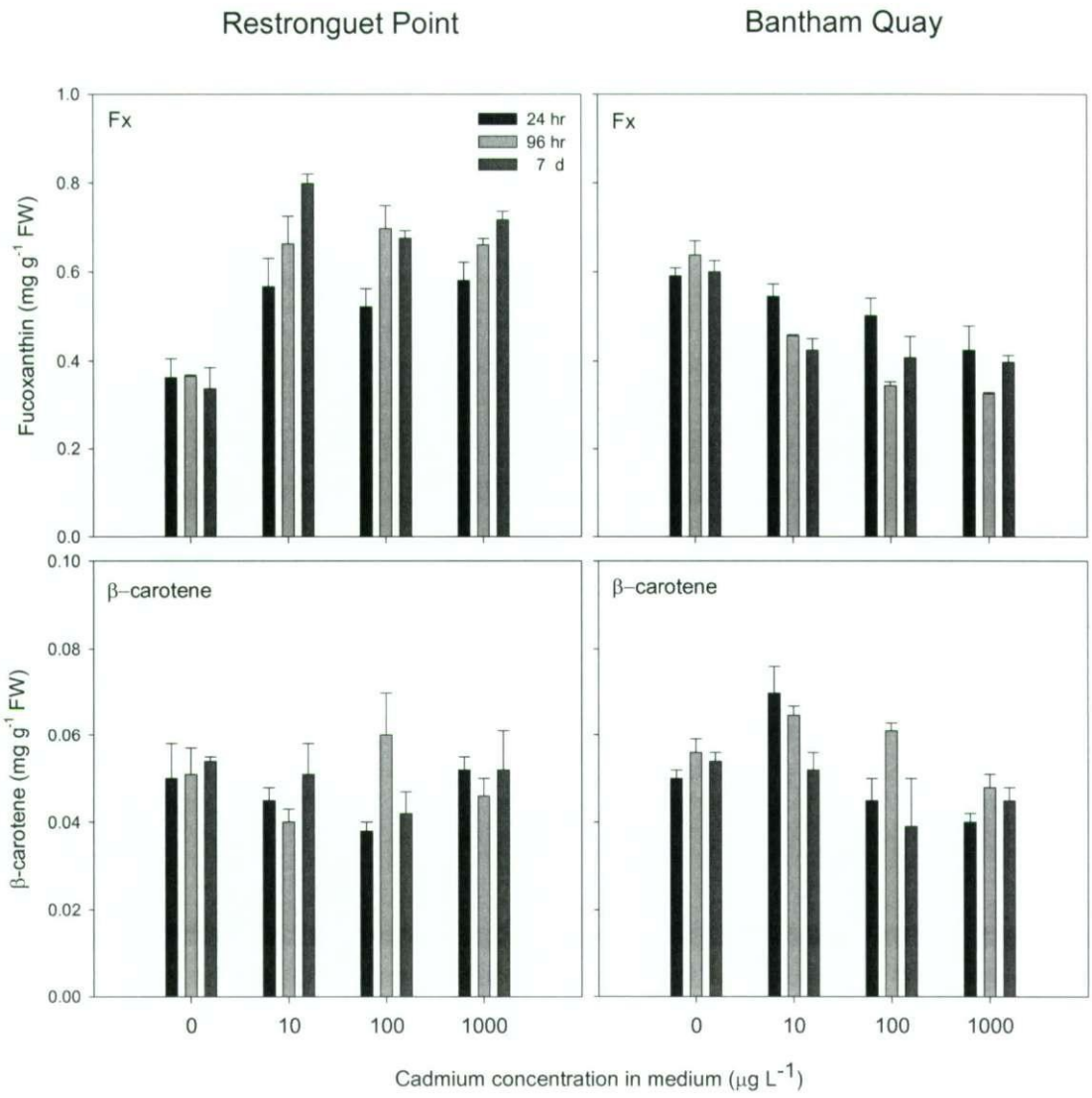

Fig. 3. 24. Fucoxanthin (Fx) and β-carotene content changes in *Fucus serratus* exposed to cadmium for 24 hr, 96 hr and 7 d. Restronguet Point is the metal-polluted site and Bantham Quay is the reference site in South West England. Values are means and standard deviations (n = 3).

3. 3. 6. 4. β-carotene

Another accessory photosynthetic pigment measured in the present study was β-carotene. Cd exposure, concentration and time of exposure were not linked to the alteration of β-carotene levels in RP ($p > 0.05$). However, Cd concentration affected β-carotene contents significantly in BQ ($p < 0.0001$). Values decreased with Cd treatment and higher Cd concentration showed lower β-carotene except 10 μg Cd L^{-1}. *Fucus* treated with 10 μg Cd L^{-1} possessed the highest β-carotene in BQ, followed by 0 μg L^{-1}, then 100 and 1000 μg L^{-1} ($10 > 0 \geq 100 \geq 1000$ μg Cd L^{-1}).

The BQ population contained significantly higher β-carotene than the RP population ($p = 0.0002$, Fig. 3. 24).

3. 4. Discussion

High concentrations of metals, including essential elements, in the environment are reported to be inhibitors of plant/algal growth and development (Kalaji and Loboda, 2007). Although many negative effects of metals on physiological and photochemical processes have been published to date, a comprehensive review on the overall photosynthetic response to metal stress has not yet been produced (Clijsters and Van Assche, 1985, Sujak, 2005, Kalaji and Loboda, 2007). In this chapter, the physiological responses of *Fucus serratus* to Cd stress were studied, with a primary focus on changes of photosynthetic parameters, as well as bioaccumulation of Cd by two different *Fucus* populations.

3. 4. 1. Interaction between stress factors

Under natural environmental conditions, organisms may experience many complex, and unpredictable situations. Marine seaweeds, by virtue of their habitat, can be exposed to a number of adverse conditions, such as high or low light, high or low temperature, desiccation, nutrients, predation or industrial pollution (Davison and Pearson, 1996, Hashim and Chu, 2004). These adverse conditions may work together in various ways and produce complex compound effects on organisms. Trace metals are among the factors that may induce various unpredictable effects when they interact, and Berry and Wallace (1981) categorized such combination results. Firstly, an independent action is one in which plants exposed to more than one factor show the same responses as plants exposed to the greater stress factor independently. Another category, an additive action, means that plants show the sum of responses produced by the single

stress factors. The third one is an antagonistic action which represents smaller combined effect than a single effect of the most active stress. The final category is a synergistic action which means that the combined effect is greater than a single effect of the most active factor.

Most research, including the present study, are focused on a single stress factor and do not often regard the combined effects in experiments. However, under natural circumstances in the field, these complex interactions between various environmental factors cannot be ignored. In the case of metals, high level of Ca is known to moderate the toxicity of some trace elements, such as Ni, Cd, Al, Mn or Cu (Rengel, 1992, Hagemeyer, 1999, Hashim and Chu, 2004). Furthermore, high levels of Mn or Zn (up to 1000 g L^{-1}) are also able to suppress the uptake of Cd, Co, Ni, Zn or Mn in *Fucus vesiculosus* (Bryan et al., 1985).

Besides metals, other environmental conditions may induce combined results. Seasonal variations of rainfall, temperature, and light intensities, or the life stage of the organism, etc. can influence the physiological responses of algae and these factors may cause combined effects. Therefore maximum efforts to explain these complex conditions are required to minimize any errors and to understand any responses and discrepancies. These combined factors have been considered in some parts of this thesis.

3. 4. 2. Bioaccumulation in natural populations of *Fucus serratus*

Seaweeds have been widely used for monitoring metal concentrations in aquatic systems since they can indicate the composition or abundance of metal species present. Brown seaweeds were reported to be unable to regulate the uptake of metals and

represented the relative and absolute contents of metals in the surrounding water (Bryan, 1969, Fuge and James, 1974). Bryan (1969) reported a linear relationship between Zn concentrations in *Laminaria digitata* and those in seawater, and also reported considerably magnified metal concentrations compared to contents in seawater.

Fucus serratus harvested from wild populations at RP contained noticeably higher values of metals except for Cd. Both total and non-exchangeable concentrations of Cu, Pb and Zn were significantly higher at RP, which must be linked to the highly metal-contaminated aquatic system at RP. As mentioned in the previous chapter (Chapter 2), Restronguet Creek is an extremely contaminated area caused by a very active mining industry that flourished for several hundred years (Bryan and Gibbs, 1983). Although all the mines are now closed, drainage water from the closed mines and the erosion of the slag heaps are still having a serious effect on the area (Bryan and Gibbs, 1983).

Contamination by metal species in Restronguet Creek can be compared with other sites in Table 3. 9. Levels of all elements of metals are extremely high at Restronguet Creek, therefore any organisms including marine macroalgae from RP must have been readily exposed to very high levels of metals for a long time. Compared with Restronguet Creek, the Avon estuary is a relatively very clean and conserved area. Bryan et al. (1987) made a comparison between Restronguet Creek and the Avon estuary and reported 3000 $\mu g\ g^{-1}$ of Cu and 2500 $\mu g\ g^{-1}$ of Zn in the sediment from the Restronguet Creek and 20 $\mu g\ g^{-1}$ of Cu and 100 $\mu g\ g^{-1}$ of Zn in the sediment from the Avon area (Table 2. 1). Compared with background levels of other sediment data (Table 3. 9), RP contained much higher levels of Cu and Zn although BQ was in the range of those data.

Total concentrations of metal species can be compared with previous data. Table 3. 10 shows the normal composition of trace elements in a plant and Table 3. 11 shows levels of trace metals in *Fucus* spp. recorded in previous studies.

Table 3. 9. Background levels in natural water and sediment (Förstner and Wittmann, 1979) and the upper limit of non-polluted soil (De Temmerman et al., 1984). The table was modified from Bryan and Gibbs (1985) and Greger (1999).

Metal	Water (μg L^{-1})			Soil (μg g^{-1})		Sediment (μg g^{-1})	
	Freshwater	Seawater	Restronguet Creek	Sandy soil	Loam	Lake	Sea
Cd	0.07	0.01 – 0.07	<0.1 – 38	1	1	0.14 – 2.5	0.02 - 0.43
Cr	0.5	0.08 – 0.15		15	30	7 – 77	11 – 90
Co	0.05	0.04		5	15		0.1 – 74
Cu	1.8	0.04 – 0.1	2 – 176	15	25	16 – 44	4 – 250
Hg	0.01	0.01		0.15	0.15	0.004 – 0.2	0.001 – 0.4
Mn	< 5	0.2	3- 1513	500	800		390 – 6700
Mo	1	10		5	5		0.2 – 27
Ni	0.3	0.2 – 0.7	1 – 18	1	1	34 – 55	2 – 225
Pb	0.2	0.001 – 0.015	<2 – 4	50	50	14 – 40	7 – 80
Zn	10	0.01 – 0.62	7 – 22460	10	150	7 - 124	16 - 165

Table 3. 10. Normal composition of trace elements in a plant (Markert, 1992). The table was modified from Markert (1992) and Greger (1999).

Trace element	$\mu g\ g^{-1}$
Aluminium	80
Cadmium	0.05
Chromium	1.5
Cobalt	0.2
Copper	10
Gold	0.001
Iron	150
Lead	1.0
Manganese	200
Mercury	0.1
Molybdenum	0.5
Nickel	1.5
Silver	0.2
Zinc	50

Table 3. 11. Reported trace metals levels in *Fucus* spp. (μg g^{-1} dry weight).

Location	Cd	Co	Cr	Cu	Fe	Ni	Pb	Zn	Authors
Goury, NW France	0.5 - 1.9	0.3 - 1.3	0.1 - 0.8	0.8 - 2.0	11 - 278	1.4 - 5.5	0.2 - 2.0	32 - 100	Greger 1999
Irish sea	1.1 - 1.4			3.2 - 10.1	127 - 249	4.1 - 6.7	2.1 - 4.0	80 - 171	Preston et al. 1972
Dorset, UK	0.3							99	Leatherland and Burton 1974
Bristol Channel[b]	3.8 - 19.5			3.8 - 14.3				88 - 262	Fuge and James 1974
Menai Straits, UK[b]	1.8 - 2.1		3.8 - 4.5	7.4 - 10	146 - 360	7.1 - 8.9	2.3 - 3.2	98 - 138	Foster 1976
Looe estuary, UK	0.9 - 2.4	0.6 - 10.5	0.6 - 3.5	3.5 - 33	121 - 3020	5.7 - 13.6		56 - 340	Bryan and Hummerstone 1977
Tamar estuary, UK[b]	1.8 - 6.4	1.8 - 9.0	1.1 - 10	20 - 107	401 - 1300	0.7 - 4.8	5.9 - 109	138- 1330	Bryan and Uysal 1978
Norway	7 - 13			39 - 150			12 - 250	1590- 4700	Melhuus et al. 1978
Estuaries, UK[a]	1 - 28	0.9 - 7.8	1.0 - 5.7	4 - 293	90 - 967	4.5 - 36	1.6 - 29	85 - 1360	Bryan 1983
Estuaries, UK[b]	0.7 - 4.8	1.9 - 7.4	2.5 - 4.8	7 - 302	1128 - 2045	2.6 - 53	2.4 - 21.6	69 - 1120	Bryan et al. 1983
Restronguet Creek[b]	0.81 – 1.41			190 - 1450				2190 - 4200	Bryan and Gibbs 1983
Mersey estuary[b]	0.5 - 2.5		0.7 - 6.6	10 - 42	227 - 1533	4.1 - 22.5	1.1 - 15.6	209 - 1964	Langston 1986
Sweden[b]	6 - 12.3	0.3 - 1.2	0.3 - 0.6	4.3 - 6.3	67 - 261	5.4 - 30.3	1.7 - 4.0	255 - 815	Forsberg et al. 1988
Baltic Sea[b]	4.1 - 17.2	0.2 - 3.2	0.11 - 1.44	2.2 - 8.0	48 - 522	4.1 - 46.4	2.0 - 11.7	181 - 877	Söderlung et al. 1988
Restronguet Point[a]	2.1 – 2.7			359 - 494			3.4 – 4.5	437 – 515	Pawlik-Skowrońska et al. 2007
Restronguet Point[b]	1.2 – 1.9			290 – 398			2.8 – 4.0	285 – 336	Pawlik-Skowrońska et al. 2007
Bantham Quay[a]	4.1 – 5.7			105 - 164			1.2 – 4.1	64 – 97	Pawlik-Skowrońska et al. 2007
Bantham Quay[b]	1.6 – 2.8			126 - 173			1.2 – 2.3	51 – 62	Pawlik-Skowrońska et al. 2007
Wembury Beach[a]	4.4 – 5.7			117 - 164			2.9 – 4.1	82 – 97	Pawlik-Skowrońska et al. 2007
Restronguet Point[a]	0.9 – 1.2			78 – 103			1.3 – 1.4	336 – 429	This study
Bantham Quay[a]	1.2 – 1.3			4.6 – 5.3			0.9- 1.0	40 - 50	This study

[a] *F. serratus*. [b] *F. vesiculosus*

Different plant species as well as different plant strains have different abilities of uptake and accumulation of metals (Greger, 1999). Therefore Markert (1992) tried to make a standard example of metal concentrations of a normal plant which can be compared with in the other research. Although the 'normal reference plant' was based on terrestrial plants, the data was useful to evaluate the contaminated levels of *F. serratus* in this study. Markert (1992) and other researchers used $\mu g\ g^{-1}$ D.W. (or $mg\ kg^{-1}$ D.W.) as the unit of metal concentrations, therefore data from the present study were re-calculated to the same unit for comparison (Table 3. 11). Concentrations of Pb and Zn in *Fucus* from BQ were in the range of Table 3. 10, although those of Cd and Cu were outside this range. However, all levels of metals in *Fucus* from RP were significantly higher than the normal reference plant (Markert, 1992).

In Table 3. 11, levels of trace metals in *Fucus* spp. were compared. Natural *Fucus* populations from RP showed high values of trace elements relative to those from other sites. However, compared with the previous report by Pawlik-Skowrońska et al. (2007), the concentrations of metal elements in *F. serratus* from both RP and BQ were significantly lower in the current study. This difference might have been caused by weather changes or collecting season since collection of algal material and analyses of metal contents were performed by the same person and instrument. One of the reasons related to the weather may be differences in rainfall. *Fucus* materials were collected in Oct. 2005 for Pawlik-Skowrońska et al. (2007) and the reported rainfall during the period in South West England was 186.6 mm (http://www.metoffice.gov.uk). For the current study, *Fucus* was collected in Oct. 2006 for the lower concentration exposure and in May 2007 for the higher concentration exposure. The rainfall values were reported as 169.2 mm (Oct. 2006) and 137.8 mm (May 2007) respectively. Therefore the higher rainfall might have released higher metal elements to marine organisms and they could have then taken up and accumulated significantly higher levels of trace

metals. In addition, the difference in seasons (autumn and spring) might be also related (e.g. temperature).

Interestingly Cd concentrations in RP materials were similar, even slightly lower, to those in BQ materials even though Cd contents in the seawater were significantly higher in RP. This discrepancy has been reported by some authors (Bryan, 1983, Bryan and Gibbs, 1983, Pawlik-Skowrońska et al., 2007). Most of them highlighted the competition between cations in ion channels and the suppression of Cd uptake by other higher metals and metalloids (Bryan and Gibbs, 1983, Bryan et al., 1985, Hashim and Chu, 2004, Pawlik-Skowrońska et al., 2007). They postulated that high levels of Zn reduce Cd absorption by algae and high levels of Mn, Cu as well as Ca also have similar ability to suppress the uptake of Cd (Bryan, 1983, Bryan and Gibbs, 1983, Hashim and Chu, 2004). The ratio of Zn and Cd in Restronguet Creek reported was 400 : 1 compared with about 10 : 1 in the Severn Estuary (Bryan, 1983). This suppression of Cd accumulation was discovered not only in marine macroalgae but at all trophic levels (Bryan, 1983). In addition, Malea (1994) reported a lower uptake of non-essential metal elements (such as Cd) in seagrass *Halophila stipulaceae* compared with a relatively higher uptake of essential metals (such as Cu and Pb). Active exclusion or sequestration of the non-essential elements were regarded for minimizing the toxic effect of metals (Ralph and Burchett, 1998).

3. 4. 3. Bioaccumulation by Cd exposed populations of *Fucus serratus*

As mentioned previously, brown algae are known to be incapable of regulating their uptake of trace metals. Therefore the concentrations of metals in seaweeds depend on prevailing concentrations in the surrounding environment (Fuge and James, 1974).

This may explain the linear increase of Cd accumulations of *F. serratus* exposed to Cd in the present study. Bryan (1969) also demonstrated a linear interaction between Zn contents in *Laminaria digitata* and those in the environment.

Bryan (1980) postulated that lower metal-permeability to Cu by tolerant seaweed helped to reduce the internal Cu concentration and this was also reported by Nielsen (2002). *F. serratus* was exposed to 42.2 ~ 844 nM [Cu^{2+}] and the RP population accumulated significantly lower levels of Cu (Nielsen, 2002). However values of total and non-exchangeable contents of Cd were the same at both polluted and reference populations after Cd exposure in this study (except non-exchangeable concentration of RP at 24 hr exposure). Landberg and Greger (1994) published differences in the accumulation of metals by 103 different clones of *Salix viminalis* and reported that the differences were not correlated with tolerance to that metal. It was related rather to the net uptake of Cd including low transport and efflux of the metal (Landberg and Greger, 1996, Landberg and Greger, 2002). Cd absorption/adsorption by *F. serratus* in this study did not show differences by locality. In addition, no visible symptom (e.g. chlorosis, necrosis or death) of Cd toxicity was recognised from either population during the experiment. Since the range of background Cd concentration in the Restronguet Creek water is known to be <0.1 ~ 38 μg L^{-1} (Table 3. 9), most of the Cd doses used in this experiment are extremely high. However, much higher doses of Cd have been used experimentally. Hu et al. (1996) exposed *Gracilaria tenuistipitata* (Rhodophyta) to 12.5 ~ 500 μM of Cd (i.e. about 1.4 ~ 56.2 mg Cd L^{-1}) and LC_{50} of the red alga was 270 μM (30.0 mg Cd L^{-1}). *G. tenuistipitata* grew normally in the medium with 75 μM Cd (8.43 mg Cd L^{-1}) and viability was not affected by 100 μM (11.24 mg Cd L^{-1}). Red algae are reported to be more sensitive than brown algae (Hashim and Chu, 2004), so the LC_{50} of *F. serratus* to Cd exposure can be expected to be much higher than that of *G. tenuistipitata*. Therefore the lack of differences in Cd accumulation

between the two different populations may be based on strong resistance mechanism of this brown alga itself and/or the less toxic characteristics of Cd. The tolerance of *F. serratus* will be discussed in the next two chapters. Moreover, the non-exchangeable fraction of Cd comprised more than 50 % of the total Cd burden in the current and the previous research (Pawlik-Skowrońska et al., 2007). This relatively high ratio of Cd accumulation must be intimately related to effective mechanisms for homeostasis and detoxification of Cd (Pawlik-Skowrońska et al., 2007). This was considered at Chapter 5 with the biosynthesis of thiol peptides.

3. 4. 4. Brown alga, *Fucus serratus* as a bioindicator and metal-remover

Many researchers have used various marine macroalgae to test their abilities to sequester metal ions from media and generally brown algae were recognised the most effective removers of metal ions (Bryan and Gibbs, 1983, Bryan et al., 1985, Hu et al., 1996, Davis et al., 2003, Hashim and Chu, 2004, Pawlik-Skowrońska et al., 2007). Hashim and Chu (2004) used seven different species of brown, green and red algae to determine their Cd sequestering ability and the Langmuir maximum adsorption capacity was red < green < brown algae in order.

The enhanced performance of brown algae is based on their basic structure and biochemical constitution, which is related to the properties of cell wall constituents (Davis et al., 2003). Polysaccharides or extracellular polymeric materials are the chief metal chelating constituents in the cell walls of brown algae, which is directly related to their biosorption capacity (Davis et al., 2003). The alginate, fucoidan and cellulose found in brown algae and the agar and carrageenan are the metal-chelating cell wall

structural polymers in red algae (Hu et al., 1996). Laminariales and Fucales are the brown algal orders with the best biosorption ability, because they produce abundant cell wall matrix polysaccharides and extracellular polymers (Davis et al., 2003). Among species in these two orders, *Fucus vesiculosus* and *Sargassum* spp. are the most frequently used as bioindicators as they are known to reflect the concentrations of dissolved metal elements in the water (Förstner and Wittmann, 1983, Bryan et al., 1985). Föstner and Wittmann (1983) and Bryan et al. (1985) concluded that other *Fucus* species including *F. serratus*, *F. spiralis* and *F. ceranoids* can also be used as bioindicators. *F. serratus* in the present study accumulated total and non-exchangeable concentrations of Cd with a linear relationship with Cd contents in the Aquil medium, which is the typical characteristic of metal uptake by plant bioindicators. In addition, *F. serratus* did not have visual symptoms of Cd stress with up to 10 mg Cd L^{-1} for 14 d, which presents a hyperaccumulation capacity against Cd exposure.

3. 4. 5. Effects of Cd on growth of *Fucus serratus*

The presence of Cd in the range of 1 ~ 10 mg L^{-1} strongly affected RGRs of *F. serratus*. The decreases of RGRs were more significant in the BQ population although the decreases in RP were also significant. RGRs of both populations decreased markedly in response to Cd treatment for 7 d, however they partially recovered after 14 d. Shrunken size and weight loss (or stable weight) of macroalgae caused by metal stress have been observed by many researchers (Bryan and Gibbs, 1983, Brown and Newman, 2003, Han et al., 2008). *Gracilariopsis longissima* (Bryan and Gibbs, 1983), *F. vesiculosus* (Brown and Newman, 2003), *Ulva pertusa* and *U. armoricana* (Han et al., 2008) showed decreased RGRs when they were exposed to Cu. Inhibition of cell

division and/or expansion are known to be induced by metals (Stauber and Florence, 1987), which may possibly be related to a decrease of turgor, an alteration of cell wall elasticity, any other trauma owing to metal toxicity (Brown and Newman, 2003) or acclimatisation.

More significantly decreased RGRs at 7 d and less recovery (still negative growth) at 14 d explained that the BQ population had more serious effects on weight growth by Cd treatment. These differences resulted from the varying resistance ability between populations, depending on their habitats. The inheritance of metal tolerance by marine macroalgae was reported by some researchers (Bryan and Gibbs, 1983, Correa et al., 1996, Nielsen, 2002, Nielsen et al., 2003b). Nielsen et al. (2003b) showed that both adult and young *F. serratus* from RP revealed higher resistance to Cu^{2+} although *F. serratus* from BQ and Wembury Beach were very sensitive to Cu^{2+}. Bryan and Gibbs (1983) reported that adult *F. serratus* from polluted areas had two- to five-fold higher RGRs than *Fucus* from uncontaminated sites. Differences in RGR with high Cd concentrations (1 ~ 10 mg L^{-1}) in this study also support those previous studies. Nevertheless, unlike higher Cd concentrations of 1 ~ 10 mg L^{-1}, changes of RGRs with 10 ~ 1000 μg Cd L^{-1} were less impressive. Clear decreases of RGRs were discovered at all Cd treatments at 24 hr and at 1000 μg Cd L^{-1} at all time intervals studied. In addition lower Cd concentrations did not show clear differences between populations, which may be more easily related to the other physiological responses of *Fucus*. Compared with the higher Cd concentrations, 10 ~ 1000 μg L^{-1} for 24 hr, 96 hr and 7 d might have not affected harmful effects, or common characteristics from both populations, i.e. antioxidative enzymes or metal chelating complexes, might have strongly performed their duty to resist the environmental stress.

3. 4. 6. Effects of Cd on Chl *a* fluorescence of *Fucus serratus*

Chl *a* fluorescence has been recognized as one of the most useful tools in plant stress physiology in the past three decades (Krause and Weis, 1984, Küpper et al., 1998). It has been also used as an estimation method for aquatic primary productivity levels *in situ* over the past two decades (Suggett et al., 2007). Chl *a* fluorescence can present various practical information, such as the excitation energy transfer among pigments and the complexes, and various electron-transfer reactions, specifically of PS II (Govindjee, 2004). Generally the higher metal dosage as well as the longer exposure period are known to cause the greater stress response (more increase or decrease of Chl *a* fluorescence parameters) of plants (Ralph and Burchett, 1998).

However it has been reported that Cd stress in terrestrial plants and marine algae do not affect the fast kinetic fluorescence parameters, especially F_v / F_m ratio (Greger and Ögren, 1991, Krupa et al., 1993, Di Cagno et al., 1999). The previous researchers used similar (but often much higher) Cd concentrations and similar exposure time to those of this study. Di Cagno et al. (1999) used 10 and 20 μM Cd^{2+} for 7 d, Greger and Ögren (1991) used 1, 5, 20, 50 or 2000 μM $CdCl_2$ for 14 d and Krupa et al. (1993) used 10, 20, and 50 μM $CdSO_4$ for 1 week. High Cd treatments (1 ~ 10 mg L^{-1}) in this study did not induce apparent stress responses in most of parameters with longer exposure (7 ~ 14 d) and lower Cd treatment also did not produce clear responses for 96 hr and 7 d. Although F_v / F_m ratio was not affected by Cd treatment, some Chl *a* fluorescence parameters showed a clear relationship with Cd concentration in these previous studies. F_0 from RP in the present research changed with higher Cd concentrations or longer time of exposure, which indicated that *Fucus* was under stress during the Cd treatment even though F_v / F_m and other parameters were not changed (Dan et al., 2000, Maxwell and Johnson, 2000). Responses of Chl *a* fluorescence of terrestrial plants and marine/freshwater algae can be derived differently by species, age, origination, metal-

exposure history of the species, metal species, concentration of metal, time of metal exposure, light intensity, temperature, nutrient state, etc. (Jensen et al., 1974, Davies, 1976, Strömgren, 1980, Boyle, 1984, Küpper et al., 1996, Wierzbicka, 1999, Dan et al., 2000, Küpper et al., 2002, Antosiewicz, 2005, Romanowska-Duda et al., 2005, Sharma and Dubey, 2005, Kalaji and Loboda, 2007, Küpper et al., 2007). Brown algae including *Fucus* spp. are known as non-sensitive marine macroalgae and, in general, Cd is relatively less toxic metal species to plant and algae than either Cu or Hg (Ralph and Burchett, 1998, Xia et al., 2004). Therefore, for these reasons, the insignificant effect of Cd on Chl *a* fluorescence of *F. serratus* may be understood, at least partially. *F. serratus* from the metal-polluted site and the clean site could survive in much higher Cd concentrations than their natural environments for up to 14 d without any serious harmful effect of Cd on Chl *a* fluorescence.

On the other hand, Cd treatment in the range of 1 ~ 10 mg L^{-1} in the current research showed an effect on Chl *a* fluorescence parameters for the first 24 hr. Therefore effects of Cd on Chl *a* fluorescence of *F. serratus* may occur so fast that most of the responses may happen within 24 hr. Ralph and Burchett (1998) used Chl *a* fluorescence successfully to reveal Cu toxicity (5 mM L^{-1}) for 1 hr exposure. In most experiments of this research, Chl *a* fluorescence was significantly affected by time of Cd exposure rather than Cd concentration. More apparent changes in PS II by exposure time using Cd and Pb was reported by Kalaji and Loboda (2007) and a different stress mechanism to different metal was mentioned since PS II activity by Chl *a* fluorescence was changed in different manner (Ciscato et al., 1999, Kalaji and Loboda, 2007). They also reported rapid effects of metal application (24 hr) for some phenomenological parameters, especially for Cd treatment. They referred to these parameters as useful indicators for the negative influence of metals at early stages of their action. Krupa et al. (1993) hypothesized that the Calvin-Benson cycle reaction was the primary target of Cd

exposure not PS II. Cd-induced inhibition of Calvin-Benson cycle reduced demand for ATP and NADPH, which brought a down-regulation of PS II photochemistry and electron transport.

Changes of Chl *a* fluorescence is a complex result of adverse effects of toxic metals (Kalaji and Loboda, 2007). Cd stress can activate the following effects which are related to Chl *a* fluorescence of plant: inhibition of photosynthesis and changes in Chl biosynthesis (Krupa et al., 1993); delayed reduction and oxidation of the reduced Q_A (Strasser et al. 1995); changes in intracellular compartmentation (Brune et al., 1995); changes in specific transport processes (Gonzalez et al., 1999); down regulation of PS II to elude reduction of Q_A and to lessen electron transport (Vassilev and Manolov, 1999); and reduced damage of thylakoid lipids in OEC and LHC II antenna system (Joshi and Mohanty, 2004).

3. 4. 7. The effect of Cd on photochemical processes of *Fucus serratus*

Photoinhibitory damage in plants, indicated by a decrease of F_v / F_m and increase of F_0 values (Maxwell and Johnson, 2000), can be caused by high or low temperature (Gamon and Pearcy, 1989, Groom and Baker, 1992), excess PFD (Ögren and Sjöström, 1990), water stress (e.g. drought) (Epron et al., 1992) and heavy metal stress (Küpper et al., 2007). Although many new parameters have been developed to measure the effects of various stress conditions on photosynthetic ability, F_v / F_m and F_0 remain trustworthy diagnostic indices of photoinhibition ((Maxwell and Johnson, 2000). The diminution of F_v / F_m is understood as interference in the reduction of the electron acceptor Q_A by PS II (Dan et al., 2000). However, changes of these parameters were limited in this research and even high Cd concentrations did not cause change of F_v / F_m and F_0 in most cases. A lack of decrease in the maximal photochemical efficiency of PS II by Cd treatment has

been reported by some researchers (Greger and Ögren, 1991, Krupa et al., 1993, Di Cagno et al., 1999). They used 10, 20 and 50 μM Cd for up to 4 weeks and did not find any significant changes in these fluorescence parameters. The primary target of Cd is still under discussion; Greger and Ögren (1991) reported the primary photochemistry of PS II as the primary target and Krupa et al. (1993) postulated Calvin-Benson cycle rather than PS II efficiency. Krupa et al. (1993) hypothesized that Cd induced an inhibition of regeneration of RuBP and/or Cd inhibited the light-activated enzymes of the carbon reduction cycle. In both cases the results show limited consumption of ATP and NADPH (decrease of qP and increase of NPQ). Since high Cd concentration in which significantly suppressed the growth of algae did not change F_v / F_m ratios, functions of the PS II reaction centre might not be affected by Cd exposure in this study (see as review Prasad and Strzałka, 1999, Küpper and Kroneck, 2005, Küpper et al., 2007). Therefore, these *in vivo* effects of Cd on the electron transport system may be understood as indirect feedback resulting in the inhibition of the Calvin-Benson cycle (Krupa et al., 1993).

F_v / F_0 is an alternative expression of F_v / F_m and sometimes F_0 / F_v can be used instead of it (Krause and Weis, 1991, Maxwell and Johnson, 2000, Mallick and Mohn, 2003). In some cases, it can express data in a better way and it is more sensitive and powerful to changes in efficiency at high values than F_v / F_m, qP and NPQ, especially at immediate exposures of metals (Maxwell and Johnson, 2000). Interestingly, the values of F_v / F_0 were not changed significantly in most cases and only 1 ~ 1000 μg Cd L^{-1} treatment in RP showed an apparent decrease for 24 hr, 96 hr and 7 d. This decrease of F_v / F_0 (or increase of F_0 / F_v) has been attributed to a severe effect on the water-splitting site, and the replacement of Mn was strongly suspected in the water-splitting apparatus of the oxidizing site, since an abrupt rise of F_0 / F_v couples with early Mn deficiency (Mallick and Mohn, 2003). However, in this study, the lack of significant changes to F_v

/ F_0 in most cases suggested no changes in the rate of electron transport from PS II to the primary electron acceptors (Dan et al., 2000). Trace metals are known to prevent the absorbed light energy from being using in the electron transport system (Dietz et al., 1999), therefore the rate of photochemistry can decrease and the pool size of the primary electron acceptor can be reduced (Krause and Weis, 1991). Hence Cd treatment did not disturb electron transport of *F. serratus* of the present study as F_v / F_m and F_v / F_0 rates were not significantly altered.

Φ_{PSII} (= ΔF / F_m') have been frequently used by some researchers since F_v / F_m was less sensitive to photosynthetic stress in certain cases (Ralph and Burchett, 1998, Macinnis-Ng and Ralph, 2002). Φ_{PSII} determines the proportion of the light captured by Chl of PS II, therefore the rate of linear electron transport and an indication of whole photosynthesis can be measured (Maxwell and Johnson, 2000). In this study, rates of light energy absorbed by photosynthetic pigments in PS II were not related to the Cd treatment and its concentration for up to 7 d in both populations, which again shows that the rate of electron transport and entire photosynthesis of *F. serratus* were not affected by Cd but were affected more probably by exposure time. However the decrease of Φ_{PSII} by exposure time cannot be regarded as the effect of exposure to Cd since the decrease was also present in the control (0 $\mu g\ L^{-1}$) of both populations. Therefore other stress factors from the culture conditions, such as high direct (or accumulated) light, etc., may be implicated.

Another parameter measuring photochemistry is qP (= (F_m' - F_s) / (F_m' - F_0')). It has very similar equation to that of Φ_{PSII} (or ΔF / F_m' = (F_m' - F_s) / F_m') however it determines the proportion of opened PS II reaction centres while Φ_{PSII} gives the proportion of energy being used in photochemistry (Maxwell and Johnson, 2000). Maxwell et al. (1994) used the alternative expression, 1-qP and it relates to the proportion of closed reaction centre (excitation pressure of PS II). The closure of the

reaction centres is due to light saturation of photosynthesis, which changes the value of qP. In the present study, Cd treatment and increase of its concentration was not related to the decrease of qP. Di Cagno et al. (1999) published Cd-induced decrease of qP and increase of qNP and this was interpreted as a lower capacity of PS II to re-oxidise Q_A. In the present research Cd did not show any direct effect to the coefficient of photochemical quenching, which may show that PS II has not been affected by Cd or *F. serratus* may have a high capacity of PS II to re-oxidise Q_A and a high turnover rate of Q_A oxidation.

3. 4. 8. Cd stress effects on non-photochemical processes of *Fucus serratus*

Under extreme environmental conditions, such as high light or excess metal concentrations, permanent photosynthetic damage can occur (Govindjee, 2004). This is called photoinhibition in the case of high light stress and strategies for protecting plants from the stress are defined as photoprotection (Holt et al., 2004). Elimination of the excess absorbed energy as a form of heat (thermal dissipation) is one of the strategies of plant survival (Govindjee, 2004) and can be measured as NPQ of Chl *a* fluorescence by the PAM instrument. NPQ represents the ability to execute non-radiative dissipation of excess energy (Xu et al., 2008) and is an indicator of the transfer to a light-adapted state from the dark-adapted state (Strasser et al., 2000). In many cases of stressful conditions, the value of NPQ increases, which may be the result of processes to protect the plant from stress-induced damage (Maxwell and Johnson, 2000). However, in this research, Cd treatment did not show a regular pattern and NPQ values decreased in RP after 24 hr of Cd treatment. NPQ in BQ increased with Cd concentration until 100 $\mu g\ L^{-1}$ and then decreased again at 1000 $\mu g\ L^{-1}$ at 24 hr exposure. NPQ values seemed more related to

the exposure time than Cd concentration in both populations. Non-photochemical parameters are related to various processes of thermal dissipation in the photochemical apparatus (Krause and Weis, 1991, Vassilev and Manolov, 1999). Hetherington et al. (1998) reported that increased NPQ value to 0.4 ~ 0.5 represented forced thermal dissipation at the pigment level since the intrathylakoid lumen had been acidified by membrane energisation. However all NPQ values in this experiment were much lower than 0.4, which suggested that the most of the absorbed energy was used for photosynthetic processes (Xu et al, 2008). Han et al. (2008) and Horton and Bowyer (1990) explained the Cu-induced NPQ decrease as a reduced electron transport rate in the route $P_{680} \rightarrow$ pheophytin $\rightarrow Q_A \rightarrow Q_B$ or increased rate of the back reaction, $Q_A^- \rightarrow P_{680}^+$. However, NPQ increase with time of Cd exposure and increase at 0 μg Cd L^{-1} for BQ cannot be explained using this view. The results presented here that Cd did not have a direct effect on NPQ, which might result from the non-effective Cd characteristics and/or the concentrations used in this study. The changes might also be related to other possible stresses from culture conditions, e.g. light intensity or medium change (from seawater to Aquil). NPQ might be related to the light, especially the accumulated light intensity from the beginning of the experiment since NPQ values increased even at 0 μg Cd L^{-1} for BQ. Although two populations that have different metal-exposed history showed similar non-photochemical quenching values, the highest values shown with exposure time were different. The RP population showed the highest values at 96 hr and the BQ population showed highest at 7 d. This could refer to different operation of the Xanthophyll cycle, the acidification of thylakoid lumen and the specific components of the antenna of PS II (inclusive of the *psbS* gene product, some other minor antenna complexes and even certain portions of LHC IIB) (Gilmore et al., 1998, Govindjee, 2004). In addition, NPQ decrease in RP at 7 d might be a result of recovery from Cd stress which may be related to any other antioxidative responses of the alga. Exudation

of Cd was less linked to this because Cd accumulation increased continuously during the experiments. These results are discussed later in other chapters in this dissertation, such as antioxidation enzymes and metal detoxifying capacity. Moreover, shorter exposures to metal ions seemed a more effective way to see toxic metal stress with Chl *a* fluorescence since 24 hr exposure showed the most clear increase/decrease pattern with the Cd concentrations used.

3. 4. 9. JIP-test

JIP-test is a relatively recently developed technique to understand the Chl *a* fluorescence signal changes (Strasser et al., 2004) and has been exploited successfully in studies using a variety of photosynthetic organisms (Tsimilli-Michael and Strasser, 2003). This test was derived from the theory of energy flow in thylakoid membranes which suggest there is a balance in the inflow/outflow of the total energy in photosynthetic pigments (Strasser et al., 2004, Kalaji and Loboda, 2007). Therefore information on the structure and function of the photosynthetic apparatus can be derived from this new test (Strasser and Strasser, 1995, Strasser et al., 1995, Sarkar et al., 2004, Strasser et al., 2004, Kalaji and Loboda, 2007). However, different stress conditions represent different physiological states and these differences can be validated by the JIP-test (Strasser et al., 2004, Romanowska-Duda et al., 2005, Kalaji and Loboda, 2007). In the present study, neither Chl *a* fluorescence transient curves nor any of the JIP-test parameters (ABS / CS, TR_0 / CS, ET_0 / CS, DI_0 / CS, RC / CS_0, RC / CS_m) showed a regular pattern or an apparent effect by high Cd concentrations ranged in 1 ~ 10 mg L^{-1}. Longer exposure (7 and 14 d) revealed much higher values than shorter exposure (6 ~ 24 hr) in both populations. Cd stress to JIP-test parameters of *F. serratus* was not obvious and recovery of the Chl *a* fluorescence transient is the evidence of a weak

correlation between Cd and the parameters of the JIP-test. Other physiological and biochemical processes commonly known in the plant system, such as detoxification or antioxidative mechanisms, and non-effective concentrations of Cd are also possibly considered as a reason for the non-significance of JIP-test results. Here, again, the possibility of Cd exudation has received little consideration. On the other hand, Gonzalez-Mendoza et al. (2007) reported Cd-induced decrease in yield for primary photochemistry, TR_0 / ABS and PI of PS II, PI_{ABS} of *Avicennia germinans*, the black mangrove. Therefore species-specific changes by Cd are also possible. Kalaji and Loboda (2007) also reported evidence of a fast shift (24 hr) in phenomenological parameters (ABS / CR_0, TR_0 / CS_0 and ET_0 / CS_0) after Cd and Pb treatment and the shift was especially quick under Cd treatment. The authors suggested these parameters could be used as efficient indicators for monitoring the negative influence at early stages of metal action. Although these parameters did not reveal significant changes under the Cd treatment and with the concentrations in the present study, some differences with exposure time and recovery of the values may be closely related to the fast response to the Cd stress and other detoxifying mechanisms of *F. serratus*.

3. 4. 10. Effect of Cd on photosynthetic pigments of *Fucus*

Heavy metals are known to reduce the biosynthesis of Chl pigments and the related enzymes (such as photochlorophyllide reductase) (Stobart et al., 1985, Somashekaraiah et al., 1992, De Filippis and Pallaghy, 1994). Metal-induced diminution of the total Chl content, the Chl *a*/*b* ratio and the Chl/carotenoid ratio have been reported (Ouzounidou, 1993, Ralph and Burchett, 1998, Di Cagno et al., 1999, Xia et al., 2004). Greger and Ögren (1991) reported the decrease of Chl concentration was associated with Mg and Fe deficiency in the processes of Chl biosynthesis. Some metals

such as Cd and Cu can substitute for Mg^{2+} in the Chl molecules of the LHC II, which is highly unstable (Küpper et al., 1996, Küpper et al., 2007). However, in the current study, effect of Cd on contents of photosynthetic pigments showed a different pattern related to a population. Cd exposure increased contents of Chl *a*, Chl *c* and Fx in the RP population, but there was no significant difference between Cd concentrations (10 ~ 1000 $\mu g\ L^{-1}$). The contents of these pigments decreased in the BQ population. However the content of β-carotene from the BQ materials were higher than those from the RP materials, and the content was not affected by Cd exposure except 10 $\mu g\ L^{-1}$ in BQ. No significant changes of Chl and carotenoids contents by Cd treatment have been reported by other researchers (Ralph and Burchett, 1998, Macinnis-Ng and Ralph, 2002, Han et al., 2008). Xia et al. (2004) reported that 50 and 100 μM Cd did not affect Chl *a* and carotenoids contents of a red alga *Gracilaria lemaneiformis* and only 200 μM Cd induced a marked decrease of Chl *a* and carotenoids contents. Ouzounidou (1993) found an increased Chl content in *Silene compacta* at low Cu exposure and decreased Chl content at elevated Cu levels. Similar results were noted with the marine microaglae *Phaeodactylum tricornutum* (Bacillariophyceae) (Cid et al., 1995). The authors suggested a high Cu demand of tolerant species. However, in the current study, algae from the polluted site increased the Chl contents and algae from the reference site showed significantly lower levels of contents. Significant increases in Chl associated with stress resistance have been noticed across a range of environmental stresses (Zhang et al., 2005). *Ulva armoricana* showed an increase in Chl content with no decrease in F_v / F_m or F_0 when the RGR was reduced by Cu stress (Han et al., 2008). The authors suggest a trade-off relationship between energetic resources for pigment biosynthesis at the expense of growth. *F. serratus* exposed to Cd in this research showed similar results. The expensive protective mechanisms might have reduced Cd stress from the reduction of photosynthetic pigments.

