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Ramesh, K

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Accumulation of silver by *Fucus* spp. (Phaeophyceae) and its toxicity to *Fucus ceranoides* under different salinity regimes

K. Ramesh[1] · S. Berry[2] · M. T. Brown[2]

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Abstract Metals constitute an important group of abiotic stressors that elicit stress responses in marine algae that include the production of reactive oxygen species (ROS). Silver (Ag) is a highly toxic metal to organisms but despite this there are relatively few studies on how it affects marine macroalgae (seaweeds). In a landmark study published in 1977 the first information was provided on the accumulation of Ag in *Fucus* spp. (Phaeophyceae) from the Looe estuary, located in south-west England, an area with a long history of mining activity. In the present study, the estuary has been re-visited and the patterns of Ag accumulation in two *Fucus* spp. and sediment re-examined after 35 years. We conclude that Ag concentrations in sediment and macroalgae from specific sites within the catchment remain high, but more generally sediment concentrations have declined by approximately 65 % and the dissolved, bioavailable fraction by 24 % over this period. In addition, from laboratory studies we provide data on the speciation and toxic effects of Ag under different salinity regimes in the euryhaline brown seaweed, *Fucus ceranoides*. From these exposure experiments, it was found that with increasing Ag concentrations growth was inhibited and lipid peroxidation associated with ROS production increased. The magnitude of the toxic effects was greater at a salinity of 10 than 28 psu which reflects the greater bioavailability of the toxic species of Ag (Ag^+ and $AgCl^0$) at reduced salinities. These findings emphasise the importance of investigating the effects of metal pollution in conjunction with other, natural, environmental stressors such as salinity.

Keywords *Fucus* spp. · Silver · Accumulation · Toxicity · Salinity

Introduction

Metals pose an immediate threat to coastal waters globally, since they are non-biodegradable and readily bio-accumulate, potentially causing a health risk to the resident biota (Esposito et al. 2001; Mamboya 2007). In near-shore waters macroalgae, such as *Fucus* species, are major primary producers and key ecosystem engineers, providing a habitat for a large diversity of organisms. Thus, any adverse effects to macroalgae would have consequences for higher trophic level species. Exposure to metals (e.g. cadmium, copper, zinc) can inhibit growth, impair photosynthetic performance, inactivate key enzyme systems and induce oxidative damage via the production of reactive oxygen species (ROS) such as singlet oxygen, superoxide ions, hydrogen peroxide and hydroxyl radicals (Pinto et al. 2003; Burzynski and Zurek 2007; Rai et al. 2008). Oxidative stress is due to the imbalance between ROS and an organism's ability to detoxify the reactive intermediates or repair the consequent damage to proteins, lipids and DNA (Halliwell and Gutteridge 1989; Collen and Davison 1999). Algae counter the production of ROS by synthesising low molecular weight antioxidants and inducing the activities of a suite of antioxidative enzymes (Pinto et al. 2003). Marine macroalgae, and especially brown algae (Phaeophyceae), accumulate metals to high concentrations that are attributed to the presence of negatively charged

✉ K. Ramesh
kramesh@geomar.de

[1] GEOMAR Helmholtz Centre for Ocean Research, 2 Hohenbergstraße, 24105 Kiel, Germany

[2] School of Marine Science and Engineering, Plymouth University, Drake Circus, Plymouth, Devon PL4 8AA, UK

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polysaccharides and physodes to which they bind (Salgado et al. 2005; Mamboya 2007). For this reason they are especially suitable for studies on metals and are frequently classed as good sentinel organisms of metal pollution in coastal and estuarine environments.

Mining activities in south west England dating back to Roman times, but particularly in the second half of the 19th century, have left legacies of metal contamination in many of the estuaries within the region (Bryan 1984; Rainbow et al. 2011) The consequences of such activities on their ecologies has been well studied and the sites remain valuable natural laboratories for eco-toxicological investigations on the biota residing therein (Bryan 1983; Bryan and Gibbs 1983; Cain et al. 2004). Silver (Ag) was one of the most important metals mined in the area; for example, outputs of more than 17 tons of Ag are recorded between 1853 and 1884 from the West Looe River, that flows into the Looe estuary, south-east Cornwall (Dewey 1921). Ag has had a long history of human usage, including as jewellery and in medicine, dentistry and now, most significantly, in electronics due its thermal and electrical conductivity properties (Luoma 2008). It is also regarded as a highly toxic metal with the main sources of contamination to the marine environment being derived from wastewater effluents and acid mine drainage (Tappin et al. 2010). Yet, despite the toxic nature of Ag to marine animals (Ratte 1999), there are relatively few empirical studies reporting on the accumulation and effects in seaweeds.

