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DIFFERENT SPECIATION FOR BROMINE IN BROWN AND RED ALGAE, REVEALED BY IN VIVO X-RAY ABSORPTION SPECTROSCOPIC STUDIES[1]

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Members of various algal lineages are known to be strong producers of atmospherically relevant halogen emissions, that is a consequence of their capability to store and metabolize halogens. This study uses a noninvasive, synchrotron-based technique, X-ray absorption spectroscopy, for addressing in vivo bromine speciation in the brown algae *Ectocarpus siliculosus*, *Ascophyllum nodosum*, and *Fucus serratus*, the red algae *Gracilaria dura*, *G. gracilis*, *Chondrus crispus*, *Osmundea pinnatifida*, *Asparagopsis armata*, *Polysiphonia elongata*, and *Corallina officinalis*, the diatom *Thalassiosira rotula*, the dinoflagellate *Lingulodinium polyedrum* and a natural phytoplankton sample. The results highlight a diversity of fundamentally different bromine storage modes: while most of the stramenopile representatives and the dinoflagellate store mostly bromide, there is evidence for Br incorporated in nonaromatic hydrocarbons in *Thalassiosira*. Red algae operate various organic bromine stores – including a possible precursor (by the haloform reaction) for bromoform in *Asparagopsis* and aromatically bound Br in *Polysiphonia* and *Corallina*. Large fractions of the bromine in the red algae *G. dura* and *C. crispus* and the brown alga *F. serratus* are present as Br^- defects in solid KCl, similar to what was reported earlier for *Laminaria* parts. These results are discussed according to different defensive strategies that are used within algal taxa to cope with biotic or abiotic stresses.

Key index words: **brown algae; extended X-ray absorption fine structure; microalgae; red algae; X-ray absorption spectroscopy**

Abbreviations: **EXAFS, extended X-ray absorption fine structure; XAS, X-ray absorption spectroscopy**

In seawater, halogens are mainly present as halide anions and also as organohalogens, the production of which is partly driven by biological processes. Halogenation of secondary metabolites reflects the availability of chloride and bromide ions in seawater. Interestingly, bromide is more frequently used by algae for organohalogen production (Gribble 2003), although chlorine occurs in higher concentrations than bromine in seawater. Marine halogenated compounds comprise a varied assembly of compounds, ranging from peptides, polyketides, indoles, terpenes, acetogenins and phenols to volatile halogenated hydrocarbons (Butler and Sandy 2009). The role of halocarbon synthesis in marine algae has recently been reviewed (Paul and Pohnert 2011). The prevalence of halogens is not similar in marine algae: chlorine and bromine appear to be

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This paper is dedicated to the memory of Johanna Fehling (October 25, 1974–February 17, 2011), an enthusiastic student at the Scottish Association for Marine Science and promising young marine scientist, who untimely passed away due to a brain tumor.
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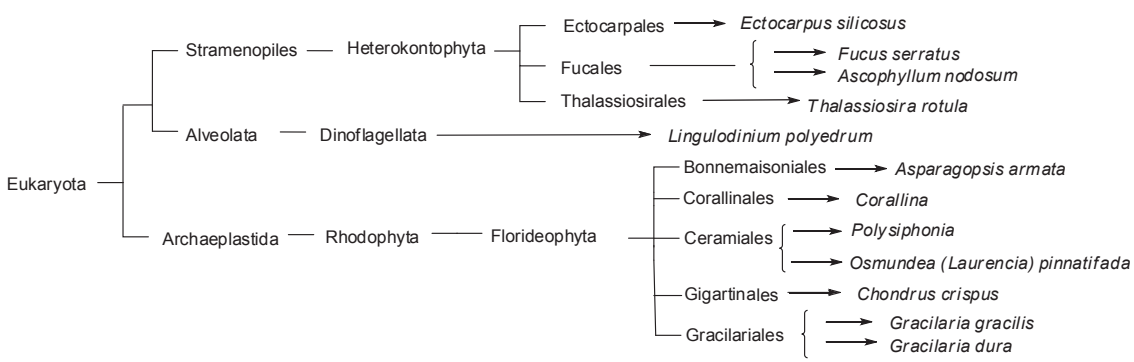
the main halogens used to increase biological activity of secondary metabolites, whereas iodine and fluorine remain quite unusual within the chemical structures (Neumann et al. 2008).

Brown and red algae have long been known to accumulate high levels of iodine and iodinated compounds (Küpper et al. 2011 for review) and iodination is more frequent in brown algae than in red and green algal metabolites (La Barre et al. 2010). As a result, only less than 1% of secondary metabolites from brown algae contain bromine or chlorine in contrast with as much as 90 and 7% of red and green algal compounds, respectively (Harper et al. 2001). Iodine as a novel element was originally discovered in the ashes of brown algae, *Laminaria* sp. and *Fucus* sp. (Courtois 1813) which is not completely surprising considering that the kelp *Laminaria digitata* accumulates iodine to more than 30,000 times the concentration found in seawater, representing an average content of 1% of dry weight (Küpper et al. 1998). Several decades after the discovery of iodine, Eschle's work (Eschle 1897) probably marked the beginning of serious scientific study of iodine accumulation in different seaweed species. Since then, kelps of the genus *Laminaria* have emerged as the best-studied models for algal halogen metabolism. Further milestones in seaweed iodine research were the discovery of the release of molecular iodine (Dangeard 1928, Kylin 1929), the establishment of an oxidative step in iodide uptake in *Laminaria* (Shaw 1959, 1960), the discovery of halocarbon emissions from kelp beds (Lovelock 1975), the formation of iodine oxides (Alicke et al. 1999) and particle bursts as a consequence of iodine emissions from kelp beds (O'Dowd et al. 2002) and, most recently, the discovery that the underlying biological significance of seaweed iodine metabolism is the provision of iodide as a simple inorganic antioxidant for protecting the algal thallus surface and apoplast against oxidative stress (Küpper et al. 2008).

