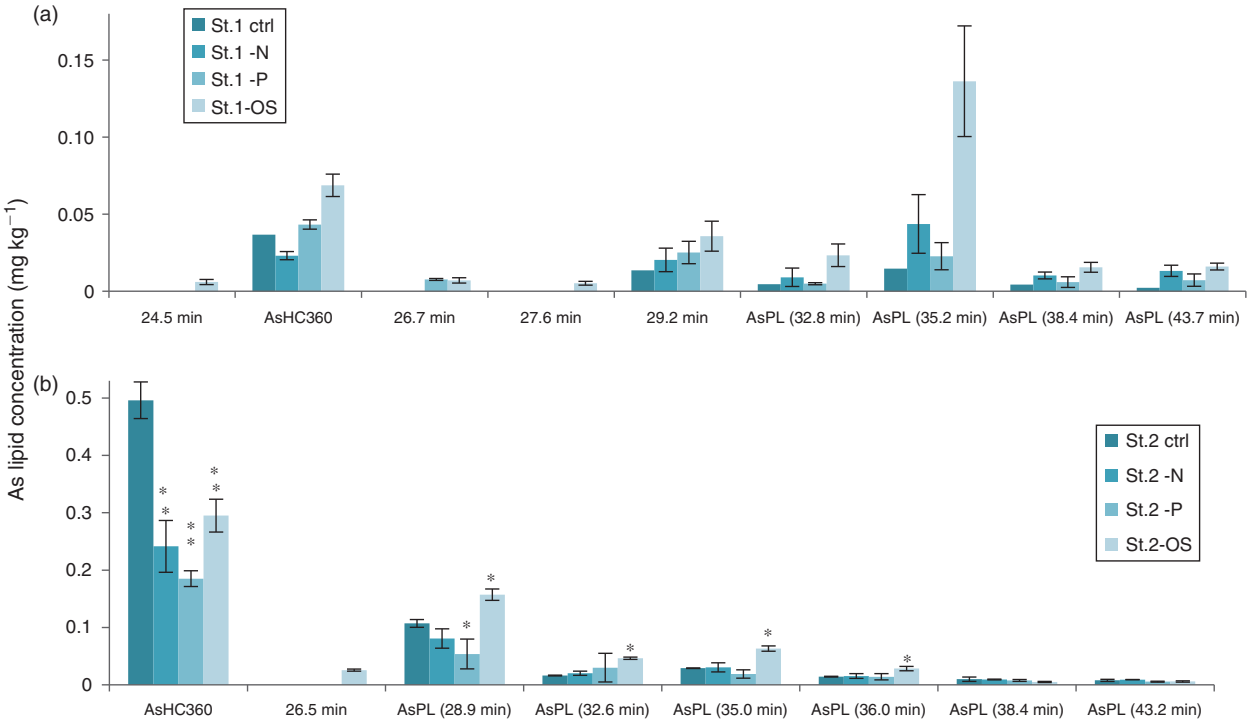


Fig. 5. Arsenolipid profiles and quantification for strains 1 and 2 under different environmental conditions. Conditions statistically significant from control using ANOVA (see text for definitions). *, $P < 0.05$; **, $P < 0.001$: strain (a) St1; (b) St2.

and As-specific detection. Because of the similarities of AsLps found in *Ectocarpus*, *Pylaiella* and *Elachista*, all filamentous brown algae, and the identification in the cultures of the same main AsHC as found in the field-collected *Ectocarpus*, it can be assumed that similar AsPLs will be present in the cultures. The lipid profile differed between the different environmental conditions as well as between the three strains. Only the cultures grown under the low-nitrate conditions generally did not differ significantly from the controls.

Oxidative stress

The cultures grown under oxidative stress had statistically significant higher concentrations for AsPLs compared with control samples, a pattern noticed for all three strains (Figs 5, 6). Seaweeds are constantly exposed to high UV radiation and radical formation, which could cause lipid peroxidation. The treatment with H_2O_2 not only caused significant physiological changes, but also an increase in AsLp synthesis. Whether the introduction of a redox-active element into a lipid environment as AsPL can be interpreted as a radical-scavenging mechanism, because trivalent and pentavalent arsenic are interconvertible at physiological conditions, remains to be studied in more detail.