Here, we investigate the effects of Ag, under two salinity regimes, on the growth, photosynthetic performance and production of and damage by ROS in *Fucus ceranoides*, an intertidal brown seaweed able to withstand the full range of salinities encountered in estuaries (Barreiro et al. 2002; Varma et al. 2013). We also report current levels of Ag pollution within the Looe estuary by analysing concentrations in sediment and two fucoid species (*Fucus vesiculosus* and *F. ceranoides*), and compare these to those recorded by Bryan and Hummerstone (1977) by sampling the exact same sites.

Materials and methods

Sample collection and processing for silver analyses

Three replicate surficial oxic sediment samples were collected from the same 11 locations of the West and East Looe Rivers and Looe estuary described in Bryan and Hummerstone (1977) (Fig. 1), using a polyethylene spatula and transferred to polypropylene centrifuge tubes. In the laboratory, each sample was sieved through 180 μm Nylon mesh and the fine fraction, collected in a 250 ml acid cleaned Pyrex beaker, was then vacuum-filtered through a 0.45 μm Whatman membrane filter to remove excess water before being freeze-dried for 48 h in a clean polypropylene centrifuge tube (Varma et al. 2011).

Five whole individuals of *F. vesiculosus* or *F. ceranoides* (i.e. where *F. vesiculosus* was not present) ranging in length between 24 and 56 cm were collected concurrently during low tide, at just above the mid-intertidal zone, and transported to the laboratory in zip-locked polythene bags within cool boxes. In the laboratory, visible epiphytes and organic debris were removed from the seaweeds by washing in filtered seawater (5 and 0.8 μm) available on tap and excess water then removed by blotting with absorbent paper. All algal samples were freeze-dried, in clean zip-locked polythene bags, for 48 h, then weighed and stored in desiccators to await digestion.

Freeze-dried sediment samples (100 mg) and certified reference material (harbour sediment; BSI-LGC6156) were weighed in triplicate into Teflon digestion vessels of 4 ml capacity, to which 4 ml nitric acid and 1 ml hydrochloric acid (both BDH, AnalaR) were added. Likewise, freeze-dried algal samples (70–100 mg) and certified reference material (*Fucus* spp.; IAEA-140) were placed in Teflon vessels containing 2.5 ml of concentrated nitric acid. The tightly capped contents were then digested in a CEM-MDS 2000 microwave digester for 40 (sediment) or 45 (algae) min. After cooling, sediment, which were first filtered (Whatman, hardened- ashless paper 11.0 cm), and algal digests were washed into 10 ml volumetric flasks, made up to the mark with Milli-Q water and then transferred to 15 ml polypropylene universal containers.

ICP-MS analyses

Sediment and algal digests were analysed for silver (Ag-107) by inductively coupled plasma mass spectrometry (ICP-MS; using a Thermo X Series II quadrupole-based benchtop instrument (Thermo Scientific, Hemel Hempstead, UK) with a concentric glass nebuliser, conical spray chamber and impact bead. Coolant, auxiliary and nebuliser gas flows were 13, 0.86 and 0.7 L min^{-1}, respectively; dwell time was 10 ms, number of sweeps 50 and number of replicates 3. The instrument was calibrated using Ag standards, prepared as above, and internal standardisation was achieved by the addition of 10 μg L^{-1} of ^{115}In and ^{193}Ir to all samples and standards (Varma et al. 2011, 2013).

Sample collection for silver toxicity experiments

The estuary catchment of the River Yealm is largely agricultural and has no recognized source of metallic contamination in contrast to other estuaries in south-west