In comparison, much less is known about bromine speciation in seaweeds. Bromine was discovered in seawater (Balard 1826). Pedersen and coworkers (e.g., von Hofsten and Pedersen 1978, Westlund et al. 1981, Pedersen and Roomans 1983) pioneered the use of X-ray microanalysis for investigating the tissue and subcellular localization of bromine and iodine. This group also found the oxidative stress-related production of bromocarbons in seaweeds (Mtolera et al. 1996, Pedersen et al. 1996). Carpenter et al. (2000) reported emissions of $CHBr_3$, CH_2Br_2, $CHBr_2Cl$, CH_3Br, C_2H_5Br, CH_2IBr and the hitherto undetected $CHIBr_2$, from seaweeds on the Irish west coast, with notably high bromoform emissions from the red alga *Asparagopsis armata*. Brominated compounds have important biological and ecological roles in algae, as for instance brominated furanones in red algae (Harder et al. 2012).

We developed X-ray absorption spectroscopy (XAS) as a noninvasive tool to investigate bromine and iodine speciation in marine algae (Feiters et al. 2005a). Its first application was the finding of a bis-brominated tyrosine residue as an endogenous component of the bromoperoxidase of the brown alga *Ascophyllum nodosum* (Feiters et al. 2005b). With extended X-ray absorption fine structure (EXAFS), we are able to distinguish incorporation of Br in aliphatic and aromatic compounds, based on the increase in bond length going from a Br atom covalently attached to a sp^2- to a sp^3-hybridized C; we observe significant differences between a Br^- ion which is hydrogen bonded to many solvent molecules or just a few heteroatoms from biomolecules, and we have identified the incorporation of Br^- ions in a KCl lattice (Küpper et al. 2013). In our studies addressing the physiology of both iodine (Küpper et al. 2008) and bromine (Küpper et al. 2013) in *Laminaria*, XAS played a central role. Using XAS in combination with other techniques, we have recently demonstrated that bromide complements iodide as an antioxidant in particular with regards to superoxide detoxification and that overall, its function is more diverse than that of iodide in algal physiology (Küpper et al. 2013). These studies confirmed the rarity of brominated organic compounds in brown algae and suggest the inducibility of bromination in brown algae using cellular bromine storage.

We here extend the use of this technique to address in situ bromine speciation in a broader phy-



SCHEME 1.

logenetic diversity (Scheme 1) of algal models: among the seaweeds, we included the first fully sequenced multicellular alga (Cock et al. 2010) and important brown algal model (Peters et al. 2004), the filamentous *Ectocarpus siliculosus* (Dillwun) Lyngbye; the morphologically rather complex brown algae *A. nodosum* Stackhouse, from which the first V bromoperoxidase had been isolated (Vilter 1995 for review) and *Fucus serratus* L.; the red algal models *Gracilaria dura* (C. Agardh) J. Agardh, *G. gracilis* (Stackhouse) M. Steentoft, L.M. Irvine & W.F. Farnham and *Chondrus crispus* Stackhouse, which have been used in a number of studies of algal innate immunity (Weinberger et al. 2005); the red alga *Osmundea pinnatifida* (Hudson) Stackhouse, which belongs to a genus well known for an active bromine metabolism (e.g. Suzuki et al. 2009); the red alga *A. armata* Harvey which was found to be a very strong emitter of $CHBr_3$ (Carpenter et al. 2000), the filamentous *Polysiphonia elongata* (Hudson) Harvey and finally the calcareous *Corallina officinalis* Linnaeus. In comparison to this, we investigated a natural phytoplankton community, the diatom *Thalassiosira rotula* Meunier and the dinoflagellate *Lingulodinium polyedrum* (F.Stein) J.D.Dodge.

MATERIALS AND METHODS

Biological material. All macroalgae were collected in the intertidal and shallow sublittoral of Roscoff (Brittany, France) just before the measurements (*Ectocarpus siliculosus, A. nodosum, F. serratus, G. dura, G. gracilis, C. crispus, O. pinnatifida, A. armata* in early December 2004*; Polysiphonia elongata* and *Corallina officinalis* in mid-February 2007). Seaweeds were transported from Roscoff to DESY-EMBL (Hamburg) in containers filled with seawater and an ~30% air headspace (maintaining a large seawater volume/seaweed biomass ratio) in insulated cool boxes kept below <5°C using freeze packs. Until being used in the XAS experiments, they were maintained in well-aerated sea water tanks. A natural phytoplankton sample was collected in Dunstaffnage Bay (Scotland, UK) using a 20 μm plankton net on December 2, 2004 and freeze dried. Two freeze-dried microalgal cultures, *Lingulodinium polyedrum* CCAP 1121/2 (grown in K minimum medium; Keller et al. 1987), and *Thalassiosira rotula* CCAP 1085/13 (grown in f/2+ Si; Guillard 1975) were obtained from the Culture Collection of Algae and Protozoa (Oban, UK).

EXAFS measurements and simulations. EXAFS measurements were carried out in the European Molecular Biology Laboratory (EMBL) Outstation in the Hamburg Synchrotron Laboratory (HASYLAB) at DORIS III (operated at 4.5 GeV with ring currents between 90 and 140 mA and X-ray beam dimensions of 1 mm by 10 mm) at the Deutsches Elektronensynchrotron (DESY) in Hamburg, Germany, at 20 K, as described before (Feiters et al. 2005a,b, Küpper et al. 2013). Details of the EXAFS instrument (Hermes et al. 1984) (Pettifer and Hermes 1985) and the data reduction (Nolting and Hermes 1992) are described elsewhere. EXAFS simulations were carried out using EXCURVE (Gurman et al. 1984, 1986, Binsted et al. 1991), release 9.331, following previously published procedures (Feiters et al. 2005a,b, Küpper et al. 2013). Briefly, the EXAFS spectra were simulated with chemically reasonable models, inspecting the Fourier transform, which in fact represents a radial distribution function of atoms around Br (Hughes et al. 2013) to judge whether additional shells of atoms should be added to the model, and including them if this led to a decrease in the fit index.

Iodine and bromine contents. Following EXAFS acquisitions, algal samples were freeze dried to determine total iodine and bromine contents at the Département d'Analyse Elémentaire, Service Central d'Analyses, Centre National de la Recherche Scientifique (Vernaison, France). After complete combustion in a Schoninger flask, the sample ashes were analyzed using established anion-exchange chromatography techniques, with UV or conductivity detection for iodide and bromide, respectively. These analyses were only conducted for algal samples with a sufficient amount of algal powder (over 200 mg dried weight).

RESULTS

Brown algae and microalgae. The Br X-ray absorption K edge spectra of the representatives of the Stramenopiles, of the dinoflagellate *Lingulodinium*, the natural phytoplankton sample, and – as a model compound – NaBr solution are presented in Figure 1.