Cd exposure had an apparent effect on the content of Fx in *F. serratus*. For brown algae, Chl *c* and Fx are known as the main light-harvesting pigments which broaden the absorption spectrum to green light relative to other algae (Charrier et al., 2008). As accessory pigments in plants, these pigment proteins transfer absorbed light energy to Chl *a* in the photosynthetic reaction centre (Grossman et al., 1995). Cd stress was shown to be related to the population. Levels of Fx of BQ decreased significantly with Cd treatment, increasing Cd concentration and exposure time until 96 hr. However, although Cd treatment had an effect, Cd stress increased contents of Fx in RP. Therefore significantly lowered levels of Fx in BQ indicate significant damage of pigment biosynthesis and its function, designating significantly reduced light harvesting ability by Cd exposure. On the other hand, increased Fx contents in RP showed a specific tolerance of this population to Cd stress, which could be derived from adaptation to the contaminated habitat and inherited tolerance from the previous generations.

3. 4. 11. Metabolic responses to Cd in tolerant and non-tolerant *Fucus* populations

Unfortunately few researchers have reported the inter-population differences of photosynthetic activity in response to metal stress. *F. serratus* had a very low sensitivity to Cd exposure and no significant difference between the polluted and the control population was revealed in most cases of the present research. Non-polluted population, BQ also showed very similar Chl *a* fluorescence with 1 ~ 10 mg Cd L^{-1} for 7 and 14 d exposure. Brown algae are known to have the ability to hyperaccumulate metal ions, for which they were used in biomonitoring programs (Stengel and Dring, 2000). Nielsen et al. (2003b) published a study indicating inherited tolerance to Cu^{2+} in *F. serratus*. They

found that the tolerant population had higher embryo and adult growth rates and lower Cu contents in thalli than the non-tolerant population. In addition the tolerant population showed more stable results of Chl *a* fluorescence parameters than the more susceptible values in the non-tolerant population. Therefore they concluded that the tolerant population from Restronguet Creek was more resistant to Cu^{2+} than non-tolerant population from BQ and Wembury Beach. However the present study showed no significant differences between polluted and non-polluted populations using both Chl *a* fluorescence parameters and bioaccumulation of Cd. Contents of Fx, on the other hand, were significantly different and Cd stress caused reduction of this pigment only in the BQ population. In addition, the contents of Chl *a* and *c* were higher in RP and the content of β-carotene was higher in BQ. Therefore *F. serratus* may have similar photosynthetic response to Cd stress and similar uptake rates of Cd regardless of their origin, although there were minor differences in tolerance. This could be derived from Cd's less toxic effects when compared with Cu (Ralph and Burchett, 1998). However many of the Chl *a* fluorescence values from BQ showed significant differences in the shorter exposure times of less than 24 hr using 1 ~ 10 mg Cd L^{-1}. Therefore the faster responses of RP population occurred for less than 24 hr. In conclusion, Chl *a* fluorescence in *F. serratus* did not respond very sensitively to Cd exposure when compared to the stress induced by other metals and this lack of response was not related to locality.

3. 5. Conclusion and further consideration

Similar concentrations of total and non-exchangeable Cd were discovered between polluted and reference populations of *Fucus serratus*. Although the RP area is seriously polluted by various metal elements and *Fucus* from RP already contained much higher amounts of trace metals, concentrations of Cd adsorbed/absorbed by *F. serratus* from RP and BQ were not significantly different. With these same Cd burdens, both populations showed similar responses in photosynthetic performance. Cd had a very limited effect on most of the parameters of Chl *a* fluorescence of *F. serratus*. Photosynthetic pigments were affected by Cd stress, however there was no significant difference between Cd concentrations (10 ~ 1000 μg Cd L^{-1}). Parameters of Chl *a* fluorescence and photosynthetic pigments are often affected by exposure time rather than Cd treatment or its concentrations. However there might be other effects from culture conditions since Chl *a* fluorescence parameters increased/decreased at 0 μg Cd L^{-1} at an extended culture period. Therefore photosynthetic responses of *F. serratus* to Cd stress are not independent and many physiological and ecological processes are incorporated into a complicated defence system. As the RGRs significantly decreased in both populations, particularly at higher Cd concentrations, the toxic effect of Cd is apparent although the harmful effect was not related to photosynthetic mechanisms.

Chl *a* fluorescence parameters were more effective at shorter exposure times of 6 ~ 24 hr than longer exposure of 96hr ~ 14 d. With longer exposures *Fucus* did not show significant damage by Cd, which indicates other physiological and biochemical protective mechanisms of detoxification causes a recovery by the algae. Metal-chelating thiol compounds, organic acids and inorganic compounds, sequestration of metal stress and increase of metabolic rate are possible mechanisms. Avoidance and exclusion may be less likely since there was a positive linear relationship between uptake and Cd

concentration. To overcome metal stress *F. serratus* maintained its photosynthetic metabolism and the increased metabolic activity limited damage to photosynthetic apparatus while RGRs decreased significantly.

In conclusion, both *Fucus* populations have the potential ability to be used as indicators of Cd content. Unlike previous reports with other algal species, *F. serratus* from the control site also had similar photosynthetic responses to those of the tolerant populations, especially when subjected to longer Cd exposure (chronic exposure).

Chapter 4. Evaluation of antioxidant defense responses to cadmium stress in *Fucus serratus* (Phaeophyceae)

4. 1. Introduction

Accumulated metals from various sources can lead to oxidative stress as well as stress on photosynthetic performances as the previous chapter (Wikfors and Ukeles, 1982, Rebhun and Ben-Amotz, 1984, Cotté-Krief et al., 2000, Bu-Olayan et al., 2001, Esser and Volpe, 2002, Pinto et al., 2003). Although this oxidative stress and the defence strategy of plants has been an interest for decades, the cellular protective mechanism of marine macroalgae is not yet clearly elucidated (Pinto et al., 2003).

4. 1. 1. Reactive oxygen species and oxidative stress

Reactive oxygen species (ROS) comprise activated oxygen, including free radicals (superoxide anion radicals, $O^{\cdot}{}_2^-$; hydroxyl radicals, $^{\cdot}OH$) and non free-radical species (hydrogen peroxide, H_2O_2; singlet oxygen $^1O_2$) produced in organisms (Gülçin et al., 2002, Gülçin et al., 2003, Güçlü et al., 2006, Halliwell and Gutteridge, 2007). These are obligatory by-products of normal aerobic metabolism, such as photosynthesis and respiration (Asada and Takahashi, 1987, Mittler, 2002), or of abiotic stresses (Mittler, 2002). Abiotic stresses that disrupt the cellular homeostasis include air pollutants (ozone or SO_2), chilling, drought and desiccation, heat shock, high light, low temperature, nutrient deprivation, salt stress, ultraviolet radiation, pathogen attack, and heavy metals (Noctor and Foyer, 1998, Collén and Davison, 1999a, Dat et al., 2000, Mittler, 2002, Dummermuth et al., 2003, Güçlü et al., 2006). Other pathways relatively recently discovered include NADPH oxidases, amine oxidases and cell-wall-bound peroxidises (Mittler, 2002).

Under benign condition the balance between the production and inactivation of ROS by the antioxidant mechanism is tightly controlled, but on exposure to

environmental stressors over-generation of ROS can lead to oxidative damage (Gülçin et al., 2003, Güçlü et al., 2006, Halliwell and Gutteridge, 2007), including alterations in cellular membranes (such as altered permeability and modification; degradation of amino acid) and in intracellular molecules (such as deletion or mutation of DNA) (El-Habit et al., 2000, Gülçin et al., 2003) as well as cell death (Hammond-Kosack and Jones, 1996, Asada, 1999, Dat et al., 2000, Mittler, 2002). Therefore, the increase of ROS synthesis is considered as an indicator of oxidative stress and the reactive oxygen scavenging mechanism for a defence response is focused on plant biology (Knight and Knight, 2001, Gülçin et al., 2002, Mittler, 2002, Gülçin et al., 2003, Güçlü et al., 2006).

4. 1. 2. Effects of metals in the environment as inducers of oxidative stress

Under natural condition, metal exposure may not pose a serious threat to organisms since the concentrations of metals encountered are often not high enough to be toxic (Pinto et al., 2003). In chronically contaminated areas, organisms are exposed to low concentrations of metals for long periods (Pinto et al., 2003). In other situations, high exposure to pollutants, mostly anthropogenic, affects organisms around the sources in a very short period. Although the mechanisms of metal toxicity are not particularly well understood, there is increasing evidence that oxidative damage is involved (for review, see Pinto et al., 2003). Oxidative damage from metal exposure can proceed in two ways: (1) increase of cellular ROS and (2) decrease of cellular antioxidant capacity (Sies, 1999, Pinto et al., 2003).

One of the main metals which have captured researcher's attention is Cd (Collén et al., 2003, Lee and Shin, 2003). Cd does not have a metabolic function in plants and algae (Pinto et al. 2003), although it was known as a cofactor of a carbonic anhydrase in

a diatom, *Thalassiosira weissflogii* (Lane and Morel, 2000) and was required for protein synthesis in a red alga, *Porphyra umbilicalis* (McLean and Wiiliamson, 1977). However the antioxidant responses of organisms to Cd stress are not yet understood (Lee and Shin, 2003). Transition metals (such as Fe^{3+} and Cu^{2+}) generate ·OH from $O_2^{\cdot-}$ and H_2O_2 in the Haber-Weiss cycle, while oxidative stress by metals without redox capacity (such as Cd^{2+}, Pb^{2+}, and Hg^{2+}) may occur via a Fenton-type reaction (Robinson, 1989, Lee and Shin, 2003, Pinto et al., 2003, Halliwell and Gutteridge, 2007). The Haber-Weiss reaction was named after Fritz Haber and Joseph Weiss and describes the generation of hydroxyl radicals ($^{\cdot}OH$) from hydrogen peroxide (H_2O_2) and superoxide ($^{\cdot}O_2^-$). This reaction is catalysed by transition metals and composed of a serious of steps, including Fenton reaction (Koppenol, 2000).

$$Fe^{3+} + {}^{\cdot}O_2^- \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \qquad \text{(Fenton reaction)}$$

Therefore the net reaction is

$$^{\cdot}O_2^- + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^- \qquad \text{(Haber-Weiss reaction)}$$

These reactions are possible sources for oxidative stress since they occur within cells (Koppenol, 2000), which is related to disruption of growth, photosynthetic electron chain, and ion / water transport and related to increase of enzyme activities and glutathione (GSH) pool (Prasad, 1996, Collén et al., 2003, Pinto et al., 2003).

4. 1. 3. Algal responses to metal-induced oxidative stress

Effects of various metals on plants and algae have been observed by many researchers to date, and chlorosis, cell lysis, necrosis, discoloration and encystment are

some of visible signs of metal toxicity (Hu et al., 1996, Okamoto et al., 1999, Küpper et al., 2002, Collén et al., 2003). In addition, oxidative damage in proteins, lipids and DNA can also be caused (Asada and Takahashi, 1987, Collén and Davison, 1999b, Halliwell and Gutteridge, 2007). Elstner et al. (1988) defined 'stress point' as the threshold level of stress for plant fitness. In the plant/algal tissue, it can be the toxic threshold point of metal toxicity and the physiological condition of the cell may be permanently changed (Van Assche et al., 1988, Van Assche and Clijsters, 1990). The over-production of ROS and imbalance of cellular oxidative status will occur at this stage (Rijstenbil et al., 1998b, Okamoto et al., 2001a, Okamoto et al., 2001b, Collén et al., 2003). Increase in activity of certain enzymes, i.e. enzyme induction, and synthesis of antioxidants describe this oxidative cellular state (Van Assche and Clijsters, 1990).

To reduce ROS in sensitive parts of the cellular machinery, low molecular weight compounds (e.g. ascorbate, carotenoids, flavonoids, GSH, phenolics, and tocopherols) and high molecular weight compounds (enzymatic catalysts) are produced as a protective mechanism (Pinto et al., 2003). A sign of the stress and some of main protective mechanisms against oxidative stress in plants and algae are outlined below.

4. 1. 3. 1. Lipid peroxidation

Polyunsaturated lipids are fundamental components for the supporting system of cells and are found in cell membranes, endoplasmic reticula and mitochondria (Muriel, 1997). Therefore oxidation of lipids by metal-induced oxidative stress will be critical to cellular function and survival. Lipid peroxidation is a complex, free radical-mediated chain reaction, composed of three phases: initiation, propagation and termination (Rice-Evans et al., 1991b). This reaction results in the oxidative degradation of lipids, chiefly

polyunsaturated fatty acids (PUFAs) containing multiple double covalent carbon-carbon bonds (Rice-Evans et al., 1991b).

Malondialdehyde (MDA) is a very reactive three carbon/dialdehyde produced from lipid hydroperoxides. It is known to be formed by degradation of polyunsaturated lipids by ROS, therefore its production has been used as a biomarker for oxidative stress (Moore and Roberts, 1998, Del Rio et al., 2005). This oxidative stress has been measured by the thiobarbituric acid (TBA) reaction (Kohn and Liversedge, 1944) in which one MDA molecule reacts with two 2-TBA molecules stoichiometrically (Sinnhuber et al., 1958, Yu et al., 1986, Rice-Evans et al., 1991b). The adduct expresses a pink chromogen which can be detected by excitation wavelength of 515 ~ 532 nm and emission wavelength of 553 nm spectrophotometrically (Rice-Evans et al., 1991b, Moore and Roberts, 1998). Since the thiobarbituric acid-reactive substances (TBARS) test is non-specific and the chromogen can be formed with several other aldehydes other than MDA, such as carbohydrates, certain antibiotics, and DNA (Halliwell and Gutteridge, 2007), more specific and accurate HPLC techniques have been developed by which true MDA and other aldehydes can be distinguished (Rice-Evans et al., 1991b). However, the TBARS test is still the one most frequently performed as it is easy and inexpensive (Moore and Roberts, 1998, Halliwell and Gutteridge, 2007).

4. 1. 3. 2. Cupric ion reducing antioxidant capacity

There have been many different methods of determining the antioxidant activity in plants, including: 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2'-diphenyl-1-picryl-hydrazyl (DPPH), oxygen radical absorbance capacity (ORAC), ferric reducing ability of plasma (FRAP), total reactive antioxidant potential (TRAP), Folin-Ciocalteu, and HPLC (Apak et al., 2004, Apak et al., 2007). These (such as ABTS and DPPH), based on the assaying radical scavenging capacity of antioxidants, encountered difficulties due to the formation and stability of coloured radicals (Miller et al., 1993, Apak et al., 2004). On the other hand, FRAP, which is based on ferric to ferrous reduction with Fe(II)-stabilizing ligand, is unrealistic since the coloured complex needs much lower pH (pH 3.6) than the physiological pH and it is not able to detect thiol-type antioxidants such as GSH (Benzie and Strain, 1996, Apak et al., 2004). Llesuy et al. (2001) have subdivided methodologies for detecting antioxidant activity into five subclasses: (1) measuring the consumption of a stable free radical (such as DPPH); (2) measuring the time required to consume all of the antioxidants (such as TRAP assay); (3) the rate decreasing after addition of the antioxidant sample (such as ORAC assay); (4) equating the total amount of antioxidants to the reducing capacity of samples (such as FRAP assay); (5) the other procedures.

In 2004, Apak and co-workers published a new methodology (cupric ion reducing antioxidant capacity, CUPRAC) for measuring antioxidant capacity of polyphenols and vitamins C and E. They used the reagent copper (II)-neocuproin [Cu (II)-Nc] as the chromogenic agent and named it as CUPRAC.

$$nCu(Nc)_2^{2+} + Ar(OH)_n \rightarrow nCu(Nc)_2^{+} + Ar(=O)_n + nH^{+}$$

where the polyphenol ($Ar(OH)_n$) is oxidized to the corresponding quinon, and the reduction product (bis(neocuproine)copper(I) chelate) shows absorption maximum at 450 nm (Apak et al., 2007).

Some advantages of the CUPRAC method were summarized by Apak et al. (2007):

(1) Fast enough to oxidize thiol-type antioxidants (such as GSH) which are the major low molecular weight thiol compounds in plant and animal cell

(2) Selective because of the lower redox potential

(3) Much more stable and easily accessible reagent than ABTS and DPPH

(4) Easy and diverse application in conventional laboratories using standard colorimeters

(5) Insensitive to air, sunlight, humidity, pH, etc.

(6) Perfectly linear relationship between the analytical absorbance and wide range of concentrations

(7) Nearly physiological pH of the redox reaction compared with unrealistic acidic conditions of FRAP

4. 1. 3. 3. 2,2-diphenyl-1-picryl-hydrazil (DPPH) free radical scavenging capacity

Free radicals are known to affect the oxidation of unsaturated lipids and DPPH radical has been used as a stable free radical to measure antioxidant activity of natural compounds (Abe et al., 1998, Connan et al., 2006, Senevirathne et al., 2006, Öztürka et al., 2007). DPPH free radical scavenging assay is one of the electron transfer (ET)-based assays (Apak et al., 2007). It was first proposed by Blois (1958) and has been widely used to evaluate the radical scavenging capacity of plant extracts and constituents (Soares et al., 1997, Wang et al., 2005). DPPH accepts an electron or

hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997) and the alcoholic DPPH solution can be reduced to form non-radical DPPH-H in the face of antioxidant (Gülçin et al., 2003, Güçlü et al., 2006, Öztürka et al., 2007).

$DPPH^{\cdot} + ArOH \rightarrow DPPH + ArO^{\cdot} + H^{+}$

where $ArOH$ is the phenol which transferred to the aryloxy radical ($ArO^{\cdot}$) and the hydrogen atom ($H^{\cdot}$) (Apak et al., 2007).

The ethanolic DPPH solution displays a strong absorption band at 517 nm (Soares et al., 1997). Lower absorbance of the reaction mixture at 517 nm represents higher free radical scavenging activity (Gülçin et al., 2003, Güçlü et al., 2006, Öztürka et al., 2007).

4. 1. 3. 4. Antioxidative stress scavenging enzymes (CAT, APX and GR)

Plants are known to have well equipped enzymatic detoxification mechanisms to eliminate and reduce ROS (Larson, 1988, Dummermuth et al., 2003) although studies on marine macroalgae are relatively limited. Catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and lipid peroxidase (LP) are the main antioxidant enzymes found in plants and algae (Rice-Evans et al., 1991a, Asada, 1999, Dummermuth et al., 2003, Pinto et al., 2003, Ratkevicius et al., 2003). Table 4. 1 and Fig. 4. 1 show the reactions and localisations of the main enzymes. CAT diminishes H_2O_2 to water and oxygen in two steps in cytosol and peroxisome, and APX, with higher affinity than CAT, also reduces it via the ascorbate-glutathione cycle in the cytosol, mitochondria and

Table 4. 1. Major antioxidant enzymes in plants and algae and their functions and localizations

Enzyme	Reaction	Location
CAT	$2H_2O_2 \rightarrow 2H_2O + O_2$	peroxisome, cytosol
APX	H_2O_2 + ascorbate $\rightarrow 2H_2O$ + DHA	cytosol, mitochondria, chloroplast
GR	GSSG + NADPH + $H^+ \rightarrow$ 2GSH + $NADP^+$	cytosol, mitochondria, chloroplast
SOD	$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$	cytosol, mitochondria, chloroplast, apoplast, peroxisome
GPX	H_2O_2 + 2GSH $\rightarrow 2H_2O$ + GSSG	peroxisome, cytosol
MDHAR	MDHA + NADPH $\rightarrow$ ascorbate + $NADP^+$	cytosol, mitochondria, chloroplast
DHAR	DHA + GSH $\rightarrow$ ascorbate + GSSG	cytosol, mitochondria, chloroplast

APX, ascorbate peroxidase; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulphide; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; SOD, superoxide dismutase

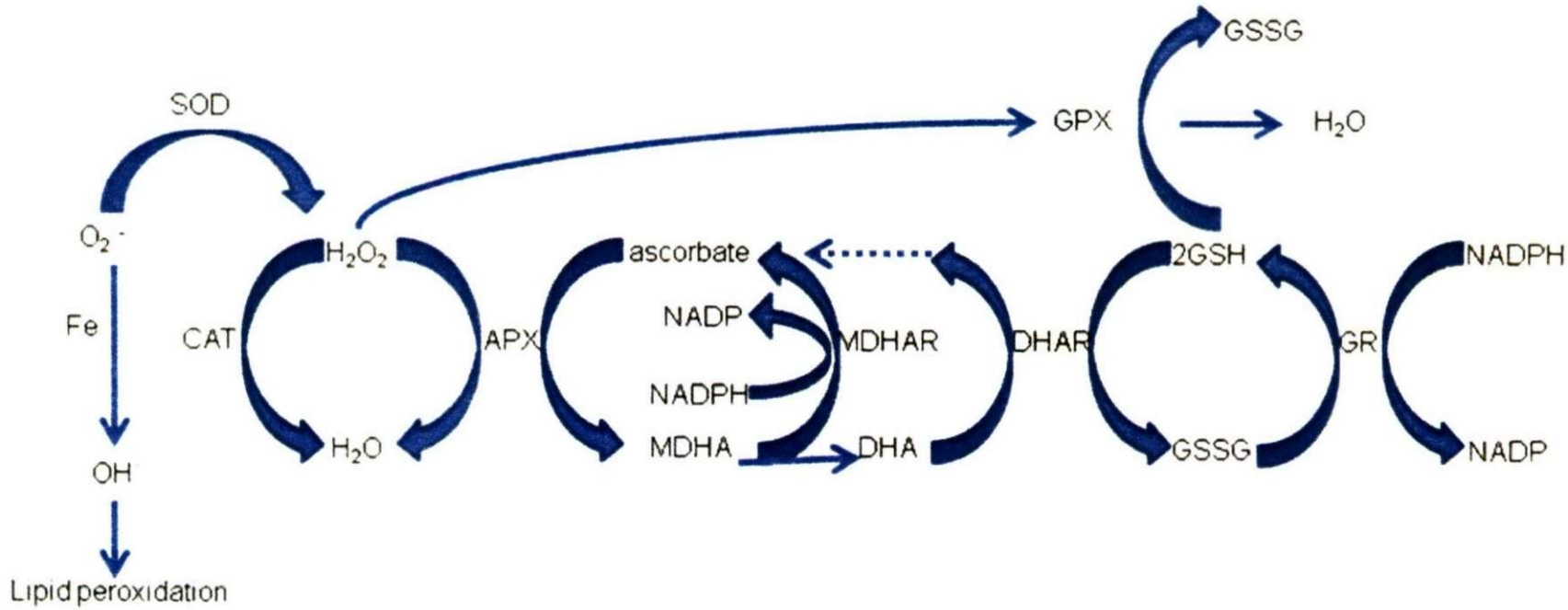

Fig. 4. 1. Reactive oxygen species scavenging pathways in plants and algae. APX, ascorbate peroxidase; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulphide; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; SOD, superoxide dismutase. Modified from Teixeira et al. (2005).

chloroplasts (Asada, 1999, Dummermuth et al., 2003, Pinto et al., 2003, Ratkevicius et al., 2003, Halliwell and Gutteridge, 2007). APX can produce oxidized glutathione (GSSG) and dehydroascorbate which can be reduced to ascorbate by activity of DHAR (Ratkevicius et al., 2003). The GSSG can be reduced by GR with consumption of NADPH (Asada, 1999, Collén and Davison, 1999b). This cycle including activities of APX, DHAR and GR was named as Halliwell-Asada cycle and functions as to detoxify H_2O_2 using NADPH (Ratkevicius et al., 2003).

Under status of metal stress, induction of antioxidant enzymes by plants and algae must be related to their metabolic roles to reduce, modulate or remove ROS (Van Assche and Clijsters, 1990, Mittler, 2002). Induction of enzymes can be discovered at low levels of toxicity, even before evident visible symptoms (Van Assche and Clijsters, 1990).

4. 1. 4. Purpose of this study

Stress tolerance and protective mechanisms have been studied in several different ways: population-specific in the same species, different parts or different development stage in an organism, transgenic plants, species-specific, etc. (Collén and Davison, 1999b). Plants increase their tolerance to oxidative stress through modification of their antioxidant defence systems (Anderson et al., 1995, Vitoria et al., 2001, Lee and Shin, 2003). However, research on antioxidative defence system of marine macroalgae is still limited (Whitton et al., 1989, Lee and Shin, 2003). In the previous chapter, photosynthetic responses of *Fucus serratus* to Cd exposure were very restricted. Apparent evidence by Cd treatment was barely evaluated with Chl *a* fluorescence parameters. However, with some parameters (i.e. growth and photosynthetic pigments), more efficient responses have been measured in the RP population. Therefore, in this

chapter, the reactive oxygen scavenging capacity of *F. serratus* was examined against Cd exposure and two different populations from different metal-exposure history were compared.

4. 2. Materials and methods

4. 2. 1. Collection of *Fucus serratus* and culture

Collection and culture of algal material was followed by Chapter 2. Non reproductive, fresh, about 1 year-old (dichotomous) algae were selected from RP and BQ. Apical tips (ca. 400 mg) were cultured in Aquil medium at 15 °C at an irradiance of 250 μmol m^{-1} s^{-1} on a 12 h light : 12 h dark cycle. Algal materials were exposed to 0 ~ 1000 μg L^{-1} $CdSO_4$ for 24 h, 96 h and 7 d and to 0 ~ 10 mg $CdSO_4$ L^{-1} for 7 and 14 d.

4. 2. 2. Procedure for lipid peroxidation analysis

10 ~ 20 mg (dry weight) of algae was homogenized with liquid nitrogen and extracted with 10% trichloroacetic acid (TCA) for 10 minutes using chilled mortar and pestle on ice. The final volume was 2 mL. The extract was centrifuged at 12,000 g (17°C). After 10 minutes centrifugation, 1 ~ 1.5 mL supernatant was collected and 1 ~ 1.5 mL of 0.6% TBA in 10% TCA was added in it. The combined reagent was incubated in an 80°C water bath for 20 minutes. The sample was moved into ice until chilled and centrifuged again at 12,000 g for 10 min (17°C). The concentration of MDA was determined spectrophotometrically (Unicam Heλios β UV-VIS spectrophotometer, Spectronic Unicam, Cambridge, U.K.) and calculated by the following equation:

$MDA\text{-}TBA\ (mmol\ mL^{-1}\ of\ extract) = \Delta A / \varepsilon$

Here, ΔA is the difference between absorbance at 532 nm and absorbance at 600 nm ($A532 - A600$) and ε is the extinction coefficient of 159,200 mM^{-1} cm^{-1}. The value of mmol mL^{-1} was then expressed as μmol g^{-1} D.W. Three replicates were used for each treatment.

4. 2. 3. Procedure for cupric ion reducing antioxidant capacity analysis

4. 2. 3. 1. Extraction of polyphenols

Harvested thalli were rinsed with running tap water to remove salt and remaining Cd and were ground to a powder using liquid nitrogen. Ground *F. serratus* (approximately 0.1 g) was extracted with 10 mL 70 : 30 MtOH : H_2O at 25 °C, in the dark, in a shaking incubator (60 rpm) for 24 hr, followed by centrifugal at 6,500 g for 10 min (25 °C). These polyphenol extracts were stored for the following analyses for antioxidant capacity (CUPRAC and DPPH free radical scavenging assay).

4. 2. 3. 2. Cupric ion reducing antioxidant capacity (CUPRAC)

CUPRAC of *F. serratus* was examined according to Apak et al. (2007). Each of 1 mL $CuCl_2$ (10 mM), neocuproine (7.5 mM), and NH_4Ac buffer (1 M, pH 7.0) was combined and aliquots of algal extracts (100 μL) were added to it. Distilled water was added to this mixture to a final volume of 4.1 mL. Stoppered tubes were kept in the dark for 1 hr at room temperature and the absorbances were recorded at 450 nm against a reagent blank. Total antioxidant capacity of *F. serratus* was expressed as Trolox equivalent antioxidant capacity (TEAC). Trolox is an antioxidant, a derivative of vitamin E, and is often used as a benchmark for the antioxidant capacity of antioxidant mixtures (Apak et al. 2007). The molar absorptivity of Trolox (ε_{Trolox}) was 1.67×10^4 L mol^{-1} cm^{-1}.

Capacity (in mmol TE g^{-1}*)* $= (A_f/\varepsilon_{TR})\ (V_f / V_s)\ r\ (V_{cup} / m)$

where A_f is the absorbance, ε_{TR} is the molar absorptivity of Trolox, V_f is the final volume of mixture (i.e. 4.1 mL), V_s is the sample volume taken for analysis from the diluted extract, r is the dilution rates, V_{cup} is the initial volume of *Fucus* extract and m is the

fresh weight (g) of algal material. The calibration curve for pure Trolox was a line passing through the origin (Fig. 4. 2).

4. 2. 4. Procedure for DPPH free radical scavenging capacity analysis

4. 2. 4. 1. Extraction of polyphenols

The same polyphenol extracts were used as CUPRAC method.

4. 2. 4. 1. DPPH free radical scavenging capacity analysis

Free radical scavenging activity of Cd-exposed *F. serratus* was assessed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) free-radical method (Connan et al., 2006). 300 μL of polyphenol extract was added to 3 mL of 1 mM solution of DPPH in MtOH : H_2O (90 : 10). Absorbance was read at 517 nm with a UV-VIS spectrophotometer against distilled water after standing in the dark for 1 hr. Lower absorbance of the mixture indicates a higher free radical scavenging activity (Öztürka et al., 2007). The DPPH radical scavenging capacity was calculated by the following equation (Gülçin et al., 2003, Öztürka et al., 2007):

$$DPPH\ Scavenging\ Effect\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100,$$

where $A_{control}$ is the absorbance of control and A_{sample} is the absorbance in the presence of the sample of *F. serratus*.

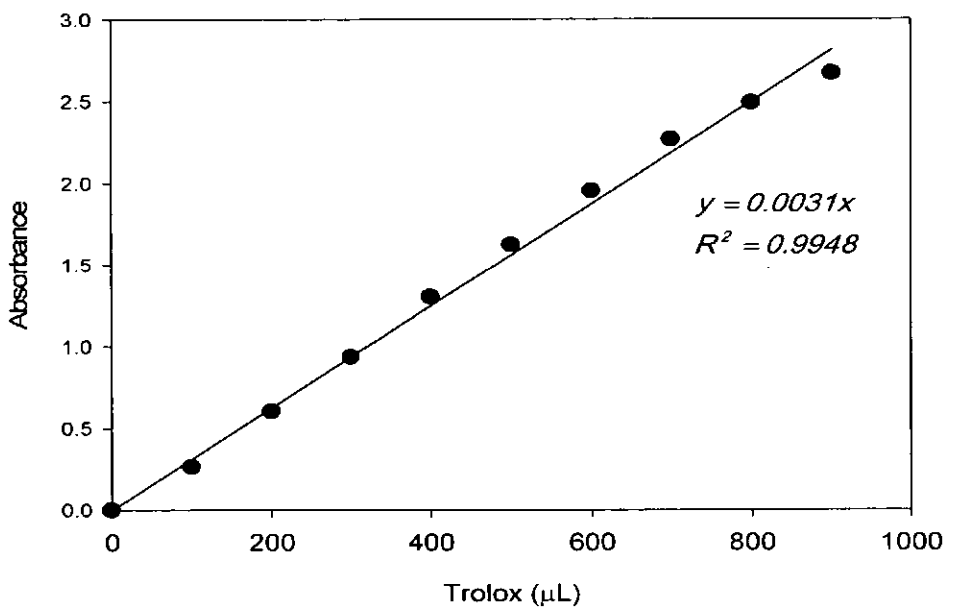

Fig. 4. 2. The calibration curve for pure Trolox.

4. 2. 5. Procedures for reactive oxygen scavenging enzyme analyses

4. 2. 5. 1. Enzyme extraction

Algal materials exposed to Cd were sealed and frozen (-20°C) until the enzyme assays. Protocols were based on Collén and Davison (1999a) and Collén and Davison (1999c) with minor modification. For the CAT and GR assays, frond pieces (approximately 0.1 g F.W.) were ground in liquid nitrogen using chilled mortar and pestles on ice and extracted for 10 minutes with five times the fresh weight of 50 mM potassium phosphate buffer (pH 7.0) containing 1 % w/v PVP-40 and 0.25% Triton X-100. APX assay was the same as above with 0.1 mM EDTA and 0.5 mM ascorbate. Extracts were centrifuged at 20,000 g at 4°C for 5 min and stored in ice during the process.

4. 2. 5. 2. Catalase assay

CAT activity was analyzed by decrease measuring in the H_2O_2. 50 μL extract was added to 700 μL 50 mM potassium phosphate buffer (pH 7.0) and 1000 μL 10 mM H_2O_2. The reaction was measured in a quartz cuvette using a UV-VIS spectrophotometer (Heλios β, Spectronic Unicam) at an absorbance of 240 nm measured at 25 °C for 1 ~ 3 min. The extinction coefficient was 39.4 mM^{-1} cm^{-1}. The CAT activity was calculated by the following equation:

$$CAT\ activity\ (\mu mole\ H_2O_2\ \min^{-1}\ mg^{-1}\ protein) = \frac{V \times dilute\ factor \times 1000}{39.4 \times d \times v \times protein\ content}$$

where V is total volume in assay mixture (mL), v is volume of enzyme in assay mixture (mL), and d is light path of cuvette (1 cm).

4. 2. 5. 3. Ascorbate peroxidase assay

For the APX assay, ascorbate consumption was monitored by a decrease in absorbance, at 290 nm for 30 s after adding 1000 μL 0.1 mM H_2O_2 to the assay mixture containing extract (30 ~ 100 μL) and potassium phosphate buffer (pH 7.0, 700 μL 50 mM) with 0.3 mM ascorbate. The assays were performed at 25°C and the extinction coefficient was 2.8 mM^{-1} cm^{-1}. The APX activity was calculated by the following equation:

$$APX\ activity\ (\mu mole\ H_2O_2\ \min^{-1}\ mg^{-1}\ protein) = \frac{V \times dilute\ factor \times 1000}{2.8 \times d \times v \times protein\ content}$$

4. 2. 5. 4. Glutathione reductase assay

Oxidation of NADPH was measured as GR assay. Changes of absorbance at 340 nm were measured by adding 20 μL extract to 1000 μL 100 mM Tris HCl buffer (pH 7.8) with 2 mM EDTA, 1 mM NADPH and 0.5 mM GSSG at 25°C for 1 min. The extinction coefficient was 6.22 mM^{-1} cm^{-1}. The GR activity was calculated by the following equation:

$$GR\ activity\ (\mu mole\ H_2O_2\ \min^{-1}\ mg^{-1}\ protein) = \frac{V \times dilute\ factor \times 1000}{6.22 \times d \times v \times protein\ content}$$

4. 2. 5. 5. Protein content assay

Protein content in the algal material was measured spectrophotometrically according to Bradford (1976) at 595 nm at room temperature. Bradford reagent was prepared with Coomassie Brilliant Blue in phosphoric acid (0.01% w/v). Bovine serum

albumin (200 μg mL^{-1}) was used as standard and duplicate tests were carried out for the standard curve of absorbance versus μg protein. Undiluted samples (10 ~ 300 μL) were added to the reagent solution (2.5 mL Bradford reagent and water, total volume 3.5 mL). The protein content of *Fucus* extract was measured from the linear part of standard curve of bovine serum albumin and calculated in mg mL^{-1}.

4. 2. 6. Statistical analysis

Data were analyzed statistically using SPSS 16.0 for windows (SPSS Inc.). Before all parametric tests, the data were tested for homogeneity of variance and normality (Sokal and Rohlf, 1995). Multivariate test of General Linear Model was carried out for analyzing the effects of locality, metal concentration and exposure time and Tukey HSD was used for the *Post Hoc* multiple comparisons. In cases where additional analyses were needed, One-way ANOVA was performed to check differences, especially within a population or under a certain condition. In all analyses, differences were considered to be significant at a probability of 5% ($p < 0.05$). Number of replicates in each experiment was from 3 to 6.

4. 3. Results

Oxidative stress expression and antioxidative reaction of *Fucus serratus* was examined with two different Cd concentration categories and time scales. 1 ~ 10 mg Cd L^{-1} with 1, 7 and 14 d exposure was named as 'high and extended Cd exposure' and 10 ~ 1000 μg Cd L^{-1} with 24 hr, 96 hr and 7 d exposure was named as 'low and short Cd exposure' for distinction. Assays for lipid peroxidation, CUPRAC, DPPH free radical scavenging capacity and reactive oxygen scavenging enzymes were explored.

4. 3. 1. Lipid peroxidation of natural populations

Lipid peroxidation of *F. serratus* collected from RP and BQ were compared without experimental Cd exposure (Fig. 4. 3). There was no statistically significant difference between the populations ($p > 0.05$).

4. 3. 2. High and extended Cd exposure

4. 3. 2. 1. Lipid peroxidation

Peroxidation of lipid of cell membrane showed apparent effects by extended time of Cd exposure (7 and 14 d) and high Cd concentration (1 ~ 10 mg Cd L^{-1}) ($p < 0.0001$, respectively) but not by locality ($p > 0.05$, Fig. 4. 4). The higher Cd concentrations (5 and 10 mg Cd L^{-1}) induced the more peroxidation of lipid in both populations with longer exposure time (14 d).

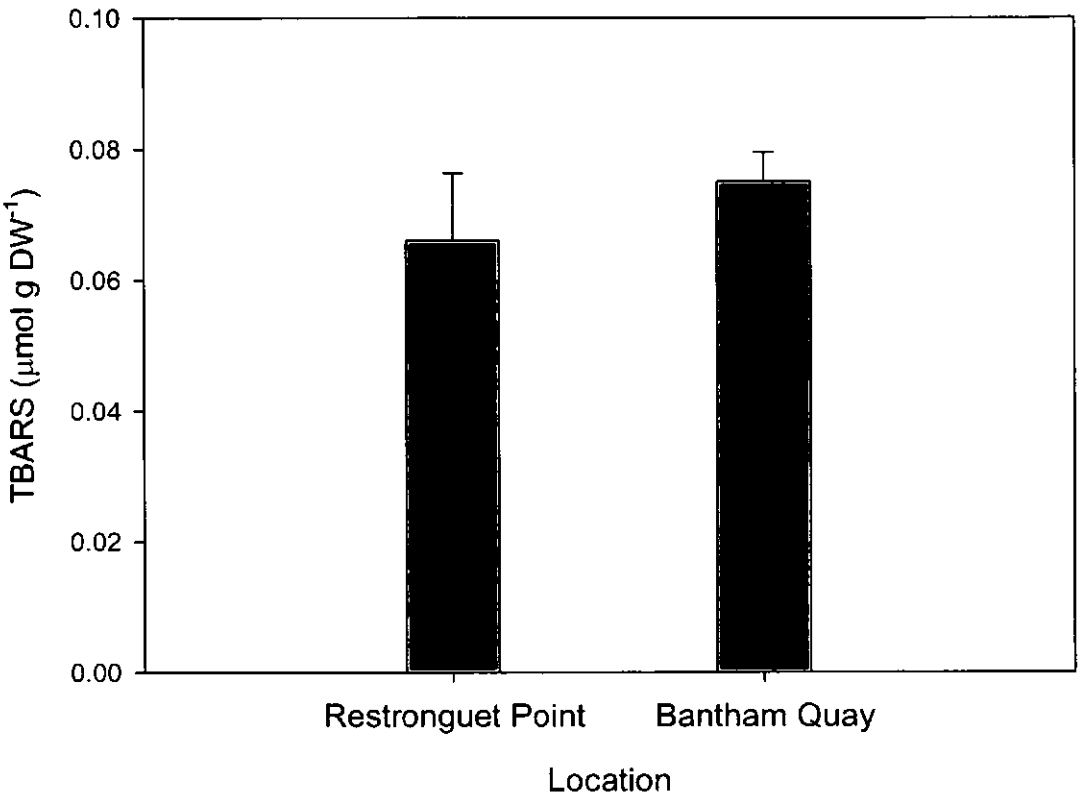

Fig. 4. 3. Thiobarbituric acid-reactive substance (TBARS) levels, mainly malondialdehyde (MDA), in *Fucus serratus* harvested from Restronguet Point and Bantham Quay. Values represent mean values of three independent replicates ± standard deviations.

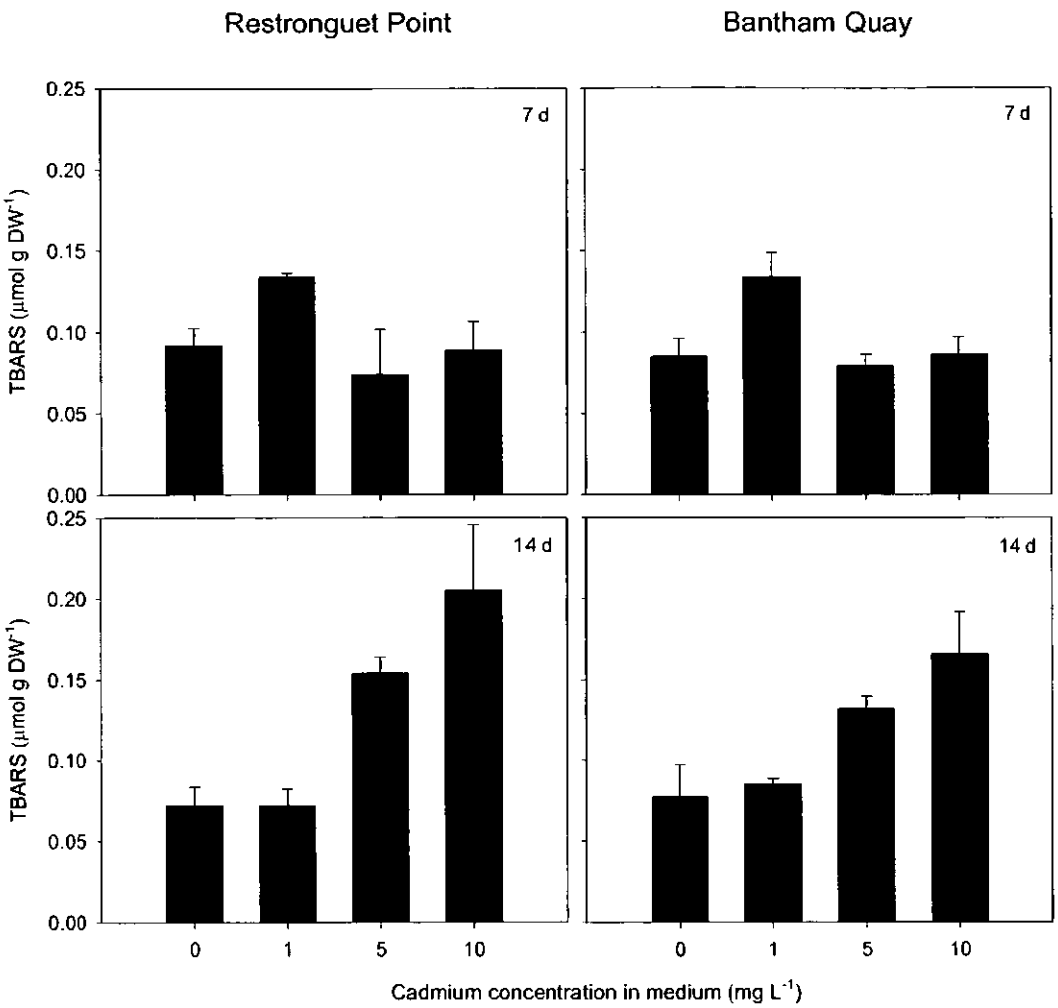

Fig. 4. 4. Thiobarbituric acid-reactive substance (TBARS) levels, mainly malondialdehyde (MDA), in *Fucus serratus* from Restronguet Point and Bantham Quay which were exposed to cadmium (7 and 14 days). Values represent mean values of three to six independent replicates ± standard deviations.