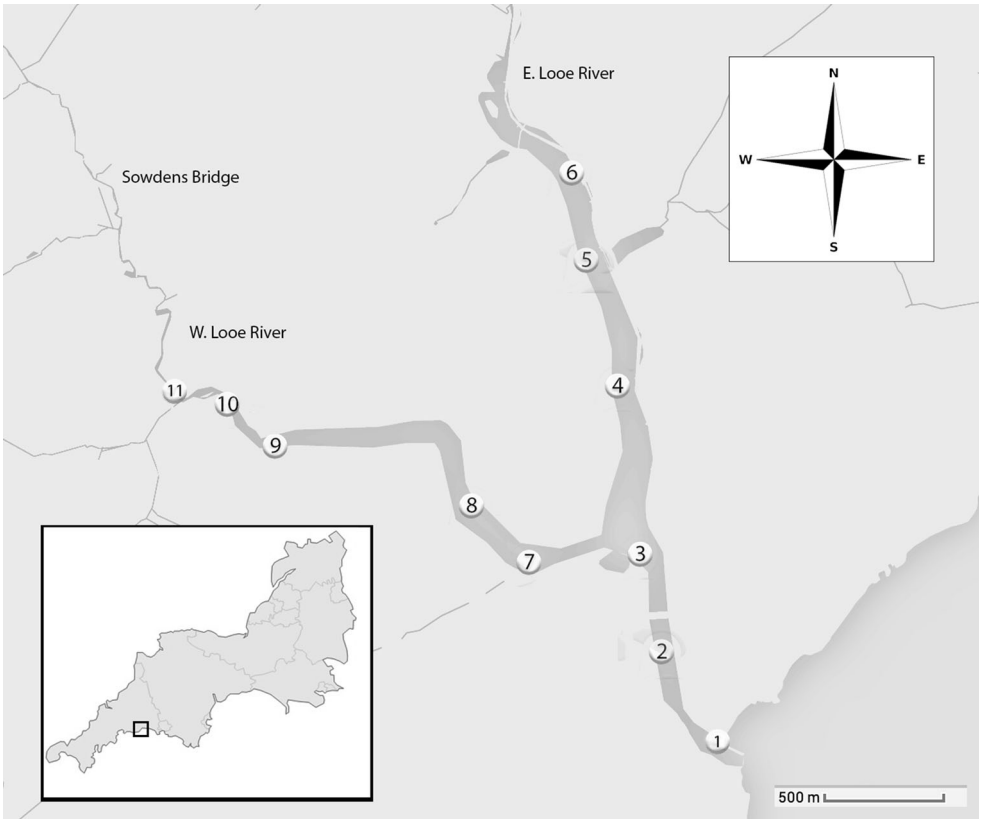

Fig. 1 Looe estuary showing the sampling areas used in Bryan and Hummerstone (1977) and the present study. Figure produced using Google Maps (2014) and Adobe Photoshop CC 2014

England (Langston et al. 2003). Thirty whole individuals (15–20 cm in length) of *F. ceranoides* were hand-picked during low tide, on 31 May 2011 from the mid-intertidal rocky shore between Noss Mayo and Newton Ferrers, on the River Yealm (50°18′51.59″N, 4°1′53.60″W) and transported in plastic containers to the laboratory. Algal samples were processed as above to remove epiphytes and debris, transferred to 2 L plastic aquaria tanks containing continuously aerated filtered (0.22 µm) seawater and maintained under controlled environmental conditions of 15 °C and 240 µmol m^{-2} s^{-1} of PAR (supplied by daylight fluorescent tubes) on a 12:12 h light/dark cycle for 5 days, prior to experimentation.

Experimental protocols

The chemically defined artificial seawater medium (ASM), Aquil was prepared at two salinities (10 and 28 psu), according to Morel et al. (1979) and Price et al. (1989); the pH of the two experimental salinities was 7.6 and 7.7, respectively. Using Aquil permitted the speciation of Ag in the two salinity regimes to be modelled (see below). A silver nitrate stock solution was prepared by dissolving silver nitrate (Fisher Scientific High Grade) salt in Milli-Q water to a concentration of 50 mg L^{-1}.

Following the period of acclimation, the algae were briefly rinsed in Milli-Q water to remove adhering salts and then split between tanks containing high strength or diluted ASM and acclimated for a further 36 h before the addition of Ag. Then, individual seaweeds were allocated, randomly, to aquaria tanks containing ASM of either 10 or 28 psu, and to which one of four nominal Ag solutions (0, 50, 100, 150 µg L^{-1}) were added. There were three replicates per concentration for both salinities. The experiment was carried out under the culture conditions described above for a period of 14 days.

Silver speciation

The inorganic equilibrium speciation of Ag under the experimental conditions was calculated using the Windermere Humic Aqueous Model (WHAM, v6; Tipping 1998) and the stability constants in its default database. The chemical composition of ASM and Milli-Q water dilutions thereof was used to define the ionic composition of the samples. Temperature was set at 288 K, pH at 7.7 and equilibrium with the atmosphere was assumed (pCO_2—3.5×10^4 atm). The Davies equation was employed to calculate ion activity coefficients.