Effect of arsenic on AsLp distribution

Higher As concentration in the media led to higher concentrations of AsLps (Fig. 6). For St2 (*E. fasciculatus*) and St3 (*E. siliculosus*), 60–80 % of the AsLps were AsHCs (Table 3), which is similar to the EC in nature (75 % ± 8). The AsHCs in the St1 (*E. crouaniorum*) control sample account for just under 50 % of the AsLp concentration, which was the sample with the lowest concentrations of AsLps. Conversely, the St3+As (*E. siliculosus*) had the highest amount of AsLps and the highest percentage of AsHCs in the control cultures. Therefore, it appears, for *Ectocarpus*, that at low concentrations of AsLps, the arsenic spreads fairly equally between all the AsLps, whereas excess arsenic results in the formation of additional AsHCs, mainly in the form of AsHC360 (Figs 5, 6).

It was observed that for both St3 (*E. siliculosus*) and St2 (*E. fasciculatus*), oxidative stress and low phosphate treatments differed from the control (statistically significant, at least $P < 0.05$, Table 3) for both AsPLs and AsHCs. These findings underline the effect the different stress conditions have on the arsenolipid profile. St1 (*E. crouaniorum*) had only one replicate for the control conditions, and hence it was not possible to evaluate if any statistical differences existed.

Low-phosphate conditions

For strain St3 (*E. siliculosus*), AsHC360 was the main AsLp peak for all conditions but under low-phosphate conditions, a shift occurred from AsHC360 to an AsPL. The AsHC360 peak was only ~1/3 compared with the other conditions, whereas the AsLp peaks are more concentrated, with an approximately four-fold increase. This could be due to mechanisms activating more efficient phosphate transporters under low-phosphate conditions, and because of the similarities between arsenate and phosphate, additional arsenate is taken up, leading to an increase of the AsPLs. The pattern of higher amounts of AsPLs in strain St3 under low-phosphate conditions can be clearly noted (Table 3).

Under low-phosphate conditions, strain St3 (*E. siliculosus*) increases the production of AsPLs but decreases AsHC production. This is different from the increase of AsLps under oxidative stress conditions, where this increase in AsLps represents a higher total AsLp concentration compared with control conditions. Here, for ECs grown under low-phosphate conditions, there is no increase in the total AsLp concentration but rather a shift in the lipids from one type of AsLp to another, i.e. from AsHCs to AsPLs.