Effects of time of exposure and Cd concentration within each population were analysed by One-way ANOVA. In RP, apparent effects of time of Cd exposure and Cd concentration were discovered ($p = 0.01$ and $p < 0.0001$, respectively). 1 mg Cd L^{-1} showed the highest peroxidised value at 7 d albeit it was not different from the control (0 mg Cd L^{-1}) at 14 d. In the lower Cd concentrations (0 and 1 mg Cd L^{-1}), 7 d exposure showed higher values than 14 d. However, the longer exposure time (14 d) and the increased Cd concentrations (5 and 10 mg Cd L^{-1}) had a clear synergistic influence ($p < 0.0001$). The BQ population showed the same pattern of lipid peroxidation to that of the RP population (Fig. 4. 3). The values were lower than RP with 5 and 10 mg Cd L^{-1} at 14 d however statistical significance was not evaluated ($p > 0.05$).

4. 3. 2. 2. Cupric ion reducing antioxidant capacity

Total antioxidant capacity was measured by reducing the cupric ion. When *F. serratus* was exposed to 1 ~ 10 mg Cd L^{-1} for 1, 7 and 14 d, the reference population, BQ, had significantly higher levels than the population from RP at each day ($p < 0.0001$, Fig. 4. 5).

Additional statistical analyses were performed for each population since time of exposure and Cd concentration were significantly related to antioxidant capacity by GLM ($p < 0.0001$ and $p = 0.001$, respectively). Antioxidant capacity of *F. serratus* from RP was the highest at 1 d, and then 14 d and 7 d in order ($p < 0.0001$). However differences between Cd concentrations were not always significant. 5 mg Cd L^{-1} showed the highest CUPRAC values at 1 d and 1 mg Cd L^{-1} had the highest values at 7 d. Meanwhile all Cd treated materials (1~10 mg Cd L^{-1}) showed higher values than 0 mg Cd L^{-1} at 14 d ($p = 0.002$).

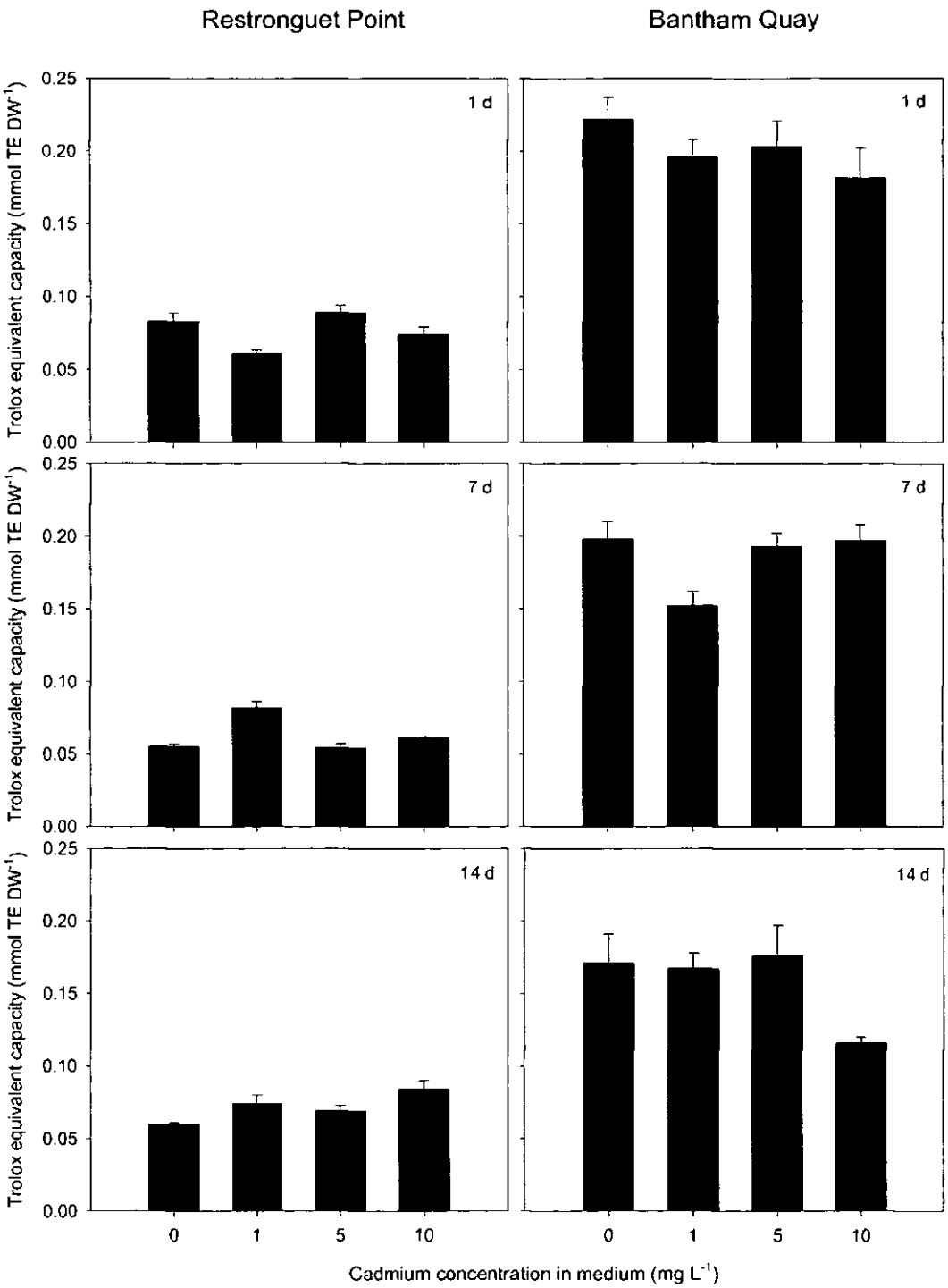

Fig. 4. 5. Cupric ion reducing antioxidant capacity (CUPRAC) in *Fucus serratus* from Restronguet Point and Bantham Quay which were exposed to cadmium (1, 7 and 14 days). Values represent mean values of three to six independent replicates ± standard deviations.

Time of Cd exposure and Cd concentration had very significant relation to total antioxidant capacity of the BQ population ($p < 0.0001$, respectively). Antioxidant capacity decreased with exposure time (1 d > 7 d > 14 d). The capacity was not significant between Cd concentrations (10 ~ 10 mg Cd L^{-1}) at 1 d and the control materials had high capacity values at each day (Fig. 4. 5). However, values of 1 mg Cd L^{-1} at 7 d and 10 mg Cd L^{-1} at 14 d were significantly lower than other treatments.

4. 3. 2. 3. DPPH free radical scavenging capacity

Free radical scavenging capacity of *F. serratus* measured by DPPH showed significant relationships to locality and time of Cd exposure (1, 7 and 14 d) ($p < 0.0001$, respectively) but not to concentration of Cd (1 ~ 10 mg L^{-1}, $p > 0.05$). The values were extremely higher at the BQ population than at the RP population (Fig. 4. 6).

Impacts of exposure time and Cd concentration were different at each population therefore they were statistically analysed again by One-way ANOVA. In RP, free radical scavenging ability did not show a regular trend with Cd concentration however Cd treated materials had significantly higher capacity than the control at 14 d ($p = 0.001$). 1 d treatment showed the highest free radical scavenging capacity and 7 d treatment had the lowest value (7 d < 14 d < 1 d, $p < 0.0001$).

In BQ, the longer time of Cd exposure showed the lower scavenging capacity for free radical (1 d < 7 d < 14 d, $p < 0.0001$). However, concentration of treated Cd did not have an apparent tendency for free radical scavenging capacity even though there were significant differences ($p = 0.006$).

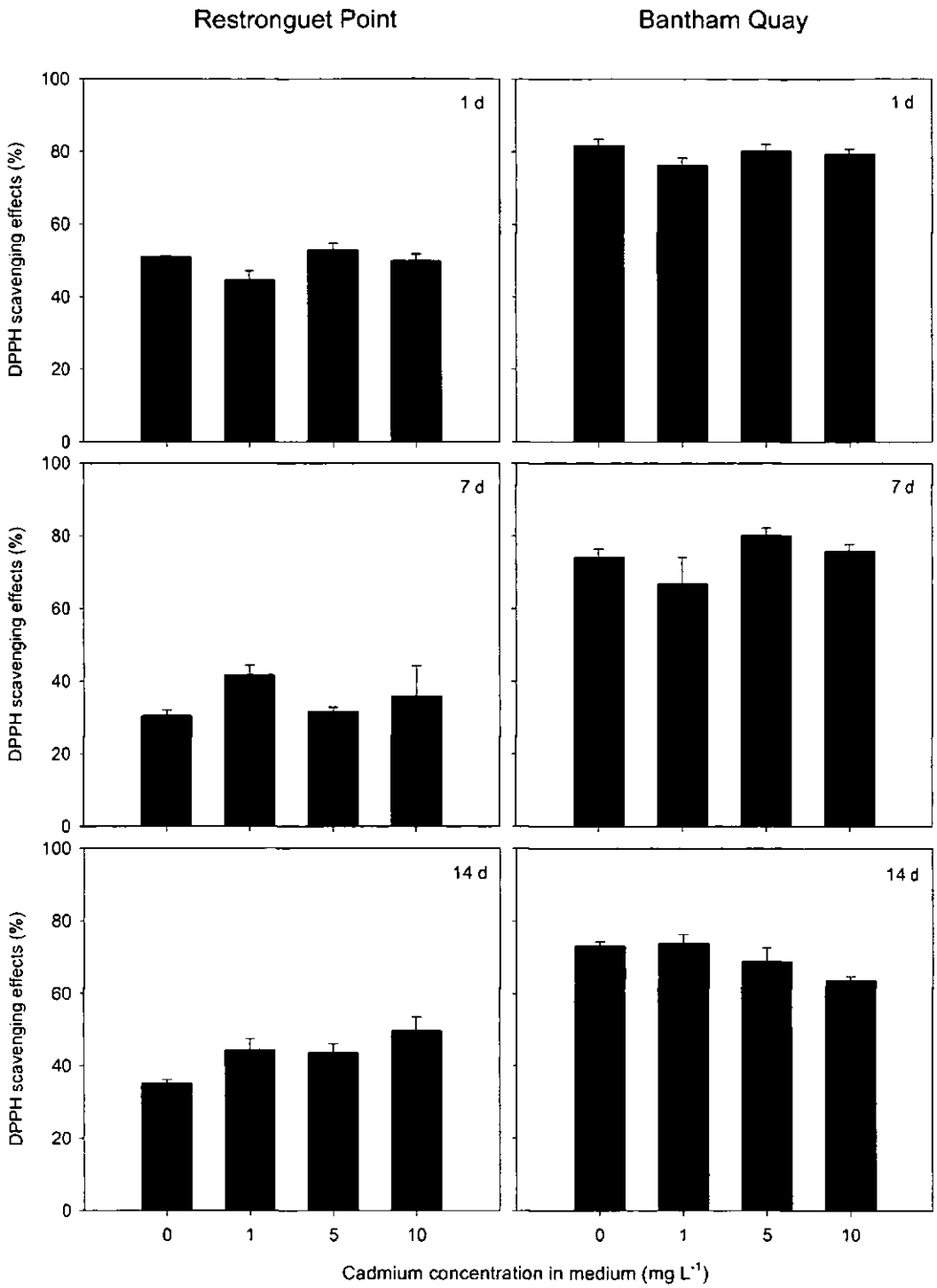

Fig. 4. 6. 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity in *Fucus serratus* from Restronguet Point and Bantham Quay which were exposed to cadmium (7 and 14 days). Values represent mean values of three to six independent replicates ± standard deviations.

4. 3. 3. Low and short Cd exposure stress

4. 3. 3. 1. Lipid peroxidation

Lipids in the cell membranes were oxidised by lower and shorter Cd exposure and were affected very significantly by locality, time of Cd exposure (24 hr, 96 hr and 7 d) and Cd concentration (10 ~ 1000 μg Cd L^{-1}) ($p < 0.0001$, respectively). RP showed significantly higher TBARS values, which represents more damage on membrane lipid by peroxidation (Fig. 4. 7).

Since patterns of TBARS contents were different in RP and BQ, effects by time of exposure and Cd concentration were analysed again within each population. In RP, TBARS contents were significantly different by time of exposure ($p < 0.0001$) and were highest at 24 hr, decreased at 96 hr and then increased again at 7 d. After 24 hr of Cd treatment, 100 μg Cd L^{-1} showed significantly higher levels of TBARS than 10 and 1000 μg Cd L^{-1}. 0 μg Cd L^{-1} had higher TBARS level than 10 and 1000 μg Cd L^{-1} at 24 hr, but the value decreased and stabilised after 96 hr. TBARS contents increased with Cd concentration at 96 hr, however 10 and 100 μg Cd L^{-1} had lower values than the control. After 7 d of treatment, all Cd exposed materials showed higher levels of TBARS than the control and 100 μg Cd L^{-1} was the highest.

In BQ, contents of TBARS were always significantly lower than those in RP (Fig. 4. 7). Like RP, exposure time and Cd concentration were significantly related to peroxidation of lipid ($p < 0.0001$, respectively). However the effects of time and Cd concentration occurred differently from the RP population. 96 hr exposure had the highest levels of TBARS and 24 hr exposure had the lowest (24 hr < 7 d < 96 hr). Significant differences by Cd concentrations were estimated ($p < 0.0001$). At 24 hr, 10 μg Cd L^{-1} showed the highest peroxidised values and over 100 μg L^{-1} showed lower

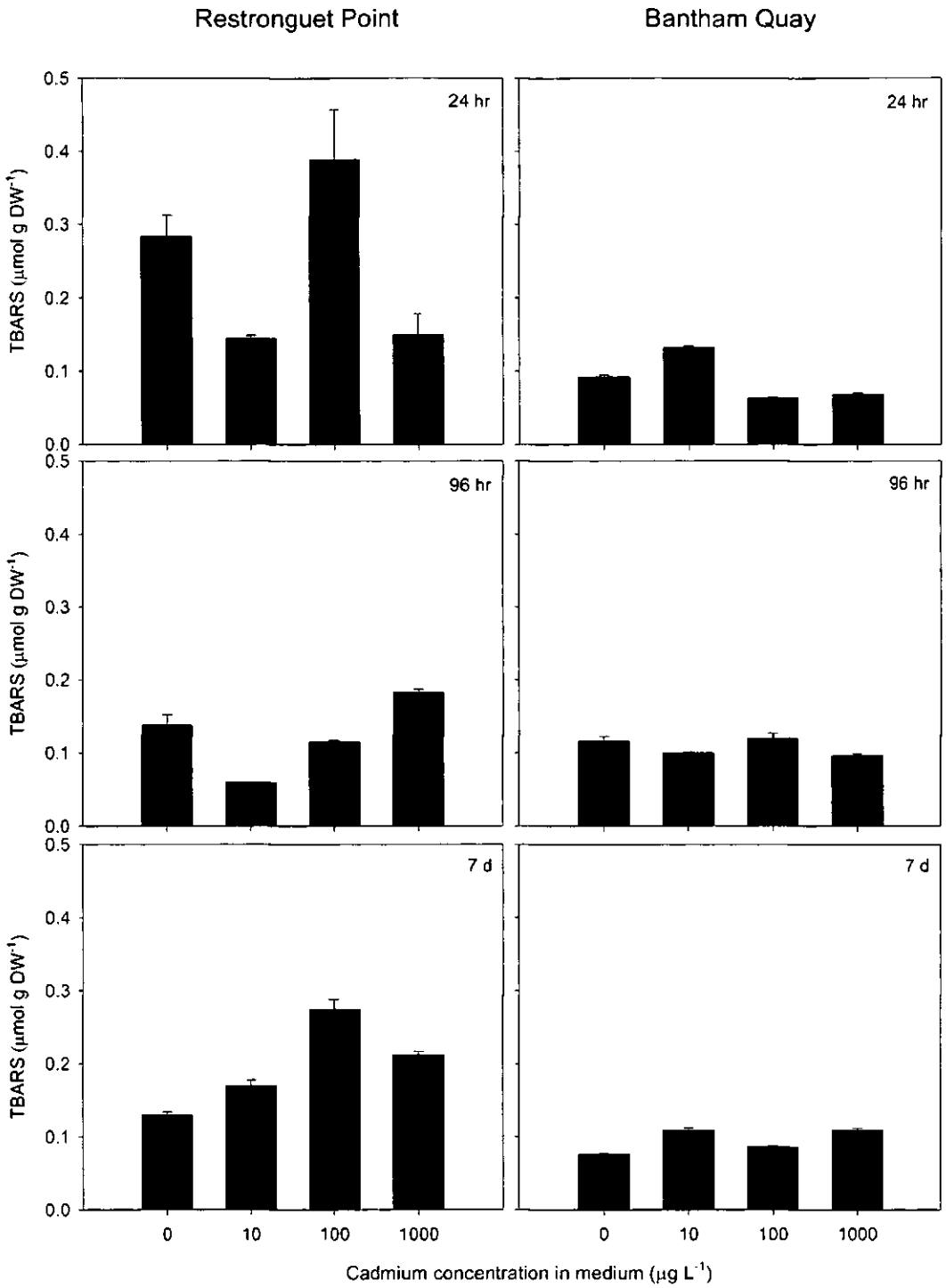

Fig. 4. 7. Thiobarbituric acid-reactive substance (TBARS) levels, mainly malondialdehyde (MDA), in *Fucus serratus* from Restronguet Point and Bantham Quay which were exposed to cadmium (24 hr, 96 hr and 7 days). Values represent mean values of three independent replicates ± standard deviations.

lipid peroxidation values than those with 10 μg L^{-1}. At 96 hr 100 μg Cd L^{-1} had the highest values and at 7 d every Cd treated material showed significantly higher value than the control ($p < 0.0001$).

4. 3. 3. 2. Cupric ion reducing antioxidant capacity

When *F. serratus* was exposed to 10 ~ 1000 μg Cd L^{-1} for 24 hr, 96 hr and 7 d, the BQ population had significantly higher levels than the RP population as the experiment with higher and extended Cd exposure ($p < 0.0001$, Fig. 4. 8).

The influences of exposure time and Cd concentration were different for each population therefore additional analyses were performed with One-way ANOVA. At 24 hr exposure in RP, 10 μg Cd L^{-1} had significantly lower values than the other concentrations ($p < 0.0001$) and the rest of them were similar ($p > 0.05$). At 96 hr in RP, 100 μg Cd L^{-1} was the lowest ($p < 0.0001$) and the others were similar one another ($p > 0.05$). However, after 7 d treatment, antioxidant ability decreased with increasing Cd concentrations in medium ($p < 0.0001$).

Time of Cd exposure as well as Cd concentration had very close relationship to total antioxidant capacity of the BQ population ($p < 0.0001$, respectively) (Fig. 4. 8). Antioxidant capacity was highest at 96 hr, 7 d and 24 hr in order ($p < 0.0001$). Differences between Cd concentrations were significant ($p < 0.0001$) however the effect of Cd concentration did not show any particular pattern. 10 μg Cd L^{-1} had the highest Trolox equivalent capacity after 96 hr and the control and 1000 μg Cd L^{-1} were similar each other at 96 hr and 7 d.

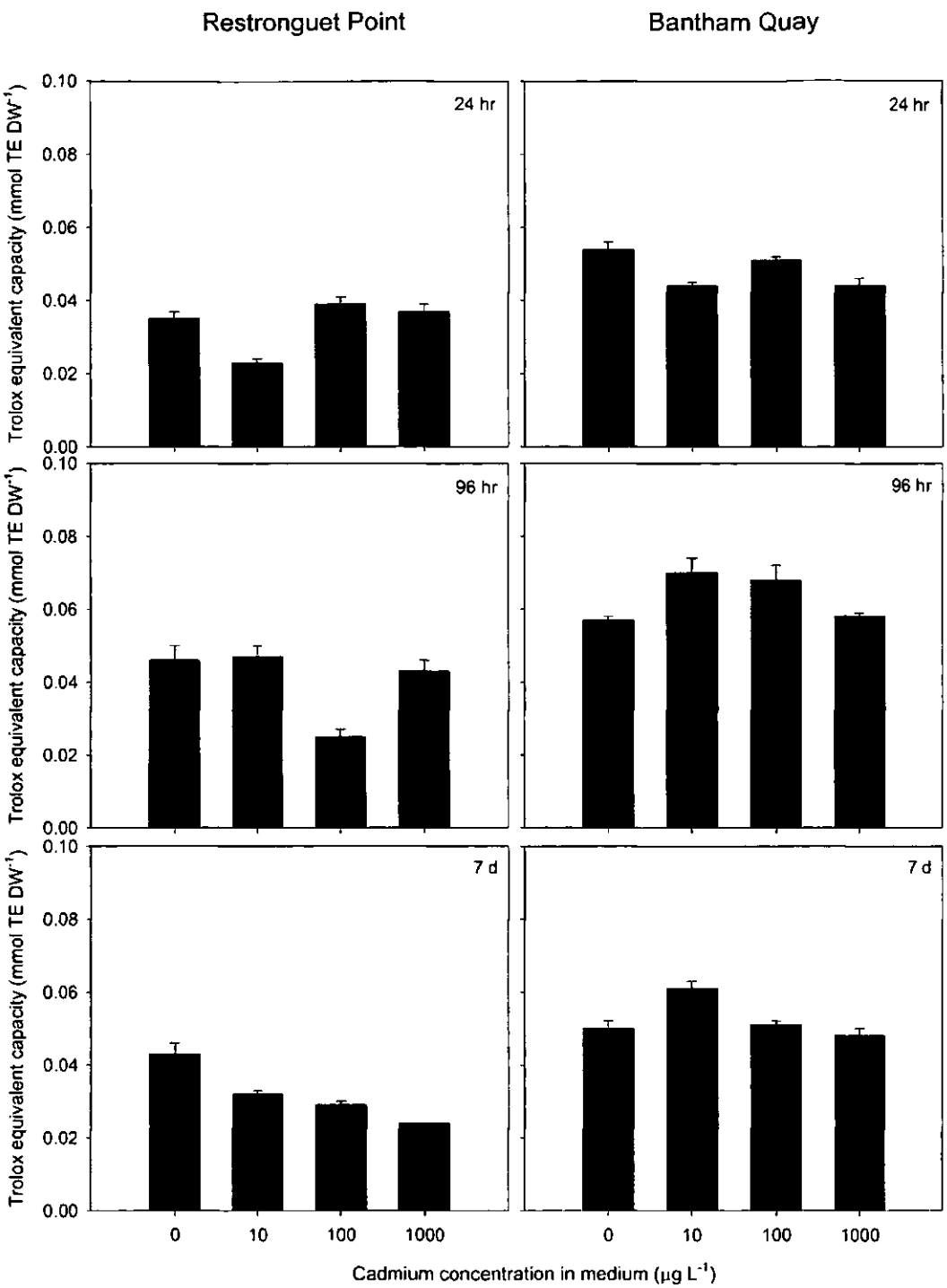

Fig. 4. 8. Cupric ion reducing antioxidant capacity (CUPRAC) in *Fucus serratus* from Restronguet Point and Bantham Quay which were exposed to cadmium (24 hr, 96 hr and 7 days). Values represent mean values of three independent replicates ± standard deviations.

4. 3. 3. 3. DPPH free radical scavenging capacity

F. serratus from the polluted site had significantly lower capacity to scavenge free radicals than the algae from the clean site ($p < 0.0001$, Fig. 4. 9). In both populations, 96 hr treatment had the highest values and 24 hr treatment had the lowest (24 hr < 7 d < 96 hr, $p < 0.0001$).

The effect of Cd concentration was analysed again since the impact was not identical for the two different populations. In RP, at 24 hr, the capacity showed a clear increase with Cd concentrations and 0 and 10 μg Cd L^{-1} were significantly lower than 100 and 1000 μg Cd L^{-1} ($p < 0.0001$). At 96 hr, all conditions have similar capacity including the control with the exception of 100 μg Cd L^{-1} ($p < 0.0001$). After 7 d of treatment, the pattern of free radical scavenging capacity of RP was exactly opposite to that of 24 hr treatment. 100 and 1000 μg Cd L^{-1} had significantly lower values than 0 and 10 μg Cd L^{-1} ($p < 0.0001$).

In BQ, the effect of Cd concentration did not have a typical pattern nor a statistical significance ($p > 0.05$). 96 hr had the highest DPPH scavenging effects (Fig. 4. 9).

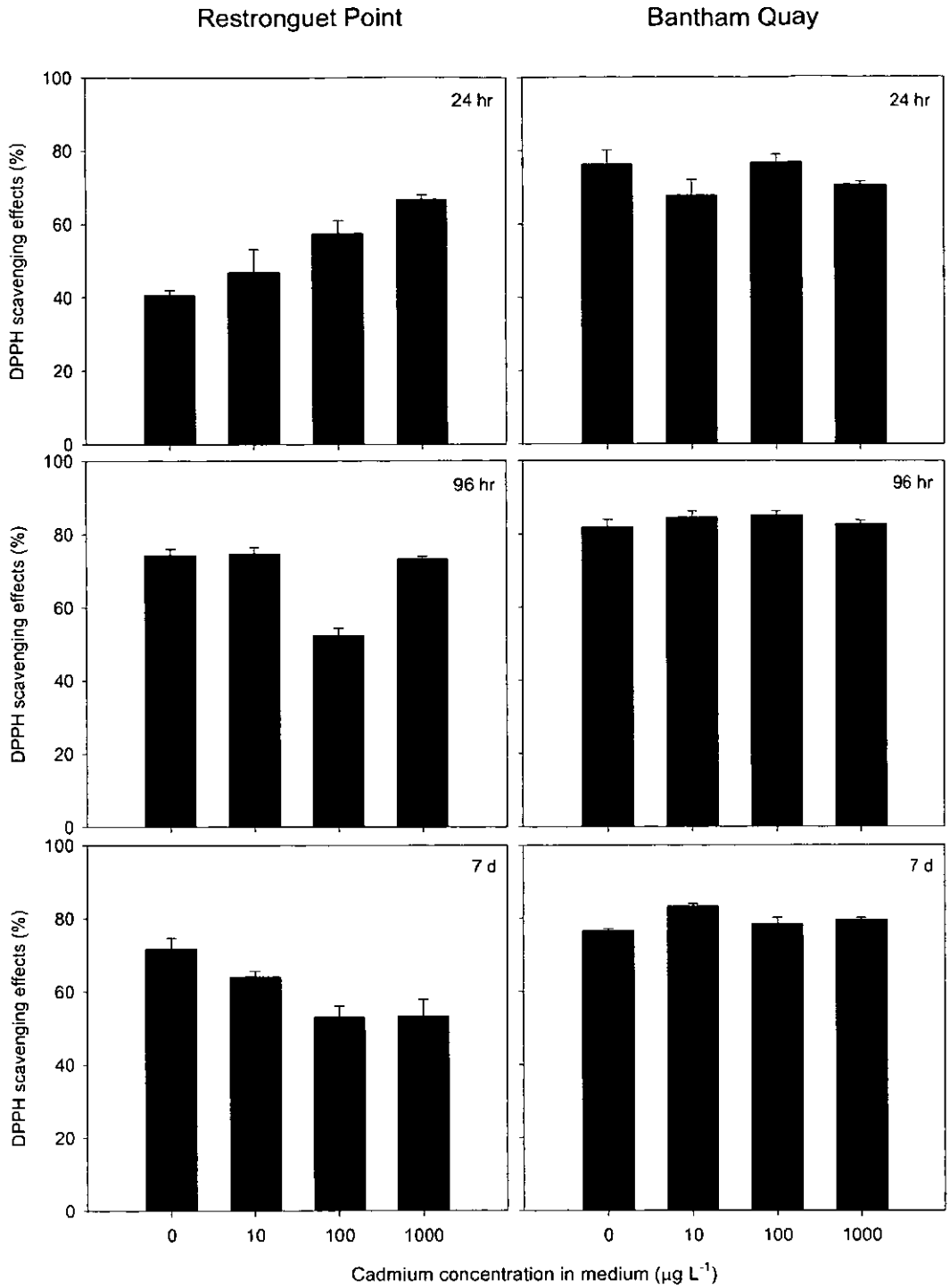

Fig. 4. 9. 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity in *Fucus serratus* from Restronguet Point and Bantham Quay which were exposed to cadmium (24 hr, 96 hr and 7 days). Values represent mean values of three independent replicates ± standard deviations.

4. 3. 4. Production of reactive oxygen scavenging enzymes under low and short Cd exposure

4. 3. 4. 1. Catalase activity

Activities of reactive oxygen scavenging enzymes were evaluated with *Fucus serratus* exposed to 10 ~ 1000 μg Cd L^{-1} for 24 hr, 96 hr and 7 d (Fig. 4. 10 ~ 4. 12). Activity of CAT was significantly higher at the RP population, especially at 24 and 96 hr ($p < 0.0001$, Fig. 4. 10).

Individual effects of exposure time and Cd concentration were analysed separately in each population since the activity of CAT in each population showed apparently different response to them. In RP, Cd treatment increased CAT activity ($p < 0.0001$) and Cd treated materials showed significantly higher CAT activities than the control, especially at 24 and 96 hr ($p < 0.0001$). Therefore statistical analysis for the effect of time of exposure was done by One-way ANOVA. CAT activities were significantly different from each time of exposure ($p < 0.0001$) and 96 hr showed extremely higher values than the other two exposure times (7 d < 24 hr < 96 hr). Activities of CAT were not different from each Cd exposure at 24 hr (10 ~ 1000 μg Cd L^{-1}, $p > 0.05$), however values of activities decreased with increasing Cd concentrations at 96 hr (10 > 100 > 1000 μg Cd L^{-1}, $p < 0.0001$). Nevertheless CAT activity increased corresponding to increasing Cd concentrations at 7 d again ($p < 0.0001$).

In BQ, generally, Cd treatment increased activities of CAT at 24 hr and 7 d ($p < 0.0001$, respectively), especially at high Cd concentration (1000 μg L^{-1}). However CAT activity decreased with increasing Cd concentration at 96 hr ($p = 0.004$). 24 hr showed the highest CAT activity and 96 hr showed the lowest values (24 hr < 7 d < 96 hr, $p < 0.0001$).

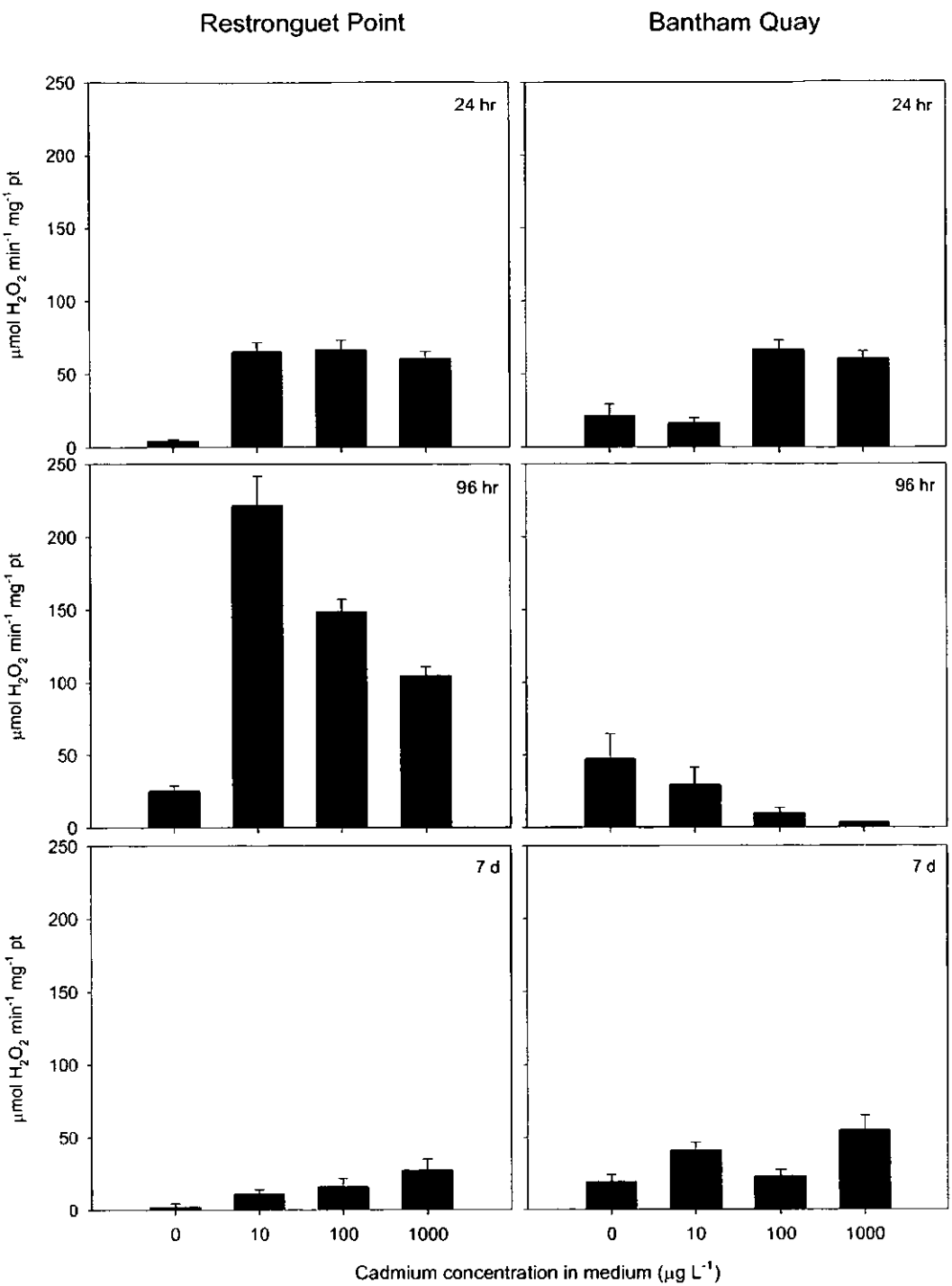

Fig. 4. 10. Activity of reactive oxygen scavenging enzyme, catalase, in *Fucus serratus* from Restronguet Point and Bantham Quay which were exposed to cadmium (24 hr, 96 hr and 7 days). Values represent mean values of three independent replicates ± standard deviations.

4. 3. 4. 2. Ascorbate peroxidase activity

Activity of APX was measured spectrophotometrically with H_2O_2 and ascorbate (Fig. 4. 11). Both the polluted and the reference populations presented similar values of APX activity on each day and no significant difference was evaluated ($p > 0.05$).

Since time of Cd exposure and Cd concentration showed significant effects on APX activity by GLM ($p < 0.0001$, respectively), effect of each factor within individual population was analysed again by One-way ANOVA. In RP, 96 hr had the lowest APX activity ($p < 0.0001$). At 24 hr of Cd treatment in RP, 10 and 1000 μg Cd L^{-1} showed extremely higher levels of APX activity than the other conditions (0 = 100 < 10 < 1000 μg Cd L^{-1}). Cd treated materials had higher antioxidant capacities at 24 hr and 96 hr except 100 μg Cd L^{-1} ($p < 0.0001$). However, 1000 μg Cd L^{-1} showed significantly lower values at 7 d although the others had similar values (1000 < 0 = 10 = 100 μg Cd L^{-1}).

In BQ, 1000 μg Cd L^{-1} showed very significantly higher values at 24 hr ($p < 0.0001$) however Cd treatment and its concentration did not affect APX activity at 96 hr ($p>0.05$. Fig. 4. 11). After 7 d of Cd treatment, 1000 μg Cd L^{-1} showed tremendously higher values and 100 μg Cd L^{-1} had exceptionally lower values than the other conditions ($p < 0.0001$).

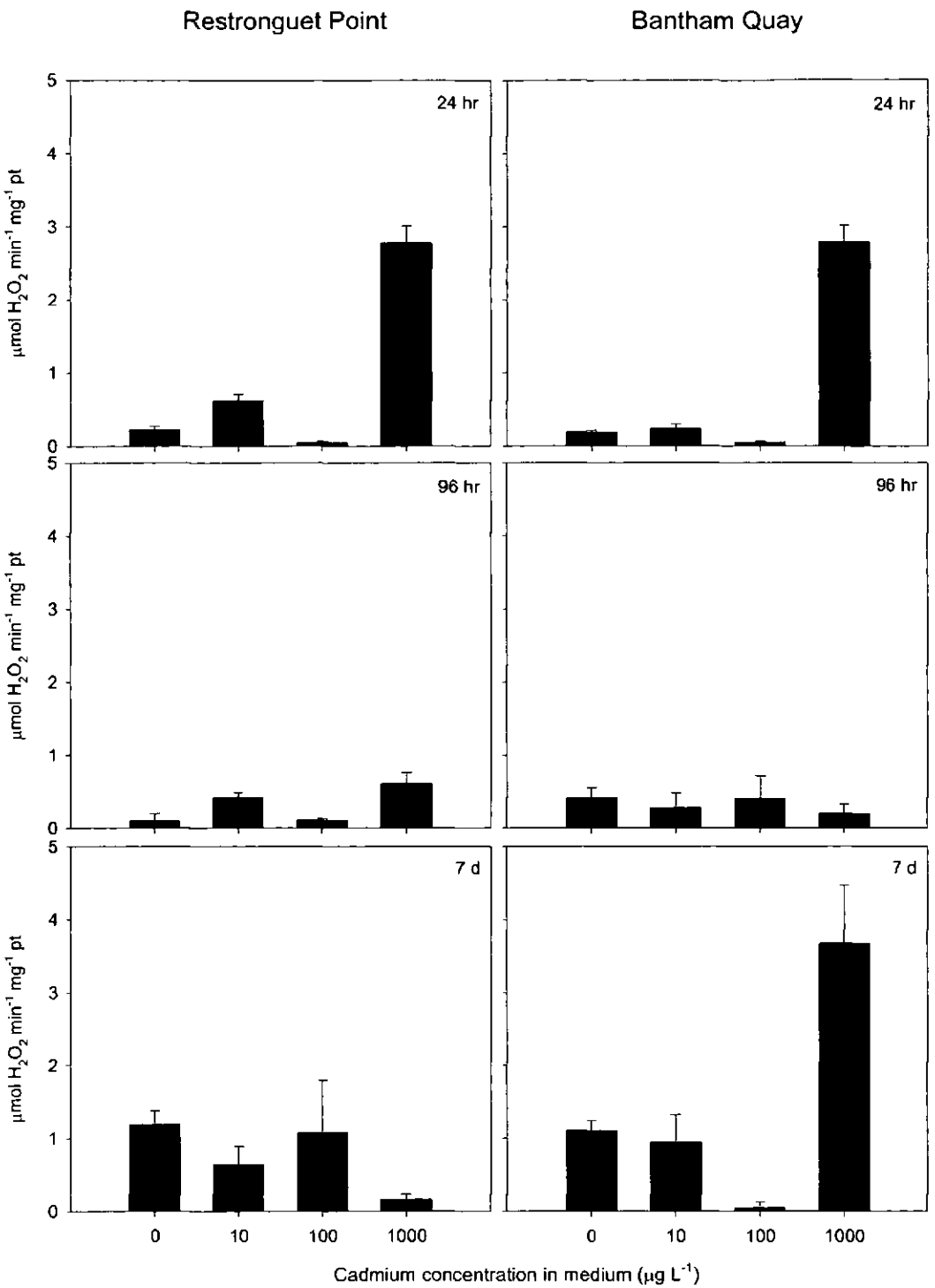

Fig. 4. 11. Activity of reactive oxygen scavenging enzyme, ascorbate peroxidase, in *Fucus serratus* from Restronguet Point and Bantham Quay which were exposed to cadmium (24 hr, 96 hr and 7 days). Values represent mean values of three independent replicates ± standard deviations.

4. 3. 4. 3. Glutathione reductase activity

Total levels of GR activities of the two populations were not significantly different ($p > 0.05$), therefore the values in the same time period were analysed again to compare the two different populations. Generally Cd treated materials of RP showed higher activities than the BQ materials on 24 hr and 96 hr but not on 7 d, although 0 μg Cd L^{-1} did not have any difference between populations (Fig. 4. 12).

Impacts of exposure time and Cd concentration were different in each population. In RP, 24 hr exposure showed the lowest activity and 96 hr exposure showed the highest (24 hr < 7 d < 96 hr, $p < 0.0001$). Generally, the controls showed lower activities than the Cd treated materials (10 ~ 1000 μg L^{-1}, $p < 0.0001$). The differences between Cd treatments and the controls were especially significant at 24 hr and 7 d.

In BQ, responses to Cd exposure were slower than the RP population (Fig. 4. 12). Only 1000 μg Cd L^{-1} showed significantly higher GR activity after 24 hr of Cd exposure ($p < 0.0001$) however no difference was found at 96 hr ($p > 0.05$). At 7 d, higher Cd concentration increased significantly higher GR activity and 100 and 1000 μg Cd L^{-1} showed significantly higher activities than the others, although 10 μg Cd L^{-1} did not show significance from the control (0 = 10 < 100 < 1000 μg Cd L^{-1}).

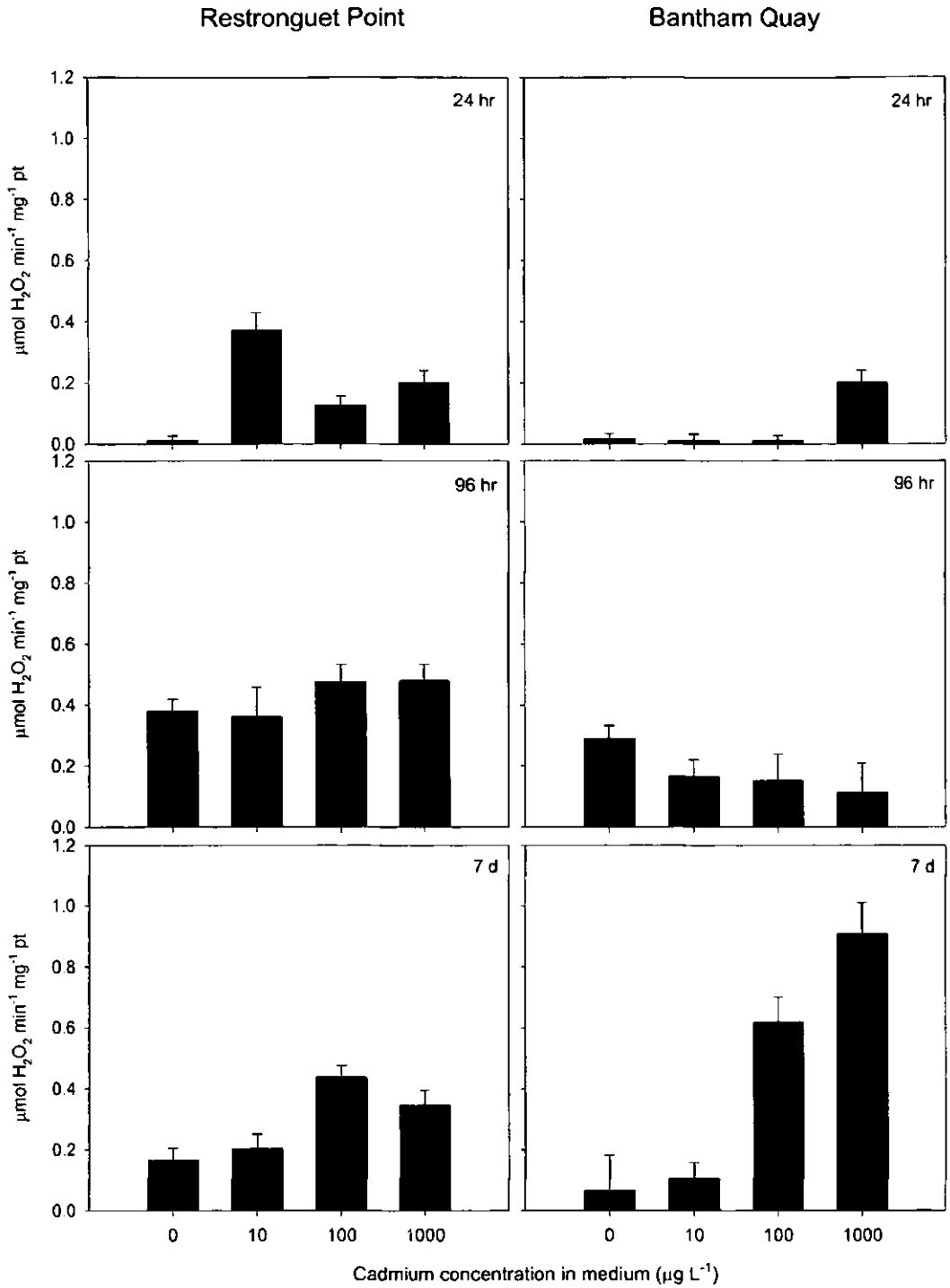

Fig. 4. 12. Activity of reactive oxygen scavenging enzyme, glutathione reductase, in *Fucus serratus* from Restronguet Point and Bantham Quay which were exposed to cadmium (24 hr, 96 hr and 7 days). Values represent mean values of three independent replicates ± standard deviations.