Measured endpoints

Relative growth rates (RGR) were measured from changes in wet biomass according to the formula:

RGR (%) = $[(\ln W_2 - \ln W_1) \div (t_2 - t_1)] \times 100$, where W is wet biomass at the beginning (t_1) and end (t_2) of the experimental period. Chlorophyll *a* fluorescence was measured using a Photosynthetic Efficiency Analyser (Hansatech Ltd, England) Samples were initially dark adapted for 30 min prior readings being taken. F_m, the maximum fluorescence yield of dark-adapted samples and F_0, the initial fluorescence yield, were recorded. The maximum quantum yield of PSII in the dark-adapted state is expressed as the ratio of variable to maximal chlorophyll a fluorescence (Fv/Fm) which is derived from $(F_m - F_0)/F_m$. Chlorophyll *a* fluorescence analysis is a non-invasive technique that provides valuable information on the physiological status of plants and algae (Maxwell and Johnson 2000). Readings were taken for each replicate sample prior to the start of the experiment at the end of the exposure period. Fluorescence was initiated by a 1 s red-light pulse with a peak wavelength of 650 nm and an intensity of 3500 µmol m^{-2} s^{-1}. Saturating irradiance was provided by six high-intensity light-emitting diodes (LEDs, Hansatech Instruments) that were focused onto the algal surface to deliver even illumination.

Concentrations of hydrogen peroxide (H_2O_2) were determined spectrophotometrically, according to Maharana et al. (2010). Algal samples (0.5 g wet weight) were homogenized with 5 ml of 10 % (w/v) TCA, centrifuged at 7000×*g* for 10 min and then 1 ml of 1 M potassium iodide and 1.5 ml of 50 mM potassium phosphate buffer (pH 7.0) was added to 0.5 ml of the supernatant. Absorbance was measured at a wavelength of 390 nm, H_2O_2 was the standard and the extinction coefficient was 43.6 M^{-1} cm^{-1}. Concentrations are expressed as mmol H_2O_2 g^{-1} wet biomass (Ye et al. 2005). Levels of lipid peroxidation were determined from measuring the concentrations of malondialdehyde (MDA) in the seaweeds. MDA is a product of lipid peroxidation which reacts with thiobarbituric acid (TBA) to form an adduct known as TBA reactive substances (TBARS). TBARS measurements were performed according to Maharana et al. (2010). Algal samples (0.5 g wet biomass) were homogenized with 5 ml of 10 % (w/v) trichloroacetic acid (TCA) and then centrifuged at 7000×*g* for 10 min using a microfuge. Two ml of 0.5 % TBA solution was added to1 ml of the supernatant, the mixture incubated at 95 °C for 45 min and then cooled to room temperature followed by centrifugation at 4000×*g* for 10 min. Absorbance was measured at a wavelength of 532 nm and 1,1,3,3, tetramethoxypropane, which breaks down to malondialdehyde (MDA) under the assay conditions, was used as the standard. Levels are expressed as nmol TBARS g^{-1} wet biomass.

Data analyses

The statistical software SPSS (version 21) was used for statistical analyses. Two way ANOVA tests were performed to test for differences in the concentrations of Ag recorded in the sediment (n = 3) and *Fucus* (n = 15) in between the different sampling sites in Looe estuary. The Spearman rank correlation coefficients were used to investigate the relationships between Ag in sediment/total Ag accumulated by the seaweeds. In addition, differences in the parameters (n = 3) between Ag treatments under the two salinity regimes were also tested. Differences between means were considered significant when $p < 0.05$.

Results

Silver pollution in the Looe estuary

The mean Ag concentrations in sediment and algae collected from 11 different sites in the West Looe River, East Looe River and the Looe estuary are presented in Table 1. *Fucus ceranoides* was present only at sites 9, 10 and 11 and is indicative of its ability to thrive under reduced salinity (Khfaji and Norton 1979). Average silver levels in the sediment were highest at sampling sites 9 and 11, both situated in the West River, and lowest at sampling site 1, situated at the estuary mouth. There was a general trend of increasing Ag concentrations with increasing distance from the mouth of the estuary. The highest level of accumulation by *F. vesiculosus* was at sites 4 and 5 in the East River, and

Table 1 Mean (±standard deviation) concentrations (mg kg^{-1}) of silver recorded in sediment and *Fucus* spp. (*F. vesiculosus* and *F. ceranoides*) sampled from 11 locations in the catchment of the Looe Estuary, south-west England