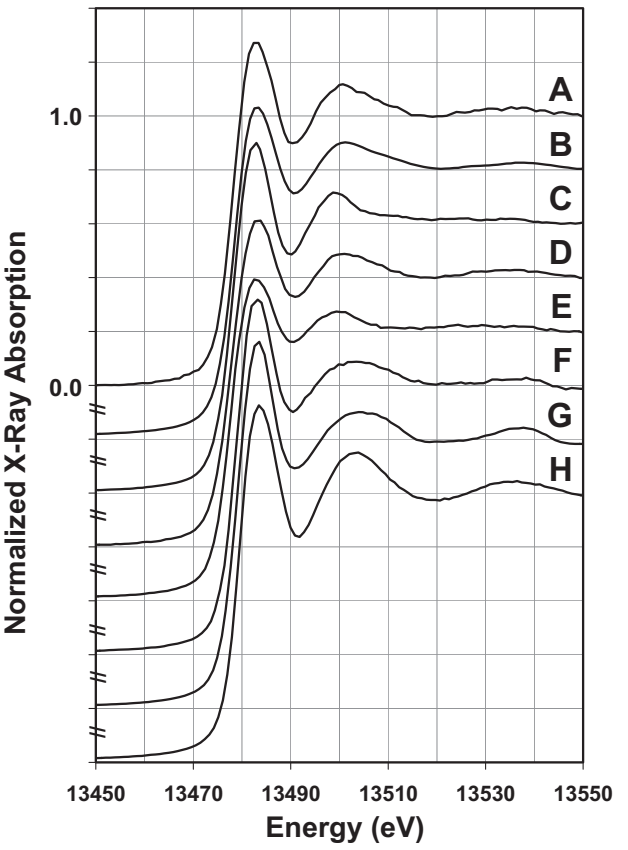


FIG. 1. Bromine K edge X-ray absorption spectra, normalized edges, of brown algae, a diatom culture and a diatom-dominated phytoplankton community: (A) *Ectocarpus siliculosus*; (B) *Laminaria digitata* (from Küpper et al. 2013); (C) *Fucus serratus*; (D) *Ascophyllum nodosum*; (E) *Thalassiosira rotula*; (F) *Lingulodinium polyedrum*; (G) Phytoplankton from Dunstaffnage Bay; (H) NaBr (aq.). Traces B and H were shifted by a few eV for alignment.

Similar to that of *Laminaria digitata* (Fig. 1, trace B; Küpper et al. 2013), the first maxima (so-called 'white line' around 14,780 eV) of most edge spectra (*Ectocarpus*, Fig. 1A; *Ascophyllum*, Fig. 1D; *Lingulodinium*, Fig. 1F; and phytoplankton, Fig. 1G) are similar in appearance to that of hydrated bromide ion, represented by the aqueous solution of NaBr (Fig. 1H); the second and third maxima (at ~13,500 and 13,535 eV) are already much weaker, however, than that of aqueous NaBr. Subtle deviations are observed for *Fucus* (Fig. 1C) and *Thalassiosira* (Fig. 1E), where the second maximum is a little closer to the white line.

The EXAFS of the brown algae and microalgal samples were relatively weak, with significant noise in the spectra already at relatively low *k*-values, and we have therefore simulated only the *k*-range corresponding to a maximum energy of 350 eV, corresponding to $k = 9.5$ Å^{-1}. Most of the EXAFS and Fourier transforms corresponding to the edges in Figure 1 are shown in Figure 2, left and right panel, respectively, and the parameters used for the simulation are in Table 1. The EXAFS and their Fourier transforms confirm that in all brown algae and related microalgal samples, bromine is predominantly present as the bromide ion, interacting by hydrogen bonds with a shell of heteroatoms (presented in the simulations as oxygens) at ~3.3 Å. For *Ectocarpus* (Fig. 2A) this is the only contribution, as attempts to include the weaker peaks in the Fourier transform in the simulation did not lead to significant improvements. Most of the Fourier transforms also contain evidence for bromine atoms covalently bound to carbon with bond lengths of ~1.9 Å (Fig. 2, B and C). It has to be kept in mind that the spectra are taken of whole algae, and that they could represent mixtures of bromine in various environments. Aside from the fraction of C-bound Br, which corresponds to the occupancy of the C shell in the refined EXAFS simulations, the relative contributions of each species are difficult to quantify.

The diatom *Thalassiosira* (Fig. 2C) is the only alga for which the carbon peak is stronger than the H-bonded heteroatom peak; the Br-C distances are in the range of 1.88–1.90 Å, but contrary to the case of some of the red algae to be discussed below, there is no evidence for aromatic rings. The spectra of *Fucus* and the natural phytoplankton sample could be simulated over a longer range (up till 530 eV, corresponding to $k = 12$ Å^{-1}) and are therefore included along with the spectra of the red algae in Figure 3. The EXAFS spectrum of *Fucus* (Fig. 3E) takes a special position among the brown algae as it has a feature just below 6 Å^{-1} which is indicative of the presence of a Br^- ion incorporated in a KCl lattice, as already shown for *L. digitata* samples (Küpper et al. 2013); the most representative of this kind of spectrum is that of *G. dura* in Figure 3D. The best simulation for the *Fucus* spectrum included contributions of C (at 1.92 Å, probably sp^3-hybridized), H-bonded heteroatoms (3.39 Å) and the K and Cl atoms of the lattice (closest Br-K contact at 3.25 Å). There is a resemblance between the spectra of the dinoflagellate *Lingulodinium* (Fig. 2D) to that of the phytoplankton sample (which probably also contains dinoflagellates, Fig. 3F) which are both dominated by a shell of H-bonded heteroatoms at 3.3 Å. For neither spectrum did attempts to simulate the shell in the Fourier transform in the Br-C range (1.9 Å)