4. 4. Discussion

Production of ROS comes from regular aerobic cell metabolisms, such as photosynthesis and respiration (Asada and Takahashi, 1987, Asada, 1999, Mittler, 2002, Contreras et al., 2005), however the net increase of ROS is considered as a clear signal of an oxidative stress (Mittler, 2002, Contreras et al., 2005). Contreras et al. (2005) measured ROS levels by determining fluorescence of 2,4-dichlorofluoresceine diacetate-incubated *Scytosiphon lomentaria* and measured antioxidative enzyme activities by monitoring oxidation of substrate. They reported the obvious increase of ROS levels in *S. lomentaria* from mine wastes and regarded the high levels of ROS and increased antioxidative enzymes (CAT, GP, APX, MDHAR, DHAR) as oxidative stress indicators in the Cu-enriched environment. However our knowledge on antioxidant mechanism of macroalgae is very limited and just a few cases have been reported to date (Collén and Pedersén, 1994, Jervis et al., 1997, Collén and Davison, 1999a, Collén and Davison, 1999c, Collén and Davison, 1999b, Ratkevicius et al., 2003). Therefore, even though there are some similar protective mechanisms between higher plants and macroalgae and ROS scavenging enzymes seems to be a normal phenomenon in algae (Ratkevicius et al., 2003), their antioxidant processes to diminish cellular damage are not yet well understood.

4. 4. 1. Lipid peroxidation

Lipid peroxidation has been used as a direct biomarker of oxidative stress (Smirnoff, 1995, Choo et al., 2004). TBARS method has widely used for determination of oxidative stress in terrestrial plant, micro- and macroalgae (Collén and Davison, 1999a, Barros et al., 2003, Pinto et al., 2003, Choo et al., 2004). ROS removes a

hydrogen atom from a methylene group in polyunsaturated fatty acids in cell membranes, which begins membrane peroxidation (Choo et al., 2004). Oxidised fatty acids react with two molecules of TBA and give a pinkish red chromogen (Senevirathne et al., 2006). Most of TBARS has been known as MDA (Senevirathne et al., 2006).

Contents of TBARS, mostly MDA, of the two natural populations from RP and BQ were similar in this study although the two natural environments have very different metal contents (See Chapter 3). Since RP area is very polluted by several metals, the similar mean value of lipid peroxidation from the natural RP population may suggest the stronger detoxifying ability or less stress from metal exposure. This may indicate they have a stronger and more efficient antioxidant protective system to reduce oxidative damage by metal exposure.

Apparent increases of TBARS levels in the present study suggest Cd-induced oxidative membrane damage. Heavy metals are known to generate ROS which can disturb common cell metabolism very rapidly. Lee and Shin (2003) reported Cd-caused lipid peroxidation and H_2O_2 occurrence in marine alga *Nannochloropsis oculata*. With 0 ~ 1000 μg Cd L^{-1}, the RP population showed a very sensitive reaction and had higher TBARS values than the BQ population. The faster and higher responses of RP at 24 hr exposure may indicate that the lipids in algal cells were disturbed by oxidative stress and the balance of cellular oxidative status has been collapsed. There can be other reasons from any trauma or shock by changing the medium, since values did not show a typical trend with Cd concentration and the control also showed a relatively high value (Fig. 4. 7). Nevertheless gradual increase with Cd concentrations at 7 d exposure of RP represents Cd-induced peroxidation in cell membrane lipids. Meanwhile most of Cd treated materials from BQ had lower TBARS values than their controls until 96 hr, they had higher values than the control at 7 d. Therefore, with lower Cd concentrations (10 ~ 1000 μg Cd L^{-1}), it took 7 days for *F. serratus* from BQ to show membrane damage by

Cd contamination. This may help to protect the algae from metal stress, compared with the rapid responses in RP.

Unlike lower Cd concentrations, higher Cd contents (1 ~ 10 mg Cd L^{-1}) did not induce a difference by locality. Values of controls were stable since the materials adjusted to the culture conditions for longer time of exposure (7 and 14 d) for which algae exposed to lower Cd concentration have also adapted to the culture conditions. Although there was no significance by locality, time of exposure and Cd concentration showed apparent effects on lipid damage. The longer exposure (7 d < 14 d) and the higher Cd concentration (0 = 1 < 5 < 10 mg Cd L^{-1}) enhanced the higher damage in cell membrane lipids.

In this study, Cd did induce lipid peroxidation although, at the beginning, additional stresses might have been included due to the change of culture media (from seawater to Aquil medium). The lipid peroxidation with higher Cd concentration was similar since the non-exchangeable Cd concentrations were similar at both populations (see Chapter 3). However, with lower Cd concentrations, the damage on membrane lipids was significantly higher in the RP population even though the non-exchangeable Cd concentrations were still similar (see Chapter 3). This inconsistency between Cd accumulation and lipid peroxidation with lower and shorter Cd exposure may results from a failure of antioxidative system. Nevertheless, similar lipid peroxidation of *F. serratus* from the two different locations may be due to over-burden of Cd stress. Since 1 ~ 10 mg Cd L^{-1} for 7 and 14 d is extremely high, long and possibly toxic situation which the alga may not experience at all in the natural aquatic environment. This exceptional status must be very threatening even to the algae from highly metal-contaminated locations like RP.

Generally, oxidative stress results in the production of ROS which induces peroxidation of lipids (Rusterucci et al., 1999, Contreras et al., 2005). The production of

TBARS is often strain-dependent as well as dose-dependent. Randhawa et al. (2001) reported that the sensitive strain of green alga *Scenedesmus acutus* f. *alternans* suffered from higher lipid peroxidation than the resistant strain under the Ni treatment. They also reported that the sensitive strain showed high lipid peroxidation when they were in absence of Ni. Jin (1998) concluded that the differences of lipid peroxidation between different strains possibly are attributed to their differences in antioxidative defence systems. However, in this research, the sensitive strain from BQ did not show any higher lipid peroxidation values than the resistant strain from RP. The expected 'sensitivity' with the membrane lipid peroxidation is not matching with the metal-exposed history of *F. serratus*. This dissimilarity of ROS production and lipid peroxidation has been reported by Collén and Davison (1999a). *F. spiralis* excreted more H_2O_2 after freezing treatment rather than *F. evanescens*, however *F. evanescens* showed significantly greater lipid peroxidation (Collén and Davison, 1999a). They concluded that *F. spiralis* could have a more efficient reaction to cease lipid peroxidation (Collén and Davison, 1999a). Therefore discrepancy of lipid peroxidation in this research may represent a potentially more efficient resistant mechanism by the *Fucus* population from BQ. Unlike the current study, relatively lower lipid peroxidation in stressed algae has been commonly reported with green, red and brown algae (Burritt et al., 2002, Choo et al., 2004, Contreras et al., 2005). This low lipid peroxidation in stressed algae was always regarded as the more efficient antioxidant system, including antioxidative enzymes and non-enzymatic antioxidative compounds, or increased degradation of ROS, or both (Burritt et al., 2002, Choo et al., 2004, Contreras et al., 2005). Meanwhile, similar TBARS synthesis in natural populations and in higher Cd-treated materials and higher TBARS synthesis in RP with lower Cd treatment are not in agreement with the previous research. This may be a population-specific characteristic of *F. serratus*. *Fucus* spp. are known to be very stress-resistant (Jiménez-Escrig et al.,

2001). Therefore, *F. serratus* from BQ may also have strong potential to defence against oxidative stress. Otherwise the RP population with lower Cd concentrations might spend their antioxidative capacity for other factors, not for reducing membrane damage. However, this result should be discussed again later with other antioxidative mechanisms, such as GSH production, since other protective mechanisms are known to reduce lipid peroxidation (Deighton et al., 1999, Thoma et al., 2003, Contreras et al., 2005).

4. 4. 2. Cupric ion reducing antioxidant capacity

Antioxidant activities can be determined by several different ways. Different results are possibly acquired according to the chosen methods, since the extracts are normally complex mixture of different compounds which have different chemical and physical characteristics (Öztürka et al., 2007). Consequently determination of antioxidant potential by multiple assays would lead to more informative and reliable results (Öztürka et al., 2007). Therefore, in this study, CUPRAC assay was performed by way of addition to DPPH free radical scavenging capacity assay.

Compared with other analysing methods for antioxidant capacity, the CUPRAC assay has not been frequently used. However, this relatively new technique has been reported as a very effective method to measure oxidative stress (Güçlü et al., 2006, Apak et al., 2007, Öztürka et al., 2007). Copper(II)-neocuproine reagent is used for the chromogenic oxidising agent in this total antioxidant capacity assay and the results were expressed as the Trolox® equivalent antioxidant capacity of the algal (or plant) materials (in mmol Trolox® per gram of matter, mmol TE g^{-1}).

Although values of CUPRAC assay were significantly related to time of Cd exposure and Cd concentrations, *F. serratus* with Cd exposure did not show any time-

dependent or dose-dependent pattern. Differences between two populations were indeed significant regardless of the Cd concentration. The reference population from BQ always had significantly higher values than the metal-contaminated population from RP, indicating the higher antioxidant response by the reference population. Meanwhile, values of CUPRAC are not likely closely related to the Cd exposure in the current study. No apparent dose-dependent pattern and highly maintained values of the BQ population (including controls) represent the loose relationship. The high values of the BQ population may present a unique response by the population and are very likely related to the other antioxidant mechanisms. Since some responses in the previous chapter were less efficient to Cd stress than the polluted population, this may compensate part of it. This will be discussed later.

4. 4. 3. DPPH free radical scavenging capacity

In the current study, compared with changeable activities of the RP population, the BQ population maintained relatively stable activities regardless of Cd concentration and time of exposure. This indicates that the BQ population has more stable capacities to combat increased Cd stress than the contaminated population although both populations had oxidative stress. On the other hand, in RP, lower Cd concentrations (10 ~ 1000 μg L^{-1}) had higher DPPH scavenging effects at 96 hr and 7 d than higher doses (1 ~ 10 mg L^{-1}) after 7 and 14 d. In addition, with lower Cd treatment (0 ~ 1000 μg Cd L^{-1}), free radical scavenging effects in RP increased at 24 hr and decreased again at 7 d corresponding to Cd concentrations. These results suggest that antioxidant capacity of *F. serratus* from RP was activated to reduce oxidative stress by Cd with shorter and lower Cd exposure and may suggest that the capacity did correspond less closely with longer

and higher Cd stress since either it was over the limit of the algal tolerance or other antioxidant activity might be related.

There have been a few reports on free radical scavenging activity of seaweeds and brown algae are known to have a superior radical scavenging capacity to other algal groups (Matsukawa et al., 1997, Yan et al., 1998). Jiménez-Escrig et al. (2001) studied three brown and two red algal species and the order of activity was *Fucus* > *Laminaria* > *Undaria* > *Porphyra* > *Chondrus*. Therefore high free radical scavenging effects of the reference population, BQ, can be understood as the natural potential of this species itself to confront environmental oxidative stress. The lower expression of RP may be correlated to other mechanisms for dealing with Cd toxicity. In the mean time, many authors have focused on the relation between free radical scavenging activity and phenolic compounds (Jiménez-Escrig et al., 2001, Connan et al., 2006, Senevirathne et al., 2006, Öztürka et al., 2007). Phenolic compounds are very important secondary metabolites in various organisms, e.g. higher plants, lichens and algae (Ragan and Glombitza, 1986, Hyvärinen et al., 2000, Connan et al., 2006, Öztürka et al., 2007). Due to their hydroxyl groups, they have been understood as strong chain breaking antioxidants (Ragan and Glombitza, 1986, Öztürka et al., 2007). Generally linear correlation was reported between concentration of phenolic compounds and free radial scavenging activity, such as DPPH free radical (Öztürka et al., 2007). The brown algae have high levels of phenols, mostly phlorotannins, in vesicles (i.e. physodes) and levels of phlorotannin in Fucales and Dictyotales was about 20 and 30% DW (Ragan and Glombitza, 1986, Targett et al., 1995, Connan et al., 2006). Some authors discovered no drastic oxidative damage in brown algal cells due to the efficient cytoprotective system of phenolic compounds (Matsukawa et al., 1997, Connan et al., 2006). Jiménez-Escrig et al. (2001) also stated strong radical scavenging activity of *F. vesiculosus* corresponding to high polyphenol contents. Therefore this supports that *F. serratus* may

also possess a high antioxidant potential related to the high polyphenol contents. High and stable levels of DPPH radical scavenging capacity in the BQ population may represent their insensitivity and high antioxidant activities against Cd exposure. In the mean time, protective capacities of other secondary metabolites are also considered, which may explain the non-correlation between phenolic contents and antioxidant activity (Deal et al., 2003). Carotenoids (especially fucoxanthin), triterpenoids, pyrophenophytin *a* and phytochelatin also reduce oxidative stress in brown algae (Anggadiredja et al., 1997, Le Tutour et al., 1998), and these compounds may be related to the changeable capacity of the RP population. One of the potential secondary metabolites will be considered in the following chapter.

4. 4. 4. Reactive oxygen scavenging enzymes

ROS produced by oxidative stress can be partially reduced by increased reactive oxygen scavenging enzymes, such as CAT, APX and GR. These enzymes have been known to play key roles in various oxidative conditions (Collén and Davison, 1999a, Collén and Davison, 1999c, Collén and Davison, 1999b). In this research, Cd exposure affected the activities of reactive oxygen scavenging enzymes, CAT, APX and GR, which represents Cd ions entered algal cells and induced processes of ROS production (Mallick and Mohn, 2000, Ratkevicius et al., 2003). CAT always showed higher antioxidant activities in RP and GR showed the higher abilities at 24 hr and 96 hr in RP. APX showed similar capacities at 24 hr and 96 hr in both populations but was higher in BQ at 7 d. High activities of these enzymes are recognised as the high capacities to withstand oxidative stress and possibly less production of ROS (Collén and Davison, 1999b). The BQ population showed the slower and less activities of enzymes, which indicates the less effective enzymatic protections and may be related to the lack of metal

contamination at the natural site. Therefore, the higher and faster enzyme activities of the RP algae could contribute to the survival of the algal population in the heavily polluted environment like Restronguet Creek. Collén and Davison (1999c) suggested that the higher reactive oxygen scavenging activity in intertidal species is an adaptation to the changeable and harsh environment. This hypothesis correlates to the antioxidant mechanism of algal species inhabiting metal-contaminated environments (Collén et al., 2003). Therefore *F. serratus* from RP has been adapted to the metal-polluted environment by increasing antioxidant enzyme capacity. In addition, the scavenging capacity of CAT was significantly higher than the other enzymes. Collén and Davison (1999b) reported that APX was the most important enzyme for *Fucus* spp. (*F. evanescens*, *F. spiralis* and *F. distichus*) in the natural habitat for reducing H_2O_2 rather than CAT and SOD. However, the conclusion may not be applied to this situation with the two different *F. serratus* populations since the activities of APX were not significantly different (at least until 96 hr) and the RP population had significantly higher levels of CAT than the BQ population. Increased activity of CAT may be more efficient to reduce the oxidative stress by Cd exposure since Cd ions will cause stress in the entire cell, not just the chloroplast (Collén et al., 2003). Some toxic metal ions, such as Cu^{2+}, Pb^{2+} and Zn^{2+}, are known to interact closely with the thylakoid membranes in the chloroplasts and an increase of APX in chloroplasts will be more efficient in controlling harmful effects by these metals (Collén et al., 2003). CAT functions as the metaboliser of the peroxide in the peroxisome after the conversion of glycolate during photorespiration, not in the chloroplast (Zutshi et al., 2008). Therefore, *Fucus* cell can be better protected by CAT than other enzymes from wide-spread effect of Cd in the entire cell.

APX and GR synthesis is known to be very useful to defend the attack of cupric ion in chloroplasts. APX and GR are vital components of the ascorbate-glutathione

pathway and scavenge ROS produced mostly in chloroplasts and other organelles (Asada, 1992, Noctor and Foyer, 1998, Asada, 1999, Zutshi et al., 2008). APX reduced potentially harmful ROS with ascorbic acid and 1000 μg Cd L^{-1} showed a large increase of APX activity in this study. Activity of GR is known to increase to supply GSH to ascorbate-glutathione cycle under oxidative condition (Zutshi et al., 2008). In this research, activity of GR increased with Cd treatment, which suggests GR could regenerate GSH from GSSG to increase the GSH/GSSG ratio and the total GSH pool (Noctor and Foyer, 1998, Zutshi et al., 2008). GSH levels of *F. serratus* will be further discussed in Chapter 5.

Generally oxidative stress increases the activity of reactive oxygen scavenging enzymes including CAT (Ratkevicius et al., 2003), however in some cases the activity declines (Schöner and Krause, 1990, Foyer and Mullineaux, 1994, Collén and Pedersén, 1996, Fadzillah et al., 1996, Combo et al., 1998, Aguilera et al., 2002, Zutshi et al., 2008). This can be understood as a reduced rate of protein turn-over by stress conditions (Hertwig et al., 1992, Zutshi et al., 2008) or direct damage by oxidative stress such as UV or metals (Foyer and Mullineaux, 1994). Although Cd treated materials had higher CAT activities than the control, the activity of CAT decreased with increasing Cd concentrations at 96 hr in RP. In BQ, Cd treated algae had lower activities rather than the control at 96 hr. MacRae and Ferguson (1985) regarded the reduced CAT activity as a general response to stresses by the inhibited enzyme synthesis or structural change in enzyme subunit. Therefore accumulation of ROS by Cd treatment may inactivate enzyme activity, possibly partially, according to the environmental conditions.

4. 5. Conclusion

A few reports have been published on Cd-induced oxidative stress in photosynthetic organisms to date. In the present study, increased lipid peroxidation, DPPH free radical scavenging effects and antioxidant enzyme activities against Cd stress suggest that this non-essential metal caused oxidative stress which was probably by generated ROS. TBARS levels, mostly MDA, of the natural *F. serratus* populations from both metal-contaminated and clean sites were similar. In the CUPRAC assay and DPPH free radical scavenging assay, the BQ population showed higher antioxidant capacities than the RP population, regardless of Cd concentrations. Moreover levels of lipid peroxidation were higher at RP than BQ under the stress of 10 ~ 1000 μg Cd L^{-1}. All of these results imply higher antioxidant capacity of the reference population from the clean site. Unlike levels of antioxidants, activities of oxidative stress scavenging enzymes were higher in the RP population, except for APX. CAT and GR increased to significantly higher levels and were produced more quickly at RP. Therefore the two different populations showed different antioxidative strategies against Cd stress, and the higher antioxidant capacity of the BQ population represents the potential strength to defeat metal stress even though the alga used to live in a clean area. In addition, the RP population presented faster (sensitive) and more efficient enzymatic responses although the antioxidant capacity measured by CUPRAC and DPPH free radical scavenging ability was lower than the BQ population.

Chapter 5. Phytochelatin and glutathione production in *Fucus serratus* (Phaeophyceae) on exposure to cadmium and copper

5. 1. Introduction

To combat the toxic effects of metals, photosynthetic organisms have evolved effective extra- and intra-cellular mechanisms for metal detoxification. In algae, poly-anionic polysaccharides associated with cell walls and inter-cellular spaces sequester cations, through an ion-exchange mechanism, forming a primary barrier to cellular uptake (De Andrade et al., 2002, Talarico, 2002, Salgado et al., 2005). This superficial binding can account for up to 95% of the total metal accumulated in some freshwater species (Pawlik-Skowrońska, 2000, Pawlik-Skowrońska et al., 2004) and between <5% and 80% in marine macroalgae, depending on the metals, their external concentrations, the species of algae, compositions of cell walls and the environmental conditions under which the algae are growing (Vasconcelos and Leal, 2001, García-Ríos et al., 2007). Metals that enter algal cells do so mainly via energy-dependent transport across the plasma-membrane (Hu et al., 1996), but once inside, the processes whereby disruption of cellular activities is prevented and metal homeostasis is maintained have not been very well elucidated. One commonly encountered intracellular mechanism for detoxification of metals in photosynthetic organisms involves chelation and sequestration of the metal ions by peptides or proteins such as glutathione (GSH), phytochelatin (PC) and metallothionein (MT) (Cobbett and Goldsbrough, 2002, Kawakami et al., 2006).

5. 1. 1. Glutathione (GSH) and its role

Reduced glutathione (GSH) and its homologues are low molecular weight polypeptides in eukaryotes (Grill et al., 1986, Zenk, 1996, Prasad, 1999, Cobbett and Goldsbrough, 2002). GSH is a thiol, -SH or sulfhydryl group, synthesized in higher plants and algae (Wei et al., 2003, Kawakami et al., 2006). Synthesis of GSH is known to be closely related to metal stress in the environment (Smith et al., 1984, Smith et al., 1985, May and Leaver, 1993, Schäfer et al., 1998, Wei et al., 2003). This one of the major non-enzymatic antioxidants can scavenge free radicals to reduce oxidative stress and chelate metals and metalloids to detoxify harmful metal stress (Prasad, 1997, Nagalakshmi and Prasad, 2001, Kawakami et al., 2006, Pawlik-Skowrońska et al., 2007).

GSH is composed of three different amino acids (Fig. 5. 1); glutamate (Glu), cysteine (Cys) and glycine (Gly) (γ-Glu-Cys-Gly) (Grill et al., 1985). γ-glutamylcysteine synthetase (EC 6.3.2.2) has been known as the enzyme of GSH synthesis and buthionine-S-sulfoximine (BSO) is reported as the inhibitor of the synthetic enzyme (Prasad, 1999). GSH is a transpeptidase to catalyse PC (Grill et al., 1986, Tomsett and Thurman, 1988, Zenk, 1996, Prasad, 1999, Cobbett and Goldsbrough, 2002, Pawlik-Skowrońska et al., 2007). GSH has some variants, such as homo-glutathione (h-GSH), hydroxymethyl-glutathione (hydroxymethyl-GSH) or γ-glutamylcysteine, to synthesise homo-phytochelatin (h-PC) (Hayashi et al., 1991, Klapheck et al., 1995, Schat et al., 2002). Depletion of GSH owing to PC induction was reported in tomato cells and *Silene cucubalus* (Coppellotti, 1989, Prasad, 1999, Pawlik-Skowrońska, 2000).

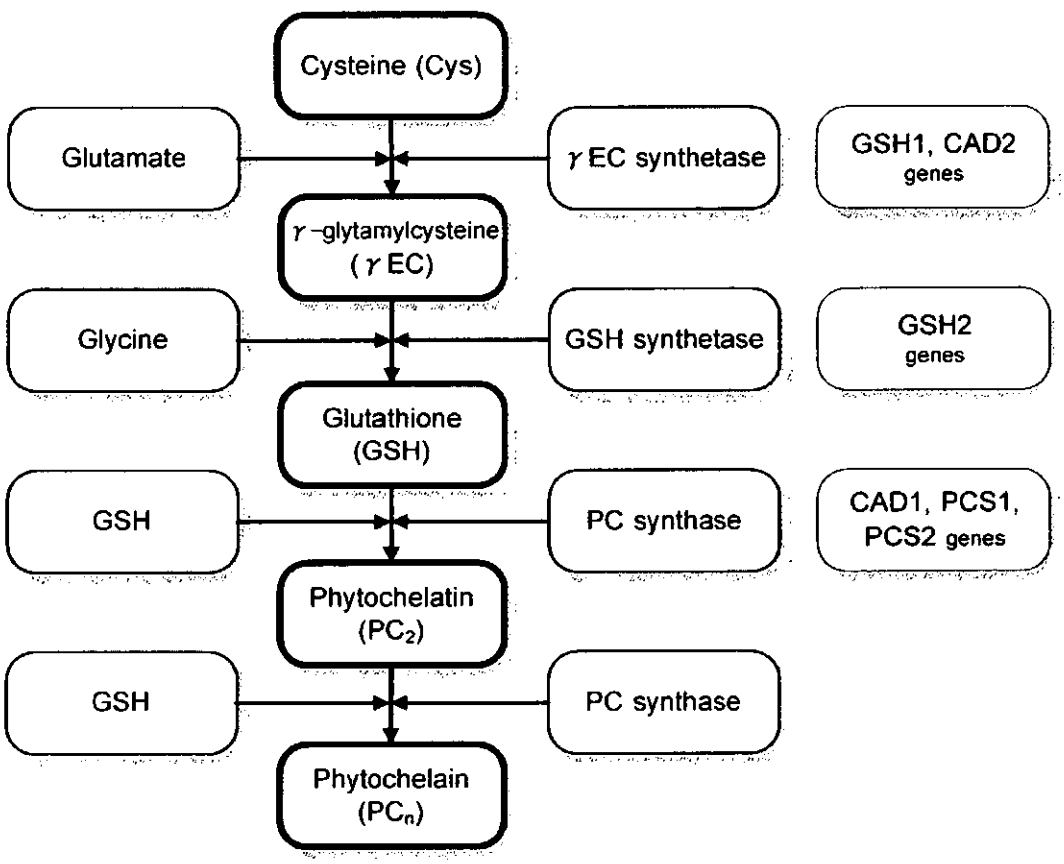

Fig. 5. 1. Biosynthetic pathway of glutathione and phytochelatins in higher plants, reproduced and modified from Hirata et al. (2001) and Inouhe (2005). Isolated genes are known to encode key enzymes of PC synthesis.

5. 1. 2. Phytochelatin (PC)

5. 1. 2. 1. Metallothionein (MT) and PC

There are two well-known metal detoxifying mechanisms; metallothionein (MT) and phytochelatin (PC) (Grill et al., 1985). Both of them are sulphur-rich polypeptides with similar intracellular function, i.e. chelating metal ions, however they have different biosynthetic pathways and structure and are often found in different kingdoms (Inouhe, 2005). MTs in vertebrates and fungi are 6.5 kD proteins and MTs in mammals are made of a single polypeptide chain with 61 amino-acid residues (Grill et al., 1985, Kägi, 1991, Inouhe, 2005). This metal-chelating protein can be observed in higher plants as well (Grill et al., 1985). MTs are known to be synthesized on ribosomes by mRNA translation (Rauser, 1990, Rauser, 1995) and were subdivided into three classes according to the structures of phenotypically related metal thiolate polypeptides (Rauser, 1990).

Class I: polypeptides with cysteine close to those in equine renal MT

Class II: polypeptides with cysteine distant to those in equine renal MT

Class III: metal thiolate polypeptides synthesized nontranslationally

PC was allocated in the Class III. It was identified from fission yeast *Schizosaccharomycese pombe* for the first time (Murasugi et al., 1981). For the following 30 years, small non-protein cysteine-rich oligopeptides, PCs, were identified from plants, algae and some fungi (Ahner and Morel, 1995, Inouhe, 2005). PC was induced by metal treatments and it was biosynthesised from GSH enzymatically by PC synthase, unlike ribosomal protein synthesis of MT (Rauser, 1990). PC is one of trivial names of these polypeptides related to GSH (Grill et al., 1985) and cadystin (Kondo et

al., 1984), poly(γ-glutamylcysteinyl)glycine or $(\gamma EC)_nG$ (Jackson et al., 1987), phytometallothionein (Rauser, 1987) and γ-glutamyl peptides (Mehra et al., 1988) were also used by some researchers. Homo-phytochelatins (h-PCs) related to homo-glutathione (h-GSH, γGlu-Cys-β-Ala) were also observed (Rauser, 1990).

5. 1. 2. 2. Biosynthesis and structure of PC

The primary structure of PC was described by Grill et al. (1985) with the Cd-exposed evergreen tree *Rauvolfia serpentina* (Apocynaceae). These small and simple γ-glutamyl peptides are composed of only three amino acids; glutamate (Glu, E), cysteine (Cys, C) and glycine (Gly, E) and form $[(\gamma\text{-Glu-Cys})_n\text{-Gly}]$ structure (n = 2~11) (Grill et al., 1985). Compound of Cys and Glu is synthesized to γ-glutamylcysteine (γ-Glu-Cys, γEC) with mediation of γEC synthetase (EC 6.3.2.2) and this compound synthesizes GSH (γ-Glu-Cys-Gly) with Gly by mediation of GSH synthetase (EC 6.3.2.3) (Inouhe, 2005). PC is synthesized from GSH with repeated γ-Glu-Cys dipeptides by γ-glutamyl cysteine dipeptidyl transpeptidase (PC synthase) (Grill et al., 1989). The whole process is described in Fig. 5. 1 and the chemical structure of PC is drawn in Fig. 5. 2. Most commonly observed repetition (n) is in the range of 2 to 4 (Rauser, 1990, Reddy and Prasad, 1990, Steffens, 1990) or to 5 (Cobbett and Goldsbrough, 2002). Moreover, there are many variations in the structure of PC, e.g. $(\gamma\text{-Glu-Cys})_n$, $(\gamma\text{-Glu-Cys})_n$-β-Ala, $(\gamma\text{-Glu-Cys})_n$-Gln, $(\gamma\text{-Glu-Cys})_n$-Glu, or $(\gamma\text{-Glu-Cys})_n$-Ser (Cobbett and Goldsbrough, 2002, Schat et al., 2002).

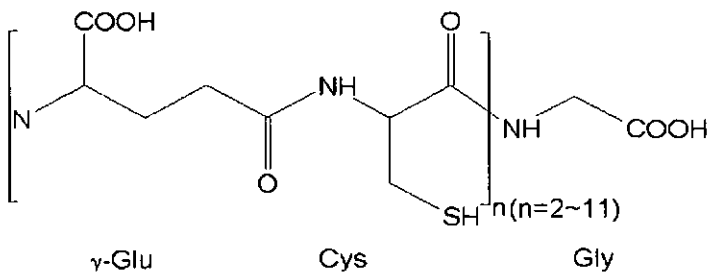

Fig. 5. 2. Chemical structure of phytochelatin.

The constitutive enzyme for catalysing biosynthesis of PC (PC synthase) was purified from various cells; e.g. *Silene cucubalus* (bladder campion, Caryophyllaceae), *Schizosaccharomyces pombe* (fission yeast, Schizosaccharomycetaceae), *Arabidopsis thaliana* (thale cress, Brassicaceae), *Beta vulgaris* (beetroot, Chenopodiaceae), *Eschscholtzia californica* (California poppy, Papaveraceae), *Equisetum giganteum* (horsetail, Pteridophyte, Equisetaceae), *Podophyllum peltatum* (mayapple, Berberidaceae), and *Triticum aestivum* (wheat, Poaceae) (Grill et al., 1989, Löeffler et al., 1989, Clemens et al., 1999, Ha et al., 1999, Prasad, 1999, Vatamaniuk et al., 1999). However, plants and some microorganisms were not the only living organisms which possessed PC synthase. Researchers identified homologous genes from nematodes *Caenorhabditis elegans* and *C. briggsae*, slime mould *Dictyostelium discoideum*, aquatic midge *Chironomus* and earth worm species (Cobbett and Goldsbrough, 2002).

To the current knowledge, on the other hand, PC or metal-PC complexes have not been discovered yet in animal cells (Cobbett and Goldsbrough, 2002).

5. 1. 2. 3. Intracellular role of PC

Formation of PC is known to be derived from metal stress of the environment (Jackson et al., 1987, Kneer and Zenk, 1992, Prasad, 1999). Other environmental impacts were not found to be related to the induction of PC, therefore synthesis of PC can be applied as a biochemical indicator for metal contamination (Pawlik-Skowrońska et al., 2002, Inouhe, 2005).

Rauser (1990) and Inouhe (2005) reported two major intracellular functions of PC; (1) metal detoxification and tolerance, and (2) metal homeostasis and sulphur metabolism. Most research on metal detoxification of plants has been carried out with high concentrations of Cd, generally over 1 μM (Cobbett and Goldsbrough, 2002). Cd-tolerant cells or plants showed greater Cd uptake than Cd-sensitive organisms, however Cd-tolerant organisms had better growth and bioactivities than Cd-sensitive organisms (Jackson et al., 1984, Rauser, 1990). Higher capability to survive in Cd-contaminated environment was found to be due to the synthesis of metal-chelating complexes of Cd-tolerant population. Cd-tolerant organisms which have metal-chelating complexes can bind more than 80% of the cellular Cd (Jackson et al., 1984, Rauser, 1990). Delhaize et al. (1989) reported that 95% of intracellular Cd was bound by PCs for 4 to 24 hr in *Datura innoxia* (moonflower, Solanaceae, Angiosperms). However Cd-sensitivity can be increased as a result of inhibited activity of γ-glutamylcysteine synthetase (γEC synthetase, EC 6.3.2.2) by BSO (Steffens et al., 1986, Grill et al., 1987, Scheller et al., 1987).

Essential micronutrients, such as Cu and Zn, can also be bound by PC (Rauser, 1990). Therefore Cu- and Zn-limited situations occur in the cells and apoenzymes (such as diamine oxidase and carbonic anhydrase) which need metal ions for their activities are inactive. Metal-chelator complexes can supply metal ions as necessary cofactors to those inactive apoenzymes (Rauser, 1990, Thumann et al., 1991, Prasad, 1999). Besides this metal homeostasis of PC, sulphur metabolism can also be counted as one of the roles of PC in the cell. Cd-PC complexes enclose acid-labile sulphide and reduction of sulphate was observed where PC was biosynthesized (Robinson, 1989). Therefore increase of Cd-PC complexes cause increase of the activities of ATP-sulfurylase (EC 2.7.7.4) and adenosine 5'-phosphosulfate sulfotransferase as a result of sulphate reduction. However this phenomenon has been discovered only with Cd-PC complexes to date (Rauser, 1990).

5. 1. 2. 4. Specificity of metal ions to the biosynthesis of PC

Induction of PC is clearly dependent on presence of excess metal ions (Löeffler et al., 1989) and addition of metal immediately activates the PC synthase (Kneer and Zenk, 1992, Prasad, 1999). Rauser (1990) reported that metal-binding complexes were detected only after the exposure to excess metal not before. *Rauvolfia serpentina* cells produced PC when they were exposed to Cd, Pb, Zn, Sb, Ag, Ni, Hg, arsenate, Cu, Sn, selenate, Au, Bi, Te, and W, however no PC induction was detected with Al, Ca, Co, Cr, Cs, K, Mg, Mn, molybdate, Na or Ba exposures (Grill et al., 1987). In cases of *Scenedesmus* and *Chlorella* cells, Cd, Pb, Zn, Ag, Cu and Hg ions induced PC production (Gekeler et al., 1988). Cd, Cu, Zn, Ni and As are the most frequently investigated metal species for PC production and their tolerance mechanisms. Grill et al. (1989) reported that Cd was the best metal activator then Ag, Bi, Pb, Zn, Cu, Hg and Au

followed. However sensitivity to metal species is different depending on plant or algal species. Most researchers agreed that Cd was the strongest PC inducer among metal species regardless of plant or algal cells (Grill et al., 1987, Grill et al., 1989, Rauser, 1990, Cobbett and Goldsbrough, 2002, Inouhe, 2005) although the order of metals totally depends on plant or algal species (Cobbett and Goldsbrough, 2002).

5. 1. 3. Purpose of this study

While the production of PC in response to metal-exposure has been extensively studied in higher plants since the early 1980s (Inouhe, 2005), the literature pertaining to algae is more limited, although there is a growing body of evidence to implicate PC in the metal resistance of marine phytoplankton and freshwater green microalgae (Pawlik-Skowrońska, 2001, Tsuji et al., 2002, Tsuji et al., 2003, Pawlik-Skowrońska et al., 2004, Torricelli et al., 2004, Kawakami et al., 2006). For seaweeds, the production of PC has been confirmed for very few species, with examples from all three phylogenetic groups: the Chlorophyceae (e.g. *Ulva* spp.) (Malea et al., 2006), Rhodophyceae (e.g. *Kappaphycus alvarezzi*) (Hu and Wu, 1998, García-Ríos et al., 2007) and Phaeophyceae (e.g. *Sargassum muticum*) (Gekeler et al., 1988). Recently Pawlik-Skowrońska et al. (2007) reported, for the first time, the presence of PC in natural assemblages of green (*Rhizoclonium tortuosum*), red (*Solieria chordalis*, *Gracilaria gracilis*) and brown (*Fucus* spp.) seaweeds growing in waters contaminated with different levels of metals/metalloids. From the study it was concluded that a combination of PC production and maintenance of high concentrations of GSH allowed *Fucus serratus*, *F. vesiculosus* and *R. tortuosum* to thrive in environments impacted by high levels of metal pollution.

To date, most studies on seaweeds have investigated inter-specific differences in the production of PC with none addressing intra-specific variation in response to metal

exposure, despite evidence for differential metal-resistance in populations of some species of seaweeds. In the present study, inter-population differences in the production of PC, and its precursor GSH, on exposure to Cd and Cu were analysed for the brown seaweed *F. serratus*. Cd was chosen for this study as it is considered to be the most effective inducer of PC (Cobbett, 2000) and it was previously shown to induce PC in this species (Pawlik-Skowrońska et al., 2007). Cu is an essential trace metal for plant growth (Göransson, 1998, Tokarnia et al., 1999), however it is also known as one of the most toxic metals to aquatic organisms above the required levels for growth and maintenance (Gledhill et al., 1999). Cu was reported as one of PC inducers for marine algae (Gekeler et al., 1988, Hu and Wu, 1998, Rijstenbil et al., 1998a), and it was used to compare with Cd stress in this study.

5. 2. Materials and methods

5. 2. 1. Algal collection and culture

Collection and preparation of algal materials were described in Chapter 2. Seaweeds were cultured under 15°C, 250 μmol photons $m^{-2}s^{-1}$ photosynthetically active radiation (PAR) and 12 : 12 h light:dark cycle in the chemically defined medium Aquil (Price et al., 1988/89, Gledhill et al., 1997), to which cadmium sulphate hydrate ($3CdSO_4 \cdot 8H_2O$) and pentahydrated cupric sulphate ($CuSO_4 5H_2O$) were added. 0 ~ 10 mg L^{-1} of Cd was added into prepared Aquil medium for the elevated Cd treatment, and 0 ~ 1000 μg Cd L^{-1} was added for lower Cd treatment. 0 and 10 μg Cd L^{-1} and 0 and 100 μg Cu L^{-1} were mixed for Cd / Cu combination effect. 10 μg Cd L^{-1} was chosen from a preliminary experiment, which showed apparent PC and GSH synthesis in *F. serratus*. 100 μg Cu L^{-1} was derived from the natural Cu concentration of Restronguet Creek area. The concentrations of metals and thiols were determined in material exposed to Cd and Cu for different time periods: 7 d and 14 d for elevated Cd treatment, 4 d and 7 d for lower Cd treatment, and 7 d for combined metal treatment. The medium was exchanged every 48 h to ensure that the seaweeds did not become nutrient limited.

5. 2. 2. Total and intracellular metal concentrations

Algal samples were frozen (-20°C), freeze-dried (Super Modulyo Freeze-drier, Girovac, United Kingdom) and then re-weighed. To determine the total concentration of metals in the samples the dried seaweed was placed in Teflon vessels containing 3 mL of concentrated nitric acid (HNO_3) and digested in a microwave oven (CEM-2000,

CEM Microwave Technology, UK) at 2 kW for 30 min. Digests were then transferred to borosilicate volumetric flasks and diluted to an exact volume of 25 mL with nano-pure water ready for analysis (Milli-Q water system ZFMQ 230 04, Millipore Corporation, France). To discriminate Cd between extracellular adsorption and intracellular uptake, duplicate seaweed samples were subjected to sequential chemical treatment in 30 mL of 5 mM ethylenediamine tetra-acetic acid (EDTA) for 10 min prior to freezing and digestion by HNO_3 (Vasconcelos and Leal, 2001). Concentrations of Cd in digests of seaweeds were determined by inductively coupled plasma-mass spectrometry (ICP-MS; PlasmaQuad PQ2+; Turbo; Thermo Elemental, UK). Metal standards were made using certified standard solutions (Merck, UK), acidified to the same pH as the samples with HNO_3. Results are expressed as means ± standard deviations of 3 replicates.

5. 2. 3. Phytochelatin and glutathione concentrations

Frozen algal samples were freeze-dried and maintained under vacuum until analysed for GSH and PC concentrations. Determination of GSH and PC followed the protocols outlined in Pawlik-Skowrońska et al. (2007). Briefly, samples (c. 20 mg DW) were extracted for 10 min with ice-cold 5% (w/v) 5-sulphosalicylic acid (SSA) containing 5 mM diethylentriamonopentaacetic acid (DTPA). Homogenised samples were centrifuged at 17 °C, 14,000 rpm for 10 min and assayed by high pressure liquid chromatography (HPLC, Beckman, USA). Post-column derivatization with 5,5'dithio(2-nitrobenzoic acid) (DTNB) was used for identifying thiol-containing peptides at 412 nm, based on their retention time with standard GSH (Merck, Germany) and PC standards from *Silene vulgaris* (Moench) Garcke. Results of PC and GSH are expressed as means ± standard deviations of 3 replicates.

5. 2. 4. Statistical analysis

Data were analysed using the statistical package SPSS version 16.0 for Windows (SPSS Inc.). Before all parametric tests, the data were tested for homogeneity of variance and normality (Sokal and Rohlf, 1995). Data for accumulation of Cd and concentrations of total PCs and GSH were subjected to the GLM three-way ANOVA (population, Cd concentration and time as main effects, and their interactions) and differences between individual means were determined by the *post hoc* Tukey's multiple comparison tests at $p < 0.05$ for this procedure.

5. 3. Results

5. 3. 1. Elevated Cd treatment (1 ~ 10 mg L^{-1})

GSH and PC production of *Fucus serratus* which was exposed to elevated Cd concentrations (1 ~ 10 mg L^{-1}) were measured (Fig. 5. 3, black bar). GSH synthesis increased with Cd concentration in both populations ($p < 0.0001$). However, time of exposure showed different effects ($p > 0.05$). With longer Cd exposure time, higher GSH was produced in the polluted population (7 d < 14 d) but lower GSH was produced in the reference population (7 d > 14 d). Both populations synthesized the same values of GSH during the experiment ($p > 0.05$). Over 14 days, the values of GSH were maintained during Cd exposure in both populations.

Total PCs were produced higher with increasing Cd concentrations ($p = 0.005$) (Fig. 5. 3, grey bar). Time of Cd exposure also had significant effect on production of total PCs ($p < 0.0001$). In both populations, 5 and 10 mg Cd L^{-1} treatments showed significantly higher PC productions than 0 and 1 mg Cd L^{-1} treatments ($p < 0.0001$). Meanwhile differences between populations were also apparent. In RP much higher PCs were synthesized than in BQ ($p < 0.0001$) and production continued with longer Cd exposure (14 d), even though the differences with time were not clear at BQ ($p > 0.05$).

