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Physiological responses of *Fucus serratus* (Phaeophyceae) to high doses of cadmium exposure

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Growth, oxidative stress and antioxidant capacity of *Fucus serratus* exposed to high doses of Cd were examined. Two sites in Southwest England (Restronguet Point and Bantham Quay) were selected since they had different histories of metal contamination. 1~10 mg Cd L^{-1} were treated to Aquil medium for up to 14 days. Similar levels of lipid peroxidation but different values of relative growth rates, cupric ion reducing antioxidant capacity (CUPRAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity indicated that *F. serratus* has population-dependent antioxidant strategies. *F. serratus* demonstrated cadmium resistance with no visual symptoms for 14 days and the population from the polluted area seemed to have more powerful antioxidant strategies. However *Fucus* from the conserved area also showed protective antioxidative mechanism.

Key words : *Fucus serratus*, Cadmium, Antioxidant response, Restronguet Point, Bantham Quay

Metals are known to encourage various physiological responses in plants and algae, of which chlorosis, cell lysis, necrosis, discoloration, encystment and death can be counted as some of visible signs (Hu *et al.*, 1996, Okamoto *et al.*, 1999, Küpper *et al.*, 2002, Collén *et al.*, 2003). Besides, as intracellular symptoms of metal stress, oxidative damage of proteins, lipids and DNA was reported (Asada and Takahashi, 1987, Collén and Davison, 1999b, Halliwell and Gutteridge, 2007). Metals are also able to induce reactive oxygen species (ROS) (Collén and Davison, 1999a, Dummermuth *et al.*, 2003, Güçlü *et al.*, 2006). ROS are obligatory by-products of common aerobic metabolism, such as photosynthesis and respiration (Noctor and Foyer, 1998, Collén and Davison, 1999a), however over-production of ROS and imbalance of cellular oxidative status can be fatal to organisms (Rijstenbil *et al.*, 1998, Okamoto *et al.*, 2001a, Okamoto *et al.*, 2001b, Collén *et al.*, 2003).

To reduce the harmful effects of metal exposure, plants and algae induces antioxidants and antioxidant enzymes (Van Assche and Clijsters, 1990, Pinto *et al.*, 2003). Contreras et al. (2005) reported the increased ROS levels in *Scytosiphon lomentaria* from mine wastes and the high levels of antioxidative enzymes against oxidative stress. However, our knowledge on antioxidant mechanism of macroalgae is relatively limited (Collén and Davison, 1999a, b, c) and most studies were focused on terrestrial higher plants to date (Ratkevicius et al., 2003).

Various macroalgae have been used to investigate their antioxidant capacity and their absorption/adsorption ability. Hashim and Chu (2004) reported that brown algae

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showed the highest Langmuir maximum adsorption ability among seven different species of brown (*Sargassum siliquosum, S. baccularia* and *Padina tetrastomatica*), green (*Chaetomorpha linum*) and red algae (*Gracilaria changii, G. edulis* and *G. salicornia*). This enhanced performance of brown algae is based on their basic structure and biochemical constitution, which is closely linked to the external/internal constituents of cell wall and antioxidant capacity (Hu *et al.*, 1996, Davis *et al.*, 2003). Although *Fucus* and *Sargassum* are known as bioindicators, *F. serratus* is not often used compared to other *Fucus* species (Förstner and Wittmann, 1983, Bryan *et al.*, 1985). Since *F. serratus* thrives in both of polluted and conserved areas in the UK, the investigation of antioxidant capacity of *F. serrutus* can reveal a relationship between metal contamination and abundant species.

Therefore, the aims of this study were to examine the oxidative stress and antioxidant capacities of *F. serratus* collected from different populations exposed at different levels of cadmium in seawater.