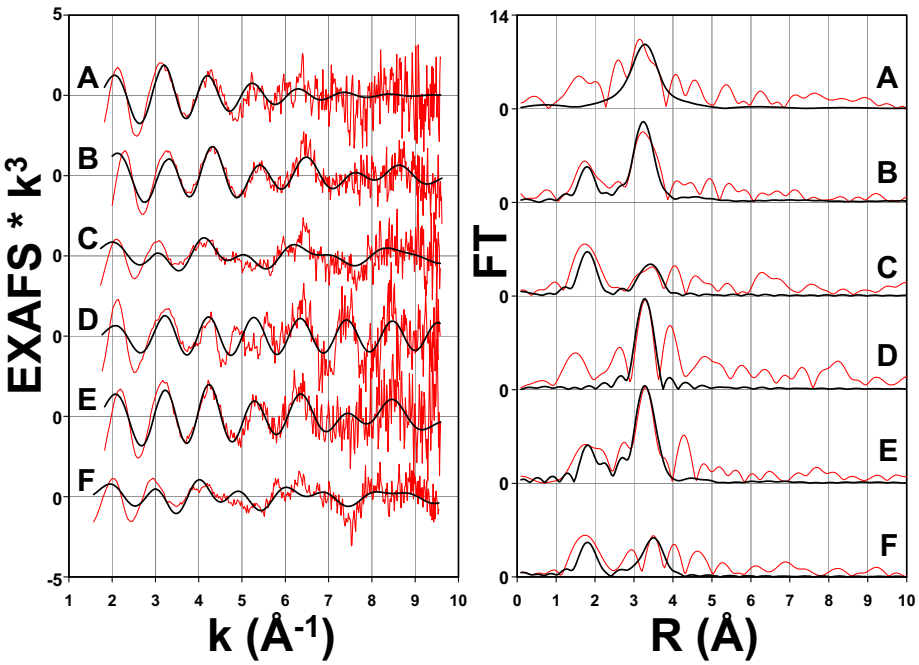


FIG. 2. Experimental (nonblack) and simulated (black, parameters in Table 1) k^3-weighted EXAFS (left panel) and its Fourier transform (right) of (top to bottom): (A) *Ectocarpus siliculosus*; (B) *Ascophyllum nodosum*; (C) *Thalassiosira rotula*; (D) *Lingulodinium polyedrum*; (E) *Osmundea* (*Laurencia*) *pinnatifada*; (F) *Gracilaria gracilis*.

TABLE 1. EXCURVE results.

Organism	ΔEF/Fit index[a] Energy range	C or Ph[b]	Br[b]	O[b]	K[b]	Cl[b]	Summary
Ectocarpus siliculosus	−11.0613/3.011 4.3623–350			7.5 at 3.367 (0.054)			Br^-
Laminaria digitata[c]	−1.0135/7.050 20–350			1.8 at 3.254 (0.023)			Br^-
Fucus serratus	−7.3338/0.8302 10.5195–537.8740	0.3 at 1.921 (0.005)		7.9 at 3.393 (0.027)	3.4 at 3.248 (0.021); 3.2 at 5.361 (0.030)	3.4 at 6.389 (0.022); 0.8 at 4.445 (0.007)	sp^3 C + Br^- + KCl
Ascophyllum nodosum	−6.0618/1.922 4.9756–350.0000	0.3 at 1.898 (0.003)		4.8 at 3.268 (0.027)			$sp^{2\text{-}3}$ C + Br^-
Thalassiosira rotula	−8.9766/5.418 4.1613–350.0000	0.4 at 1.889 (0.003)		2.4 at 3.440 (0.030)			$sp^{2\text{-}3}$ C + Br^-
Lingulodinium polyedrum	−8.8997/3.910 4.6167–350.0000			3.1 at 3.332 (0.007)			Br^-[d]
Phytoplankton	−7.7779/3.301 10.5612–535.6064			5.6 @ 3.291 (0.017)			Br^-[d]
Asparagopsis armata	−6.0491/0.5471 4.3491–534.8223	0.6 at 1.929 (0.004)	1.4 at 3.158 (0.008)				Geminal Br
Aqueous $CHBr_3$	−8.2884/0.5560 4.3927–520.3867	0.7 at 1.914 (0.004)	2.2 at 3.196 (0.009)				Geminal Br
Aqueous 2,2-diBr-propane	−12.2502/0.9733 3.7620–533.9775	0.8 at 1.963 (0.003)	1.6 at 3.185 (0.011)				Geminal Br
2-Br-phenol in 1 M KOH	−11.7625/1.379 4.0703–538.0361	1.0 Ph at 1.908 (0.003) 2.838 (0.007) 4.229 (0.012) 4.692 (0.009)					sp^2 C (aromatic)
Corallina officinalis	−1.4877/3.580 7.3351–551.3867	1.0 Ph at 1.852 (0.004) 2.731 (0.015); 4.121 (0.015); 4.660 (0.002)					sp^2 C (aromatic)
Polysiphonia lanosa	−3.6893/1.655 7.5032–551.8691	1.0 Ph at 1.862 (0.006) 2.810 (0.005); 4.158 (0.016); 4.668 (0.007)		2.2 at 3.394 (0.003)			sp^2 C (aromatic) + Br^-
Chondrus crispus	−10.1084/1.444 10.5612–536.3271	0.5 at 1.904 (0.003)		3.1 at 3.477 (0.018)	2 at 3.287 (0.030); 2 at 5.402 (0.024)	2 at 6.328 (0.022); 2 at 4.477 (0.017)	KCl + Br^-+ $sp^{2\text{-}3}$ C
Osmundea pinnatifada	−8.0140/1.753 4.3491–350.0000	0.2 at 1.908 (0.013)		6.0 at 3.330 (0.026)			Br^- + $sp^{2\text{-}3}$ C
Gracilaria dura	−12.2538/0.9518 10.5195–533.4141	0.4 at 1.910 (0.003)			2.8 at 3.224 (0.012); 2.8 at 5.393 (0.008)	2.8 at 6.338 (0.016); 5.1 at 4.454 (0.016)	KCl + $sp^{2\text{-}3}$ C
Gracilaria gracilis	−11.1561/4.292 4.3886–350.0000	0.3 at 1.902 (0.003)		3.1 at 3.527 (0.030)			Br^- + $sp^{2\text{-}3}$ C

[a]EF and energy range in eV; fit index to be multiplied by 10^{-3}.
[b]Occupancy @ distance (Å) with Debye–Waller factor as $2\sigma^2$ in $Å^2$ in parentheses.
[c]From Küpper et al. (2013).
[d]Including a shell of unidentified halogen atoms (Cl or Br) at ~4 Å.