$PC_{2\sim5}$ were identified from both *Fucus* populations and all of them were increased by rising Cd concentrations (Fig. 5. 4), however up to 7 days there was no difference in PC production between populations. RP produced much higher values of each PC chain in 14 d than BQ ($p < 0.0001$) since the latter has showed the same PC values to 7 d exposure ($p > 0.05$). With increasing time of exposure, longer PC chains increased. In both populations, PC_3 was the most highly produced thiol. Fig. 5. 5 shows

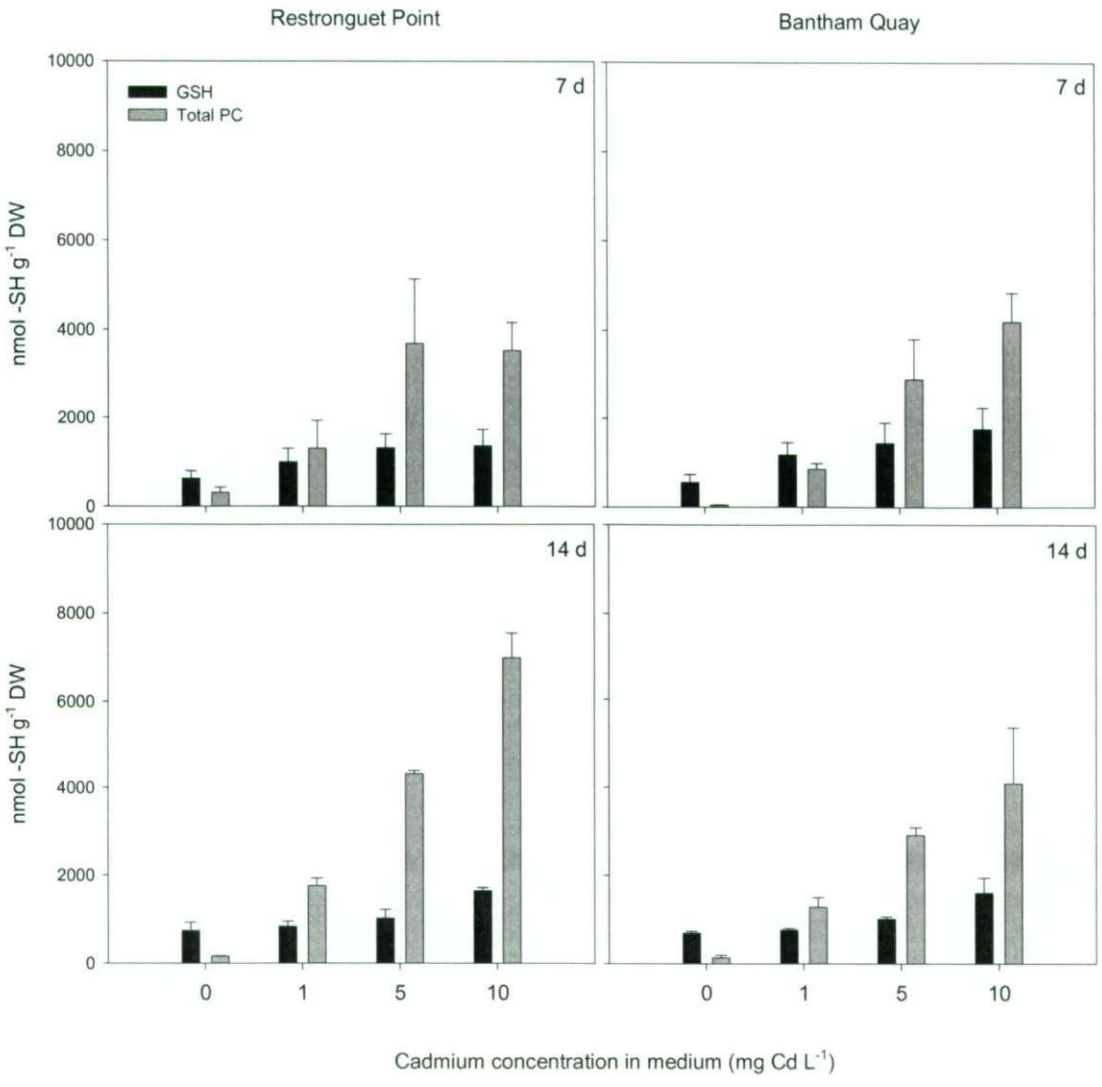

Fig. 5. 3. Concentrations of glutathione (GSH) and total phytochelatin (PC) of *Fucus serratus* from Restronguet Point and Bantham Quay exposed to cadmium for 7 days (7 d) and 14 days (14 d). Values are means and standard deviations (n = 3).

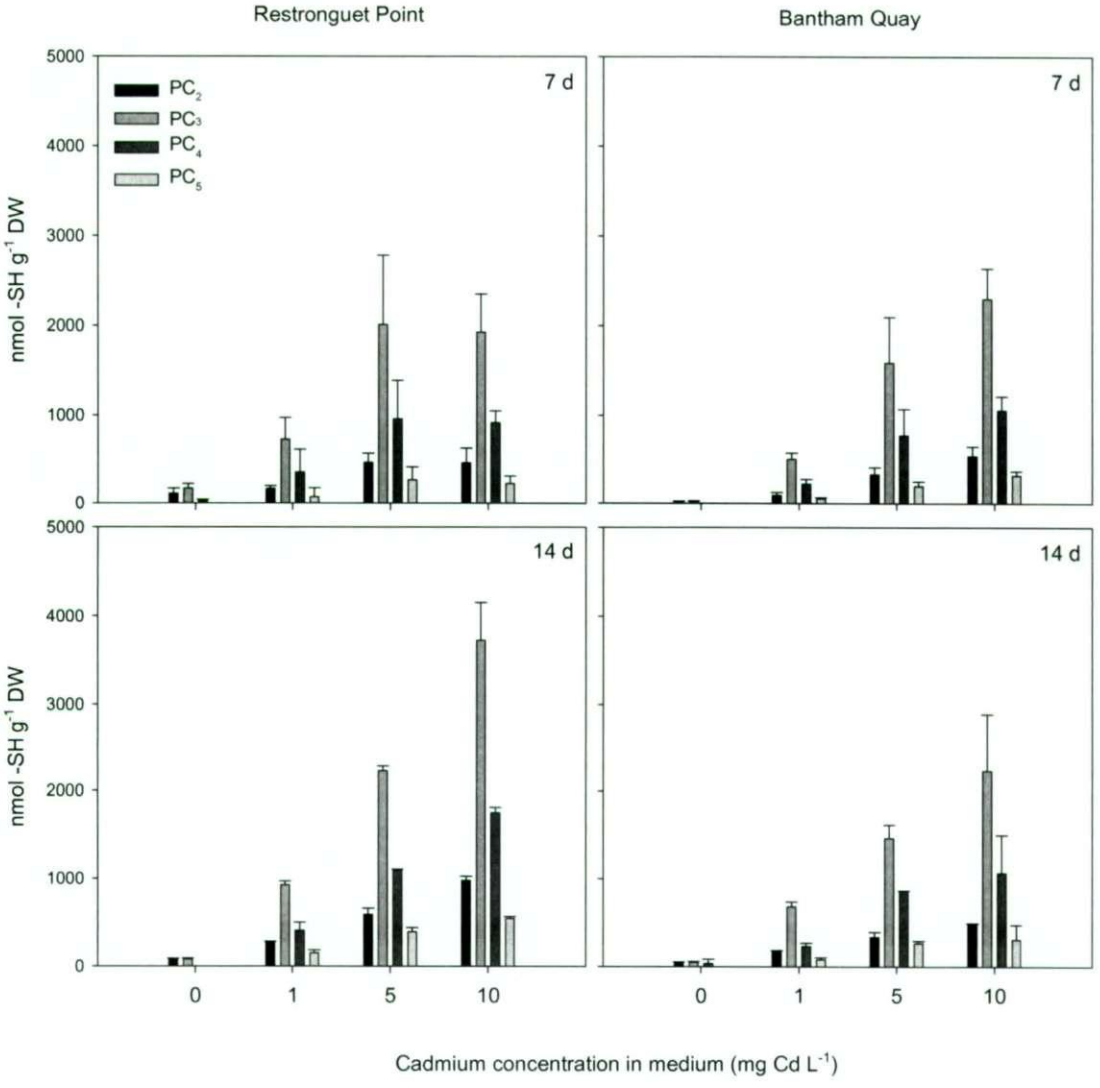

Fig. 5. 4. Production of different phytochelatin chains by *Fucus serratus* collected from Restronguet Point and Bantham Quay after 7 days (7 d) and 14 days (14 d) exposure to cadmium. Values were expressed by means and standard deviations (n = 3).

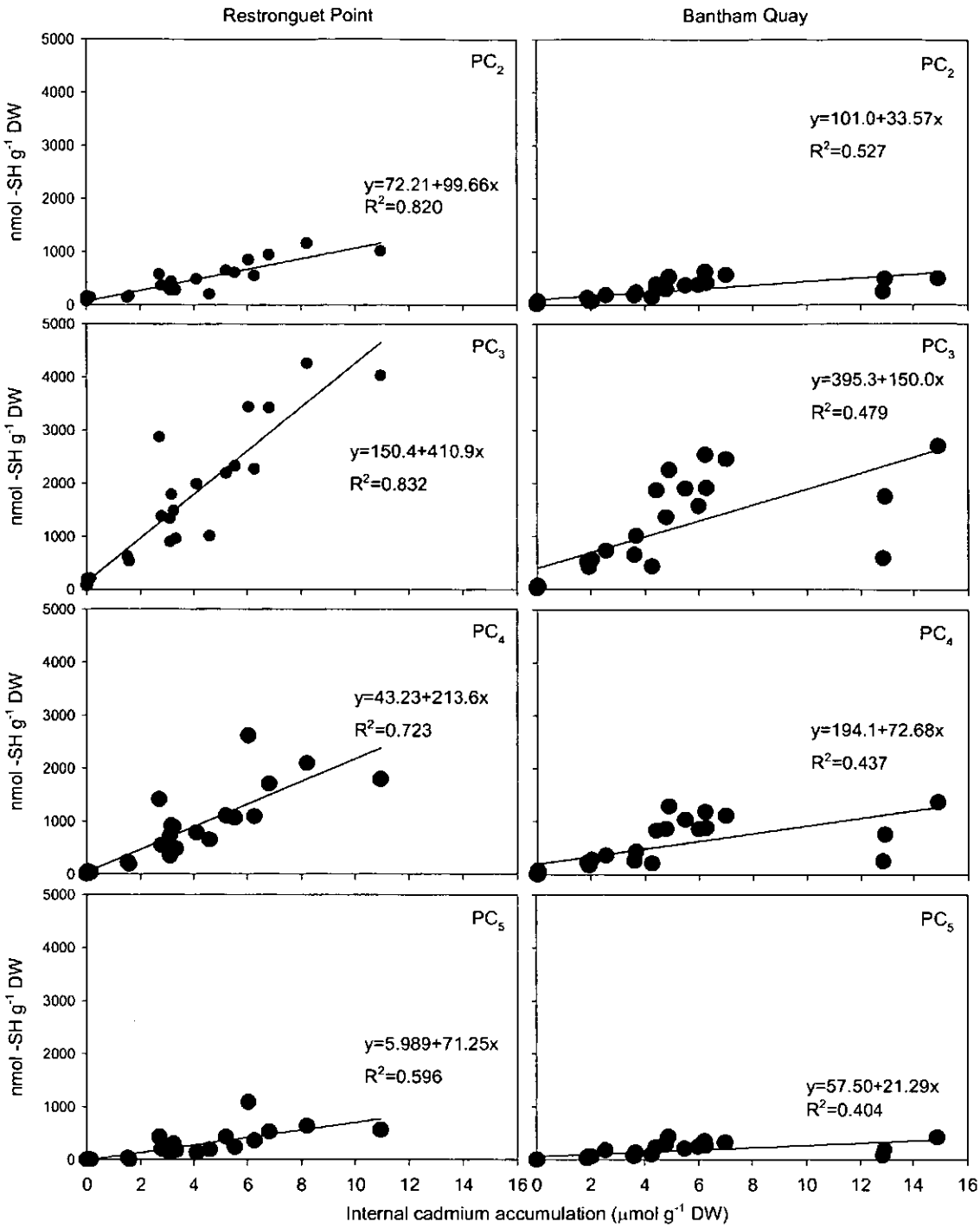

Fig. 5. 5. The relationship between the concentrations of phytochelatins of different chain-length and non-exchangeable cadmium accumulation in *Fucus serratus* from Restronguet Point and Bantham Quay exposed to cadmium in the range of 0 ~ 10 mg L^{-1} for 7 and 14 days. Data of cadmium concentrations were taken from Chapter 3.

the relationship between the concentrations of PCs of different chain-length and internal Cd concentrations in *F. serratus*. Data of internal Cd concentrations were taken from Chapter 3. RP, the polluted population, produced higher PC chains than BQ in the response to relatively less internal Cd contents (Fig. 5. 5). The slopes of trend lines in RP were steeper than in BQ, which indicates higher PC production in lower internal Cd contents (Fig. 5. 5).

With high Cd treatments (5 and 10 mg Cd L^{-1}), an unidentified additional peak was discovered around 23 ~ 24 min of retention time in both populations (Fig. 5. 6 and 5. 7). RP population had higher values (Fig. 5. 6) than BQ (Fig. 5. 7) with 5 and 10 mg Cd L^{-1} ($p < 0.05$). It was found on both 7 d and 14 d ($p > 0.05$) and increased with rising Cd concentrations ($p < 0.0001$) (data not shown).

5. 3. 2. Lower Cd treatment (10 ~ 1000 μg L^{-1})

To confirm the effect of Cd on GSH and PC production in *F. serratus*, Cd concentrations of lower than 1 mg Cd L^{-1} were used with the same procedure as elevated concentrations but with shorter time of Cd exposure. GSH synthesis was enhanced by time of Cd exposure and higher GSH was measured at 7 d than 4 d in both locations (Fig. 5. 8, black bar). The level of GSH was generally increased by Cd concentration in medium at 4 d, however levels were not affected by Cd concentration at 7 d in both populations ($p > 0.05$). The polluted population has similar values of GSH contents to the reference population at each exposure time ($p > 0.05$). During the experiment, GSH levels were maintained and did not decrease, however the values were higher than elevated Cd treatment. While GSH levels with 0 μg Cd L^{-1} were maintained

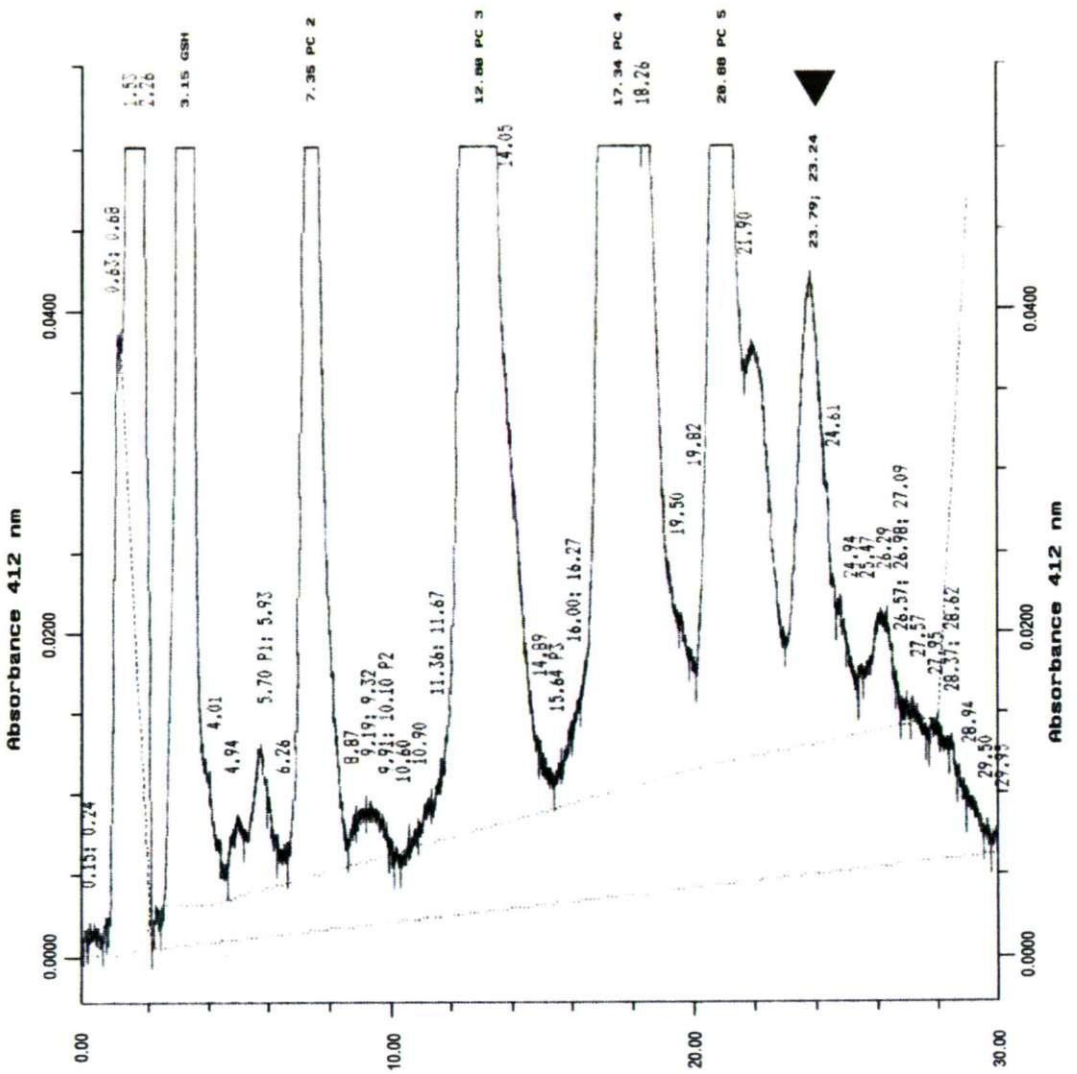

Fig. 5. 6. HPLC chromatogram of phytochelatin from *Fucus serratus* collected from Restronguet Point exposed to 10 mg Cd L^{-1} for 7 days. The arrow head shows unidentified peak around 23 ~ 24 minutes of running.

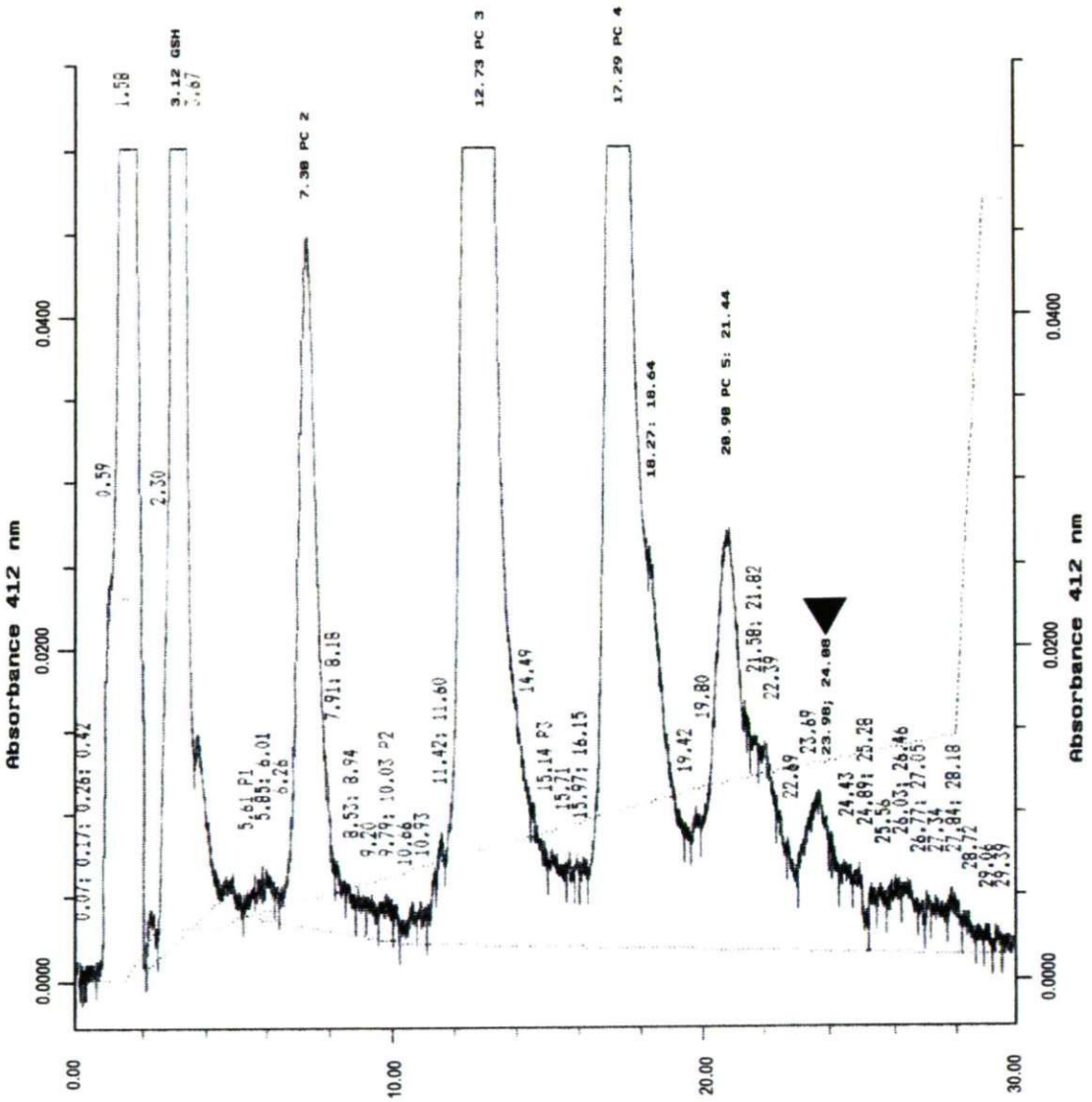

Fig. 5. 7. HPLC chromatogram of phytochelatin from *Fucus serratus* collected from Bantham Quay exposed to 10 mg Cd L^{-1} for 14 days. The arrow head shows unidentified peak around 23 ~ 24 minutes of running.

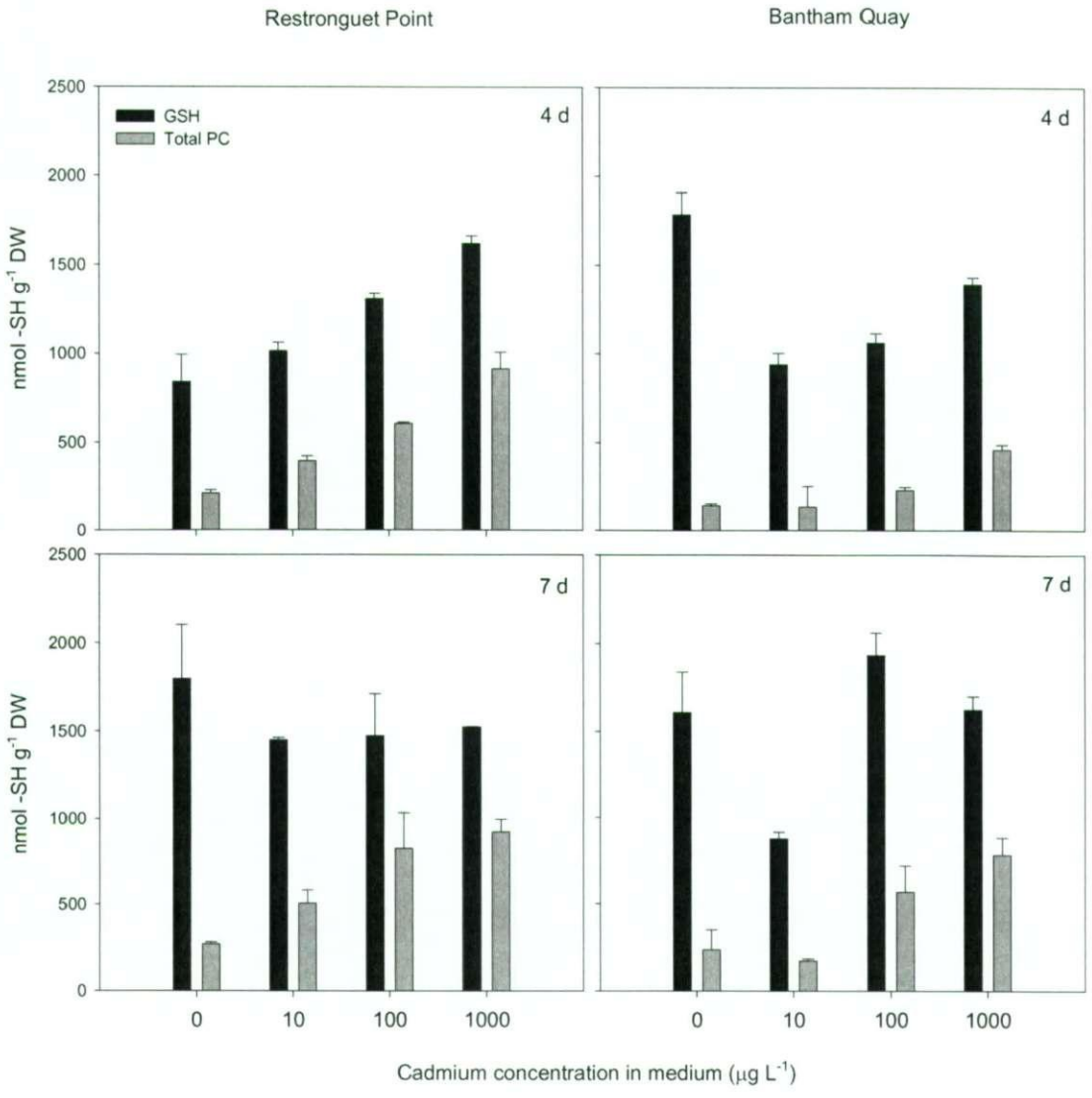

Fig. 5. 8. Concentrations of glutathione (GSH) and total phytochelatin (PC) of *Fucus serratus* from Restronguet Point and Bantham Quay exposed to cadmium for 4 days (4 d) and 7 days (7 d) at lower cadmium concentration range up to 1000 µg Cd L^{-1}. Values are means and standard deviations (n = 3).

without change with extended exposure time in BQ, the values in RP increased significantly at 14 d.

Total PC contents increased with Cd concentrations in the medium, so higher Cd treatment encouraged higher PC production (Fig. 5. 8, grey bar). Significantly higher PC was produced over 100 μg Cd L^{-1} than 0 and 10 μg L^{-1} in both populations ($p < 0.0001$). However, even control treatments (0 μg Cd L^{-1}) contained PC in both polluted and unpolluted populations. Longer time of Cd exposure also enhanced more PC production and this was shown more apparently in BQ. Algae from RP produced significantly higher PC than BQ, especially at 4 d, and differences between populations were significant as elevated Cd treatment (RP > BQ). With Cd concentration, RP presented significantly higher PC contents over 100 μg L^{-1} after 4 d and at 10 μg L^{-1} after 7 d.

$PC_{2\sim5}$ were identified from both of the populations with Cd treatment after 4 d and $PC_{2\sim4}$ were isolated from 0 μg L^{-1} treatment from both RP and BQ populations (Fig. 5. 9). Rising Cd concentrations and exposure times increased each PC chain level and enhanced longer PC chains. In RP, higher PC values were produced than in BQ during Cd exposure and PC_3 was always the highest PC chain with all Cd treatments (10 ~ 1000 μg Cd L^{-1}). BQ, however, showed much slower and lower PC production and 7 d exposure had lower PC values even than 4 d exposure of RP. PC_3 was the highest at 1000 μg Cd L^{-1} of 4 d and at 100 ~ 1000 μg Cd L^{-1} of 7 d exposure in BQ. 1000 μg L^{-1} of 7 d produced much lower each PC level when they were compared with those of the same conditions with elevated Cd experiment (1 mg L^{-1} with 7 d exposure). PC production vs. non-exchangeable Cd contents shows the same pattern as elevated Cd treatment (Fig. 5. 10). The contaminated population responding to less non-

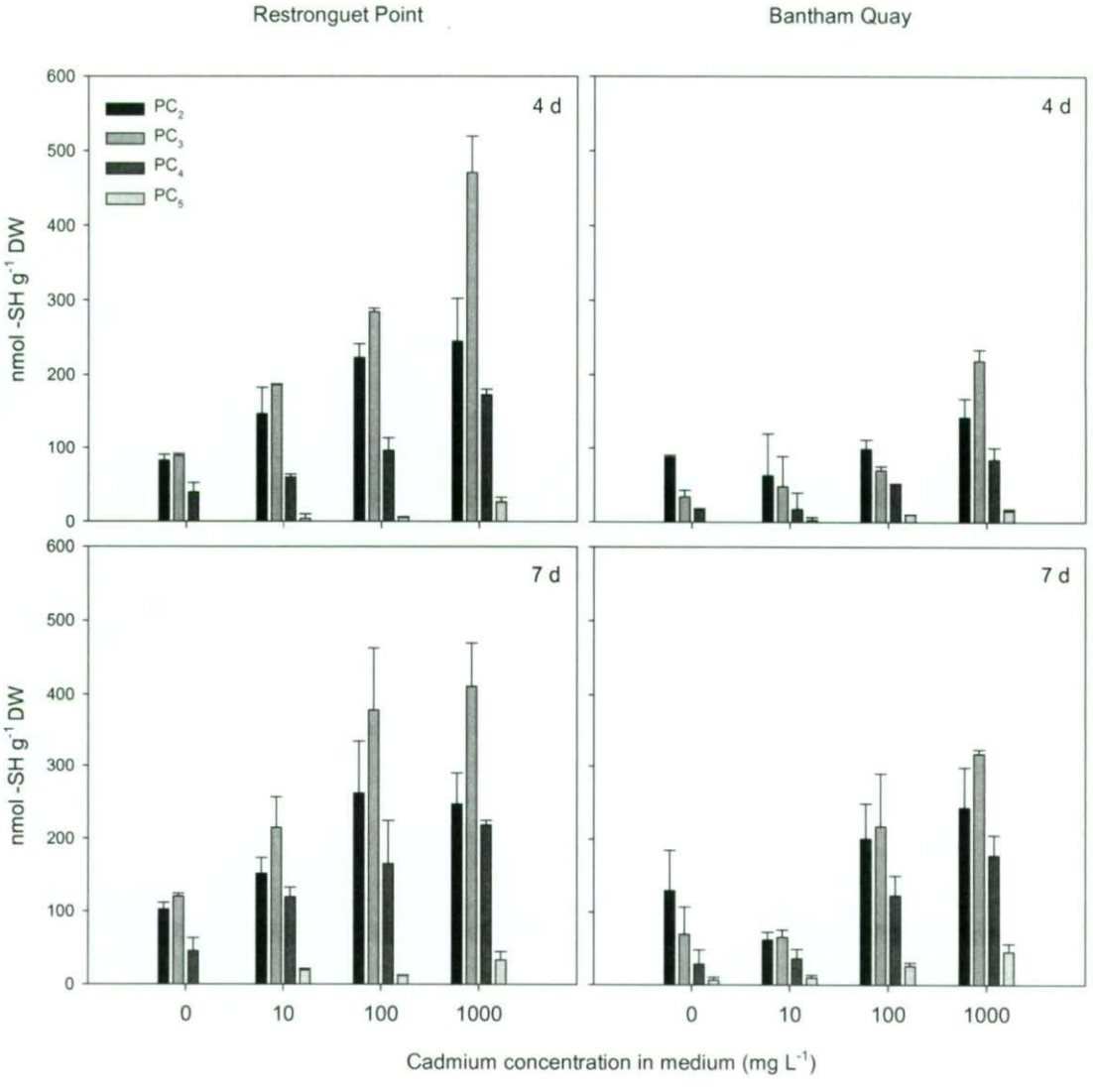

Fig. 5. 9. Production of different phytochelatin chains by *Fucus serratus* collected from Restronguet Point and Bantham Quay after 4 days (4 d) and 7 days (7 d) exposure to lower concentration of cadmium in the range of 10 ~ 1000 μg L^{-1}. Values were expressed by means and standard deviations (n = 3).

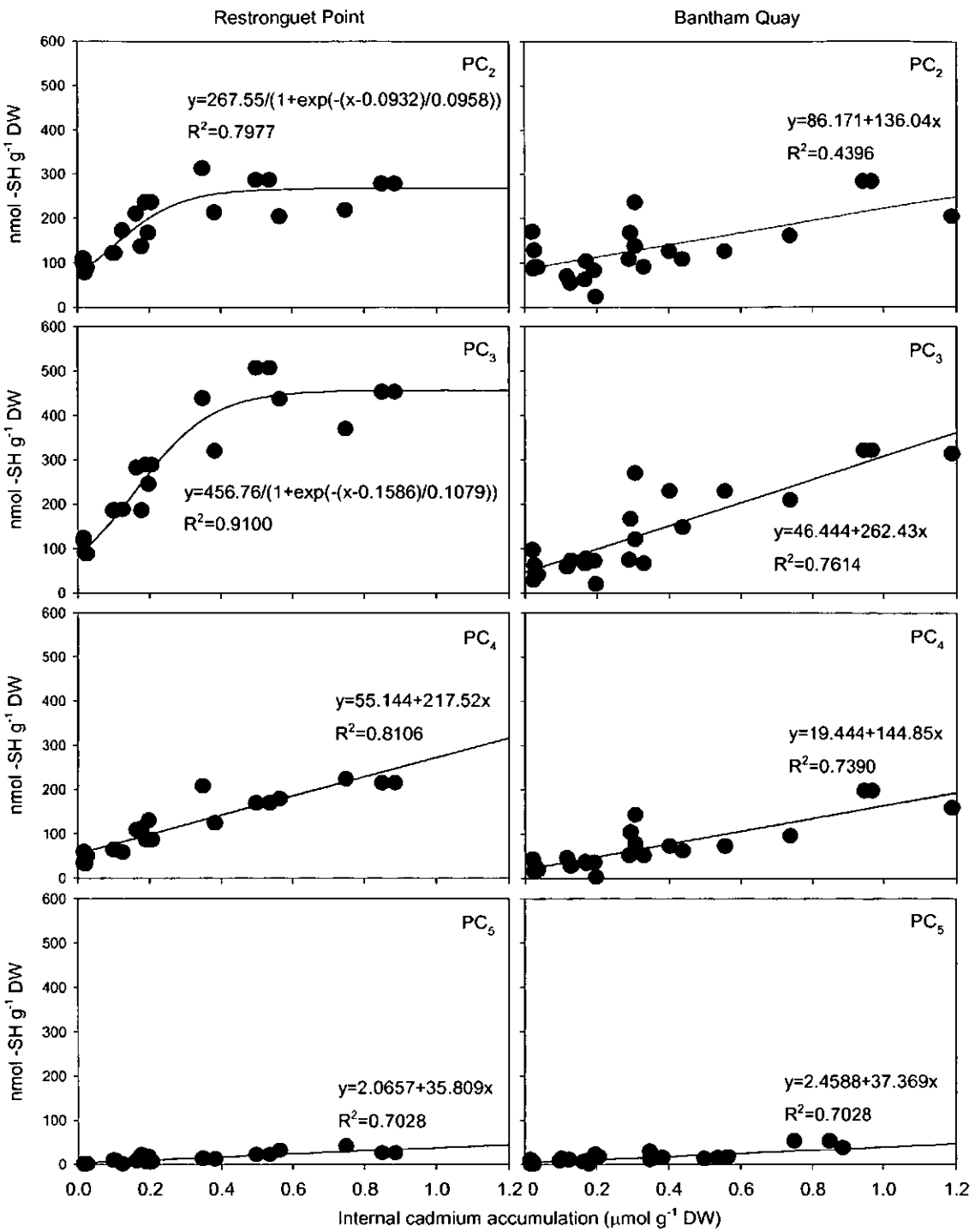

Fig. 5. 10. The relationship between the concentrations of phytochelatins of different chain-length and non-exchangeable cadmium accumulation in *Fucus serratus* from Restronguet Point and Bantham Quay exposed to cadmium in the range of 0 ~ 1000 μg L^{-1} for 4 and 7 days. Data of cadmium concentrations were taken from Chapter 3.

exchangeable Cd contents produced higher $PC_{2\sim4}$ chains than the reference population. Nevertheless the contents of produced PC_5 were not different ($p > 0.05$).

5. 3. 3. Cd and Cu interaction

Induction of PC and GSH by Cu and by interaction of Cd and Cu were also studied with *Fucus serratus* from both polluted and non-polluted sites. Similar levels of GSH were produced by RP and BQ materials after 7 days of Cd and/or Cu exposure (Fig. 5. 11, black bar). GSH values were maintained with/without metal treatments in both populations. In RP, PC was already produced at 0 μg Cd·Cu L^{-1} (Fig. 5. 11, grey bar). The values of total PC at RP were not significantly different from those with/without Cd or Cu exposure, except with the combined metal treatment (10 μg Cd L^{-1} + 100 μg Cu L^{-1}) ($p \leq 0.016$). Algae from BQ had also already synthesized PC without metal treatment as algae from RP, however the levels were significantly lower than those in RP (Fig. 5. 11). Cd treatment induced higher levels of PC than Cu treatment in BQ. The highest PC production was found in combined metal treatment in BQ as in RP.

Fig. 5. 12 shows production of each PC chain ($PC_2 \sim PC_5$) in both populations. Trimer (PC_3) was the highest PC chain, except with Cu treatment, in both populations. RP contained higher levels of each PC chain than BQ in most cases (Fig. 5. 12). Algae from RP already possessed considerable $PC_{2\sim4}$ without experimental metal exposure. Unlike algae from RP, algae from BQ contained significantly lower and shorter PC chains at 0 μg Cd·Cu L^{-1}. However the lower values of each PC chain in the BQ population were enhanced by metal treatment and Cd showed a larger effect than Cu.

BQ population showed similar values of PC production to RP population with combined metal treatment. Interestingly Cd treatment encouraged the production of PC_5 in BQ materials, which was not identified in any case of RP materials.

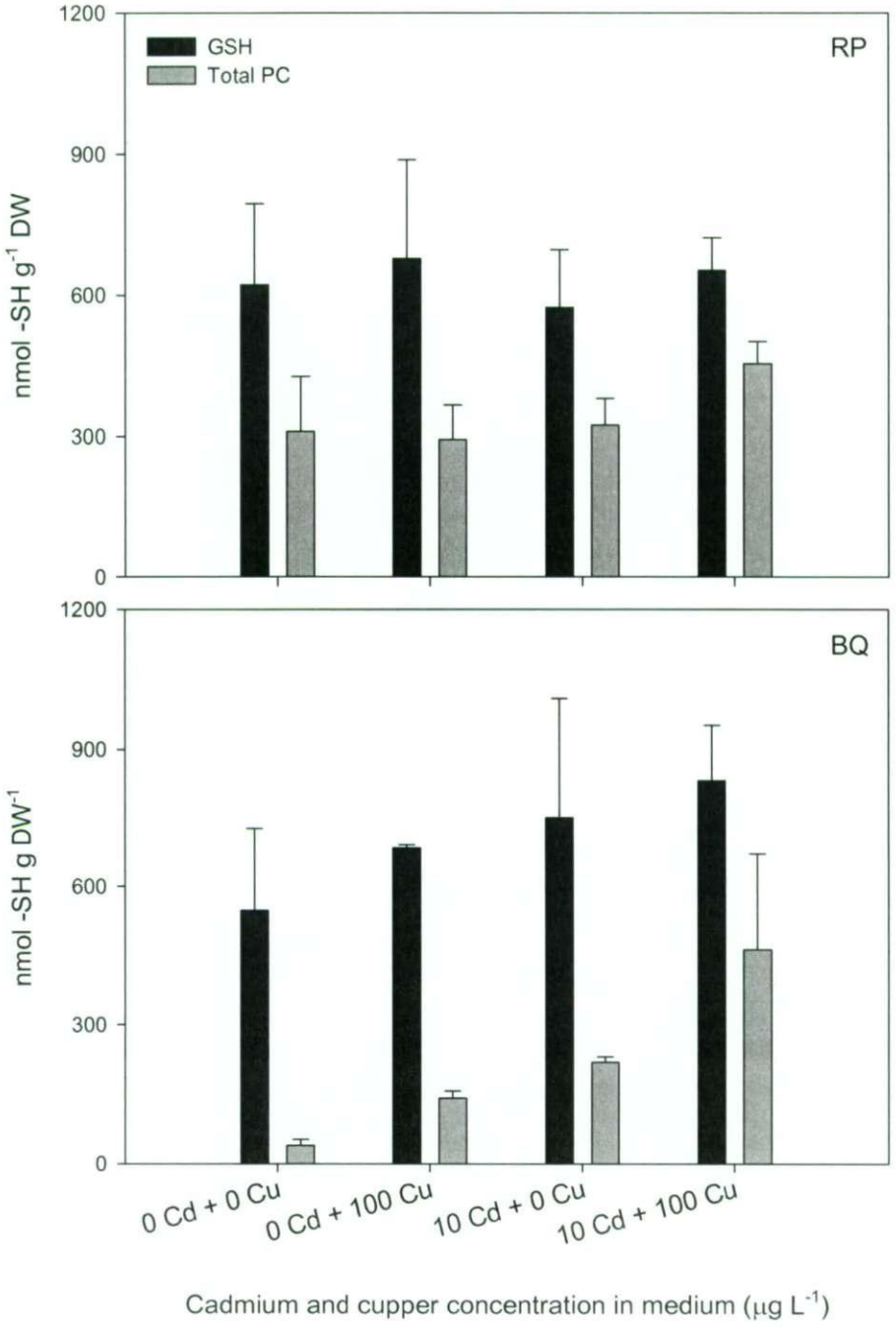

Fig. 5. 11. Concentrations of glutathione (GSH) and total phytochelatin (PC) of *Fucus serratus* from Restronguet Point and Bantham Quay exposed to combined metal (cadmium and copper) for 7 days. Values are means and standard deviations (n = 3 ~ 6).

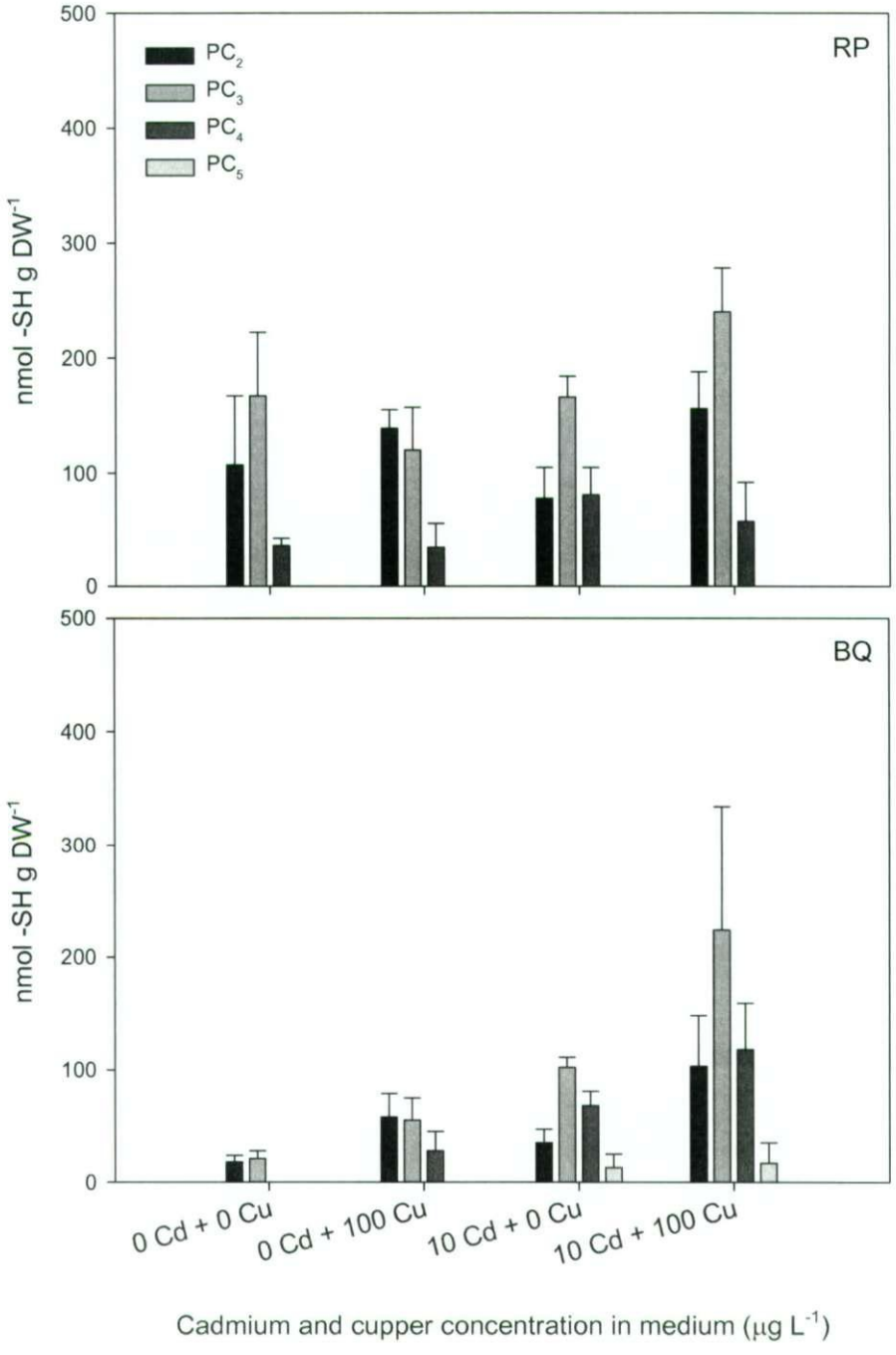

Fig. 5. 12. Production of different phytochelatin chains by *Fucus serratus* collected from Restronguet Point and Bantham Quay after 7 day-exposure to combined metals (cadmium and copper). Values were expressed by means and standard deviations (n = 3 ~ 6).