Material and methods

Fucus serratus was collected from two sites with different history of metal contamination (Fig. 1). Bantham Quay (BQ; 266710OE, 43795N), at the mouth of the river Avon, has been known as one of the least polluted coastal areas in the UK in which 18 μg g^{-1} of total Cu was measured in sediment (Table 1). On the contrary, Restronguet Point (RP; 18150OE, 37435N), the entrance to Restronguet Creek, is a part of the most metal-contaminated area in the Fal Estuary (Pirrie et al., 2003). In this estuary, hard rock mining had prevailed from the 13th century (Gerrard, 2000), and drainage from the old mine adits and erosion of the spoil heaps by water continues (Bryan and Gibbs, 1983, Pirrie *et al.*, 2003).

Culture conditions were maintained for 14 days 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. Culture tanks containing artificial culture medium, Aquil, were mixed and aerated by shakers (60 rpm) for 24 h.

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

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

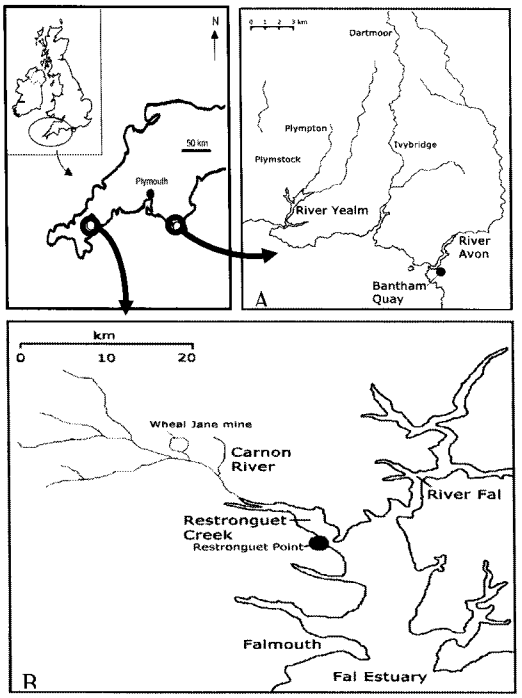

Fig. 1. Two target sites in which *Fucus serratus* was collected. A, Bantham Quay; B, Restronguet Point, South West England.

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

Lipid peroxidaton using 10% trichloroacetic acid (TCA) and 0.6% thiobarbituric acid (TBA) was followed by Lee (2009). The concentration of malondialdehyde (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, 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.

Table 1. Concentrations of metals in sediments and water column of sampling area

Metals	Water (μg L^{-1})	Sediment (μg g^{-1} D.W.)	
	Restronguet Creek	Restronguet Creek	Avon Estuary
Ag		3.76	0.06
As		1740	13.0
Cd	<0.1-38	1.53	0.08
Co		21	10
Cr		32	28
Cu	2-176	2398	18
Fe		49071	18361
Hg		0.46	0.12
Mn	3 - 1513	485	326
Ni	1 - 18	58	23
Pb	<2 - 4	341	68
Sn		55.9	3.9
Zn	7 - 22460	2821	82
Reference	Bryan and Gibbs (1983)	Bryan and Langston (1992)	

For cupric ion reducing antioxidant capacity (CUPRAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity analysis, extraction of polyphenols was preceded. Harvested thalli were thoroughly rinsed with running tap water to remove salt and remaining Cd on the surface of thalli 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 ℃, in the dark, in a shaking incubator (60 rpm) for 24 hr, followed by centrifugal at 6,500 g for 10 min (25 ℃). These polyphenol extracts were stored for the following analyses for antioxidant capacity.

CUPRAC of *F. serratus* was examined according to Apak et al. (2007). Total antioxidant capacity of *F. serratus* was expressed as trolox equivalent antioxidant capacity (TEAC).

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

where A_f is the absorbance, ε_{TR} is the molar absorptivity of Trolox (1.67×10^4 L mol^{-1} cm^{-1}), V_f is the final volume of mixture, 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.

Free radical scavenging activity of Cd-exposed *F. serratus* was assessed by the DPPH free-radical method (Connan et al., 2006). Lower absorbance of the mixture indicates a higher free radical scavenging activity (Oztürka et al., 2007). The DPPH free radical scavenging capacity was calculated by the following equation (Gülçin *et al.*, 2003, Oztürka *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*.