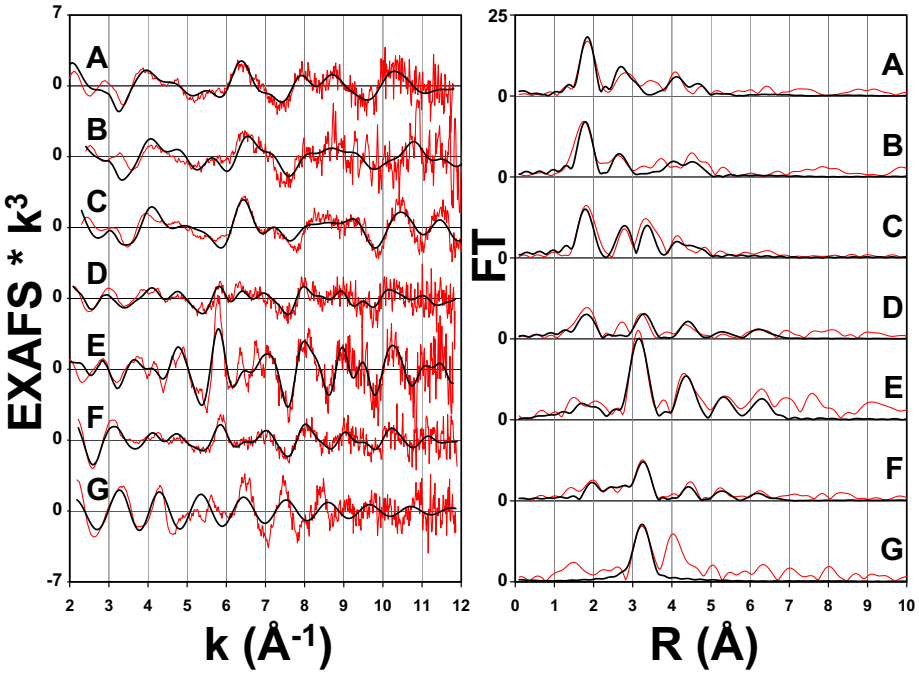


FIG. 3. Experimental (nonblack) and simulated (black, parameters in Table 1) k^3-weighted EXAFS (left panel) and its Fourier transform (right) of (top to bottom): (A) 10 mM 2-Br-phenol in 1 M aqueous KOH, (B) *Corallina officinalis*; (C) *Polysiphonia elongata*; (D) *Chondrus crispus*; (E) *Gracilaria dura*; (F) *Fucus serratus*; (G) Phytoplankton.

significantly improve the fits. Interestingly, the fit could be improved by simulating the contribution of the shell on the high-R side of the major shell; the reason that this is not included in the final presented simulation is that it was impossible to decide whether this should be a chlorine or a bromine shell. We have great difficulty in rationalizing a molecular model that combines a shell of H-bonded heteroatom atoms (3.3 Å) with a halogen at 4.0 Å. Depending on the conformation of the molecule, this could be a reasonable distance between halogens attached to vicinal carbon atoms, which are known form other algae (Katsui et al. 1967, Woolard et al. 1976, Paul et al. 2006b, McConnell and Fenical 1977, Scarratt and Moore 1996). In that case, however, the absence of a contribution from C, in particular the one directly bound to Br, is hard to explain. Another possibility would be that the C-bound Br interacts with another C-bound halogen by a noncovalent halogen bond (Metrangolo et al. 2005) but for that the distance is too long; there is crystallographic evidence for I···Br halogen bonds in the range 3.09–3.28 Å (Kilah et al. 2010) which is clearly much shorter than 4 Å.

Red algae. The Br X-ray absorption K edge spectra of red algae and related model compounds are presented in Figure 4. The spectra of the representatives of the Florideophycaeae family (*Osmundea*, Fig. 4H; *G. gracilis*, Fig. 4I; and – subtly different in the positions of the second and third maxima – *G. dura*, Fig. 4J) and that of one of the Rhodophycaeae (*Chondrus*, Fig. 4G) are similar to those of most brown algae and aqueous NaBr in Figure 1, and indicate the presence of Br^-. The edges of the

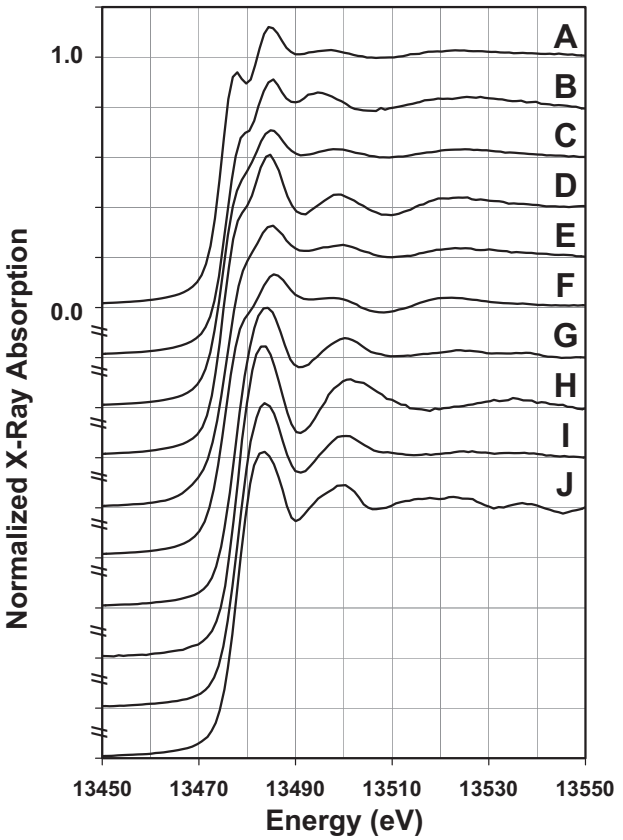


FIG. 4. Bromine K edge X-ray absorption edge spectra of red algae and relevant model compounds: (A) $CHBr_3$ (aq., from Feiters et al. 2005a); (B) 2,2-dibromopropane (aq.); (C) *Asparagopsis armata*; (D) 10 mM 2-Br-phenol in 1 M aqueous KOH, (E) *Corallina officinalis*; (F) *Polysiphonia elongata*; (G) *Chondrus crispus*; (H) *Osmundea* (*Laurencia*) *pinnatifada*; (I) *Gracilaria gracilis*; (J) *Gracilaria dura*.