5. 4. Discussion

5. 4. 1. GSH and PC production by *Fucus serratus* from two different locations

To survive in metal-contaminated environment, plants and algae have adapted with a wide range of strategies (Prasad, 1999). Synthesis of PC and MT may be the most major mechanisms to overcome the stress and to thrive in the polluted ecosystem (Rauser, 1990, Reddy and Prasad, 1990, Steffens, 1990, Prasad, 1999). However, to date, research on PC production of marine macroalgal species are yet very limited (Inouhe, 2005, Pawlik-Skowrońska et al., 2007). Most of studies have been focused on terrestrial plant cells and freshwater microalgae although a few were reported on PC production in seaweed, e.g. *Sargassum muticum* (Gekeler et al., 1988), *Kappaphycus alvarezii* (Hu and Wu, 1998) and *Enteromorpha prolifera* (Rijstenbil et al., 1998a). Recently Pawlik-Skowrońska et al. (2007) reported metal-complexing thiol peptides in natural populations of some marine macroalgae. Eight species of red, green and brown algae from four natural habitats (RP and its upstream as contaminated sites; Wembury Beach and BQ as reference sites) were analysed. GSH production was observed in all of the eight algal species and PC production was verified in five species among them. *Fucus serratus* was one of the five PC producing brown algae. The polluted population contained higher PC levels and longer PC chains than the reference population, which shows the relationship between PC contents and contamination history of algal habitats or total metal stress of the species. Historically the Restronguet Creek area is contaminated by Cu and Sn mining works (Bryan and Gibbs, 1983, Bryan et al., 1987). At the present time all mines have been closed even though the area is still highly contaminated by many metals from old mines and other industrial sources (Bryan and Gibbs, 1983, Bryan et al., 1987, Nielsen, 2002, Pawlik-Skowrońska et al., 2007).

Therefore higher and longer PC synthesis by *F. serratus* from the contaminated area without metal exposure in the present research is regarded as a result of acclimatisation to the metal-polluted natural habitat. PCs verified at 0 μg Cd L^{-1} may be the result of a defence system to defeat stress from the retained metals in the thalli which were accumulated prior to the experiment. Therefore lower PC contents and shorter PC chains in BQ materials at 0 μg Cd L^{-1} can be explained by a lower metal burden from the natural environment which is not contaminated by metals. The reason of PC induction in the BQ control (0 μg L^{-1}) with significantly lower metal contents can be understood by Grill et al. (1988), at least partially. They reported that low concentrations of essential micronutrients, Cu and Zn (0.1~1 μM Cu, 2.1~37 μM Zn), could also cause the appearance of PC. The contents of Cu and Zn in the current study were in the range of their scale (Table 2. 1 and 2. 2).

5. 4. 2. Effects of time of Cd exposure and Cd concentrations on PC production

Rising Cd concentration and prolonged time of Cd exposure increased synthesis of PC in *F. serratus* regardless of the collected site. In this study, however, *Fucus* from RP presented higher and faster PC synthesis than *Fucus* from BQ with similar non-exchangeable Cd concentrations. This indicates that RP materials might have inherited more effective strategies for a metal stressful environment. The metal resistance process by seaweed is not yet well understood, even though some researchers commented on avoidance and tolerance (Hall et al., 1979, Correa et al., 1996), metallothionein-encoding gene (Morris et al., 1999), internal and external complexation with ligands (Smith et al., 1986, Gledhill et al., 1999), population-specificity with metal-exposed history of habitats, and exclusion mechanisms (Nielsen et al., 2003b). With PC

synthesis *F. serratus* presented strong tolerance by detoxifying Cd, and more effectual PC activation by the contaminated population can be understood as one of their surviving strategies.

5. 4. 3. Chain length of PC in *Fucus serratus*

Metal chelating ability of PC is known to be related to the length of its chain. The longer chain possesses the stronger metal-binding capacity than the shorter (Zenk, 1996). The longest PC chain to be synthesised is known as PC_{11} (Gekeler et al., 1988) however most of studies on freshwater/marine algae have revealed that algae produce generally up to PC_4 (Hu and Wu, 1998, Pawlik-Skowrońska, 2000, Hirata et al., 2001, Pawlik-Skowrońska, 2001, Pawlik-Skowrońska, 2002, Pawlik-Skowrońska, 2003, Wei et al., 2003, Pawlik-Skowrońska et al., 2004, Malea et al., 2006, García-Ríos et al., 2007). In the current study, $PC_{2\sim5}$ were identified by HPLC in most cases. Therefore, this brown alga possesses vigorous chelating potentials than previously studied algal species. The potential use of PC production and PC chain lengths for *in situ* biomarkers of metal exposure and bioavailability has been considered by some researchers (Pawlik-Skowrońska et al., 2007). Therefore, it may be possible to use this brown alga as a metal remover or a bioindicator in coastal waters. Brown algae *Fucus serratus* and *F. vesiculosus* produced significantly higher PCs than red algae *Solieria chordalis* and *Gracilaria gracilis* and green alga *Rhizoclonium tortuosum* (Pawlik-Skowrońska et al., 2007). Since PC production of marine macroalgae has not been studied in enough detail yet, however, there may be other algal species possessing the greater ability to chelate metal ions.

In the mean time, complex polysaccharides in cell walls of red and brown algae are known as effective barriers to metal toxicity (García-Ríos et al., 2007). Among them,

alginic acid in brown macroalgae was thought to be more competent to chelate Cd^{2+} than agar and carrageenan in red algae (Hashim and Chu, 2004). On these grounds there is high potential to use this brown alga as an environmental metal remover.

The unidentified additional peak after PC_5 around 23 ~ 24 min could possibly be PC_6 or other thiols along with some proteins (Pawlik-Skowrońska, peronal. communication). Therefore additional further work on identification is required with liquid chromatography-electrospray mass spectrometry (LC-ESIMS).

5. 4. 4. GSH production by *Fucus serratus* exposed to Cd

Unlike differential PC generation, GSH was maintained equally in both populations whether elevated or lower Cd concentration. GSH is one of the well-known antioxidants in algae (Rijstenbil, 2002) and an important thiol in eukaryotic cells to maintain reduced states for amino acids and proteins (Kawakami et al., 2006). Generally it decreases with increasing PC formation since it is consumed for PC production as the precursor of PC in freshwater (Coppellotti, 1989, Pawlik-Skowrońska, 2000) and marine microalgae (Ahner et al., 2002), and marine macroalgae (Pawlik-Skowrońska et al., 2007). *F. serratus* maintained the GSH values for up to 14 days without decrease in the present study, which suggests their consistent high antioxidant potentials to respond to metal exposure. Maintaining high GSH values was considered as a tightly regulated intracellular procedure for other cellular functions (Ahner et al., 2002, Pawlik-Skowrońska et al., 2007). Therefore highly sustained GSH levels can be another useful strategy of *F. serratus* to thrive in metal-contaminated environment. Some other researchers also reported constant intracellular GSH levels and their role for production of PC by healthy phytoplankton (e.g. *Phaeodactylum tricornutum*) (Tang et al., 2000, Ahner et al., 2002, Kawakami et al., 2006). Kawakami et al. (2006) indicated that GSH

is not an appropriate parameter to use as a biomarker of metal stress in phytoplankton since intracellular GSH contents can either increase or decrease depending on which phytoplankton species. However they also mentioned that intracellular GSH contents are controlled by different mechanisms in different species and in different strains of the same species (Kawakami et al., 2006). GSH concentrations increased and/or sustained in this study and the maintained GSH concentration can be regarded as consistent antioxidant ability and potential to be converted to PC.

5. 4. 5. Effects of Cu and combined metals (Cu + Cd) on PC production by *Fucus serratus*

The natural marine environment does not contain single metal species and more than two metal species often make up complicated interactions in various physiological, chemical and biological situations. Combined metals frequently cause synergistic or antagonistic interactions, which makes it hard to forecast their effects and algal responses (Wei et al., 2003). Among various trace metals, Cu, Zn and Cd are the most common contaminants and excess of these metals are toxic to marine organisms (Wei et al., 2003). Cu is also known to be one of the PC inducing metals and the strength to induce PC depends on the algal/plant species (Ahner and Morel, 1995). In the present research, 100 μg L^{-1} of Cu induced significant GSH and PC production in both populations. However the PCs induced by Cu in BQ were lower and shorter than PCs induced by 10 μg L^{-1} of Cd. Like in many plants and other algal species, Cd showed a stronger capability in PC induction than Cu (Rauser, 1990, Ahner and Morel, 1995, Pawlik-Skowrońska et al., 2002, Kawakami et al., 2006). PC and GSH produced in 0 μg Cd·Cu L^{-1} of both populations are regarded as responses to metals which were accumulated in thalli prior to this study. PC production with combined metal effect with

BQ algae was similar to the sum of each PC production from the two single metal effects (i.e. additive effect), on the other hand, RP algae showed a slight increase with both metals compared to each alone (i.e. synergistic effect). *F. serratus* is known to inherit Cu^{2+} resistant character (Nielsen et al., 2003b). Algae from polluted site have been shown to resist harmful effects of Cu significantly more than algae from reference site (Nielsen et al., 2003b). Considering results from the present study, the inherited Cu resistance character of *F. serratus* may be related to the highly sustained GSH levels and higher PC production in polluted site.

5. 4. 6. Differences in PC and GSH production depending on season

Fucus serratus produced higher PC and GSH levels with 1 mg Cd L^{-1} in the elevated Cd treatment experiment than 1000 μg Cd L^{-1} in the lower Cd treatment experiment at the same time period (7 d). With the same Cd concentration and the same time of exposure, this alga synthesised significantly different values of metal-binding peptides and GSH. There may be seasonal factors which affect PC and GSH production, since collections were done in two different seasons (October and May). These could account for the overall different levels of induction of the PC and GSH. Future research should examine any trends.

5. 5. Conclusion

In conclusion, this is the first report of differences in PC production between populations of *F. serratus* in response to various experimental Cd treatments and Cu exposure. Metal resistance in *F. serratus* involves effective cellular detoxification of the metal by thiols. Greater production of intracellular PC (of longer chain length), and maintenance of concentrations of GSH are responsible, at least in part, for tolerance of the population growing in Restronguet Point which is a metal-contaminated area. Although this study covered both elevated and lower Cd concentrations and Cd·Cu combined exposure for PC production by *F. serratus* in laboratory conditions, the natural populations will be exposed to much lower and extremely complex metal conditions. Therefore further research with various metal conditions more closely representing natural environments would be desirable.

Chapter 6. General discussion

Environmental pollution affecting marine organisms is not a recent issue. It has been of global concern since the 1950s (Kennish, 1996). Pollution can occur from various different sources including industry, mining activities, urban sewage, and agricultural runoff. Metals, including Cd, are one group of pollutants that have been prevalent since the late 19th century (Pinto et al., 2003). Metal pollution changes the chemical conditions of the marine environment, which affects the constituents of the ecosystem. Organisms living in different environmental backgrounds have their own strategies for adaptation and adjustment to stress. However, some species are found in many different locations regardless of the contamination. The genus *Fucus* is one of the marine macroalgae found in both contaminated and clean areas and thrives in the coastal waters of South West England. Therefore, in the present study, the physiological and biochemical responses of *Fucus serratus* from Restronguet Point (RP) and Bantham Quay (BQ) in South West England were determined under a wide range of Cd exposure. RP is known to be a part of the most metal-contaminated area in the Fal Estuary, although BQ is regarded as one of the least polluted coastal areas in the UK (Bryan and Langston, 1992, Pirrie et al., 2003). The objectives of this research were to investigate the effects of non-essential Cd which have received less attention in studies of marine algae than metals such as Cu and Zn (Eklund and Kautsky, 2003). More specifically, the study examined the photosynthetic and antioxidative responses of *F. serratus*, the metal chelating ability of thiols, the responses of two populations with different metal-exposure histories, and the potential of this alga as a biological indicator.

Natural populations of *F. serratus* of RP and BQ with no experimental Cd exposure have similar total and non-exchangeable Cd concentrations. Since RP is heavily contaminated by various metals from a long history of mining activity, the

accumulated concentrations of other metals (i.e. Cu, Pb and Zn) in *F. serratus* were significantly higher than those of BQ. All metal species, including Cd, showed significantly higher levels in RP seawater compared with data from other sites (Table 3. 9). Therefore relatively less accumulated Cd by *F. serratus* might result from the suppressed interaction by other metal species, the competition for binding sites between cations in ion channels of cell membranes, and/or the binding to cell walls (Ralph and Burchett, 1998, Pawlik-Skowrońska et al., 2007). Moreover, since Cd is not required for algal growth and development, it may be relatively less actively accumulated by plants and algae. A lower uptake of non-essential metal elements (e.g. Cd) was reported in the seagrass *Halophila stipulacea* (Malea, 1994). Interestingly, although the native RP population has suffered from significantly higher levels of various metals in the habitat, oxidative stress expressed by lipid peroxidation indicating membrane damage was similar in each population. Different antioxidative responses to different metal conditions were anticipated and the RP population may have a unique and efficient protective mechanism. Inherited tolerance characteristics of *F. serratus* against Cu have been reported and adaptations to the photosynthetic apparatus and exclusion of cupric ions were considered as the tolerance mechanisms (Nielsen et al., 2003b).

Although Cd is not an essential metal for the physiology of marine macroalgae, Cd is known to be readily taken-up by plants and algae (Pinto et al., 2003, Clemens, 2006). Both total and non-exchangeable Cd concentrations increased with exposure to increasing Cd concentrations in the medium and with time of exposure. Lack of metal regulation ability has been reported in brown seaweeds (Bryan, 1976). For this reason brown algae have been used as bioindicators and the relative composition of metals in the surrounding seawater can be determined for measurements of metal contents in the seaweed (Fuge and James, 1974). In the present study, a limitation for Cd accumulation

was not discovered up to 10 mg Cd L^{-1} for 14 d. In addition, no visual symptoms of metal stress (such as chlorosis, discoloration, and necrosis) were observed for up to 14 d, which agrees with the previous reports that brown algae are a very tolerant algal group to external abiotic stress (Phillips, 1994, Hashim and Chu, 2004). Regardless of the Cd range, over 50% of Cd was accumulated within the cells (non-exchangeable vs. total). The ratio of intracellular accumulation of metals is species-specific. It depends on the cell wall composition (i.e. polysaccharides) and internal concentrations of thiol peptides (García-Ríos et al., 2007). Marine macroalgae can internally accumulate <5 ~ 80% of total metal burden and algae which can produce glutathione (GSH) and phytochelatin (PC) are known to accumulate more metals internally (Hu et al., 1996, García-Ríos et al., 2007). Therefore, over 50% of Cd was accumulated intracellularly and might have sequestered into the vacuoles as a tolerance mechanism. The rest was bound to extracellular polysaccharides of *F. serratus*. However, Cd ions are less likely to be excluded in *F. serratus*, if so very partially, since the non-exchangeable Cd concentration increased without inhibition up to 10 mg Cd L^{-1}.

Cd accumulation by *F. serratus* was the same for both populations, regardless of the Cd range (10 μg L^{-1} ~ 10 mg L^{-1}). However responses of growth were significantly different depending on populations and Cd concentrations. Cd exposure inhibited the algal growth. Response of reduced growth rate occurred within 24 hr. However, in the range of 1 ~ 10 mg Cd L^{-1}, significantly different relative growth rates (RGRs) were obtained for the two populations although non-exchangeable Cd concentrations were similar. Both populations showed negative growth after 7 d exposure at 1 ~ 10 mg L^{-1} although the reduction was greater in BQ materials and there was no sign of recovery after 14 d. By contrast in RP materials recovery was apparent with positive RGRs after 14 d exposure at 1 ~ 10 mg Cd L^{-1}. Therefore, Cd inhibited the growth of *F. serratus*

and higher concentrations caused more inhibition, although the alga did not show visual signs of Cd stress. The high RGRs and faster recovery in growth in terms of weight of the RP population indicate higher tolerance to Cd stress in the RP population. Metal stress can induce shrinking and weight loss of macroalgae, which is related to decreased turgor, the changed elasticity of the cell wall and indirect impact (e.g. the decreased photosynthesis, protein biosynthesis, and uptake of inorganic nutrients) of metal toxicity (Bryan and Gibbs, 1983, Boyle, 1984, Brown and Newman, 2003, Pinto et al., 2003, Han et al., 2008).

Metal-induced changes in pigment contents have often been observed in macroalgae (Han et al., 2008, Lobban and Harrison, 1994). In most cases metals decrease the pigment content as a result of the inhibition of biosynthesis of photosynthetic pigments (De Filippis and Pallaghy, 1994, Xia et al., 2004). However, in some cases, an increase in pigment contents resulting from metal exposure results in resistance to the stressful condition (Cid et al., 1995, Han et al., 2008). Besides, Cd and Cu are known to substitute for Mg^{2+} in the chloroplast (Chl) molecules, and this consequently reduces photosynthetic efficiency (Küpper et al., 1996, Küpper et al., 2007). Cd treatments over the range of 10 ~ 1000 μg L^{-1} in the present study increased the Chl *a*, Chl *c* and fucoxanthin (Fx) in RP, but it did not significantly alter the contents of either of these pigments in BQ, nor β-carotene in either population. There have been previous reports of Cd treatments in the range of 50 and 100 μM (5.6 and 11.2 mg L^{-1}); for example Xia et al. (2004) reported the lack of effect on Chl *a* and carotenoid concentrations in *Gracilaria lemaneiformis*. Han et al. (2008) also reported the increased contents of Chl *a* and Chl *b* in *Ulva armoricana* with no decrease in F_v / F_m or F_0 with Cu exposure (50 and 100 μg L^{-1}), although the RGR was significantly reduced. Therefore the increased contents of photosynthetic pigments with the

significantly decreased RGRs of *F. serratus* of RP in the present research and *U. armoricana* in Han et al. (2008) represent a tolerance mechanism against metal stresses with an expensive energy trade-off. These two marine macroalgae spend the available energy from photosynthetic activity for the biosynthesis of photosynthetic pigments at the expense of growth. However, this hypothesis cannot explain the much lower RGRs and the unchanged pigment contents of BQ compared with the relatively higher RGRs and the increased pigment contents in RP. Therefore the BQ population seems to be less tolerant to Cd exposure than the population from the more polluted site. The significantly higher contents of the accessory pigments in RP support this view. Higher concentrations of Chl *c* and Fx in RP indicate a greater and more efficient transfer of the absorbed light to the Chl *a* of the reaction centre, which is most likely related to tolerance to the contaminated waters of its natural habitat.

Most parameters of Chl *a* fluorescence measured by Handy PEA and PAM FMS did not show effects of Cd exposure or increased Cd concentration. Chl *a* fluorescence of plants and algae has been known as a practical, non-invasive technique to measure primary productivity and environmental stress, however responses of the parameters are closely related to metal elements, concentration of metals, plant/algal species, exposure time to stress, etc. (Küpper et al., 1998, MacFarlane and Burchett, 2001, Suggett et al., 2007). No significant changes of Chl *a* fluorescence by Cd exposure has been reported (Greger and Ögren, 1991, Krupa et al., 1993, Di Cagno et al., 1999). The lack of effect on most of Chl *a* fluorescence parameters and no significant differences between populations in the present research may imply the strong tolerant character of this brown alga regardless of their natural habitats, and the effectiveness of the metal chelation and antioxidative strategies. In addition, the main target of Cd in plant photosynthesis was suggested to be the process of Calvin-Benson cycle rather than the

light reaction (Clijsters and Van Assche, 1985, Van Assche and Clijsters, 1990, Krupa and Baszynski, 1995, Joshi and Mohanty, 2004).

Chl *a* fluorescence transient parameters were altered by time of Cd exposure rather than by the Cd treatment or the Cd concentrations. This was shown by changes of the controls. Therefore, Chl *a* fluorescence parameters might be affected by other factors (most likely culture conditions) rather than by the Cd treatment. *F. serratus* responded to Cd exposure rapidly since fluorescence parameters showed clear differences at 24 hr. After 24 hr treatment, changes in Chl *a* fluorescence did not show any noticeable pattern or differences. Cd seems, therefore, to have a rapid significant effect related to the growth and photosynthetic performances of *F. serratus*. Conclusively acute Cd effect and shorter time intervals between measurements are required to produce conclusive evidence of the effects of Cd exposure on the photosynthetic performances of *F. serratus*.

One of the most effective protective mechanisms in plants and algae against oxidative stress is reactive oxygen scavenging enzymes, such as catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase and superoxide dismutase (SOD). They eliminate reactive oxygen species (ROS) in cytosol, peroxisome, mitochondria and chloroplasts of plant/algal cells and reduce harmful impacts of ROS (Dummermuth et al., 2003, Ratkevicius et al., 2003, Halliwell and Gutteridge, 2007). *F. serratus* produced CAT, APX and GR and generally higher Cd concentrations and longer times of exposure induced higher activities of antioxidative enzymes. The RP population produced significantly higher levels of CAT and GR at 24 hr and 96 hr than the BQ population. Therefore *F. serratus* from the polluted area is able to remove more ROS and diminish the toxic effects better than the alga from the clean area, since higher activities of enzymes imply higher antioxidative capacity and an

advanced protective mechanism. Intertidal algal species are known to produce greater activities of antioxidative enzymes than subtidal species since they are normally exposed to more changeable environmental conditions. Collén and Davison (1999c) postulated that the effective response with antioxidant enzymes was an adaptation to the prevailing environmental conditions and Collén et al. (2003) added Cd as one of the environmental stresses, including mechanical disruption, CO_2 limitation, high light, freezing and desiccation, epiphytic bacteria and algae, and Cu (Collén and Pedersén, 1994, Collén et al., 1995, Collén and Pedersén, 1996, Collén and Davison, 1997, Rijstenbil et al., 1998a). Therefore, *F. serratus* from RP, which has been exposed to relatively high concentration of metals, is better adapted to deal with exposure to Cd by having greater activities of reactive oxygen scavenging enzymes.

However the levels of APX were similar at 24 hr and 96 hr in both populations. Collén and Davison (1999b) suggested that APX was the main enzyme for reducing ROS (i.e. H_2O_2) in the three *Fucus* species (*F. evanescens*, *F. spiralis* and *F. distichus*) in the natural habitat. Their finding is not supported for this species in the current study. The activity of APX was similar in both populations with the pattern of the synthesis irregular with Cd concentrations. However, exposure to 1000 μg Cd L^{-1} did result in significantly increased activity of the enzyme. In the case of Cu, Pb and Zn stress, APX in chloroplasts might have been important since these metals would affect the thylakoid membranes of chloroplasts (Collén et al., 2003). In contrast, when the alga was exposed to Cd, CAT may be the most important antioxidant enzymes. As a metaboliser of peroxide in the peroxisome, CAT may be a more effective enzyme for reducing Cd stress since this metal interferes with metabolic processes in the entire cell (Collén et al., 2003). However there may be other enzymes which have better reactive oxygen scavenging abilities than CAT since only three major enzymes were measured in this study; other enzymes may also be involved in scavenging ROS.

Oxidative stress has frequently been measured by peroxidised membrane lipid and the levels of thiobarbituric acid-reactive substances (TBARS), mostly malondialdehyde (MDA), which can show the degree of oxidative stress in plants, micro- and macroalgae (Collén and Davison, 1999a, Pinto et al., 2003, Choo et al., 2004). Higher Cd concentrations and longer exposure times induced higher lipid peroxidation in *F. serratus*. The alga from the RP population showed higher lipid peroxidation than the BQ materials under the lower Cd concentrations (10 ~ 1000 μg L^{-1}) although no significant difference was found under the higher Cd concentrations (1 ~ 10 mg L^{-1}). Results from CUPRAC and DPPH free radical scavenging activity were similar to the results from lipid peroxidation. The BQ population had higher antioxidant capacity than the RP population. However all values of CUPRAC and DPPH free radical scavenging activity including the controls were always higher at BQ and the patterns of changes were not linked to Cd treatment nor to Cd concentrations. These two parameters provide evidence of the higher and effective antioxidative mechanism in the BQ population, and the lipid peroxidation under lower Cd concentrations reveals the less sensitive response in membrane lipid peroxidation of the BQ population. Therefore the reference population may have its own strategies to confront oxidative stress.

Research on the chelation and sequestration of metal ions by thiol group peptides in marine macroalgae is currently in the early stages. This is the first report of PC production in *F. serratus* on exposure to Cu and to various concentrations of Cd. PC is a polypeptide with metal detoxifying function (Grill et al., 1985, Rauser, 1990). PC has been found in terrestrial plants, freshwater and marine algae and some fungi (Ahner et al., 1995, Inouhe, 2005), however, information on PC production by marine macroalgae is very limited (Inouhe, 2005, Pawlik-Skowrońska et al., 2007). Cd is the

most frequently investigated metal element in studies of PC production since it has been shown to be the strongest PC inducer in plants and algae (Grill et al., 1987, Grill et al., 1989, Rauser, 1990, Inouhe, 2005). Although some other metals, e.g. Cu, Pb, Zn, Ni and Ar, are also known to induce PC in plants and freshwater microalgae, the effects of these metal elements on PC synthesis by marine macroalgae are not yet clear (Grill et al., 1987, Gekeler et al., 1988, Pawlik-Skowrońska, 2002, Pawlik-Skowrońska, 2003, Pawlik-Skowrońska et al., 2004). In the present study, *F. serratus* from both populations produced considerable concentrations of PC with Cd exposure; higher Cd concentrations and longer exposure times induced more PCs with longer PC chain lengths. The RP population responded faster to Cd exposure and produced higher total PCs at lower internal Cd concentrations. Interestingly the controls (0 μg Cd L^{-1}) of *F. serratus* from both populations also contained the metal-chelating peptides and the control algae of RP possessed higher and longer PCs than the control of BQ. Production of PC by the control is most likely the result of metals accumulated from their natural habitats prior to the experiment. Therefore, the biosynthesis of higher PC values and longer PC chain lengths by the RP population is a protective mechanism to chelate and sequester metal ions accumulated from the surrounding environment. The population from the polluted site possessed a faster and greater ability to reduce metal burden as one of their survival strategies. Although the BQ population also produced considerable amounts of PC and the same chain lengths under Cd stress, it did so at a slower rate and totals accumulated were lower. This greater detoxifying characteristic in the RP population might have been inherited from predecessors as an adaptation to a contaminated environment, since the total and non-exchangeable Cd accumulations were similar to those of the BQ population.

GSH and its homologues are low molecular thiols that act as antioxidants (Landberg and Greger, 2002, Rijstenbil, 2002, Kawakami et al., 2006). In addition, GSH is a precursor of biosynthesis of PC (Grill et al., 1986, Zenk, 1996, Pawlik-Skowrońska et al., 2007). Therefore the levels of GSH are closely related to the antioxidative ability and the production of metal-chelating PC. GSH is a part of the ascorbate-glutathione cycle and is generated from oxidised glutathione (GSSG) by GR under oxidised situations (Fig. 4. 1) (Zutshi et al., 2008). Higher GSH values represent higher antioxidant potential. In the present study, *F. serratus* produced GSH with or without Cd exposure and Cd treatments from 10 $\mu g\ L^{-1}$ to 10 $mg\ L^{-1}$ increased the levels of GSH. Both populations had similar values of GSH and the values were maintained without decreasing for up to 14 d. Since GSH is consumed as a precursor in PC synthesis, the concentrations commonly decrease with PC production (Pawlik-Skowrońska, 2000, Coppellotti, 1989). Highly maintained GSH concentrations in *F. serratus* represent consistent antioxidant ability, potential biosynthesis of PC and metal-sequestration. This potential was not population-specific since both possessed the sustained high concentrations of GSH. *F. serratus* from RP and BQ accumulated similar Cd concentrations within the cells and produced similar levels of GSH. However the population from the polluted site produced higher concentrations of PC more rapidly than the reference population but not at the expense of GSH which remained similar to those of BQ with the aid of higher GR activity (at least at 24 hr and 96 hr). Consequently both populations have antioxidant capacity against Cd stress, but the RP population possessed a more effective mechanism.

Metal-specific activation of PC synthase *in vitro* has been reported for various plants and yeasts, e.g. fission yeast *Schizosaccharomyces pombe*, budding yeast *Saccharomyces cerevisiae*, and bladder campion *Silene cucubalus* (Cobbett and

Goldsbrough, 2002). Induction of PC *in vivo* also showed a metal-specific formation of PC-metal complexes (Cobbett and Goldsbrough, 2002). Cd, Cu, Ag, Hg, Zn, Pb, Au and As were reported as activators of the PC synthase and Cd, Cu, Ag, Pb and Zn produced the PC complexes *in vivo* (Pawlik-Skowrońska, 2000, Cobbett and Goldsbrough, 2002, Pawlik-Skowrońska, 2003, Pawlik-Skowrońska et al., 2004, Pawlik-Skowrońska et al., 2007). In most cases Cd was the strongest activator for the enzyme and inducer for the PC complexes (Grill et al., 1985, Grill et al., 1989, Cobbett and Goldsbrough, 2002, Inouhe, 2005). Activation of the enzyme and formation of the PC-metal complexes are not only metal-specific but also strain-specific (Grill et al., 1985, Ahner and Morel, 1995, Cobbett and Goldsbrough, 2002, Inouhe, 2005). In the present study, PC was produced upon exposure to both 10 μg Cd L^{-1} and 100 μg Cu L^{-1} and there was an additive effect of the combined metals (Cu + Cd) in BQ. On the other hand, in RP, the single metal treatments of Cd and Cu did not show increased PC production with concentrations similar to the control. The combined metal treatment in RP showed a synergistic effect in PC production. The PC production by Cu was lower than that by Cd in BQ; therefore Cu has a weaker influence than Cd in production of PC by *F. serratus*. Vatamaniuk et al. (1999) reported that PC synthase 1 of *Arabidopsis thaliana* (AtPCS1) had a higher affinity (K_d = 0.54 ± 0.20 μM) and higher capacity (stoichiometric ratio = 7.09 ± 0.94) for binding Cd ions than for other metals (such as Cu). Higher sensitivity to Cd exposure than other metal species has been found in PC synthase-deficient mutants of *Arabidopsis* and *S. pombe* (Ha et al., 1999). These two PC-deficient mutants have high sensitivity to Cd and As, but display little or no increased sensitivity to other metals, such as Ag, Cu, Hg, Ni and Zn. Similar results have been reported with PC synthase of *Caenorhabditis elegans*, with the suppressed PC synthase having a Cd-sensitive response (Vatamaniuk et al., 2001). However, response of the suppressed PC synthase in *C. elegans* to other metals has not been

reported (Cobbett and Goldsbrough, 2002). These reports support the role of PC in detoxification and strong sequestration of Cd ions rather than other metal species. PC-Cu complexes may be sequestered relatively poorly to the cell vacuole, or there may be a more effective detoxifying mechanism against Cu (Cobbett and Goldsbrough, 2002).

According to habitats in which an alga has been adapted or transplanted, the physiological and biochemical responses can be different and a new or modified mechanism may be developed for adaptation or acclimatisation. Different responses or mechanisms by different populations to the same factors have been reported (Dietz et al., 1999, Hall, 2002, Nielsen et al., 2003b). Some researchers have postulated that tolerant species or ecotypes are less likely to have a better oxidative defence but more likely have better avoidance and homeostatic mechanisms to prevent the stress (Dietz et al., 1999, Hall, 2002). However, avoidance and exclusion mechanisms of Cd ions were not identified with similar non-exchangeable and total Cd concentration in the two populations of *F. serratus*. Equal accumulation of Cu in ship-fouling thalli and non-fouling thalli has been reported and tolerance was suggested as a primary internal detoxification rather than an exclusion mechanism (Lobban and Harrison, 1994). It should also be noted that Fucales and Dictyotales are known to contain the highest phlorotannin levels in Phaeophyceae (Targett et al., 1995, Connan et al., 2006). Since levels of phenolic compounds (mainly phlorotannins) and polysaccharides (mainly alginic acids) have not been measured in the present research, other potential effects cannot be discussed. As free radical scavenging activity is relevant to the presence and abundance of polyphenol (Jiménez-Escrig et al., 2001, Connan et al., 2006), the contents of polyphenol of *F. serratus* from both populations should be examined under the stress of various ranges of Cd in a future study. Therefore the relatively high DPPH

free radical scavenging effect of BQ and lack of visual effects of metal stress may be concerned with the phlorotannin levels.

Hall (2002) concluded that no single mechanism can account for tolerance to a wide range of metals. RP population was characterised by higher tolerance and oxidative defences as shown by the higher activities of reactive oxygen scavenging enzymes (CAT and GR). The better homeostatic potential of the RP population was indicated by higher and faster PC synthesis. Faster growth and quicker recovery from Cd stress, and higher levels of Chl *a*, Chl *c* and Fx were also components of the tolerance mechanism of the population from the polluted site. However Chl *a* fluorescence, the activity of APX for 24 hr and 96 hr, the total and non-exchangeable Cd concentrations and GSH level were the same in both populations. On the other hand, the BQ population showed higher antioxidant ability measured by CUPRAC and DPPH free radical scavenging ability test. Therefore the BQ population also appears to have developed strategies against Cd stress. Although the reference population has lower efficiencies in some parameters, it has considerable levels of GSH and PC. Consequently *F. serratus* has a strong tolerance and homeostatic capacity against Cd exposure whether it is growing in a polluted or a clean location, although the population from the polluted site does possess higher tolerance, faster recovery, and better homeostatic control.

Since Cd accumulation increased with Cd concentration in the medium and the time of exposure, *F. serratus* did not regulate the uptake of Cd. In addition, the alga took up Cd ions without demonstrating visual stress symptoms or mortality across a wide range of Cd exposures. Therefore the linear increase of internal Cd contents and the strong tolerance to it suggest *F. serratus* may be a useful biological indicator and

hyperaccumulator in the coastal environment. Even though further studies are required, *F. serratus* may be able to be used to remove Cd from the environment. The hyperaccumulating character of this species, along with polyphenol, alginic acid and PC could make this alga an effective environmental protector.

The present research presented the basic data on Cd accumulation and antioxidative defence, further information on PC production by this marine macroalga, and potential uses of this species. In addition some topics for further studies were also discussed. Firstly, shorter time intervals for measurement and comparisons between acute and chronic Cd exposure should be performed. This is because of the fast effects of Cd and the quick responses of *F. serratus*. The alga responded very quickly within a 24 hr time span. Although Chl *a* fluorescence could not estimate the effects of Cd, antioxidative enzymes and PC production are required to be estimated with shorter exposure. Secondly, the location of PC-metal complexes in the cell and the molecular approach on PC synthase gene of *F. serratus* will fill the knowledge gaps. Thirdly, antioxidant enzymes which were not determined in this study (such as superoxide dismutase), ROS production, and contents of polyphenol and polysaccharides against Cd exposure will provide a useful discussion with the data in the present study. Fourthly, transplantation and studies on the early life stages of development may confirm the inheritance of tolerance to Cd. Cross transplantation or transplanting to a third area might provide changed responses to changed environmental conditions. The early stages of *F. serratus* may possess inherited tolerance to Cd, since a similar effect has been observed with Cu (Nielsen et al. 2003). Furthermore, algal responses to multi-metal and lower metal levels close to real field conditions should be determined. The final goal of the research is to find an applicable realistic parameter or assay to examine the complex actual environment.

LIST OF REFERENCES

ABE, N., MURATA, T. & HIROT, A. (1998) Novel DPPH radical scavengers, bisorbicillinol and demethyltrichodimerol, from a fungus. *Bioscience, Biotechnology, and Biochemistry,* 62, 661-666.

ADRIANO, D. C. (1986) *Trace elements in the terrestrial environment,* New York, Springer-Verlag.

AGUILERA, J., BISCHOF, K., KARSTEN, U., HANELT, D. & WIENCKE, C. (2002) Seasonal variation in ecophysiological patterns in macroalgae from an Arctic fjord. II. Pigment accumulation and biochemical defence systems against high light stress. *Marine Biology,* 140, 1087-1095.

AHNER, B. A., KONG, S. & MOREL, F. M. M. (1995) Phytochelatin production in marine algae. 1. An interspecies comparison. *Limnology and Oceanography,* 40, 649-657.

AHNER, B. A. & MOREL, F. M. M. (1995) Phytochelatin production in marine algae. 2. Induction by various metals. *Limnology and Oceanography,* 40, 658-665.

AHNER, B. A., WEI, L., OLESON, J. R. & OGURA, N. (2002) Glutathione and other low molecular weight thiols in marine phytoplankton under metal stress. *Marine Ecology Progress Series,* 232, 93-103.

ANDERSON, B. S., HUNT, J. W., TURPEN, S. L., COULON, A. R. & MARTIN, M. (1990) Copper toxicity to microscopic stages of giant kelp *Macrocystis pyrifera*: interpopulation comparisons and temporal variability. *Marine Ecology Progress Series,* 68, 147-156.

ANDERSON, M. D., PRASAD, T. K. & STEWART , C. R. (1995) Changes in isozyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. *Plant Physiology,* 109, 1247-1257.

ANGGADIREDJA, J., ANDYANI, R., HAYATI & MUAWANAH (1997) Antioxidant activity of *Sargassum polycystum* (Phaeophyta) and *Laurencia obtusa* (Rhodophyta) from Scribu Islands. *Journal of Applied Phycology,* 9, 477-479.

ANTOSIEWICZ, D. M. (2005) Study of calcium-dependent lead-tolerance on plants differing in their level of Ca-deficiency tolerance. *Environmental Pollution,* 134, 23-34.

APAK, R., G L , K., DEMIRATA, B., ÖZY REK, M., ÇELIK, S. E., BEKTAŞOĞLU, B., BERKER, K. I. & ÖZYURT, D. (2007) Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules,* 12, 1496-1547.

APAK, R., G L , K., ÖZY REK, M. & KARADEMIR, S. E. (2004) Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *Journal of Agricultural and Food Chemistry,* 52, 7970-7981.

ASADA, K. (1992) Ascorbate peroxidase - a hydrogen peroxide-scavenging enzyme in plants. *Physiologia Plantarum,* 85, 235-241.

ASADA, K. (1999) The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annual Review of Plant Physiology and Plant Molecular Biology,* 50, 601-639.

ASADA, K. & TAKAHASHI, M. (1987) Production and scavenging of active oxygen species in photosynthesis. IN KYLE, D. J., OSMOND, C. & ARNTZEN, C. (Eds.) *Photoinhibition.* New York, Elsevier Science Publishers.

BAKER, A. J. M. (1981) Accumulators and excluders -strategies in the response of plants to heavy metals. *Journal of Plant Nutrition,* 3, 643 - 654.

BAKER, A. J. M. & WALKER, P. L. (1990) Ecophysiology of metal uptake by tolerant plants. IN SHAW, A. J. (Ed.) *Heavy metal tolerance in plants: evolutionary aspects.* Boca Raton, Florida, USA, CRC Press, .

BAKER, N. R. & OXBOROUGH, K. (2004) Chlorophyll fluorescence as a probe of photosynthetic productivity. IN PAPAGEORGIOU, G. C. & GOVINDJEE (Eds.) *Chlorophyll a Fluorescence.* Dordrecht, The Netherlands, Springer.

BAMBER, S. D. & DEPLEDGE, M. H. (1997) Evaluation of changes in the adaptive physiology of shore crabs (*Carcinus maenas*) as an indicator of pollution in estuarine environments. *Marine Biology,* 129, 667-672.

BANKS, D., YOUNGER, P. L., ARNESEN, R.-T., IVERSEN, E. R. & BANKS, S. B. (1997) Mine-water chemistry: the good, the bad and the ugly. *Environmental Geology,* 32, 157-174.

BARROS, M. P., PEDERS N, M., COLEPICOLO, P. & SNOEIJS, P. (2003) Self-shading protects phytoplankton communities against H_2O_2-induced oxidative damage *Aquatic Microbial Ecology,* 30, 275-282.

BASS, E. L. (1977) Influence of temperature and salinity on oxygen consumption of tissues in the American oyster (*Crassostrea virginica*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 58B, 125–130.

BASZYŃKI, T., WAJDA, L., KR L, M., WOLIŃSKA, D., KRUPA, Z. & TUKENDORF, A. (1980) Photosynthetic activities of cadmium-treated tomato plants. *Physiologia Plantarum,* 48, 365-370.

BENZIE, I. F. F. & STRAIN, J. J. (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry,* 239, 70-76.

BERRY, W. L. & WALLACE, A. (1981) Toxicity: the concept and relationship to the dose response curve. *Journal of Plant Nutrition,* 3, 13-19.

BJ RKMAN, O. & DEMMIG, B. (1987) Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta,* 170, 489-504.

BLOIS, M. S. (1958) Antioxidant determinations by the use of a stable free radical. *Nature,* 181, 1199-1200.

BOND, P. R., BROWN, M. T., MOATE, R. M., GLEDHILL, M., HILL, S. J. & NIMMO, M. (1999) Arrested development in *Fucus spiralis* (Phaeophyceae) germlings exposed to copper. *European Journal of Phycology,* 34, 513-521.

BOYLE, T. P. (1984) The effect of environmental contaminants on aquatic algae. IN SHUBERT, L. E. (Ed.) *Algae as Ecological Indicators.* London, Academic Press.

BRADFORD, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* 72, 248-254.

BRAITHWAITE, R. A. & FLETCHER, R. L. (2005) The toxicity of Irgarol 1051 and Sea-Nine 211 to the non-target macroalga *Fucus serratus* Linnaeus, with the aid of an image capture and analysis system. *Journal of Experimental Marine Biology and Ecology,* 322, 111-121.

BRINKHUIS, B. H., PENELLO, W. F. & CHURCHILL, A. C. (1980) Cadmium and manganese flux in eelgrass *Zostera marina* II. Metal uptake by leaf and root-rhizome tissues. *Marine Biology,* 58, 187-196.

BROWN, M. T. & NEWMAN, J. E. (2003) Physiological responses of *Gracilariopsis longissima* (S.G. Gmelin) Steentoft, L.M. Irvine and Farnham (Rhodophyceae) to sub-lethal copper concentrations. *Aquatic Toxicology,* 64, 201-213.

BRUCE, D. & VASIL'EV, S. (2004) Excess light stress: multiple dissipative processes of excess excitation. IN PAPAGEORGIOU, G. C. & GOVINDJEE (Eds.) *Chlorophyll a Fluorescence.* Dordrecht, The Netherlands, Springer.

BRUNE, A., URBACH, W. & DIETZ, K.-J. (1995) Differential toxicity of heavy metals is partly related to a loss of preferential extraplasmic compartmentation: a comparison of Cd-, Mo-, Ni- and Zn-stress. *New Phytologist,* 129, 403-409.

BRYAN, G. (1980) Recent trends in research on heavy-metal contamination in the sea. *Helgoland Marine Research,* 33, 6-25.