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.

Results

The effect of high concentrations of Cd (1～10 mg L^{-1}) on RGRs of *Fucus serratus* was measured on the basis of weight change for 7～14 d exposure (Fig. 2). In the RP population, RGRs of *F. serratus* decreased for the first 7 d in all treatments except 0 μg Cd L^{-1}. After 14 d of Cd exposure, recovery of weight was shown as increased RGRs in RP. 5 and 10 mg Cd L^{-1} exposure increased RGRs of *F. serratus* after 14 d even though they were lower than RGRs of 0 mg L^{-1}. 1 mg L^{-1} showed the last recovery of growth in RP.

The BQ population also showed drastic decrease of 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 RGR values from Cd-exposed BQ materials were significantly lower than RGRs of the RP population ($p < 0.0001$).

Lipid peroxidation of *Fucus* thalli from two natural sites was evaluated without additional metal exposure (Fig. 3). *F. serratus* from RP showed lower thiobarbituric acid-reactive substance (TBARS) values than *F. serratus* from BQ, but statistical differences were not significant ($p > 0.05$).

When high doses of Cd (1~10 mg Cd L^{-1}) were treated, lipid peroxidation of cell membrane occurred by time of exposure and Cd concentrations ($p < 0.0001$, respectively) but not by locality ($p > 0.05$, Fig. 4). The longer exposure of Cd showed the higher TBARS values (7 d < 14 d). The higher Cd concentrations (5 and 10 mg Cd L^{-1}) induced the higher TBARS values in both populations at 14 d.

Total antioxidant capacity of Cd treated *Fucus* was studied by reducing a cupric ion (Fig. 5). When *F. serratus* was exposed to Cd, the BQ population showed significantly higher levels of trolox equivalent capacity (mmol TE DW^{-1}) than the RP population at each day ($p < 0.0001$). Time of Cd exposure and Cd concentration significantly affected antioxidant capacity of *F. serratus* from RP ($p < 0.0001$ and $p = 0.001$, respectively). However any clear pattern of trolox equivalent capacity was not discovered according to Cd concentrations at 1 and 7 d. Meanwhile, at 14 d, Cd treated materials revealed higher values than 0 mg Cd L^{-1} ($p = 0.002$). On the other hand, time of exposure and Cd concentration revealed a significant effect on CUPRAC values of the BQ population ($p < 0.0001$, respectively). The capacity was not significant between Cd concentrations (1~10 mg Cd L^{-1}) at 1 d and the control materials showed high values at each day (Fig. 5).

Free radical scavenging capacity of *F. serratus* was evaluated using DPPH (Fig. 6). Locality and time of exposure revealed a significant effect on DPPH free radical scavenging capacity ($p < 0.0001$, respectively), however Cd concentration did not have a noticeable effect ($p > 0.05$). Same as CUPRAC, the BQ population showed much higher values than the RP population.

Effects of exposure time and Cd concentration on DPPH free radical scavenging ability were different at each population. In RP, free radical scavenging ability did not show a regular trend with Cd concentrations however Cd treated materials had significantly higher capacities than the control at 14 d ($p = 0.001$). In BQ, the longer time of Cd exposure showed the lower scavenging capacity for free radical ($p < 0.0001$). However, concentration of treated Cd did not have an apparent tendency for free radical scavenging capacity even though there were significant differences ($p = 0.006$).

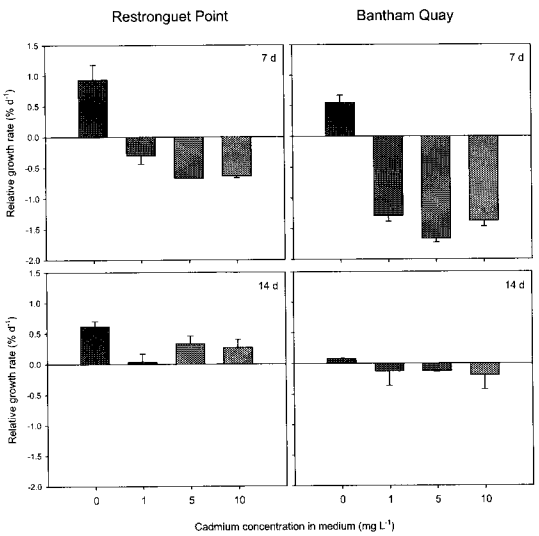

Fig. 2. Relative growth rates of *Fucus serratus* from Restronguet Point and Bantham Quay exposed to cadmium for 7 and 14 days. Values were expressed by means and standard deviations (n = 3).