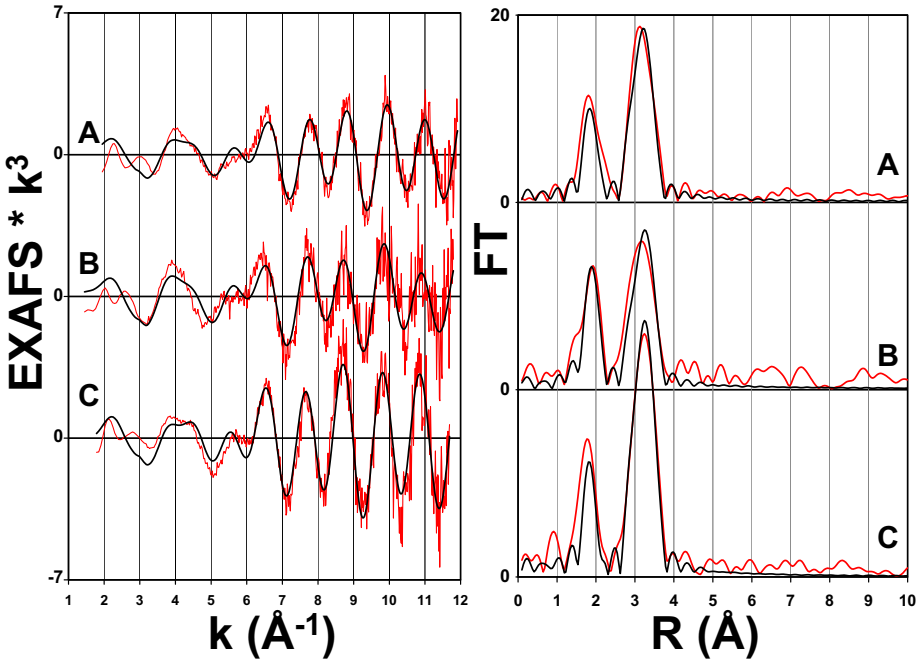
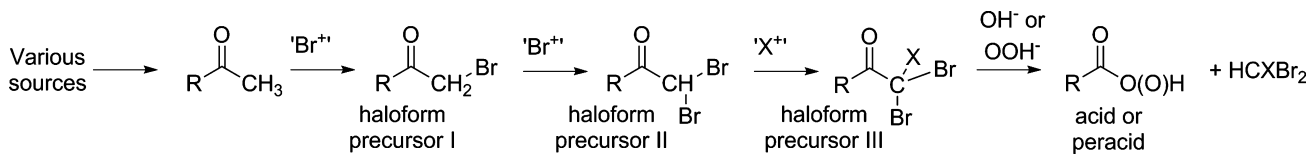


FIG. 5. Experimental (nonblack) and simulated (black; parameters in Table 1) k^3-weighted EXAFS (left) and its Fourier transform (right) of (top to bottom): (A) *Asparagopsis armata*; (B) aqueous 2,2-dibromopropane; (C) aqueous $CHBr_3$.

other Rhodophycaeae (*Asparagopsis,* Fig. 4C; *Corallina,* Fig. 4E; *Polysiphonia*, Fig. 4F) are similar to those of model compounds in which Br is covalently bound to carbon, such as $CHBr_3$ (Fig. 4A), 2,2-dibromopropane (Fig. 4B), 2-bromophenol (Fig. 4D), and 4-bromophenylalanine (Feiters et al. 2005a).

The Br EXAFS of *A. armata* and its Fourier transform are very similar (Fig. 5; Table 1) to those of bromoform ($CHBr_3$, Feiters et al. 2005a) and 2,2-dibromopropane, as they all feature a Br-C bond of 1.91–1.96 Å (indicative of sp^3 C) and a Br shell at 3.15–3.20 Å (i.e., one or more "geminal" bromine scatterers, attached to the same carbon as the absorber). This is in line with the observation that this alga is by orders of magnitude more active in evolving bromoform and analogs than the others (Carpenter et al. 2000, Mata et al. 2011). *Asparagopsis* species contain many different geminal dibromo and tribromo compounds (e.g., Woolard et al. 1976).

When simulated and iteratively refined over the same range as is available for the *Asparagopsis* sample, the numbers of C and Br in the model compounds are slightly under- and over-estimated, respectively. From a direct comparison, it can be concluded that *Asparagopsis* is more likely to contain a compound like 2,2-dibromopropane, such as the haloform precursor II in Scheme 2, than bromoform or another 1,1,1-tribromo compound; the spectrum is also consistent with what would be expected for other geminal dibromo compounds such as dibromoacetic acid (Paul et al. 2006b). It has to be kept in mind, however, that on the basis of the EXAFS, it is also possible that *Asparagopsis* contains a mixture of the haloform precursor I and III (or bromoform) in Scheme 2. The 1,1-bis-brominated precursor (haloform precursor II) in Scheme 2 would require just one more halogenation (which could also be by Cl or I, explaining the observed diversity in the haloform analogs formed (Carpenter et al. 2000) to form haloform precursor III which is more susceptible to attack by OH^- or OOH^- than II. In studies on both red (Theiler et al. 1978) and green (Beissner et al. 1981) algae, 3-oxo-octanoic acid was identified by mass spectrometry as a likely source for the haloform precursor in a pathway where the haloform precursor II from Scheme 2 would correspond to 1,1-dibromo-heptan-2-one. Our study provides the first ever, potentially significant in vivo evidence, obtained by a noninvasive technique, that the haloform reaction, which has been suggested to occur in the synthesis of bromoform



SCHEME 2.

by haloperoxidases in marine algae (Wever and van der Horst 2013), is operative as a biosynthetic pathway of halocarbons in a marine alga.