BRYAN, G. W. (1969) The absorption of zinc and other metals by the brown seaweed Laminaria digitata. *Journal of the Marine Biological Association of the UK,* 49, 225-243.

BRYAN, G. W. (1971) The effects of heavy metals (other than mercury) on marine and estuarine organisms. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* 177, 389–410.

BRYAN, G. W. (1974) Adaptation of an estuarine polychaete to sediments containing high concentrations of heavy metals. IN VERNBERG, F. J. & VERNBERG, W. B. (Eds.) *Pollution and Physiology of Marine Organisms.* New York, U.S.A., Academic Press.

BRYAN, G. W. (1976) Heavy metal contamination in the sea. IN JOHNSTON, R. (Ed.) *Marine Pollution.* London, U.K., Academic Press.

BRYAN, G. W. (1983) Brown seaweed, *Fucus vesiculosus*, and the gastropod, *Littorina littoralis*, as indicators of trace-metal availability in estuaries. *Science of The Total Environment,* 28, 91-104.

BRYAN, G. W. & GIBBS, P. E. (1983) Heavy metals in the Fal estuary, Cornwall: a study of long-term contamination by mining waste and its effects on estuarine organisms. *Occasional Publication of Marine Biological Association of the United Kingdom,* 2, 1-112.

BRYAN, G. W. & GIBBS, P. E. (1985) Polychaetes as indicators of heavy-metal availability in marine deposits. IN CAPUZZO, J. M. & KESTER, D. R. (Eds.) *Oceanic Processes in Marine Pollution. Vol. I, Biological Processes and Wastes in the Oceans.* Melbourne, FL Krieger Publishing Co.

BRYAN, G. W., GIBBS, P. F., HUMMERSTONE, L. G. & BURT, G. R. (1987) Copper, zinc, and organotin as long-term factors governing the distribution of organisms in the Fal Estuary in southwest England. *Estuaries,* 10, 208-219.

BRYAN, G. W. & HUMMERSTONE, L. G. (1971) Adaptation of the polychaete *Nereis diversicolor* to estuarine sediments containing high concentations of heavy metals. I. General obersvations and adaptation to copper. *Journal of the Marine Biological Association of the United Kingdom,* 51, 845-863.

BRYAN, G. W. & HUMMERSTONE, L. G. (1973) Brown seaweed as an indicator of heavy metals in estuaries in South-West England. *Journal of Marine Biological Association of U.K.,* 53, 705-720.

BRYAN, G. W. & LANGSTON, W. J. (1992) Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. *Environmental Pollution,* 76, 89-131.

BRYAN, G. W., LANGSTON, W. J., HUMMERSTONE, L. G. & BURT, G. R. (1985) A guide to the assessment of heavy metal contamination in estuaries using biological indicators. *Marine Biological Association of the United Kingdom,* 4, 92p.

BU-OLAYAN, A. H., AL-HASSAN, R., THOMAS, B. V. & SUBRAHMANYAM, M. N. V. (2001) Impact of trace metals and nutrients levels on phytoplankton from the Kuwait Coast. *Environment International,* 26, 199-203.

BUKHOV, N. G. & CARPENTIER, R. (2004) Effects of water stress on the photosynthetic efficiency of plants. IN PAPAGEORGIOU, G. C. & GOVINDJEE (Eds.) *Chlorophyll a Fluorescence: A Signature of Photosynthesis. Advances in Photosynthesis and Respiration.* Dordrecht, The Netherlands, Springer.

BURRITT, D. J., LARKINDALE, J. & HURD, C. L. (2002) Antioxidant metabolism in the intertidal red seaweed *Stictosiphonia arbuscula* following desiccation. *Planta,* 215, 829-838.

BURROWS, E. M. (1971) Assessment of pollution effects by the use of algae. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* 177, 295-306.

BURT, R. (1998) History of metalliferous mining. IN SELLWOOD, E. B., DURRANCE, E. M. & BRISTOW, C. M. (Eds.) *The Geology of Cornwall.* Exeter, University of Exeter Press.

BUSSOTTI, F., STRASSER, R. J. & SCHAUB, M. (2007) Photosynthetic behavior of woody species under high ozone exposure probed with the JIP-test: A review. *Environmental Pollution,* 147, 430-437.

CAIRR O, E., COUDERCHET, M., SOARES, A. M. V. M. & GUILHERMINO, L. (2004) Glutathione-S-transferase activity of *Fucus* spp. as a biomarker of environmental contamination. *Aquatic Toxicology,* 70, 277-286.

CHAERLE, L. & VAN DER STRAETEN, D. (2000) Imaging techniques and the early detection of plant stress *Trends in Plant Science,* 5, 495-501.

CHARRIER, B., COELHO, S. M., LE BAIL, A., TONON, T., MICHEL, G., POTIN, P., KLOAREG, B., BOYEN, C., PETERS, A. F. & COCK, J. M. (2008) Development and physiology of the brown alga *Ectocarpus siliculosus*: two centuries of research. *New Phytologist,* 177, 319-332.

CHOO, K.-S., SNOEIJS, P. & PEDERSEN, M. (2004) Oxidative stress tolerance in the filamentous green algae *Cladophora glomerata* and *Enteromorpha ahlneriana. Journal of Experimental Marine Biology and Ecology,* 298, 111-123.

CHRISTEN, D., SCH NMANN, S., JERMINI, M., STRASSER, R. J. & D FAGO, G. (2007) Characterization and early detection of grapevine (*Vitis vinifera*) stress responses to esca disease by in situ chlorophyll fluorescence and comparison with drought stress. *Environmental and Experimental Botany,* 60, 504-514.

CID, A., HERRERO, C., TORRES, E. & ABALDE, J. (1995) Copper toxicity on the marine microalga *Phaeodactylum tricornutum*: effects on photosynthesis and related parameters. *Aquatic Toxicology,* 31, 165-174.

CISCATO, M., VANGRONSVELDB, J. & VALCKEA, R. (1999) Effects of heavy metals on the fast chlorophyll fluorescence induction kinetics of Photosystem II: a comparative study. *Zeitschrift für Naturforschung,* 54c, 735-739.

CLEMENS, S. (2001) Molecular mechanisms of plant metal tolerance and homeostasis. *Planta,* 212, 475-486.

CLEMENS, S. (2006) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie,* 88, 1707-1719.

CLEMENS, S., KIM, E. J., NEUMANN, D. & SCHROEDER, J. I. (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *The EMBO Journal* 18, 3325-3333.

CLIJSTERS, H. & VAN ASSCHE, F. (1985) Inhibition of photosynthesis by heavy metals. *Photosynthesis Research,* 7, 31-40.

COBBETT, C. & GOLDSBROUGH, P. (2002) Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology,* 53, 159-182.

COBBETT, C. S. (2000) Phytochelatins and their roles in heavy metal detoxification. *Plant Physiology,* 123, 825-832.

COLL N, J. & DAVISON, I. R. (1997) *In vivo* measurement of active oxygen production in the brown alga *Fucus evanescens* using 2',7'-dichlorohydrofluoresein diacetate. *Journal of Phycology,* 33, 643-648.

COLL N, J. & DAVISON, I. R. (1999a) Reactive oxygen metabolism in intertidal *Fucus* spp. (Phaeophyceae). *Journal of Phycology,* 35, 62-69.

COLL N, J. & DAVISON, I. R. (1999b) Reactive oxygen production and damage in intertidal *Fucus* spp. (Phaeophyceae). *Journal of Phycology,* 35, 54-61.

COLL N, J. & DAVISON, I. R. (1999c) Stress tolerance and reactive oxygen metabolism in the intertidal red seaweeds *Mastocarpus stellatus* and *Chondrus crispus*. *Plant, Cell & Environment,* 22, 1143-1151.

COLL N, J., DEL RIO, M. J., GARC A-REINA, G. & PEDERS N, M. (1995) Photosynthetic production of hydrogen peroxide by *Ulva rigida* C. Ag. (Chlorophyta). *Planta,* 196, 225-230.

COLL N, J. & PEDERS N, M. (1994) A stress-induced oxidative burst in *Eucheuma platycladum* (Rhodophyta). *Physiologia Plantarum,* 92, 417–422.

COLL N, J. & PEDERS N, M. (1996) Production, scavenging and toxicity of hydrogen peroxide in the green seaweed *Ulva rigida*. *European Journal of Phycology,* 31, 265-271.

COLL N, J., PINTO, E., PEDERSEN, M. & COLEPICOLO, P. (2003) Induction of oxidative stress in the red macroalga *Gracilaria tenuistipitata* by pollutant metals. *Archives of Environmental Contamination and Toxicology,* 45, 337-342.

COMBO, M. E., BENAVIDES, M. P. & TOMARO, M. L. (1998) Effect of salt stress on antioxidant defence system in soybean root nodules. *Journal of Plant Physiology,* 25, 665-671.

CONNAN, S., DELISLE, F., DESLANDES, E. & GALL, E. A. (2006) Intra-thallus phlorotannin content and antioxidant activity in Phaeophyceae of temperate waters. *Botanica Marina,* 49, 39-46.

CONTRERAS, L., MOENNE, A. & CORREA, J. A. (2005) Antioxidant responses in *Scytosiphon lomentaria* (Phaeophyceae) inhabiting copper-enriched coastal environments. *Journal of Phycology,* 41, 1184-1195.

COOMBS, T. L. (1979) Cadmium in aquatic organisms. IN WEBB, M. (Ed.) *The Chemistry, Biochemistry and Biology of Cadmium.* Amsterdam, Elsevier/North-Holland Biomedical Press.

COPPELLOTTI, O. (1989) Glutathione, cysteine and acid-soluble thiol levels in *Euglena gracilis* cells exposed to copper and cadmium. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology,* 94, 35-40.

CORREA, J. A., GONZ LEZ, P., S NCHEZ, P., MU OZ, J. & ORELLANA, M. C. (1996) Copper-algae interactions: inheritance or adaptation? *Environmental Monitoring and Assessment,* 40, 41-54.

COSTANZO, S. D., O'DONOHUE, M. J. & DENNISON, W. C. (2000) *Gracilaria edulis* (Rhodophyta) as a biological indicator of pulsed nutrients in oligotrophic waters. *Journal of Phycology,* 36, 680-685.

COTT -KRIEF, M.-H., GUIEU, C., THOMAS, A. J. & MARTIN, J.-M. (2000) Sources of Cd, Cu, Ni and Zn in Portuguese coastal waters. *Marine Chemistry,* 71, 199-214.

DAN, T. V., KRISHNARAJ, S. & SAXENA, P. K. (2000) Metal tolerance of scented geranium (*Pelargonium* sp. 'Frensham'): effects of cadmium and nickel on chlorophyll fluorescence kinetics. *International Journal of Phytoremediation,* 2, 91-104.

DAT, J., VANDENABEELE, S., VRANOV , E., VAN MONTAGU, M., INZ , D. & VAN BREUSEGEM, F. (2000) Dual action of the active oxygen species during plant stress responses *Cellular and Molecular Life Sciences,* 57, 779-795.

DAVIES, A. G. (1976) An assessment of the basis of mercury tolerance in *Dunaliella tertiolecta. Journal of Marine Biological Association of the U.K.,* 56, 39-57.

DAVIS, T. A., VOLESKY, B. & MUCCI, A. (2003) A review of the biochemistry of heavy metal biosorption by brown algae. *Water Research,* 37, 4311-4330.

DAVISON, I. R. & PEARSON, G. A. (1996) Stress tolerance in intertidal seaweeds. *Journal of Phycology,* 32, 197-211.

DE ANDRADE, L. R., FARINA, M. & FILHO, G. M. A. (2002) Role of *Padina gymnospora* (Dictyotales, Phaeophyceae) cell walls in cadmium accumulation. *Phycologia,* 41, 39-48.

DE FILIPPIS, L. F. & PALLAGHY, C. K. (1994) Heavy metals: source and biological effects. IN RAI, L. C., GAUR, J. P. & SOEDER, C. J. (Eds.) *Algae and Water Pollution.* Stuttgart, Germany, E. Schweizerbart'sche Verlagsbuchhandlung.

DE TEMMERMAN, L. O., HOENIG, M. & SCOKART, P. O. (1984) Determination of "normal" levels and upper limit values of trace elements in soils. *Zeitschrift für Pflanzenernährung und Bodenkunde,* 147, 687-694.

DEAL, M. S., HAY, M. E., WILSON, D. & FENICAL, W. (2003) Galactolipids rather than phlorotannins as herbivore deterrents in the brown seaweed *Fucus vesiculosus. Oecologia,* 136, 107-114.

DEELL, J. R. & TOIVONEN, P. M. A. (2003) Use of chlorophyll fluorescence in postharvest quality assessments of fruits and vegetables. IN DEELL, J. R. & TOIVONEN, P. M. A. (Eds.) *Practical Applications of Chlorophyll Fluorescence in Plant Biology.* Dordrecht, The Netherlands, Kluwer Academic Publishers.

DEIGHTON, N., MUCKENSCHNABEL, I., GOODMAN, B. A. & WILLIAMSON, B. (1999) Lipid peroxidation and the oxidative burst associated with infection of Capsicum annuum by Botrytis cinerea. *The Plant Journal,* 20, 485-492.

DEL RIO, D., STEWART, A. J. & PELLEGRINI, N. (2005) A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism and Cardiovascular Diseases,* 15, 316-328.

DELHAIZE, E., JACKSON, P. J., LUJAN, L. D. & ROBINSON, N. J. (1989) Poly(γ-glutamylcysteinyl)glycine synthesis in *Datura innoxia* and binding with cadmium : Role in cadmium tolerance. *Plant Physiol.,* 89, 700-706.

DENTON, G. R. W. & BURDON-JONES, C. (1981) Influence of temperature and salinity on the uptake, distribution and depuration of mercury, cadmium and lead by the black-lip oyster *Saccostrea echinata. Marine Biology,* 64, 317-326.

DEWEZ, D., DIDUR, O., VINCENT-H ROUX, J. & POPOVIC, R. (2008) Validation of photosynthetic-fluorescence parameters as biomarkers for isoproturon toxic effect on alga *Scenedesmus obliquus. Environmental Pollution,* 151, 93-100.

DI CAGNO, R., GUIDI, L., STEFANI, A. & SOLDATINI, G. F. (1999) Effects of cadmium on growth of *Helianthus annuus* seedlings: physiological aspects. *New Phytologist,* 144, 65-71.

DIETZ, K.-J., BAIER, M. & KR MER, U. (1999) Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. IN PRASAD, M. N. V. & HAGEMEYER, J. (Eds.) *Heavy metal stress in plants. From molecules to ecosystems.* Heidelberg, Germany, Springer-Verlag.

DINES, H. G. (1969) *The metalliferous mining region of south-west England,* London, U.K., H.M.S.O.

DUMMERMUTH, A. L., KARSTEN, U., FISCH, K. M., KONIG, G. M. & WIENCKE, C. (2003) Responses of marine macroalgae to hydrogen-peroxide stress. *Journal of Experimental Marine Biology and Ecology,* 289, 103-121.

EKLUND, B. T. & KAUTSKY, L. (2003) Review on toxicity testing with marine macroalgae and the need for method standardization--exemplified with copper and phenol. *Marine Pollution Bulletin,* 46, 171-181.

EL-HABIT, O. H. M., SAADA, H. N., AZABB, K. S., ABDEL-RAHMANA, M. & EL-MALAH, D. F. (2000) The modifying effect of β-carotene on gamma radiation-induced elevation of oxidative reactions and genotoxicity in male rats *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 466, 179-186.

ELSTNER, E. F., WAGNER, G. A. & SCHUTZ, W. (1988) Activated oxygen in green plants in relation to stress situations. *Plant Biochemistry and Physiology,* 7, 159-187.

ENGEL, D. W., SUNDA, W. G. & FOWLER, B. A. (1981) Factors affecting trace metal uptake and toxicity to estuarine organisms. I. Environmental parameters. IN VERNNBERG, F., CALABRESE, A., THURBERG, F. P. & VERNBERG, W. B. (Eds.) *Biological Monitoring of Marine Pollutants.* New York, Academic Press.

EPRON, D., E., D. & BR DA, N. (1992) Photosynthesis of oak trees [*Quercus petraea* (Matt.) Liebl.] during drought under field conditions: diurnal course of net CO_2 assimilation and photochemical efficiency of photosystem II. *Plant, Cell and Environment,* 15, 809-820.

ESSER, B. K. & VOLPE, A. (2002) At-sea high-resolution trace element mapping: San Diego Bay and its plume in the adjacent coastal ocean. *Environmental Science & Technology,* 36, 2826-2832.

EVANS, L. V. (1988) The effects of spectral composition and irradiance level on pigment levels in seaweeds. IN LOBBAN, C. S., CHAPMAN, D. J. & KREMER, B. P. (Eds.) *Experimental Phycology: a Laboratory Manual.* Cambridge, Cambridge University Press.

F RSTNER, U. & WITTMANN, G. T. W. (1979) *Metal Pollution in the Aquatic Environment,* New York, Springer-Verlag, Inc.

F RSTNER, U. & WITTMANN, G. T. W. (1983) *Metal Pollution in the Aquatic Environment,* Berlin, Germany, Springer-Verlag.

FADZILLAH, N. A. M., GILL, V., FINCH, R. P. & BURDON, R. H. (1996) Chilling, oxidative stress and antioxidant responses in shoot cultures of rice. *Planta,* 199, 552-556.

FERNANDES, J. & HENRIQUES, F. (1991) Biochemical, physiological, and structural effects of excess copper in plants. *The Botanical Review,* 57, 246-273.

FERRAT, L., PERGENT-MARTINI, C. & ROM O, M. (2003) Assessment of the use of biomarkers in aquatic plants for the evaluation of environmental quality: application to seagrasses. *Aquatic Toxicology,* 65, 187-204.

FORSBERG, Å., S DERLUND, S., FRANK, A., PETERSSON, L. R. & PEDERS N, M. (1988) Studies on metal content in the brown seaweed, *Fucus vesiculosus,* from the Archipelago of Stockholm. *Environmental Pollution,* 49, 245-263.

FOYER, C. H. & MULLINEAUX, P. (1994) *Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants,* Boca Raton, Florida, CRC Press.

FRACHEBOUD, Y., HALDIMANN, P., LEIPNER, J. & STAMP, P. (1999) Chlorophyll fluorescence as a selection tool for cold tolerance of photosynthesis in maize (*Zea mays* L.). *Journal of Experimental Botany,* 50, 1533-1540.

FUGE, R. & JAMES, K. H. (1974) Trace metal concentrations in *Fucus* from the Bristol channel. *Marine Pollution Bulletin,* 5, 9-12.

G L , K., ALTUN, M., ÖZY REK, M., KARADEMIR, S. E. & APAK, R. (2006) Antioxidant capacity of fresh, sun- and sulphited-dried Malatya apricot (*Prunus armeniaca*) assayed by CUPRAC, ABTS/TEAC and folin methods. *International Journal of Food Science & Technology,* 41, 76-85.

G L IN, İ., OKTAY, M., K FREVIOĞLU, Ö. İ. & ALI, A. (2002) Determination of antioxidant activity of lichen *Cetraria islandica* (L) Ach *Journal of Ethnopharmacology,* 79, 325-329.

G L IN, İ., OKTAY, M., KIRE CI, E. & K FREVIOĞLU, Ö. İ. (2003) Screening of antioxidant and antimicrobial activities of anise (*Pimpinella anisum* L.) seed extracts. *Food Chemistry,* 83, 371-382.

G RANSSON, A. (1998) Steady-state nutrition and growth responses of *Betula pendula* to different relative supply rates of copper. *Plant Cell and Environment,* 21, 937-944.

GAMON, J. A. & PEARCY, R. W. (1989) Leaf movement, stress avoidance and photosynthesis in *Vitis californica*. *Oecologia,* 79, 475-481.

GARC A-R OS, V., FREILE-PELEGR N, Y., ROBLEDO, D., MENDOZA-C ZATL, D., MORENO-S NCHEZ, R. & GOLD-BOUCHOT, G. (2007) Cell wall composition affects Cd^{2+} accumulation and intracellular thiol peptides in marine red algae. *Aquatic Toxicology,* 81, 65-72.

GAVRILESCU, M. (2004) Removal of heavy metals from the environment by biosorption. *Engineering in Life Sciences,* 4, 219-232.

GEKELER, W., GRILL, E., WINNACKER, E.-L. & ZENK, M. H. (1988) Algae sequester heavy metals via synthesis of phytochelatin complexes. *Archives of Microbiology,* 150, 197-202.

GERRARD, S. (2000) *The Early British Tin Industry,* Stroud, Tempus Publishing Ltd.

GILMORE, A. M., SHINKAREV, V. P., HAZLETT, T. L. & GOVINDJEE (1998) Quantitative analysis of the effects of intrathylakoid pH and xanthophyll cycle pigments on chlorophyll *a* fluorescence lifetime distributions and intensity in thylakoids. *Biochemistry,* 37, 13582-13593.

GLEDHILL, M., NIMMO, M., HILL, S. J. & BROWN, M. T. (1997) The toxicity of copper (II) species to marine algae, with particular reference to macroalgae. *Journal of Phycology,* 33, 2-11.

GLEDHILL, M., NIMMO, M., HILL, S. J. & BROWN, M. T. (1999) The release of copper-complexing ligands by the brown alga *Fucus vesiculosus* (Phaeophyceae) in response to increasing total copper levels. *Journal of Phycology,* 35, 501-509.

GONZALEZ-MENDOZA, D., GILL, F. E. Y., SANTAMAR A, J. M. & ZAPATA-PEREZ, O. (2007) Multiple effects of cadmium on the photosynthetic apparatus of *Avicennia germinans* L. as probed by OJIP chlorophyll fluorescence measurements. *Zeitschrift für Naturforschung,* 62c, 265-272.

GONZALEZ, A., KOREN'KOV, V. & WAGNER, G. J. (1999) A comparison of Zn, Mn, Cd, and Ca transport mechanisms in oat root tonoplast vesicles. *Physiologia Plantarum,* 106, 203-209.

GOVINDJEE (2004) Chlorophyll a fluorescence: a bit of basics and history. IN GOVINDJEE (Ed.) *Chlorophyll a Fluorescence.* Dordrecht, The Netherlands, Springer.

GRANT, A., HATELEY, J. G. & JONES, N. V. (1989) Mapping the ecological impact of heavy metals on the estuarine polychaete *Nereis diversicolor* using inherited metal tolerance. *Marine Pollution Bulletin,* 20, 235-238.

GREGER, M. (1999) Metal availability and bioconcentration in plants. IN PRASAD, M. N. V. & HAGEMEYER, J. (Eds.) *Heavy metal stress in plants. From molecules to ecosystems.* Heidelberg, Germany, Springer-Verlag.

GREGER, M. & ÖGREN, E. (1991) Direct and indirect effects of Cd^{2+} on photosynthesis in sugar beet (*Beta vulgaris*). *Physiologia Plantarum,* 83, 129-135.

GRILL, E., GEKELER, W., WINNACKER, E. L. & ZENK, H. H. (1986) Homo-phytochelatins are heavy metal-binding peptides of homo-glutathione containing Fabales. *FEBS Letters,* 205, 47-50.

GRILL, E., LOFFLER, S., WINNACKER, E.-L. & ZENK, M. H. (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific gamma -glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). *Proceedings of the National Academy of Sciences of the United States of America,* 86, 6838-6842.

GRILL, E., THUMANN, J., WINNACKER, E.-L. & ZENK, M. H. (1988) Induction of heavy-metal binding phytochelatins by inoculation of cell cultures in standard media. *Plant Cell Reports,* 7, 375-378.

GRILL, E., WINNACKER, E.-L. & ZENK, M. H. (1985) Phytochelatins: The principal heavy-metal complexing peptides of higher plants. *Science,* 230, 674-676.

GRILL, E., WINNACKER, E.-L. & ZENK, M. H. (1987) Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. *Proceedings of the National Academy of Sciences of the United States of America,* 84, 439-443.

GROOM, Q. J. & BAKER, N. R. (1992) Analysis of light-induced depressions of photosynthesis in leaves of a wheat crop during the winter. *Plant Physiology,* 100, 1217-1223.

GROSSMAN, A. R., BHAYA, D., APT, K. E. & KEHOE, D. M. (1995) Light-harvesting complexes in oxygenic photosynthesis: Diversity, control, and evolution. *Annual Review of Genetics,* 29, 231-288.

H DOUIN, L., BUSTAMANTE, P., FICHEZ, R. & WARNAU, M. (2008) The tropical brown alga *Lobophora variegata* as a bioindicator of mining contamination in the New Caledonia lagoon: A field transplantation study. *Marine Environmental Research,* 66, 438-444.

HA, S.-B., SMITH, A. P., HOWDEN, R., DIETRICH, W. M., BUGG, S., O'CONNELL, M. J., GOLDSBROUGH, P. B. & COBBETT, C. S. (1999) Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe. Plant Cell,* 11, 1153-1164.

HAGEMEYER, J. (1999) Ecophysiology of plant growth under heavy metal stress. IN PRASAD, M. N. V. & HAGEMEYER, J. (Eds.) *Heavy metal stress in plants. From molecules to ecosystems.* Heidelberg, Germany, Springer-Verlag.

HALDIMANN, P., FRACHEBOUD, Y. & STAMP, P. (1996) Photosynthetic performance and resistance to photoinhibition of *Zea mays* L. leaves grown at sub-optimal temperature. *Plant, Cell and Environment,* 19, 85-92.

HALL, A., FIELDING, A. H. & BUTLER, M. (1979) Mechanisms of copper tolerance in the marine fouling alga *Ectocarpus siliculosus* — Evidence for an exclusion mechanism. *Marine Biology,* 54, 195-199.

HALL, J. L. (2002) Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot.,* 53, 1-11.

HALLIWELL, B. & GUTTERIDGE, J. M. C. (2007) *Free radicals in biology and medicine,* Oxford, Oxford University Press.

HAMMOND-KOSACK, K. E. & JONES, J. D. G. (1996) Resistance Gene-Dependent Plant Defense Responses *Plant Cell,* 8, 1773-1791. .

HAN, T. & CHOI, G.-W. (2005) A novel marine algal toxicity bioassay based on sporulation inhibition in the green macroalga *Ulva pertusa* (Chlorophyta). *Aquatic Toxicology,* 75, 202-212.

HAN, T., KANG, S.-H., PARK, J.-S., LEE, H.-K. & BROWN, M. T. (2008) Physiological responses of *Ulva pertusa* and *U. armoricana* to copper exposure. *Aquatic Toxicology,* 86, 176-184.

HARRISON, P. J., WATERS, R. E. & TAYLOR, F. J. R. (1980) A broad spectrum artificial sea water medium for coastal and open ocean phytoplankton. *Journal of Phycology,* 16, 28-35.

HASHIM, M. A. & CHU, K. H. (2004) Biosorption of cadmium by brown, green, and red seaweeds. *Chemical Engineering Journal,* 97, 249-255.

HAUPT-HERTING, S. & FOCK, H. P. (2000) Exchange of oxygen and its role in energy dissipation during drought stress in tomato plants. *Physiologia Plantarum,* 110, 489-495.

HAYASHI, Y., NAKAGAWA, C. W., MUTOH, N., ISOBE, M. & GOTO, T. (1991) Two pathways in the biosynthesis of cadystins $(\gamma EC)_nG$ in the cell-free system of the fission yeast. *biochemistry and Cell Biology,* 69, 115-121.

HERTWIG, B., STREB, P. & FEIERABEND, J. (1992) Light dependence of catalase synthesis and degradation in leaves and the influence of interfering stress conditions. *Plant Physiology,* 100, 1547-1553.

HETHERINGTON, S., SMILLIE, R. & DAVIES, W. (1998) Photosynthetic activities of vegetative and fruiting tissues of tomato. *Journal of Experimental Botany,* 49, 1173-1181.

HIRATA, K., TSUJIMOTO, Y., NAMBA, T., OHTA, T., HIRAYANAGI, N., MIYASAKA, H., ZENK, M. H. & MIYAMOTO, K. (2001) Strong induction of phytochelatin synthesis by zinc in marine green alga, *Dunaliella tertiolecta. Journal of Bioscience and Bioengineering,* 92, 24-29.

HOLT, N. E., FLEMING, G. R. & NIYOGI, K. K. (2004) Toward an understanding of the mechanism of nonphotochemical quenching in green plants. *Biochemistry,* 43, 8281-8289.

HORTON, P. & BOWYER, J. R. (1990) Chlorophyll fluorescence transients. IN HARWOOD, J. & BOWYER, J. R. (Eds.) *Methods in Plant Biochemistry.* London, Academic Press.

HU, S., TANG, C. H. & WU, M. (1996) Cadmium accumulation by several seaweeds. *Science of The Total Environment,* 187, 65-71.

HU, S. & WU, M. (1998) Cadmium sequestration in the marine macroalga *Kappaphycus alvarezii. Molecular Marine Biology and Biotechnology,* 7, 97-104.

HUNG, Y.-W. (1982) Effects of temperature and chelating agents on the cadmium uptake in the American oyster. *Bulletin of Environmental Contamination and Toxicology,* 28, 546-551.

HUNT, R. (1982) *Plant Growth Curves,* London, U.K., Edward Arnold Publisher Ltd.

HYV RINEN, M., KOOPMANN, R. & HORMI, O. T., JUHA (2000) Phenols in reproductive and somatic structures of lichens: a case of optimal defence? *Oikos,* 91, 371-375.

INOUHE, M. (2005) Phytochelatins. *Brazilian Journal of Plant Physiology,* 17, 1-24.

JACKIM, E., MORRISON, G. & STEELE, R. (1977) Effects of environmental factors on radiocadmium uptake by four species of marine bivalves. *Marine Biology,* 40, 303-308.

JACKSON, P. J., ROTH, E. J., MCCLURE, P. R. & NARANJO, C. M. (1984) Selection, isolation, and characterization of cadmium-resistant *Datura innoxia* suspension cultures. *Plant Physiol.,* 75, 914-918.

JACKSON, P. J., UNKEFER, C. J., DOOLEN, J. A., WATT, K. & ROBINSON, N. J. (1987) Poly(gamma-glutamylcysteinyl)glycine: its role in cadmium resistance in plant cells. *Proceedings of the National Academy of Sciences of the United States of America,* 84, 6619-6623.

JENSEN, A., RYSTAD, B. & MELSOM, S. (1974) Heavy metal tolerance of marine phytoplankton. 1. Tolerance of three algal species to zinc in coastal sea water. *Journal of Experimental Marine Biology and Ecology,* 15, 145-157.

JERVIS, L., REES-NAESBORG, R. & BROWN, M. T. (1997) Biochemical responses of the marine macroalgae *Ulva lactuca* and *Fucus vesiculosus* to cadmium and copper from sequestration to oxidative stress. *Biochemical Society Transactions,* 25, 63S.

JIM NEZ-ESCRIG, A., JIM NEZ-JIM NEZ, I., PULIDO, R. & SAURA-CALIXTO, F. (2001) Antioxidant activity of fresh and processed edible seaweeds. *Journal of the Science of Food and Agriculture,* 81, 530-534(5).

JIN, X. (1998) Nickel toxicity and mechanisms of nickel resistance in *Scenedesmus acutus* F. *alternans.* Toronto, Ontario, University of Toronto.

JOHNSON, G. N., YOUNG, A. J., SCHOLES, J. D. & HORTON, P. (1993) The dissipation of excess excitation energy in British plant species. *Plant, Cell and Environment,* 16, 673-679.

JOSHI, M. K. & MOHANTY, P. (2004) Chlorophyll a fluorescence as a probe of heavy metal ion toxicity in plants. IN PAPAGEORGIOU, G. C. & GOVINDJEE (Eds.) *Chlorophyll a Fluorescence.* Dordrecht, The Netherlands, Springer.

K GI, J. H. R. (1991) Overview of metallothioncin. *Methods in Enzymology,* 205, 613-626.

K PPER, H., K PPER, F. & MARTIN, S. (1998) In situ detection of heavy metal substituted chlorophylls in water plants. *Photosynthesis Research,* 58, 123-133.

K PPER, H., K PPER, F. & SPILLER, M. (1996) Environmental relevance of heavy metal-substituted chlorophylls using the example of water plants *Journal of Experimental Biology,* 47, 259-266.

K PPER, H. & KRONECK, P. M. H. (2005) Heavy metal uptake by plants and cyanobacteria. IN SIGEL, A., SIGEL, H. & SIGEL, R. K. O. (Eds.) *Metal Ions in Biological Systems. Biogeochemistry, Availability, and Transport of Metals in the Environment.* New York, USA, Marcel Dekker, Inc.

K PPER, H., PARAMESWARAN, A., LEITENMAIER, B., TRTILEK, M. & ŠETL K, I. (2007) Cadmium-induced inhibition of photosynthesis and long-term acclimation to cadmium stress in the hyperaccumulator *Thlaspi caerulescens. New Phytologist,* 175, 655-674.

K PPER, H., ŠETL K, I., SPILLER, M., K PPER, F. C. & PR ŠIL, O. (2002) Heavy metal-induced inhibition of photosynthesis: targets of *in vivo* heavy metal chlorophyll formation. *Journal of Phycology,* 38, 429-441.

KALAJI, H. M. & LOBODA, T. (2007) Photosystem II of barley seedlings under cadmium and lead stress. *Plant, Soil and Environment,* 53, 511-516.

KASTORI, R., PLESNIČAR, M., SAKAČ, Z., PANKOVIČ, D. & ARSENIJEVIČ-MAKSIMOVIČ, I. (1998) Effect of excess lead on sunflower growth and photosynthesis. *Journal of Plant Nutrition,* 21, 75 - 85.

KAUTSKY, H., APPEL, W. & AMANN, H. (1960) Chlorophyllfluorescenz und kohlensaureassimilation. *Biochemische Zeitschrift,* 322, 277-292.

KAUTSKY, L. (1998) Monitoring eutrophication and pollution in estuarine environments - focusing on the use of benthic communities. *Pure and Applied Chemistry,* 70, 2313-2318.

KAWAKAMI, S. K., GLEDHILL, M. & ACHTERBERG, E. P. (2006) Effects of metal combinations on the production of phytochelatins and glutathione by the marine diatom *Phaeodactylum tricornutum. BioMetals,* 19, 51-60.

KENNISH, M. J. (1992) *Ecology of Estuaries: Anthropogenic Effects,* Boca Raton, FL, CRC Press.

KENNISH, M. J. (1996) *Practical Handbook of Estuarine and Marine Pollution,* CRC Press.

KLAPHECK, S., SCHLUNZ, S. & BERGMANN, L. (1995) Synthesis of phytochelatins and homo-phytochelatins in *Pisum sativum* L. *Plant Physiology,* 107, 515-521.

KNAUER, G. A. & MARTIN , J. H. (1973) Seasonal variations of cadmium, copper,manganese, lead, and zinc in water and phytoplankton in Monterey Bay, California. *Limnology and Oceanography,* 18, 597-604.

KNEER, R. & ZENK, M. H. (1992) Phytochelatins protect plant enzymes from heavy metal poisoning. *Phytochemistry,* 31, 2663-2667.

KNIGHT, B. & PARKE, M. (1950) A Biological Study of *Fucus vesiculosus* L. and *F. serratus* L. *Journal of the Marine Biological Association of the United Kingdom,* 26, 439-515.

KNIGHT, H. & KNIGHT, M. R. (2001) Abiotic stress signalling pathways: specificity and cross-talk. *Trends in Plant Science,* 6, 262-267.

KOHN, H. I. & LIVERSEDGE, M. (1944) On a new aerobic metabolite whose production by brain is inhibited by apomorphine, emetine, ergotamine, epinephrine, and menadione. *Journal of Pharmacology And Experimental Therapeutics,* 82, 292-300.

KONDO, N., IMAI, K., ISOBE, M., GOTO, T., MURASUGI, A., WADA-NAKAGAWA, C. & HAYASHI, Y. (1984) Cadystin a and b, major unit peptides comprising cadmium binding peptides induced in a fission yeast - separation, revision of structures and synthesis. *Tetrahedron Letters,* 25, 3869-3872.

KOPPENOL, W. H. (2000) Facts and fiction in free radical biochemistry: The Haber-Weiss Cycle. *Oxygen Society Annual Meeting,.* San Diego, CA.

KRAUSE, G. H. & WEIS, E. (1984) Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals. *Photosynthesis Research,* 5, 139-157.

KRAUSE, G. H. & WEIS, E. (1991) Chlorophyll fluorescence and photosynthesis: The basics. *Annual Review of Plant Physiology and Plant Molecular Biology,* 42, 313-349.

KREMER, B. P. & MARKHAM, J. W. (1982) Primary metabolic effects of cadmium in the brown alga, *Laminaria saccharina. Z. Pflanzenphysiol.,* 108, 125-130.

KRUPA, Z. & BASZYNSKI, T. (1995) Some aspects of heavy metals toxicity towards photosynthetic apparatus - direct and indirect effects on light and dark reactions. *Acta Physiologiae Plantarum,* 17, 177-190.

KRUPA, Z., ÖQUIST, G. & HUNER, N. P. A. (1993) The effects of cadmium on photosynthesis of *Phaseolus vulgaris* - a fluorescence analysis. *Physiologia Plantarum,* 88, 626-630.

KRUPA, Z., ÖQUIST, G. & HUNTER, N. P. A. (1992) The influence of cadmium on primary photosystem II photochemistry in bean as revealed by chlorophyll *a* fluorescence - a preliminary study. *Acta Physiologiae Plantarum,* 14, 71-76.

KRUPA, Z., SZORZYNSKA, E., MAKSYMIEC, W. & BASZYNSKI, T. (1987) Effect of cadmium treatment on the photosynthetic apparatus and its photochemical activities in greening radish seedlings. *Photosynthetica,* 21, 156-164.

L EFFLER, S., HOCHBERGER, A., GRILL, E., WINNACKER, E.-L. & ZENK, M. H. (1989) Termination of the phytochelatin synthase reaction through sequestration of heavy metals by the reaction product. *FEBS Letters,* 258, 42-46.

LANDBERG, T. & GREGER, M. (1994) Can heavy metal tolerant clones of Salix be used as vegetation filters on heavy metal contaminated land? IN ARONSSON, P. & PERTTU, K. (Eds.) *Report from Symposium: Willow Vegetation Filters for Municipal Waste-waters and Sludges — a Biological Purification System.* Uppsala, Sweden, SLU Info/Repro.

LANDBERG, T. & GREGER, M. (1996) Differences in uptake and tolerance to heavy metals in *Salix* from unpolluted and polluted areas. *Applied Geochemistry,* 11, 175-180.

LANDBERG, T. & GREGER, M. (2002) Differences in oxidative stress in heavy metal resistant and sensitive clones of *Salix viminalis. Journal of Plant Physiology,* 159, 69-75.

LANE, T. W. & MOREL, F. M. M. (2000) A biological function for cadmium in marine diatoms. *Proceedings of the National Academy of Sciences of the United States of America,* 97, 4627-4631.

LARSON, R. (1988) The antioxidants of higher plants. *Phytochemistry,* 27, 969-978.

LE TUTOUR, B., BENSLIMANE, F., GOULEAU, M., GOUYGOU, J., SAADAN, B. & QUEMENEUR, F. (1998) Antioxidant and pro-oxidant activities of the brown algae, *Laminaria digitata, Himanthalia elongata, Fucus vesiculosus, Fucus serratus* and *Ascophyllum nodosum. Journal of Applied Phycology,* 10, 121-129.

LEE, J. G., ROBERTS, S. B. & MOREL, F. M. M. (1995) Cadmium: A nutrient for the marine diatom *Thalassiosira weissflogii. Limnology and Oceanography,* 40, 1056-1063.

LEE, M. Y. & SHIN, H. W. (2003) Cadmium-induced changes in antioxidant enzymes from the marine alga *Nannochloropsis oculata. Journal of Applied Phycology,* 15, 13-19.

LEWIS, J. R. (1964) *The Ecology of Rocky Shores,* London, Hodder and Stoughton.

LLESUY, S., EVELSON, P., CAMPOS, A. M. & LISSI, E. (2001) Methodologies for evaluation of total antioxidant activities in complex mixtures. A critical review. *Biological Research,* 34, 51-73.

LOBBAN, C. S. & HARRISON, P. J. (1994) *Seaweed Ecology and Physiology,* Cambridge, University of Cambridge.

LOCATELLI, C. (1999) Sea water quality criteria by bioindicators. Possible classification based on metal contents in *Ulva rigida* and *Tapes philippinarum. Toxicological and Environmental Chemistry,* 71, 435-446.

LUOMA, S. N., BRYAN, G. W. & LANGSTON, W. J. (1982) Scavenging of heavy metals from particulates by brown seaweed. *Marine Pollution Bulletin,* 13, 394-396.

LYNGBY, J. E. (1990) Monitoring of nutrient availability and limitation using the marine macroalga *Ceramium rubrum* (Huds.) C. Ag. *Aquatic Botany,* 38, 153-161.

LYNGBY, J. E. & BRIX, H. (1982) Seasonal and environmental variation in cadmium, copper, lead and zinc concentrations in eelgrass (*Zostera marina* L.) in the Limfjord, Denmark *Aquatic Botany,* 14, 59-74.

MA, M., ZHU, W., WANG, Z. & WITKAMP, G. J. (2003) Accumulation, assimilation and growth inhibition of copper on freshwater alga (*Scenedesmus subspicatus* 86.81 SAG) in the presence of EDTA and fulvic acid. *Aquatic Toxicology,* 63, 221-228.

MACFARLANE, G. R. & BURCHETT, M. D. (2001) Photosynthetic pigments and peroxidase activity as indicators of heavy metal stress in the grey mangrove, *Avicennia marina* (Forsk.) Vierh. *Marine Pollution Bulletin,* 42, 233-240.

MACINNIS-NG, C. M. O. & RALPH, P. J. (2002) Towards a more ecologically relevant assessment of the impact of heavy metals on the photosynthesis of the seagrass, *Zostera capricorni. Marine Pollution Bulletin,* 45, 100-106.

MACRAE, E. A. & FERGUSON, I. B. (1985) Changes in catalase activity and hydrogen peroxide concentration in plants in response to low temperature. *Physiologia Plantarum,* 65, 51-56.

MALEA, P. (1994) Uptake of cadmium and the effect on viability of leaf cells in the seagrass *Halophila stipulacea* (Forsk.) Aschers. *Botanica Marina,* 37, 67-73.

MALEA, P., RIJSTENBIL, J. W. & HARITONIDIS, S. (2006) Effects of cadmium, zinc and nitrogen status on non-protein thiols in the macroalgae *Enteromorpha* spp. from the Scheldt Estuary (SW Netherlands, Belgium) and Thermaikos Gulf (N Aegean Sea, Greece). *Marine Environmental Research,* 62, 45-60.

MALLICK, N. & MOHN, F. H. (2000) Reactive oxygen species: response of algal cells. *Journal of Plant Physiology,* 157, 183-193.

MALLICK, N. & MOHN, F. H. (2003) Use of chlorophyll fluorescence in metal-stress research: a case study with the green microalga *Scenedesmus. Ecotoxicology and Environmental Safety,* 55, 64-69.

MALM, T. & KAUTSKY, L. (2003) Differences in life history characteristics are consistent with the vertical distribution pattern of *Fucus serratus* and *Fucus vesiculosus* (Fucales, Phaeophyceae) in the central Baltic sea. *Journal of Phycology,* 39, 880-887.

MALM, T., KAUTSKY, L. & ENGKVIST, R. (2001) Reproduction, recruitment and geographical distribution of *Fucus serratus* L. in the Baltic Sea. *Botanica Marina,* 44, 101-108.

MANTOURA, R. F. C., DICKSON, A. & RILEY, J. P. (1978) The complexation of metals with humic materials in natural waters. *Estuarine, Coastal and Shelf Science,* 6, 387-408.

MARKERT, B. (1992) Establishing of 'Reference Plant' for inorganic characterization of different plant species by chemical fingerprinting. *Water, Air, & Soil Pollution,* 64, 533-538.

MARKHAM, J. W., KREMER, B. P. & SPERLING, K.-R. (1980a) Cadmium effects on growth and physiology of *Ulva lactuca. Helgolander Meeresuntersuchungen,* 33, 103-110.