Sampling site	Sediment	$Algae_{total}$
1 (M)	0.07 ± 0.01	0.27 ± 0.09
2 (M)	0.19 ± 0.03	0.66 ± 1.01
3 (M)	0.36 ± 0.04	0.32 ± 0.10
4 (E)	0.42 ± 0.03	1.19 ± 0.50
5 (E)	0.48 ± 0.07	1.28 ± 0.50
6 (E)	0.52 ± 0.04	0.51 ± 0.32
7 (W)	0.50 ± 0.09	0.40 ± 0.16
8 (W)	0.28 ± 0.08	0.63 ± 0.49
9 (W)*	1.16 ± 0.21	0.82 ± 0.77
10 (W)*	0.68 ± 0.06	0.43 ± 0.96
11 (W)*	1.23 ± 0.36	0.33 ± 0.42

Refer to Fig. 1 for locations of the sampling sites (*M* estuary mouth, *E* East Looe river, *W* West Looe river)

Asterisk denotes the sites where Fucus ceranoides was collected

by *F. ceranoides* at site 9 in the West River. There was no significant relationship between Ag levels in sediment and total concentrations in the seaweed.

Toxic effects

Growth rates of *F. ceranoides* were found to be significantly higher ($p < 0.001$) in the lower salinity treatment (10 psu), under control conditions (Fig. 2). Differences in growth rates were highly significant between both salinities for each concentration of Ag and within each salinity for all concentrations of Ag ($p < 0.001$). There was also a significant interaction between salinity and Ag concentration. Growth of *F. ceranoides* was significantly affected by increasing concentrations of Ag under both salinity regimes (Fig. 2). Growth decreased significantly with increasing concentrations of Ag under both salinity regimes but the decline occurred at a lower concentration at the lower salinity treatment (Fig. 2). At 150 µg/L, decline in growth was greater under 10 psu than 28 psu.

After the 14 days exposure to Ag, there was no evidence for a significant decrease in the photosynthetic efficiency of *F. ceranoides* (Fig. 3). Initial mean values of F_v/F_m were 0.73 and these were maintained under both salinity regimes and all Ag concentrations.

Hydrogen peroxide content

Differences in H_2O_2 concentrations were highly significant between both salinities for each concentration of silver and within in each salinity for all concentrations of silver ($p < 0.001$; Fig. 4). The interaction between both salinities was also tested to be highly significant for H_2O_2 content ($p < 0.001$). H_2O_2 showed a pattern of increasing content with increasing concentrations of Ag at 10 psu. In contrast, at 28 psu, there was a small, but significant ($p < 0.001$) decrease in H_2O_2 content at 50 and 100 µg/L from control values. However, at this salinity, a large increase in H_2O_2 content was observed at the highest exposure concentration.

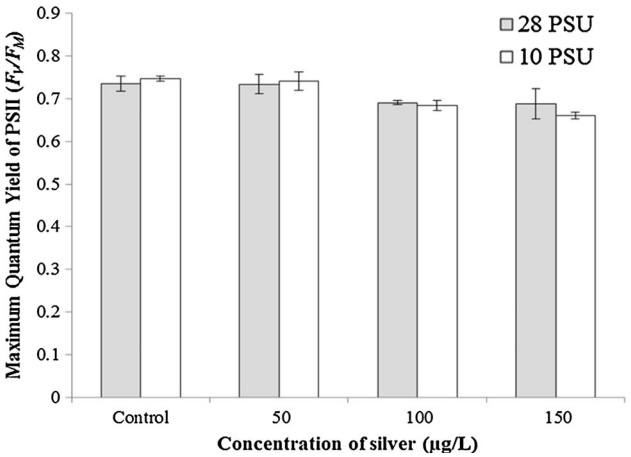

Fig. 3 Efficiency of photochemical energy conversion (F_v/F_m) of *Fucus ceranoides* following 14 day exposure to Ag under two salinity regimes (10, 28 psu). *Error bars* indicate ± standard deviation (n = 3)

Lipid peroxidation

MDA concentrations are good indicators of lipid peroxidation. Under control conditions, lipid peroxidation was found to be similar at both salinities. Differences in MDA

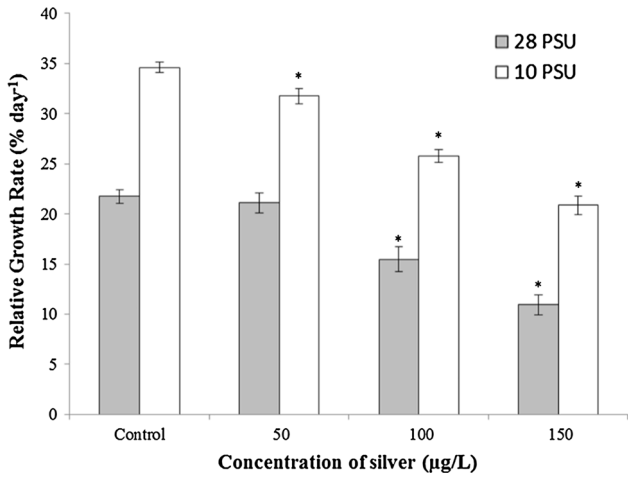

Fig. 2 The effects of Ag on the relative growth rate (% day^{-1}) of *Fucus ceranoides* following 14 day exposure under two salinity regimes (10, 28 psu). *Error bars* indicate ± standard deviation (n = 3). *Asterisks* indicate treatments that are significantly different from controls ($p < 0.05$)