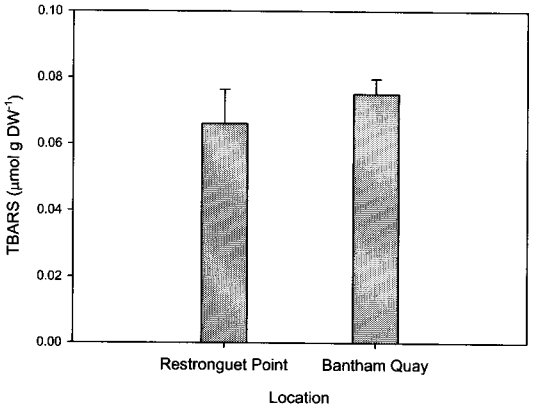

Fig. 3. Thiobarbituric acid-reactive substance (TBARS) levels, mainly malondialdehyde, in *Fucus serratus* harvested from Restronguet Point and Bantham Quay. Values represent mean values of three independent replicates ± standard deviations.

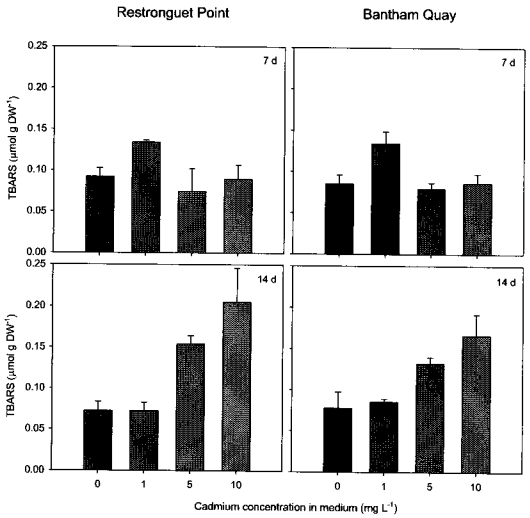

Fig. 4. Thiobarbituric acid-reactive substance (TBARS) levels, mainly malondialdehyde, in *Fucus serratus* from Restronguet Point and Bantham Quay which were exposed to cadmium for 7 and 14 days. Values represent mean values of three to six independent replicates ± standard deviations.

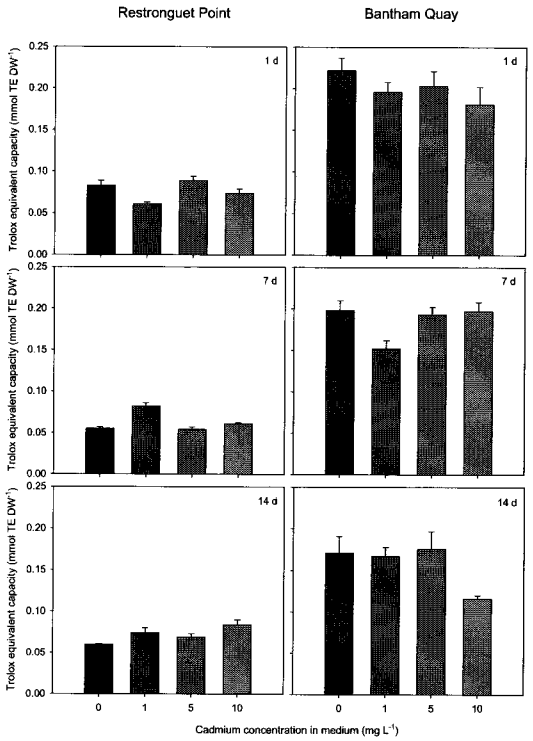

Fig. 5. Cupric ion reducing antioxidant capacity (CUPRAC) in Fucus serratus from Restronguet Point and Bantham Quay which were exposed to cadmium for 1, 7 and 14 days. Values represent mean values of three to six independent replicates ± standard deviations.