MARKHAM, J. W., KREMER, B. P. & SPERLING, K. R. (1980b) Effects of cadmium on *Laminaria saccharina* in culture. *Marine Ecology Progress Series,* 3, 31-39.

MATSUKAWA, R., DUBINSKY, Z., KISHIMOTO, E., MASAKI, K., MASUDA, Y., TAKEUCHI, T., CHIHARA, M., YAMAMOTO, Y., NIKI, E. & KARUBE, I. (1997) A comparison of screening methods for antioxidant activity in seaweeds *Journal of Applied Phycology,* 9, 29-35.

MAXWELL, D. P., FALK, S., TRICK, C. G. & HUNER, N. P. A. (1994) Growth at low temperature mimics high-light acclimation in *Chlorella vulgaris. Plant Physiology,* 105, 535-543.

MAXWELL, K. & JOHNSON, G. N. (2000) Perspectives in experimental botany. Chlorophyll fluorescence - a practical guide. *Journal of Experimental Botany,* 51, 659-668.

MAY, M. J. & LEAVER, C. J. (1993) Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. *Plant Physiology,* 103, 621-627.

MCLEAN, M. W. & WILLIAMSON, P. B. (1977) Cadmium accumulation by the marine red alga *Porphyra umbilicalis. Physiologia Plantarum,* 41, 268-272.

MEHRA, R. K., TARBET, E. B., GRAY, W. R. & WINGE, D. R. (1988) Metal-specific synthesis of two metallothioneins and γ-glutamyl peptides in *Candida glabrata*. *Proceedings of the National Academy of Sciences of the United States of America*, 85, 8815-8819.

MILLER, N. J., RICE-EVANS, C., DAVIES, M. J., GOPINATHAN, V. & MILNER, A. (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science*, 84, 407-412.

MILLWARD, R. N. & GRANT, A. (2000) Pollution-induced tolerance to copper of nematode communities in the severely contaminated Restronguet Creek and adjacent estuaries, Cornwall, United Kingdom. *Environmental Toxicology and Chemistry*, 19, 454–461.

MITTLER, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, 7, 405-410.

MOORE, K. & ROBERTS, L. J. (1998) Measurement of lipid peroxidation. *Free Radical Research*, 28, 659 - 671.

MORALES, F., BELKHODJA, R., ABAD A, A. & ABAD A, J. (2000) Photosystem II efficiency and mechanisms of energy dissipation in iron-deficient, field-grown pear trees (*Pyrus communis* L.). *Photosynthesis Research*, 63, 9-21.

MOREL, F. M. M., REINFELDER, J. R., ROBERTS, S. B., CHAMBERLAIN, C. P., LEE, J. G. & YEE, D. (1994) Zinc and carbon co-limitation of marine phytoplankton. *Nature*, 369, 740-742.

MOREL, F. M. M., RUETER, J. G., ANDERSON, D. M. & GUILLARD, R. R. L. (1979) Aquil: a chemically defined phytoplankton culture medium for trace metal studies. *Journal of Phycology*, 15, 135-141.

MORRIS, A. W. & BALE, A. J. (1975) The accumulation of cadmium, copper, manganese and zinc by *Fucus vesiculosus* in the Bristol Channel. *Estuarine and Coastal Marine Science*, 3, 153-163.

MORRIS, C. A., NICOLAUS, B., SAMPSON, V., HARWOOD, J. L. & KILLE, P. (1999) Identification and characterization of a recombinant metallothionein protein from a marine alga, *Fucus vesiculosus*. *Biochemical Journal*, 338, 553-560.

MURASUGI, A., WADA, C. & HAYASHI, Y. (1981) Cadmium-binding peptide induced in fission yeast, *Schizosaccharomyces pombe*. *The Journal of Biochemistry*, 90, 1561-1565.

MURIEL, P. (1997) Peroxidation of lipids and liver damage. IN BASKIN, S. I. & SALEM, H. (Eds.) *Oxidants, Antioxidants, and Free Radicals*. London, UK, Taylor & Francis Ltd.

MUSE, J. O., CARDUCCI, C. N., STRIPEIKIS, J. D., TUDINO, M. B. & FERNANDEZ, F. M. (2006) A link between lead and cadmium kinetic speciation in seawater and accumulation by the green alga *Ulva lactuca*. *Environmental Pollution*, 141, 126-130.

MYKLEBUST, I. & PEDERSEN, H. C. (1999) Accumulation and distribution of cadmium in willow ptarmigan. *Ecotoxicology*, 8, 457-465.

NAGALAKSHMI, N. & PRASAD, M. N. V. (2001) Responses of glutathione cycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus*. *Plant Science*, 160, 291-299.

NATIONAL RESEARCH COUNCIL CANADA (1979) *Effects of Cadmium in the Canadian Environment*, NRCC/CNRC, Ottawa, Canada.

NICKLESS, G., STENNER, R. & TERRILLE, N. (1972) Distribution of cadmium, lead and zinc in the Bristol Channel. *Marine Pollution Bulletin*, 3, 188-190.

NIELSEN, H. D. (2002) Copper toxicity in the physiology and early development of *Fucus serratus*. *Department of Biological Sciences*. Plymouth, UK, University of Plymouth.

NIELSEN, H. D., BROWN, M. T. & BROWNLEE, C. (2003a) Cellular responses of developing *Fucus serratus* embryos exposed to elevated concentrations of Cu^{2+}. *Plant, Cell & Environment*, 26, 1737-1747.

NIELSEN, H. D., BROWNLEE, C., COELHO, S. M. & BROWN, M. T. (2003b) Inter-population differences in inherited copper tolerance involve photosynthetic adaptation and exclusion mechanisms in *Fucus serratus*. *New Phytologist*, 160, 157-165.

NOCTOR, G. & FOYER, C. H. (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology,* 49, 249-279.

NRIAGU, J. O. (1988) Silent epidemic of environmental metal poisoning *Environmental Pollution,* 50, 139-161.

ÖGREN, E. & SJ STR M, M. (1990) Estimation of the effect of photoinhibition on the carbon gain in leaves of a willow canopy. *Planta,* 181, 560-567.

OKAMOTO, O. K., ASANO, C. S., AIDAR, E. & COLEPICOLO, P. (1996) Effects of cadmium on growth and superoxide dismutase activity of the marine microalga *Tetraselmis gracilis* (Prasinophyceae). *Journal of Phycology,* 32, 74-79.

OKAMOTO, O. K. & COLEPICOLO, P. (2001) Circadian protection against reactive oxygen species involves changes in daily levels of the manganese- and iron-containing superoxide dismutase isoforms in *Lingulodinium polyedrum. Biological Rhythm Research,* 32, 439 - 448.

OKAMOTO, O. K., PINTO, E., LATORRE, L. R., BECHARA, E. J. H. & COLEPICOLO, P. (2001a) Antioxidant modulation in response to metal-induced oxidative stress in algal chloroplasts. *Archives of Environmental Contamination and Toxicology,* 40, 18-24.

OKAMOTO, O. K., ROBERTSON, D. L., FAGAN, T. F., HASTINGS, J. W. & COLEPICOLO, P. (2001b) Different regulatory mechanisms modulate the expression of a dinoflagellate iron-superoxide dismutase. *Journal of Biologica Chemistry,* 276, 19989-19993.

OKAMOTO, O. K., SHAO, L., HASTINGS, J. W. & COLEPICOLO, P. (1999) Acute and chronic effects of toxic metals on viability, encystment and bioluminescence in the dinoflagellate *Gonyaulax polyedra. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology,* 123, 75-83.

OUZOUNIDOU, G. (1993) Changes in variable chlorophyll fluorescence as a result of Cu-treatment dose response relations in *Silene* and *Thlaspi. Photosynthetica,* 29, 455-462.

ÖZT RKA, M., AYDOĞMUŞ-ÖZT RKB, F., DURU, M. E. & TOP UD, G. (2007) Antioxidant activity of stem and root extracts of Rhubarb (*Rheum ribes*): An edible medicinal plant. *Food Chemistry,* 103, 623-630.

PAWLIK-SKOWROŃSKA, B. (2000) Relationships between acid-soluble thiol peptides and accumulated Pb in the green alga *Stichococcus bacillaris. Aquatic Toxicology,* 50, 221-230.

PAWLIK-SKOWROŃSKA, B. (2001) Phytochelatin production in freshwater algae *Stigeoclonium* in response to heavy metals contained in mining water; effects of some environmental factors. *Aquatic Toxicology,* 52, 241-249.

PAWLIK-SKOWROŃSKA, B. (2002) Correlations between toxic Pb effects and production of Pb-induced thiol peptides in the microalga *Stichococcus bacillaris. Environmental Pollution,* 119, 119-127.

PAWLIK-SKOWROŃSKA, B. (2003) When adapted to high zinc concentrations the periphytic green alga *Stigeoclonium tenue* produces high amounts of novel phytochelatin-related peptides. *Aquatic Toxicology,* 62, 155-163.

PAWLIK-SKOWROŃSKA, B., DI TOPPI, L. S., FAVALI, M. A., FOSSATI, F., PIRSZEL, J. & SKOWROŃSKI, T. (2002) Lichens respond to heavy metals by phytochelatin synthesis. *New Phytologist,* 156, 95-102.

PAWLIK-SKOWROŃSKA, B., PIRSZEL, J. & BROWN, M. T. (2007) Concentrations of phytochelatins and glutathione found in natural assemblages of seaweeds depend on species and metal concentrations of the habitat. *Aquatic Toxicology,* 83, 190-199.

PAWLIK-SKOWROŃSKA, B., PIRSZEL, J., KALINOWSKA, R. & SKOWROŃSKI, T. (2004) Arsenic availability, toxicity and direct role of GSH and phytochelatins in As detoxification in the green alga *Stichococcus bacillaris. Aquatic Toxicology,* 70, 201-212.

PAYNE, C. D. & PRICE, N. M. (1999) Effects of cadmium toxicity on growth and elemental composition of marine phytoplankton. *Journal of Phycology,* 35, 293-302.

PERRYMAN, S. A. M. (1996) The effect of heavy metal contamination on estuarine benthic fauna at varying levels of biological organisation. *Department of Biological Sciences.* Plymouth, U.K., University of Plymouth.

PF NDEL, E. (2003) Action of UV and visible radiation on chlorophyll fluorescence from dark-adapted grape leaves (*Vitis vinifera* L.). *Photosynthesis Research,* 75, 29-39.

PHILLIPS, D. J. H. (1994) Macrophytes as biomonitors of trace metals. IN KRAMER, K. J. M. (Ed.) *Biomonitoring of Coastal Waters and Estuaries.* Boca Raton, U.S.A., CRC Press, Inc.

PINTO, E., SIGAUD-KUTNER, T. C. S., LEIT O, M. A. S., OKAMOTO, O. K., MORSE, D. & COLEPICOLO, P. (2003) Heavy metal-induced oxidative stress in algae. *Journal of Phycology,* 39, 1008-1018.

PIRRIE, D., POWER, M. R., ROLLINSON, G., CAMM, G. S., HUGHES, S. H., BUTCHER, A. R. & HUGHES, P. (2003) The spatial distribution and source of arsenic, copper, tin and zinc within the surface sediments of the Fal Estuary, Cornwall, UK. *Sedimentology,* 50, 579-595.

POPOVIC, R., DEWEZ, D. & JUNEAU, P. (2003) Applications of chlorophyll fluorescence in ecotoxicology: heavy metals, herbicides and air pollutants. IN DEELL, J. R. & TOIVONEN, P. M. A. (Eds.) *Practical Applications of Chlorophyll Fluorescence in Plant Biology.* Dordrecht, The Netherlands, Kluwer Academic Publishers.

PRASAD, M. N. V. (1996) *Trace metals,* New York, U.S.A., John Wiley & Sons, Inc.

PRASAD, M. N. V. (1997) Trace metals. IN PRASAD, M. N. V. (Ed.) *Plant Ecophysiology.* New York, Wiley.

PRASAD, M. N. V. (1999) Metallothioneins and metal binding complexes in plants. IN PRASAD, M. N. V. & HAGEMEYER, J. (Eds.) *Heavy metal stress in plants: From molecules to ecosystems.* Heidelberg, Germany, Springer-Verlag.

PRASAD, M. N. V. & STRZAŁKA, K. (1999) Impact of heavy metals on photosynthesis. IN PRASAD, M. N. V. & HAGEMEYER, J. (Eds.) *Heavy metal stress in plants. From molecules to ecosystems.* Heidelberg, Germany, Springer-Verlag.

PRESTON, A. (1973) Cadmium in the marine environment of the United Kingdom *Marine Pollution Bulletin,* 4, 105-107.

PRESTON, A., JEFFRIES, D. F., DUTTON, J. W. R., HARVEY, B. R. & STEELE, A. K. (1972) British Isles coastal waters: The concentrations of selected heavy metals in sea water, suspended matter and biological indicators - A pilot survey. *Environmental Pollution,* 3, 69-82.

PRICE, N. M., HARRISON, G. I., HERING, J. G., HUDSON, R. J., NIREL, P. M. V., PALENIK, B. & MOREL, F. M. M. (1988/89) Preparation and chemistry of the artificial algal culture medium Aquil. *Biological Oceanography,* 6, 443-461.

PRICE, N. M. & MOREL, F. M. M. (1990) Cadmium and cobalt substitution for zinc in a marine diatom. *Nature,* 344, 658-660.

QUATRANO, R. S. (1974) Developmental biology: development in marine organisms. IN MARISCAL, R. (Ed.) *Experimental Marine Biology.* London, Academic Press.

QUATRANO, R. S. (1980) Gamete release, fertilization, and embryogenesis in the Fucales. IN PHYCOLOGICAL SOCIETY OF AMERICA, I. (Ed.) *Handbook of Phycological Methods.* Cambridge, Cambridge University Press.

QUATRANO, R. S. & STEVENS, P. T. (1976) Cell wall assembly in *Fucus* zygotes *Plant Physiology,* 58, 224-231.

R NNBERG, O., ADJERS, K., RUOKOLAHTI, C. & BONDESTAM, M. (1990) *Fucus vesiculosus* as an indicator of heavy metal availability in a fish farm recipient in the northern Baltic Sea. *Marine Pollution Bulletin,* 21, 388-392.

RAGAN, M. A. & GLOMBITZA, K. W. (1986) Phlorotannins, brown algal polyphenols. IN ROUND, F. E. & CHAPMAN, D. J. (Eds.) *Progress in Phycological Research.* Bristol, Biopress Litd.

RAI, L. C., GAUR, J. P. & KUMAR, H. D. (1981) Phycology and heavy-metal pollution. *Biological Reviews,* 56, 99-151.

RAI, L. C., TYAGI, B., MALLICK, N. & RAI, P. K. (1995) Interactive effects of UV-B and copper on photosynthetic activity of the cyanobacterium *Anabaena doliolum. Environmental and Experimental Botany,* 35, 177-185.

RALPH, P. J. & BURCHETT, M. D. (1998) Photosynthetic response of *Halophila ovalis* to heavy metal stress. *Environmental Pollution,* 103, 91-101.

RANDHAWA, V. K., ZHOU, F., JIN, X., NALEWAJKO, C. & KUSHNER, D. J. (2001) Role of oxidative stress and thiol antioxidant enzymes in nickel toxicity and resistance in strains of the green alga *Scenedesmus acutus* f. *alternans. Canadian Journal of Microbiology,* 47, 987-993.

RATKEVICIUS, N., CORREA, J. A. & MOENNE, A. (2003) Copper accumulation, synthesis of ascorbate and activation of ascorbate peroxidase in *Enteromorpha compressa* (L.) Grev. (Chlorophyta) from heavy metal-enriched environments in northern Chile. *Plant, Cell & Environment,* 26, 1599-1608.

RAUSER, W. E. (1987) Compartmental efflux analysis and removal of extracellular cadmium from roots. *Plant Physiology,* 85, 62-65.

RAUSER, W. E. (1990) Phytochelatins. *Annual Review of Biochemistry,* 59, 61-86.

RAUSER, W. E. (1995) Phytochelatins and related peptides. *Plant Physiology,* 109, 1141-1149.

RAUSER, W. E. (1999) Structure and function of metal chelators produced by plants. The case for organic acids, amino acids, phytin, and metallothioneins. *Cell Biochemistry and Biophysics,* 31, 19-48.

RAY, S. & MCLEESE, D. W. (1987) Biological cycling of cadmium in marine environment. IN NRIAGU, J. O. & SPRAGUE, J. B. (Eds.) *Cadmium in the Aquatic Environment.* New York, U.S.A., John Wiley & Sons.

REBHUN, S. & BEN-AMOTZ, A. (1984) The distribution of cadmium between the marine alga *Chlorella stigmatophora* and sea water medium: Effect on algal growth. *Water Research,* 18, 173-178.

REDDY, A. R. & STRASSER, R. J. (2000) Probing the vitality of plants by the JIP-test, a novel non-invasive phenotypic screening technique for performance under water-limited conditions *Workshop on Molecular Approaches for the genetic Improvement of Cereals for Stable Production in Water-Limited Environments. A Strategic Planning Workshop, A Strategic Planning Workshop.* El Batan, Mexico, CIMMYT, International Maize and Wheat Improvement Center.

REDDY, G. N. & PRASAD, M. N. V. (1990) Heavy metal-binding proteins/peptides: Occurrence, structure, synthesis and functions. A review. *Environmental and Experimental Botany,* 30, 251-264.

REED, R. H. & GADD, G. M. (1990) Metal tolerance in eukaryotic and prokaryotic algae. IN SHAW, A. J. (Ed.) *Heavy Metal Tolerance in Plants: Evolutionary Aspects.* Boca Raton, Florida, CRC Press Inc.

REES, T. K. (1932) A note on the logevity of certain species of the Fucaceae. *Annals of Botany,* 46, 1062-1064.

RENGEL, Z. (1992) Role of calcium in aluminium toxicity. *New Phytologist,* 121, 499-513.

RICE-EVANS, C. A., DIPLOCK, A. T. & SYMONS, M. C. R. (1991a) Assay of antioxidant nutrients and antioxidant enzymes. IN RICE-EVANS, C. A., DIPLOCK, A. T. & SYMONS, M. C. R. (Eds.) *Techniques in Free Radical Research.* 1 ed. Amsterdam, the Netherlands, Elsevier Science Publishers BV.

RICE-EVANS, C. A., DIPLOCK, A. T. & SYMONS, M. C. R. (1991b) Investigation of the consequences of free radical attack on lipids. IN RICE-EVANS, C. A., DIPLOCK, A. T. & SYMONS, M. C. R. (Eds.) *Techniques in Free Radical Research.* 1 ed. Amsterdam, the Netherlands, Elsevier Science Publishers BV.

RIJSTENBIL, J. W. (2002) Assessment of oxidative stress in the planktonic diatom *Thalassiosira pseudonana* in response to UVA and UVB radiation. *Journal of Plankton Research,* 24, 1277-1288.

RIJSTENBIL, J. W., DEHAIRS, F., EHRLICH, R. & WIJNHOLDS, J. A. (1998a) Effect of the nitrogen status on copper accumulation and pools of metal-binding peptides in the planktonic diatom *Thalassiosira pseudonana. Aquatic Toxicology,* 42, 187-209.

RIJSTENBIL, J. W., DERKSEN, J. W. M., GERRINGA, L. J. A., POORTVLIET, T. C. W., SANDEE, A., VAN DEN BERG, M., VAN DRIE, J. & WIJNHOLDS, J. A. (1994) Oxidative stress induced by copper: defense and damage in the marine planktonic diatom *Ditylum brightwellii,* grown in continuous cultures with high and low zinc levels *Marine Biology,* 119, 583-590.

RIJSTENBIL, J. W., HARITONIDIS, S., MALEA, P., SEFERLIS, M. & WIJNHOLDS, J. A. (1998b) Thiol pools and glutathione redox ratios as possible indicators of copper toxicity in the green macroalgae

Enteromorpha spp. from the Scheldt Estuary (SW Netherlands, Belgium) and Thermaikos Gulf (Greece, N Aegean Sea). *Hydrobiologia*, 385, 171-181.

ROBINSON, N. J. (1989) Algal metallothioneins: secondary metabolites and proteins. *Journal of Applied Phycology*, 1, 5-18.

ROMANOWSKA-DUDA, B., KALAJI, M. H. & STRASSER, R. J. (2005) The use of PSII activity of *Spirodela oligorrhiza* plants as an indicator for water toxicity. IN VAN DER EST A., B. (Ed.) *Photosynthesis: Fundamental Aspects to Global Perspectives.* Lawrence, Allen Press.

RUSTERUCCI, C., MONTILLET, J.-L., AGNEL, J.-P., BATTESTI, C., ALONSO, B., KNOLL, A., BESSOULE, J.-J., ETIENNE, P., SUTY, L., BLEIN, J.-P. & TRIANTAPHYLIDES, C. (1999) Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. *Journal of Biological Chemistry*, 274, 36446-36455.

SALGADO, L. T., ANDRADE, L. R. & FILHO, G. M. A. (2005) Localization of specific monosaccharides in cells of the brown alga *Padina gymnospora* and the relation to heavy-metal accumulation. *Protoplasma*, 225, 123-128.

SARKAR, R. K., PANDA, D., RAO, D. N. & SHARMA, S. G. (2004) Chlorophyll fluorescence parameters as indicators of submergence tolerance in rice. *Crop Management & Physiology.*

SAY, P. J., BURROWS, I. G. & WHITTON, B. A. (1990) *Enteromorpha* as a monitor of heavy metals in estuaries. *Hydrobiologia*, 195, 119-126.

SCH FER, H. J., HAAG-KERWER, A. & RAUSCH, T. (1998) cDNA cloning and expression analysis of genes encoding GSH synthesis in roots of the heavy-metal accumulator *Brassica juncea* L.: evidence for Cd-induction of a putative mitochondrial γ-glutamylcysteine synthetase isoform. *Plant Molecular Biology*, 37, 87-97.

SCH NER, S. & KRAUSE, H. G. (1990) Protective systems against active oxygen species in spinach: response to cold acclimation in excess light. *Planta*, 180, 383-389.

SCHACHTSCHABEL, P., BLUME, H.-P., HARTGE, K.-H. & SCHWERTMANN, U. (1984) *Lehrbuch der Bodenkunde*, Stuttgart, Ferdinand Enke Verlag.

SCHAT, H., LLUGANY, M., VOOIJS, R., HARTLEY-WHITAKER, J. & BLEEKER, P. M. (2002) The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. *Journal of Experimental Botany*, 53, 2381-2392.

SCHELLER, H. V., HUANG, B., HATCH, E. & GOLDSBROUGH, P. B. (1987) Phytochelatin synthesis and glutathione levels in response to heavy metals in tomato cells. *Plant Physiol.*, 85, 1031-1035.

SCHREIBER, U., BILGER, W., HARMAN, H. & NEUBAUER, C. (1998) Chlorophyll fluorescence as a diagnostic tool: basics and some aspects of practical relevance. IN RAGHAVENDRA, A. S. (Ed.) *Photosynthesis: a comprehensive treatise.* Cambridge, Cambridge University Press

SENEVIRATHNE, M., KIM, S.-H., SIRIWARDHANA, N., HA, J.-H., LEE, K.-W. & JEON, Y.-J. (2006) Antioxidant potential of *Ecklonia cavaon* on reactive oxygen species scavenging, metal chelating, reducing power and lipid peroxidation inhibition. *Food Science and Technology International*, 12, 27-38.

SGARDELIS, S., COOK, C. M., PANTIS, J. D. & LANARAS, T. (1994) Comparison of chlorophyll fluorescence and some heavy metal concentrations in *Sonchus* spp. and *Taraxacum* spp. along an urban pollution gradient. *The Science of The Total Environment*, 158, 157-164.

SHARMA, P. & DUBEY, R. S. (2005) Lead toxicity in plants. *Brazilian Journal of Plant Physiology*, 17, 35-52.

SHIOI, Y., TAMAI, H. & SASA, T. (1978) Effects of copper on photosynthetic electron transport systems in spinach chloroplasts. *Plant & Cell Physiology*, 19, 203-209.

SIES, H. (1999) Glutathione and its role in cellular functions. *Free Radical Biology and Medicine*, 27, 916-921.

SIMKISS, K. & TAYLOR, M. G. (1995) Transport of metals across membranes. IN TESSIER, A. & TURNER, D. R. (Eds.) *Metal Speciation and Bioavailability in Aquatic Systems.* 1 ed. Chichester, John Wiley & Sons Ltd.

SIMPSON, W. R. (1981) A critical review of cadmium in the marine environment. *Progr. Oceanogr.*, 10, 1-70.

SINNHUBER, R. O., YU, T. C. & YU, T. C. (1958) Characterization of the red pigment formed in the 2-thiobarbituric acid determination of oxidative rancidity. *Journal of Food Science*, 23, 626-634.

SKORZYNSKA-POLIT, E. & BASZYNSKI, T. (1997) Differences in sensitivity of the photosynthetic apparatus in Cd-stressed runner bean plants in relation to their age. *Plant science*, 128, 11-21.

SKOWROŃSKI, T. (1986) Influence of some physico-chemical factors on cadmium uptake by the green alga *Stichococcus bacillaris*. *Applied Microbiology and Biotechnology*, 24, 423-425.

SKOWROŃSKI, T., DE KNECHT, J. A., SIMONS, J. & VERKLEIJ, J. A. C. (1998) Phytochelatin synthesis in response to cadmium uptake in *Vaucheria* (Xanthophyceae). *European Journal of Phycology*, 33, 87-91.

SMIRNOFF, N. (1995) Antioxidant systems and plant response to the environment. IN SMIRNOFF, N. (Ed.) *Environment and plant metabolism*. Oxford, Bios Scientific Publishers.

SMITH, I. K., KENDALL, A. C., KEYS, A. J., TURNER, J. C. & LEA, P. J. (1984) Increased levels of glutathione in a catalase-deficient mutant of barley (*Hordeum vulgare* L.). *Plant Science Letters*, 37, 29-33.

SMITH, I. K., KENDALL, A. C., KEYS, A. J., TURNER, J. C. & LEA, P. J. (1985) The regulation of the biosynthesis of glutathione in leaves of barley (*Hordeum vulgare* L.). *Plant Science*, 41, 11-17.

SMITH, K. L., HANN, A. C. & HARWOOD, J. L. (1986) The subcellular localisation of absorbed copper in *Fucus*. *Physiologia Plantarum*, 66, 692-698.

SOARES, J. R., DINIS, T. C. P., CUNHA, A. P. & ALMEIDA, L. M. (1997) Antioxidant activities of some extracts of *Thymus zygis*. *Free Radical Research*, 26, 469 - 478.

SOKAL, R. R. & ROHLF, F. J. (1995) *Biometry : the principles and practice of statistics in biological research*, New York ; Oxford, Freeman.

SOMASHEKARAIAH, B. V., PADMAJA, K. & PRASAD, A. R. K. (1992) Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseolus vulgaris*): Involvement of lipid peroxides in chlorphyll degradation. *Physiologia Plantarum*, 85, 85-89.

SOMERFIELD, P. J., MICHAEL GEE, J. & WARWICK, R. M. (1994) Benthic community structure in relation to an instantaneous discharge of waste water from a tin mine. *Marine Pollution Bulletin*, 28, 363-369.

STAUBER, J. L. & FLORENCE, T. M. (1987) Mechanism of toxicity of ionic copper and copper complexes to algae. *Marine Biology*, 94, 511-519.

STEFFENS, J. C. (1990) The heavy metal-binding peptides of plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 41, 553-75.

STEFFENS, J. C., HUNT, D. F. & WILLIAMS, B. G. (1986) Accumulation of non-protein metal-binding polypeptides (gamma-glutamyl- cysteinyl)n-glycine in selected cadmium-resistant tomato cells. *J. Biol. Chem.*, 261, 13879-13882.

STENGEL, D. B. & DRING, M. J. (2000) Copper and iron concentrations in *Ascophyllum nodosum* (Fucales, Phacophyta) from different sites in Ireland and after culture experiments in relation to thallus age and epiphytism. *Journal of Experimental Marine Biology and Ecology*, 246, 145-161.

STENGEL, D. B., MACKEN, A., MORRISON, L. & MORLEY, N. (2004) Zinc concentrations in marine macroalgae and a lichen from western Ireland in relation to phylogenetic grouping, habitat and morphology. *Marine Pollution Bulletin*, 48, 902-909.

STENNER, R. D. & NICKLESS, G. (1974) Distribution of some heavy metals in organisms in Hardangerfjord and Skjerstadfjord, Norway. *Water, Air, & Soil Pollution*, 3, 279-291.

STIBOROVA, M., DOUBRAVOVA, M., BREZINOVA, A. & FRIEDRICH, A. (1986) Effect of heavy metal ions on growth and biochemical characteristics of photosynthesis of barley (*Hordeum vulgare* L.). *Photosynthetica*, 20, 418-425.

STOBART, A. K., GRIFFITHS, W. T., AMEEN-BUKHARI, I. & SHERWOOD, R. P. (1985) The effect of Cd^{2+} on the biosynthesis of chlorophyll in leaves of barley. *Physiologia Plantarum*, 63, 293-298.

STR MGREN, T. (1979) The effect of copper on length increase in *Ascophyllum nodosum* (L.) Le Jolis. *Journal of Experimental Marine Biology and Ecology,* 37, 153-159.

STR MGREN, T. (1980) The effect of lead, cadmium, and mercury on the increase in length of five intertidal fucales. *Journal of Experimental Marine Biology and Ecology,* 43, 107-119.

STRASSER, B. J. & STRASSER, R. J. (1995) Measuring fast fluorescence transients to address environmental questions: the JIP-test. IN MATHIS, P. (Ed.) *Photosynthesis: From Light to Biosphere. Proceedings of the Xth International Photosynthesis Congress.* Montpellier, France, Kluwer Academic Publishers, Dordrecht, The Netherlands.

STRASSER, R. J., SRIVASTAVA, A. & GOVINDJEE (1995) Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. *Photochemistry and Photobiology,* 61, 32-42.

STRASSER, R. J., SRIVASTAVA, A. & TSIMILLI-MICHAEL, M. (2000) The fluorescence transient as a tool to characterize and screen photosynthetic samples. IN M. YUNUS, U. P., P. MOHANTY (EDS.) (Ed.) *Probing Photosynthesis: Mechanisms, Regulation and Adaptation.* London, GB, Taylor & Francis.

STRASSER, R. J., TSIMILLI-MICHAEL, M. & SRIVASTAVA, A. (2004) Analysis of the chlorophyll *a* fluorescence transient. IN GOVINDJEE (Ed.) *Chlorophyll a Fluorescence.* Dordrecht, The Netherlands, Springer.

SUGGETT, D. J., LE FLOC'H, E., HARRIS, G. N., LEONARDOS, N. & GEIDER, R. J. (2007) Differnet strategies of photoacclimation by two strains of *Emiliania huxley* (Haptophyta). *Journal of Phycology,* 43, 1209-1222.

SUJAK, A. (2005) Interaction between cadmium, zinc and silver-substituted plastocyanin and cytochrome b_6f complex — heavy metals toxicity towards photosynthetic apparatus. *Acta Physiologiae Plantarum,* 27, 61-69.

TALARICO, L. L. (2002) Fine structure and X-ray microanalysis of a red macrophyte cultured under cadmium stress. *Environmental Pollution,* 120, 813-821.

TANG, D., HUNG, C.-C., WARNKEN, K. W. & SANTSCHI, P. H. (2000) The distribution of biogenic thiols in surface waters of Galveston Bay. *Limnology and Oceanography,* 45, 1289–1297

TANG, J. Y., ZIELINSKI, R. E., ZANGERL, A. R., CROFTS, A. R., BERENBAUM, M. R. & DELUCIA, E. H. (2006) The differential effects of herbivory by first and fourth instars of *Trichoplusia ni* (Lepidoptera: Noctuidae) on photosynthesis in *Arabidopsis thaliana. Journal of Experimental Botany,* 57, 527-536.

TARGETT, N. M., BOETTCHER, A. A., TARGETT, T. E. & VROLIJK, N. H. (1995) Tropical marine herbivore assimilation of phenolic-rich plants. *Oecologia,* 103, 170-179.

TAYLOR, A. C. (1977) The respiratory responses of *Carcinus maenas* (L.) to changes in environmental salinity *Journal of Experimental Marine Biology and Ecology,* 29, 197-210.

TEIXEIRA, F. K., MENEZES-BENAVENTE, L., GALV O, V. C. & MARGIS-PINHEIRO, M. (2005) Multigene families encode the major enzymes of antioxidant metabolism in *Eucalyptus grandis* L. *Genetics and Molecular Biology,* 28, 529-538.

THEEDE, H., SCHOLZ, N. & FISCHER, H. (1977) Temperature and salinity effects on the acute toxicity of cadmium to *Laomedea loveni* (hydrozoa). *Marine Ecology Progress Series,* 1, 13-19.

THOMA, I., LOEFFLER, C., SINHA, A. K., GUPTA, M., KRISCHKE, M., STEFFAN, B., ROITSCH, T. & MUELLER, M. J. (2003) Cyclopentenone isoprostanes induced by reactive oxygen species trigger defense gene activation and phytoalexin accumulation in plants. *The Plant Journal,* 34, 363-375.

THUMANN, J., GRILL, E., WINNACKER, E.-L. & ZENK, M. H. (1991) Reactivation of metal-requiring apoenzymes by phytochelatin--metal complexes. *FEBS Letters,* 284, 66-69.

TOKARNIA, C. H., DOBEREINER, J. & MORAES, S. S. (1999) Mineral deficiencies and imbalances in cattle and sheep - a review of Brazilian studies made between 1987 and 1998. *Pesquisa Veterinária Brasileira,* 19, 47-62.

TOMSETT, A. B. & THURMAN, D. A. (1988) Molecular biology of metal tolerances of plants. *Plant, Cell and Environment,* 11, 383-394.

TOPCUOĞLU, S., G VEN, K. C., BALKıS, N. & KıRBAŞOĞLU, Ç. (2003) Heavy metal monitoring of marine algae from the Turkish Coast of the Black Sea, 1998-2000. *Chemosphere,* 52, 1683-1688.

TORRICELLI, E., GORBI, G., PAWLIK-SKOWROŃSKA, B., DI TOPPI, L. S. & CORRADI, M. G. (2004) Cadmium tolerance, cysteine and thiol peptide levels in wild type and chromium-tolerant strains of *Scenedesmus acutus* (Chlorophyceae). *Aquatic Toxicology,* 68, 315-323.

TRIPATHY, B. C. & MOHANTY, P. (1980) Zinc-inhibited electron transport of photosynthesis in isolated barley chloroplasts. *Plant Physiology,* 66, 1174-1178.

TSIMILLI-MICHAEL, M., EGGENBERG, P., BIRO, B., K VES-PECHY, K., V R S, I. & STRASSER, R. J. (2000) Synergistic and antagonistic effects of arbuscular mycorrhizal fungi and *Azospirillum* and *Rhizobium* nitrogen-fixers on the photosynthetic activity of alfalfa, probed by the polyphasic chlorophyll a fluorescence transient O-J-I-P. *Applied Soil Ecology,* 15, 169-182.

TSIMILLI-MICHAEL, M., KR GER, G. H. J. & STRASSER, R. J. (1995) Suboptimality as driving force for adaptation: a study about the correlation of excitation light intensity and the dynamic fluorescence emission in plants. IN MATHIS, P. (Ed.) *Photosynthesis: from Light to Biosphere.* Dordrecht, Kluwer Academic Publishers.

TSIMILLI-MICHAEL, M., KRÜGER, G. H. J. & STRASSER, R. J. (1996) About the perpetual state changes in plants approaching harmony with their environment. *Archives des Sciences et Compte Rendu Seances de la Societe,* 49, 173-203.

TSIMILLI-MICHAEL, M. & STRASSER, R. J. (2003) Screening of plant tolerance to soil and water pollutants by analysing the polyphasic chlorophyll *a* fluorescence transient O-J-I-P. IN 837, C. A. (Ed.) *Plant biotechnology for the removal of organic pollutants and toxic metals from wastewaters and contaminated sites*

TSUJI, N., HIRAYANAGI, N., IWABE, O., NAMBA, T., TAGAWA, M., MIYAMOTO, S., MIYASAKA, H., TAKAGI, M., HIRATA, K. & MIYAMOTO, K. (2003) Regulation of phytochelatin synthesis by zinc and cadmium in marine green alga, *Dunaliella tertiolecta. Phytochemistry,* 62, 453-459.

TSUJI, N., HIRAYANAGI, N., OKADA, M., MIYASAKA, H., HIRATA, K., ZENK, M. H. & MIYAMOTO, K. (2002) Enhancement of tolerance to heavy metals and oxidative stress in *Dunaliella tertiolecta* by Zn-induced phytochelatin synthesis. *Biochemical and Biophysical Research Communications,* 293, 653-659.

VALLEE, B. L. & ULMER, D. D. (1972) Biochemical Effects of Mercury, Cadmium, and Lead *Annual Review of Biochemistry,* 41, 91-128.

VAN ASSCHE, F., CARDINAELS, C. & CLIJSTERS, H. (1988) Induction of enzyme capacity in plants as a result of heavy metal toxicity: Dose-response relations in *Phaseolus vulgaris* L., treated with zinc and cadmium *Environmental Pollution,* 52, 103-115.

VAN ASSCHE, F. & CLIJSTERS, H. (1990) Effects of metals on enzyme activity in plants. *Plant, Cell and Environment,* 13, 195-206.

VAN KOOTEN, O. & SNELL, J. F. H. (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynthesis Research,* 25, 147-150.

VASCONCELOS, M. T. S. D. & LEAL, M. F. C. (2001) Seasonal variability in the kinetics of Cu, Pb, Cd and Hg accumulation by macroalgae. *Marine Chemistry,* 74, 65-85.

VASSILEV, A. & MANOLOV, P. (1999) Chlorophyll fluorescence of barley (*H. vulgare* L.) seedlings grown in excess of Cd. *Bulgarian Journal of Plant Physiology,* 25, 67-76.

VATAMANIUK, O. K., BUCHER, E. A., WARD, J. T. & REA, P. A. (2001) A new pathway for heavy metal detoxification in animals. Phytochelatin synthase is required for cadmium tolerance in *Caenorhabditis elegans. Journal of Biological Chemistry,* 276, 20817-20820.

VATAMANIUK, O. K., MARI, S., LU, Y.-P. & REA, P. A. (1999) $AtPCS_1$, a phytochelatin synthase from *Arabidopsis*: Isolation and in vitro reconstitution. *Proceedings of the National Academy of Sciences of the United States of America,* 96, 7110-7115.

VITORIA, A. P., LEA, P. J. & AZEVEDO, R. A. (2001) Antioxidant enzymes responses to cadmium in radish tissues. *Phytochemistry,* 57, 701-710.

WAHBEH, M. I. (1984) Levels of zinc, manganese, magnesium, iron and cadmium in three species of seagrass from Aqaba (Jordan) *Aquatic Botany,* 40, 179-183.

WANG, K.-J., ZHANG, Y.-J. & YANG, C.-R. (2005) Antioxidant phenolic compounds from rhizomes of *Polygonum paleaceum. Journal of Ethnopharmacology,* 96, 483-487.

WATANABE, T., MACHIDA, K., SUZUKI, H., KOBAYASHI, M. & HONDA, K. (1985) Photoelectrochemistry of metallochlorophylls. *Coordination Chemistry Reviews,* 64, 207-224.

WEI, L., DONAT, J. R., FONES, G. & AHNER, B. A. (2003) Interactions between Cd, Cu, and Zn influence particulate phytochelatin concentrations in marine phytoplankton: laboratory results and preliminary field data. *Environmental Science and Technology,* 37, 3609-3618.

WHITTON, B. A., BURROWS, I. G. & KELLY, M. G. (1989) Use of *Cladophora glomerata* to monitor heavy metals in rivers *Journal of Applied Phycology,* 1, 293-299.

WIERZBICKA, M. (1999) Comparison of lead tolerance in *Allium cepa* with other plant species. *Environmental Pollution,* 104, 41-52.

WIKFORS, G. H. & UKELES, R. (1982) Growth and adaptation of estuarine unicellular algae in media with excess copper, cadmium or zinc, and effects of metal-contaminated algal food on *Crassostrea virginica* larvae. *Marine Ecology Progress Series,* 7, 191-206

WILLIAMS, G. A. (1996) Seasonal variation in a low shore *Fucus serratus* (Fucales, Phaeophyta) population and its epiphytic fauna. *Hydrobiologia,* 326/327, 191-197.

WRIGHT, D. A. (1977a) The Effect of salinity on cadmium uptake by the tissues of the shore crab *Carcinus maenas. Journal of Experimental Biology,* 67, 137-146.

WRIGHT, D. A. (1977b) The uptake of cadmium into the haemolymph of the shore crab *Carcinus maenas*: the relationship with copper and other divalent cations. *Journal of Experimental Biology,* 67, 147-161.

XIA, J. R., LI, Y. J., LU, J. & CHEN, B. (2004) Effects of copper and cadmium on growth, photosynthesis, and pigment content in *Gracilaria lemaneiformis. Bulletin of Environmental Contamination and Toxicology,* 73, 979-986.

XU, P., ZOU, J., MENG, Q., ZOU, J., JIANG, W. & LIU, D. (2008) Effects of Cd^{2+} on seedling growth of garlic (*Allium sativum* L.) and selected physiological and biochemical characters. *Bioresource Technology,* 99, 6372-6378.

YAN, X., NAGATA, T. & FAN, X. (1998) Antioxidative activities in some common seaweeds. *Plant Foods for Human Nutrition (Formerly Qualitas Plantarum),* 52, 253-262.

YOUNGER, P. L. (2002) Mine water pollution from Kernow to Kwazulu-Natal: geochemical remedial options and their selection in practice. *Geoscience in south-west England,* 10, 255-266.

YRUELA, I., ALFONSO, M., BAR N, M. & PICOREL, R. (2000) Copper effect on the protein composition of photosystem II. *Physiologia Plantarum,* 110, 551-557.

YU, L. W., LATRIANO, L., DUNCAN, S., A., H. R. & WITZ, G. (1986) High-performance liquid chromatography analysis of the thiobarbituric acid adducts of malonaldehyde and *trans,trans*-muconaldehyde *Analytical Biochemistry,* 156, 326-333.

ZANGERL, A. R., HAMILTON, J. G., MILLER, T. J., CROFTS, A. R., OXBOROUGH, K., BERENBAUM, M. R. & DE LUCIA, E. H. (2002) Impact of folivory on photosynthesis is greater than the sum of its holes. *Proceedings of the National Academy of Sciences of the United States of America,* 99, 1088-1091.

ZENK, M. H. (1996) Heavy metal detoxification in higher plants - a review. *Gene,* 179, 21-30.

ZHANG, H., XU, W., GUO, J., HE, Z. & MA, M. (2005) Coordinated responses of phytochelatins and metallothioneins to heavy metals in garlic seedlings. *Plant Science,* 169, 1059-1065.

ZUTSHI, S., CHOUDHARY, M., BHARAT, N., ABDIN, M. Z. & FATMA, T. (2008) Evaluation of antioxidant defense responses to lead stress in *Hapaloshiphon fontinalis. Journal of Phycology,* 44, 889-896.

APPENDICES

APX	ascorbate peroxidase
BQ	Bantham Quay
BSO	L-buthionine-[S,R]-sulphoximine
CAT	catalase
Cd	cadmium
Chl	chlorophyll
Cu	copper
CUPRAC	copper reducing antioxidant capacity
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picryl-hydrazil
FMS	fluorescence monitoring system
Fx	fucoxanthin
GLM	general linear model in SPSS
GR	glutathione reductase
GSH	reduced glutathione
h-GSH	homo glutathione
h-PC	homo phytochelatin
kD	kiloDalton
LHC	light harvesting complex
MDA	malondialdehyde
MT	metallothionein
OEC	oxygen evolution complex
PC	phytochelatin
PEA	plant efficiency analyser
PFD	photon flux density
PS	photosystem
QA	plastoquinone A
RGR	relative growth rate
ROS	reactive oxygen species
RP	Restronguet Point
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid-reactive substances
TCA	trichloroacetic acid
TWCA1	an isoform of carbonic anhydrase (CA) in the marine diatom *Thalassiosira weissflogii*