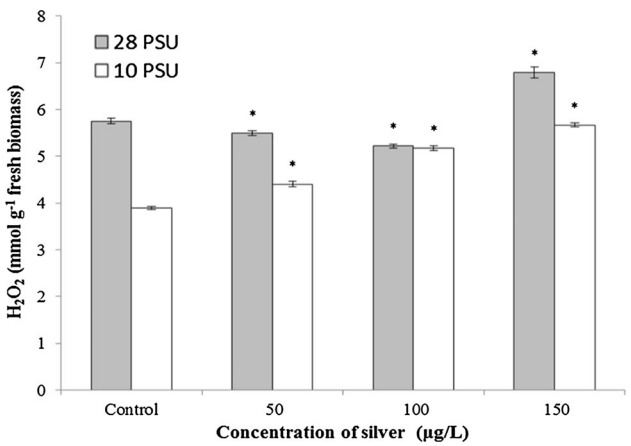

Fig. 4 Concentrations of hydrogen peroxide in *Fucus ceranoides* following 14 day exposure to Ag under two salinity regimes (10, 28 psu). *Error bars* indicate ± standard deviation (n = 3). *Asterisks* indicate treatments that are significantly different from the controls ($p < 0.05$)

concentrations were highly significant between both salinities for each concentration of silver and within in each salinity for all concentrations of silver ($p < 0.0001$) (Fig. 5). The interaction between both salinities at 50 µg/L was also tested to be highly significant for MDA content ($p < 0.001$). At 10 psu, the levels of lipid peroxidation increased with increasing concentrations of Ag. At 28 psu, levels of lipid peroxidation observed were slightly lower at 100 µg/L in comparison to 50 µg/L, but higher than under control conditions. At both salinities, highest lipid peroxidation occurred at the highest concentration of Ag (150 µg/L), over twofold higher than control levels.

Silver speciation

The inorganic speciation of aqueous silver under the experimental conditions is presented in Table 2. Charged chloro-complexes ($AgCl_2^-$, $AgCl_3^{2-}$, $AgCl_4^{3-}$) were calculated to be the most abundant forms of inorganic Ag present, accounting for 96 and 99 % of total aqueous Ag in the 10 and 28 psu salinity regimes, respectively. Neutral chloride and the free ion comprised <1 % and about 4 % in the 28 and 10 psu media, respectively.

Discussion

Recently reported regional baseline concentrations (<33 % percentile point of the dataset) for Ag in estuaries of south west England ranged between 0.07 and 0.18 mg kg^{-1} (Rainbow et al. 2011). The mean (0.53 mg kg^{-1}) and range (0.07–1.23 mg kg^{-1}) of silver concentrations in sediment of the Looe rivers and estuary recorded in this study are higher than these baseline levels but do not exceed the 75 % quartile (indicative of serious levels of contamination) which was the case in 2003 (Rainbow et al. 2011). A comparison of our results with those from the same sampling sites recorded in 1977 by Bryan and Hummerstone reveal that while the highest concentrations are to be found in the West Looe River, they are approximately 65 % lower than they were 35 years ago. However, since there has been no routine sampling of these sites, information on possible annual variability is lacking.

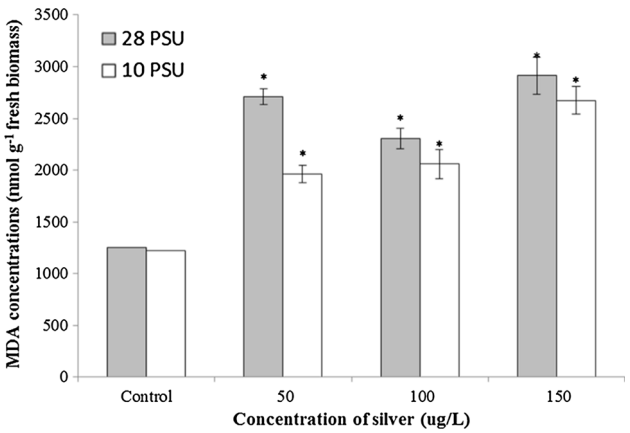

Fig. 5 Levels of lipid peroxidation (measured as MDA concentrations) in *Fucus ceranoides* following 14 day exposure to Ag under two salinities regimes (10, 28 psu). *Error bars* indicate ± standard deviation (n = 3). *Asterisks* indicate treatments that are significantly different from the controls ($p < 0.05$)