Discussion

Brown macroalga *F. serratus* was cultured with high doses of Cd in Aquil medium for up to 14 d. *Fucus* species has been known as bioindicators since they accumulated metals with a positive linear correlation with concentrations (Förstner and Wittmann, 1983, Bryan *et al.*, 1985). Although no visible symptoms of metal stress (e.g. discolouration, dark spot or death) were recognized for 14 d, growth of *F. serratus* was significantly affected by Cd treatment. Both populations showed drastic decreases of RGRs at 7 d, however the decrease of RGRs in the BQ population was more

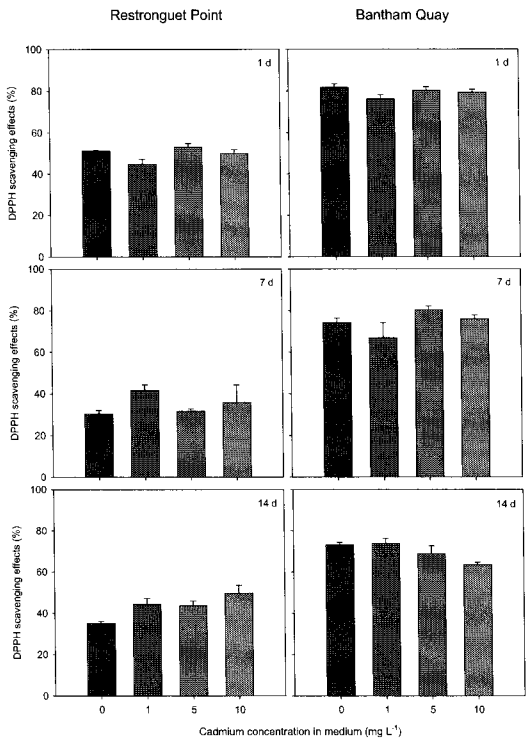

Fig. 6. 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity in *Fucus serratus* from Restronguet Point and Bantham Quay which were exposed to cadmium for 7 and 14 days. Values represent mean values of three to six independent replicates ± standard deviations.

significant. RGRs of both populations were partially recovered after 14 d, but RGRs of BQ still showed negative growth compared with the initial states. It is not the first report of shrunken size and weight loss (or stable weight) of macroalgae after metal treatment (Bryan and Gibbs, 1983, Brown and Newman, 2003, Han *et al.*, 2008). Metal-induced stress, such as inhibition of cell division and/or expansion, may be connected to a decrease of cell turgor, and alteration of cell wall elasticity or acclimatization (Stauber and Florence, 1987, Brown and Newman, 2003).

More significant decrease and less recovery of RGRs in the BQ population showed more serious effects on weight growth by Cd, which might be resulted from the different resistance ability due to the population or adaptation to their habitats. Some researchers have reported the inheritance of metal tolerance of marine macroalgae (Bryan and Gibbs, 1983, Correa *et al.*, 1996, Nielsen, 2002, Nielsen *et al.*, 2003). *F. serratus* from polluted areas revealed higher RGRs and metal resistance than *F. serratus* from unpolluted areas (Bryan and Gibbs, 1983, Nielsen *et al.*, 2003).

Polyunsaturated lipids are essential components of cell membranes, endoplasmic reticula and mitochondria (Muriel, 1997). Consequently lipid peroxidation by metal stress can be crucial to cellular function and survival. Although the TBARS test is non-specific and HPLC techniques may be more accurate, it has widely used for determination of oxidative stress in terrestrial plants, micro- and macroalgae as one of the most frequently operated tests (Halliwell and Gutteridge, 2007, Lee, 2009). Before measuring oxidative stress in *Fucus* from Cd-treated medium, contents of TBARS, mostly malondialdehyde, of two natural populations from RP and BQ were measured. Since the RP area is significantly polluted by metals, including Cd (Table 1), two populations showed similar values in lipid peroxidation. It implies the RP population has the stronger detoxifying ability, less stress from metal exposure and/or adaptation. This indicates they may have more efficient antioxidant protective systems than the BQ population.