The EXAFS and Fourier transforms of most of the other red algae are shown in Figure 3, along with that of 2-bromophenol. Although similar to *Asparagopsis* in the appearance of the edge (Fig. 4), the EXAFS shows that *Corallina* (Fig. 3A) and *Polysiphonia* (Fig. 3B) contain Br covalently bound to C in a different form, viz. a phenyl ring. The Br-C bond length (1.85–6 Å) is in reasonable agreement with that found for an sp^2 carbon in the aromatic amino acids 4-bromophenylalanine and 3,5-dibromotyrosine (1.87–8 Å, Feiters et al. 2005a), but a little shorter than the model compound 2-bromophenol (1.91 Å) included in this study. In crystallographic studies on a di-bromotyrosine analog (Stewart et al. 2004), Br-C distances of 1.896 and 1.899 Å were found. 2-Bromophenol is of interest since this has actually been isolated from *Corallina*, along with larger amounts of bis- and tris-brominated phenols (Whitfield et al. 1999). We have been able to detect any 1,3-dibromo substituted aromates in our previous work on 3,4-dibromotyrosine (Feiters et al. 2005a) and bromoperoxidase (Feiters et al. 2005b), but we do not detect them in *Corallina*. The apparent discrepancy may be explained by the fact that our current EXAFS study is noninvasive, with no possibility of additional halogenations during isolation. The presence of aromatic bromine compounds in *Polysiphonia* species is well established (Craigie and Gruenig 1967). A weak contribution for H-bonded Br^- was included for *Polysiphonia* but did not improve the simulation significantly for *Corallina*. The EXAFS and Fourier transform of *G. dura* (Fig. 3D) and, to a lesser extent, of *C. crispus* (Fig. 3C) and the brown alga *F. serratus* (Fig. 3E) are reminiscent of a spectrum first encountered in the EXAFS studies of parts of *Laminaria digitata* (Küpper et al. 2013), in particular the meristem, which was interpreted as due to the occurrence of a Br^- ion in the site of a Cl^- defect in a KCl lattice. The EXAFS spectrum shows a strong feature just below 6 $Å^{-1}$, and the Fourier transform has a characteristic pattern of K, Cl, K, and Cl shells at ~3.2, 4.5, 5.4, and 6.4 Å, respectively. In all three cases, a relatively weak C shell in the range 1.90–1.94 Å was also included in the simulation. Inclusion of a shell of H-bonded O atoms (as present in hydrogen-bonded bromide) was found to improve the quality of the simulations for *Chondrus* and *Fucus*, but not for *G. dura*. It is not possible to quantify both the amounts of Br involved in KCl defects and H-bonding, because of the variability in the numbers of closest atoms (K and O, respectively) involved, and the superposition combined with destructive interference of the K and O contributions (which are out of phase for most of the range of the EXAFS) at just above 3 Å in the Fourier transform.

Because their EXAFS is very weak, and relatively noisy at high k, the spectra of *Osmundea* (*Laurencia*) *pinnatifida* and *G. gracilis* are included in Figure 2 as traces E and F, respectively. Both contain H-bonded bromide and bromine covalently bound to carbon (at 1.90–1 Å) to varying extents, with the fraction of C-bound Br corresponding to the occupancy of the C shell.

DISCUSSION

XAS versus other microscopic techniques using chemical fixation or chromatographic techniques after chemical extraction. Up to now, it is difficult to draw a general conclusion about halide contents, especially in relation with phylogenetic data. If red and brown algae differed according to their iodine contents, especially for Laminariales, the differences appeared indeed less significant according to total bromine content (Hou and Yan 1998, Romaris-Hortas et al. 2012a,b). Following EXAFS acquisitions, the total iodine (from 0.025 to 1.6 $mg \cdot g^{-1}$ of dry weight) and bromine (from 0.24 to 0.99 $mg \cdot g^{-1}$ of dry weight) contents of algal samples showed similar patterns with higher iodine contents in Fucales, such as *A. nodosum*, and a smaller range for bromine contents (see Table 2). Most speciation studies of bromine in red and brown algae were focused on the chemistry and localization of haloge-

TABLE 2. Iodine and bromine contents of selected algal XAS samples.

XAS samples	Iodine		Bromine	
	$mg \cdot g^{-1}$ DW	$mg \cdot g^{-1}$ FW	$mg \cdot g^{-1}$ DW	$mg \cdot g^{-1}$ FW
Heterokontophyta				
Ectocarpus siliculosus	0.078	0.028	0.24	0.09
Fucus serratus	0.890	0.150	0.99	0.17
Ascophyllum nodosum	1.600	0.459	0.36	0.10
Rhodophyta				
Chondrus crispus	0.134	0.026	0.98	0.19
Osmundea (Laurencia) pinnatifida	0.430	0.056	0.74	0.10
Gracilaria gracilis	0.160	0.035	0.71	0.15
Gracilaria dura	0.071	0.014	0.89	0.17
Others				
Natural phytoplankton sample	0.025	nd	0.71	nd

nd, non-determined.

nated compounds. They were often based on selective chemical extraction using organic solvents (Fenical 1975). Most of these speciation studies remain somewhat questionable since they rely on extracting bromine species from frozen or dehydrated seaweed samples. During the extraction process, the modification of the redox potential induced by osmotic shock, the dilution and the nonphysiological pH of aqueous buffers may have altered bromine speciation (Wright et al. 2003).

In this study, XAS has once more proven to be a powerful tool to noninvasively investigate the in vivo speciation of a bioelement that had hitherto been poorly studied in this respect. Other studies have successfully employed XAS to investigate the in vivo speciation of bromine (Küpper et al. 2013) and iodine (Küpper et al. 2008) in *Laminaria*, iron in *Ectocarpus* (Böttger et al. 2012), copper in the first documented hyperaccumulator of this element, *Crassula helmsii* (Küpper et al. 2009), cadmium and zinc in the hyperaccumulator *Thlaspi caerulescens* (Küpper et al. 2004) and selenium (Lee et al. 2001). Obviously and while being a powerful qualitative analytical technique, XAS is not a quantitative technique.

Other techniques have been applied for the in situ study of halogenated compounds in marine algae. Desorption electrospray ionization mass spectrometry (DESI-MS) and some applications, such as imaging, have very recently started to be used for natural products detection on intact algal tissue surfaces (Lane et al. 2009, Nyadong et al. 2009). DESI-MS is an ambient ionization technique coupled with mass spectrometry, allowing probing the surface of solids, liquids, frozen solutions, and adsorbed gases (Wiseman and Laughlin 2007). DESI-MS has been shown to be a sensitive and effective approach to detect algal diterpene-benzoate macrolide natural products, namely bromophycolides, directly on the surface and interior of the red alga, *Callophycus serratus* (Nyadong et al. 2009). Imaging, one of DESI-MS possible applications, has been shown to provide an exceptional capability to map secondary metabolites to distinct algal surface sites (Lane et al. 2009).