The concentrations of Ag measured in the two *Fucus* spp. provide information on the bioavailability of dissolved Ag within the catchment. The highest mean concentrations were recorded in *Fucus* spp from the East Looe River and the lowest from the estuary mouth. The concentrations in *F. ceranoides* sampled at the limits of the tidal influence in the West Looe River were also higher than in *F. vesiculosus* form the estuary mouth. These results are consistent with the strong influence of salinity on the bioavailability of free Ag, due to its affinity for chloride ions (Luoma et al. 1995). Additionally, the bioavailability of other metals such as zinc and copper influence the accumulation of Ag (Ratte 1999). Hence, the accumulation of Ag by brown seaweeds in estuaries is the result of complex interactions with other metals and with salinity. Overall, the bioavailability of dissolved Ag has decreased by about 24 % since 1977, but values remain similar to those recorded in 2006 by Rainbow et al. (2011).

The results obtained from the experimental study reflect this complexity and show that salinity modified Ag speciation and consequently its bioavailability and toxicity to the euryhaline brown seaweed *F. ceranoides*. Toxicity of dissolved Ag is usually attributed to the uptake and cellular effects of the free ionic silver (Ag^+) and the moderately hydrophobic, neutral chloro-complex ($AgCl^0$) (Wood et al. 2010).The bioavailability of toxic Ag species (Ag^+ and $AgCl^0$) is almost six times higher at the lower salinity, 10 psu in comparison to the higher salinity regime, 28 psu. Despite the less bioavailability of toxic Ag forms, growth is observed to decrease at higher concentrations of Ag under 28 psu, indicative of energy re-allocation towards reactive oxygen metabolism. Greater decreases in relative growth rates and higher increase in H_2O_2 content were observed at the lower salinity regime, whilst no significant changes were detected at 150 µg/L of Ag between the two salinity treatments, suggesting that the energy re-directed toward reactive oxygen metabolism is higher at the lower salinity, 10 psu. Growth was adversely affected by Ag under both salinity regimes but significant negative impacts were first observed at the lowest experimental concentration (50 µg/L) only in 10 psu and the adverse effects of exposure to 150 µg/L were greater also under this salinity treatment. This may be explained by the higher bioavailability of the toxic Ag^+ at the 10 psu. The lowest growth rates recorded

Table 2 The inorganic equilibrium speciation (%) of Ag in Aquil of two salinities

Salinity	Ag^+	$AgCl^0$	Charged silver chloro-complexes ($AgCl_2^-$, $AgCl_3^{2-}$, $AgCl_4^{3-}$)
10 %	0.04	3.59	96.37
28 %	0.01	0.61	99.38

Speciation was calculated using the Windermere Humic Aqueous Model (WHAM, v6; Tipping 1998)

in the experiment were at 28 psu. Moreover, under control conditions, *F. ceranoides* exhibited lower growth rates at 28 psu suggesting that the species' presence in low salinity environments may be due to an adaptive success under low salinity conditions.

In contrast to the growth results, no significant changes in maximum quantum yield of photosystem II were observed over the range of Ag concentrations used. However, previous studies have reported a decline in the yield of photosystem II following exposure for shorter periods of time. For example, in the freshwater microalga, *Chlamydomonas reinhardtii* have a reported a decline in the yield of photosystem II following a 5 h exposure to 53.5 µg/L Ag (e.g. Navarro et al. 2008) and Turner et al. (2012) reported a significant decline in the effective quantum yield of PSII (F_v/F_m) in *Ulva lactuca* following 48 h exposure to 2.5 µg/L Ag. Therefore, it is possible that any transitory inhibition was compensated for by increasing the number of reaction centres as observed for other abiotic stressors in e.g. cyanobacteria (Vass et al. 2000). It is also possible that the parameter measured (F_v/F_m), was not the most sensitive (see review by Ralph et al. 2007). For example, Zhang et al. (2014) observed that photosystem I (PSI) has a higher sensitivity to ROS and the sensitivity of PSII was lower in comparison to PSI in the leaves of cucumber.