Increases of TBARS levels with Cd treatment at 14 d suggested Cd-induced oxidative damage in the present study. Even though time of exposure and Cd concentration had clear relations with TBARS values, especially at

14 d, both populations did not reveal a difference by locality. Similar values of TBARS from the two populations may include some causes. 1) Similar lipid peroxidation due to over-burden of Cd stress, regardless of their history of metal exposure. Indeed 1~10 mg Cd L^{-1} for 7 and 14 d is a toxic situation that the alga may not experience at all in the natural environment. 2) Population-dependent antioxidant response. Sensitive populations revealed higher lipid peroxidation than resistant populations (Randhawa *et al.*, 2001), which were attributed to their different antioxidative defence systems. In this research, the RP thalli still contained significantly higher concentrations of metals that were absorbed from the natural seawater before the experiment (data not shown). Nevertheless the RP population represented similar TBARS values to the BQ population, and this implies the higher and powerful antioxidant mechanism of the polluted population. 3) Species-specific stress-resistant mechanism. Similar TBARS values may not describe the higher antioxidant capacity of the RP population or the over-burden of Cd stress. *Fucus* spp. are well-known as a stress-tolerant species (Jiménez-Escrig et al., 2001). Therefore, *F. serratus* from BQ may also have strong potential against oxidative stress and showed sustained high levels of antioxidant activities (Lee, 2009).

Antioxidant activities can be evaluated by many different ways. Determination of antioxidant potential with different assays would give more informative and reliable results (Oztürka et al., 2007). Consequently CUPRAC assay was processed with DPPH free radical scavenging capacity assay in this study.

The BQ population showed significantly higher antioxidant capacity in CUPRAC assay, however the values were not likely related to the Cd exposure in the research. Highly sustained values and no clear dose-dependent pattern of the BQ population (including controls) represent a loose relationship between CUPRAC and Cd treatment. However, differences between the two populations were evident that the two populations were in significantly different status.

DPPH free radical scavenging activity represented similar patterns with CUPRAC assay in the current study. The BQ population showed stable antioxidant activities regardless of Cd doses and time of exposure than the RP population. This indicates the BQ population has more stable activities to defense oxidative stress. Since *F. serratus* exposed to lower and shorter Cd showed dose-related activities in our previous study (manuscript in preparation), less relationship with high dose of Cd may be due to the over-limit of the algal tolerance on Cd or other antioxidant activities, such as antioxidative enzymes or antioxidants.

Brown algae were reported to have excellent radical scavenging capacities compared with other algal groups (Matsukawa *et al.*, 1997, Yan *et al.*, 1998). The free radical scavenging activity is known to be closely related to phenolic compounds which are abundant in brown algae (Jiménez-Escrig *et al.*, 2001, Connan *et al.*, 2006, Oztürka *et al.*, 2007). *F. vesiculosus* was also reported to have a strong radical scavenging activity due to the high contents of polyphenol (Jiménez-Escrig et al., 2001). Therefore *F. serratus* in this study may also contain high antioxidant potentials due to the high polyphenol contents, which was represented by high and stable levels of free radical scavenging activities. In the mean while, the lower values of the RP population represent population-specific strategy for oxidative stress.

The RP population showed the lower free radical scavenging ability and higher RGRs with the same Cd exposure and the much higher other metal contents from the natural habitat. It can be regarded that the RP population may possess other efficient antioxidant strategies (manuscript in preparation).

In conclusion, *F. serratus* had strong Cd resistance and represented the population-specific antioxidant capacities. The BQ population from the reference area also possessed high antioxidant ability and the RP population from the contaminated area seems to have higher and stronger ability against metal stress.

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