Halogen storage pools in different algal classes and defense strategies. In conclusion, the results of this study show that most of the bromine in the Ectocarpales and Fucales (represented by *F. serratus* and *A. nodosum*) is present as bromide via H bonds in a heteroatom environment, but they also show evidence for smaller amounts of bromine incorporated in organic compounds by covalent bonds to carbon. In contrast, the diatom *Thalassiosira* shows a predominance of Br bound to nonaromatic hydrocarbons, while the *Lingulodinium* culture and the natural phytoplankton sample contain bromide surrounded by heteroatoms of biomolecules via H bonds. In addition, *Lingulodinium* and phytoplankton also feature an as yet unassigned shell of atoms at 4 Å from Br. This is consistent with the sparse literature available – *Thalassiosira* has indeed been reported to produce methyl halides (Scarratt and Moore 1996, Carpenter et al. 2012) impacting atmospheric processes (in particular, $CHBr_3$; Hughes et al. 2013), while no knowledge seems to exist whether dinoflagellates have a halogen metabolism.

Likewise, the bromine pool of the red algae *Osmundea*, *C. crispus*, *G. gracilis*, and *G. dura* is dominated by bromide. The spectra of *G. dura*, like those of *F. serratus*, show the incorporation of at least part of the bromide pool in a KCl lattice, while *G. gracilis* and *O. pinnatifida* also have measurable amounts of organically bound Br. In contrast, *Asparagopsis*, *Polysiphonia*, and *Corallina* have mostly organically bound bromine. While most of the Br pool of *Asparagopsis* is aliphatically bound, Br in *Polysiphonia* and *Corallina* is mostly bound to aromatic molecules.

Organohalogens can cause adverse effects and their production in living organisms requires a tight regulation or a capability to partition compounds into specialized storage structures to avoid autotoxicity (McKey 1979). They include gland cells (or vesicle cells), in which the vesicle occupies practically the entire cellular space (Young and West 1979, Dworjanyn et al. 1999), and cells with refractile inclusions, such as corps en cerise (CC) or cherry bodies (Young et al. 1980) and physodes in brown algae (Schoenwaelder 2002). Most specialized storage cells are located at, or close to, the surface cell layer. Among the different classes of marine macroalgae, red algae are the major producers of halogenated compounds (Cabrita et al. 2010). *Laurencia* (*Osmundea*, Rhodomelaceae) is considered one of the most prolific genera in this respect (Faulkner 2001, Wright et al. 2003). It has been intensively studied over the last five decades, even though new halogenated compounds are still being reported from this genus. Typically, the degree of halogenation found in compounds from *Laurencia* is rather high (Hill 2007). Major secondary metabolites of this genus are diterpenes, sesquiterpenes, triterpenes, and C15-acetogenins (Erickson 1983, Faulkner 1995), associated with cytotoxic (Juagdan et al. 1997, Sun et al. 2005), antimicrobial (König and Wright 1997), antigrazing (Kurata et al. 1998), and antihelmintic (Davyt et al. 2001, Topcu et al. 2003) activities. Bromoform produced by the red algae *Lithophyllum yessoense* and *Corallina pilulifera* was found to inhibit the growth of epiphytic diatoms on algal surfaces, and this activity was dependent on vBPO activity (Ohsawa et al. 2001). The Australian red alga *Delisea pulchra* produces halogenated furanones, structural analogs to *N*-acyl homoserine lactones (AHL), which disrupt quorum sensing in biofilm-forming bacteria, thus protecting algal surfaces by selective inhibition of bacterial colonization and biofilm formation (Maximilien et al. 1998, Rasmussen et al. 2000, Manefield et al. 2002). In

C. crispus gametophytes, three VHOCs, upregulated after defense elicitation, were toxic against algal spores and germlings of *Acrochaete operculata* (K. Bourab and P. Potin, personal communication). In red algae, VHOCs seem to have an important physiological role in activated defense responses, acting as biocidal or repelling substances against microorganisms and herbivores (Paul et al. 2006b), but also preventing the settlement of epiphytic (Ohsawa et al. 2001) or pathogenic algae (Bouarab et al. personal communication). In the red alga *A. armata*, bromoform and dibromoacetic acid are produced in high quantity and stored with other brominated compounds in specialized gland cells. When released at the surface of the thallus, they displayed antibiotic activity against epiphytic bacteria (Paul et al. 2006b). In the same alga, brominated compounds have also a role in feeding deterrence of mesograzers, because more intensive consumption of algae was observed, when brominating metabolism was less intense by bromide starvation of algal cultures (Paul et al. 2006c). One of the most interesting aspects of the present study is that the in vivo results from *Asparagopsis* provide novel support for a bromoform biosynthetic pathway via the haloform reaction. In fact, over 100 volatile halogenated compounds were identified in both *Asparagopsis taxiformis* and *A. armata*, including haloforms, haloacids and haloketones, some of the compounds being only described for the genus (McConnell and Fenical 1977, Woolard et al. 1979). Besides their high diversity, high quantities of these metabolites are stored in specialized structures known as gland cells, which may represent 2%–6% of the algal dry weight (Wolk 1968, Fenical 1975, Marshall et al. 2003, Paul et al. 2006a).

Therefore, it appears that two distinct defense strategies occur in red algae that rely on the one hand on the compartmentation of halogenated metabolites that are constitutively stored in specialized vesicles, and on the other hand on an activated mechanism of bromination that is operative upon oxidative stress in red algae that may be concomitant with biotic or abiotic perturbations.

General conclusions for algal halogen metabolism. Based on the results presented here and in light of existing literature, it is possible to draw the following conclusions:

1 Brown algae such as *Ectocarpus* store bromide ions and some brominated compounds. Bromide storage is mostly extracellular in kelps (Verhaeghe et al. 2008). It is tempting to hypothesize that this is likely linked to the activities of apoplastic VHPO and this system at least in kelps favors iodine accumulation. This evolutionary pathway is likely linked to the antioxidant potential of iodide, partly complemented by bromide.

2 Red algae display a large diversity of brominated organic compounds the abundance of which is probably linked to compartmentation – as highlighted especially by the case of *A. armata*. 90% of secondary metabolites in red algae are brominated (Harper et al. 2001), storage of bromide is probably in most cases intracellular and may provide substrate for the bromination (Weinberger et al. 2007) and biosynthesis of secondary metabolite (Butler and Carter-Franklin 2004, Butler and Sandy 2009).

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