The apparent uncoupling of growth from photosynthetic activity has been observed previously for other metals (e.g. Brown and Newman 2003; Han et al. 2008). It has been suggested this phenomenon could be related to the re-allocation of the energy captured by the light reactions of PSII and PS1 away from carbon assimilation and growth towards maintaining cell integrity, including offsetting the production of ROS. For example, Li and Brawley (2004) have reported that generation of antioxidants in *Fucus* species was energetically expensive and diverted metabolic resources away from growth and reproduction.

Compared with other metals, the relationship between Ag and reactive oxygen metabolism in macroalgae is very limited; however information is available for other photoautotrophs. For example, an empirical study on the freshwater alga, *Chlamydomonas reinhardtii* revealed that Ag was a strong inducer of ROS and this was due to the high affinity of Ag ions for thiol groups Szivak et al. (2009). Using A*rabidopsis thaliana* to investigate ROS generation by Ag in plants, Navabpour et al. (2003) observed the increased expression of senescence-enhanced genes such as the *LC54* gene. By combining treatments with quenchers of ROS such as ascorbate, tiron and benzoic acid, they found the expression of the *LC54* gene and other senescence-enhanced genes to be directly related to elevated levels of oxidative stress in the plant tissues. Thus, ROS generated due to Ag exposure could play a role in regulating cell death. Further studies are required to assess whether this type of mechanism can help explain the declining growth rates in *F. ceranoides* with increasing Ag concentrations.

Salinity, in the absence of Ag, also significantly affected relative growth rates of *F. ceranoides*, with values consistently lower at 28 psu. Growth is regarded to be a good measure of a plants ability to tolerate salt stress because growth requires maintenance of cell turgor (Hellebust 1976; Huang and Redmann 1995). Under reduced salinities that are encountered at the upper reaches of estuaries, *F. ceranoides* is able to out-compete other *Fucus* spp; for example, germlings of *F. ceranoides* can develop from zygotes under salinities down to 8.5 psu, whilst those of *F. vesiculosus* do not survive below 24 psu (Khfaji and Norton 1979). The higher concentrations of H_2O_2 and levels of lipid peroxidation at 28 psu further indicate that salinity poses an additional stress for this alga. Cairrao et al. (2004) founds that in *F. ceranoides* collected from sites with higher salinity, in comparison to sites with low salinity, exhibited significantly higher concentrations of glutathione S-transferase (GST), an enzyme that plays an important role in the cellular detoxification of peroxidised lipids. Increased antioxidant activity at high salinities lends support to the hypothesis that metabolic resources are being re-allocated towards reactive oxygen metabolism under 28 psu treatment. Thus the ability *of F. ceranoides* to outcompete *F. vesiculosus* at salinities below 17 psu is reversed under full salinity which in part is due to negating the cellular effects of ROS.

The stress posed by higher salinity and exposure to Ag, decreases the growth potential in *F. ceranoides* with energetic resources potentially being utilized to combat cellular stress. The results indicate that the levels of lipid peroxidation and H_2O_2 are higher at the higher salinity regime, 28 psu for each treatment of Ag whilst growth rates are also lower for each treatment of Ag at this salinity. This result suggests that although energy derived from photosynthesis is going towards countering the adverse effects of ROS production at 10 psu, *F. ceranoides* is able

to allocate resources for the purpose of growth at low salinities. This consequently reduces a populations ability to respond to short-term favourable conditions and reducing its potential for success. Therefore studying algal growth rates can present a sensitive measurement of toxicity and also provide realistic insight to the actual inhibition within the system Suter and Lewis (1989).

The variability in concentrations of H_2O_2 and MDA at the 28 psu salinity regime may also be explained the additional stress faced by the alga at this salinity. The results indicate variable concentration of H_2O_2 at 28 psu, different to the clear pattern of increase with increasing concentrations of Ag at the 10 psu salinity regime. Therefore, in ecotoxicological studies using euryhaline species, it is important to investigate the interaction with salinity as an additional environmental stressor. Salinity can be lowered by freshwater input from rivers to estuaries and algae such as *F. ceranoides* can be negatively affected by the higher abundance of Ag in its toxic and bioavailable (Ag^+, $AgCl^0$) forms.

Conclusions

Concurrent analyses of *Fucus* spp and sediments indicate that while Ag concentrations remain high in some sections of Looe estuary, there has been a general decline over the past 35 years. Results from the experimental study indicate that Ag is toxic to the euryhaline species *F. ceranoides* as observed by a decrease in growth rates, increase in ROS and lipid peroxidation at both salinity regimes studied. Toxicity is influenced by salinity which alters the bioavailability of toxic Ag forms.

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Conflict of interest The authors declare that they have no conflicts of interest.

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