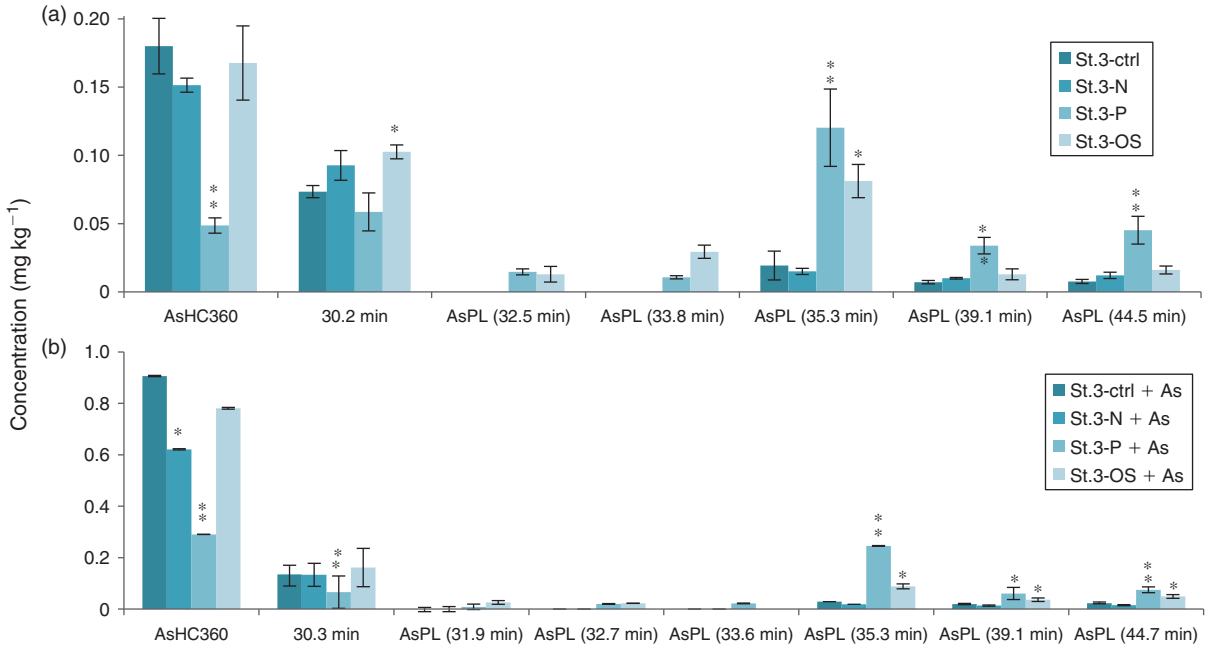


Fig. 6. Arsenolipid profiles and quantification for St3. Condition statistically significant from control using ANOVA: $*P < 0.05$, $**P < 0.001$; (a) St3; (b) St3 under enriched arsenic conditions.

Table 3. Summary of the percentages of arsenohydrocarbons (AsHCs) and arsenophospholipids (AsPLs) in the samples as well as arsenosugars (AsSugars) (mean ± s.d., $n = 3$, replicate cultures)

Samples listed are all three strains of Ectocarpus cultures (EC) at different conditions and field collected samples (*Elachista*, *Pylaiella*, *Ectocarpus* from Aberdeen (EC Abdn) and *Ectocarpus* from Reykjavik (EC Rvk)). Significance refers to whether the percentage of AsPLs and AsHCs differs statistically from control conditions using a one-way ANOVA (multiple comparisons versus control). NA, not applicable; NS, not significant. Only one replicate for St1 was used for control conditions, statistical evaluation not computed

		AsHC (%)	Significance	AsPL (%)	Significance	AsSugOH (%)	AsSugPO$_4$ (%)	AsSugSO$_3$ (%)
Nature	*Elachista*	57 ± 9	NA	28 ± 10	NA	33 ± 5	26 ± 1	37 ± 3
	Pylaiella	38 ± 9	NA	34 ± 12	NA	8.6 ± 0.1	16.8 ± 0.2	69.0 ± 0.3
	EC Abdn	75 ± 8	NA	6 ± 11	NA	44 ± 7	13 ± 2	16 ± 2
	EC Rvk	75 ± 5	NA	8 ± 11	NA	19 ± 3	20 ± 14	23 ± 10
St2	EC control	72 ± 1		11 ± 1		19 ± 3	22 ± 2	57 ± 8
	EC N	40 ± 1	$P < 0.05$	19 ± 2	$P < 0.01$	14 ± 4	15 ± 3	69 ± 4
	EC P	59 ± 2	$P < 0.05$	17 ± 3	$P < 0.01$	33 ± 7	1.2 ± 0.5	62 ± 15
	EC H_2O_2	50 ± 3	$P < 0.01$	25 ± 1	$P < 0.01$	17 ± 2	12 ± 1	68 ± 6
St1	EC control	48	–	34	–	52 ± 5	7 ± 2	29 ± 6
	EC N	20 ± 4	–	62 ± 12	–	30 ± 1	30 ± 8	27 ± 5
	EC P	38 ± 5	–	36 ± 5	–	48 ± 7	0	27 ± 2
	EC H_2O_2	23 ± 3	–	60 ± 5	–	3.4 ± 0.1	30 ± 2	2.3 ± 0.3
St3	EC control	62 ± 6		12 ± 4		43 ± 13	0	31 ± 12
	EC N	54 ± 2	NS	13 ± 1	NS	32 ± 7	13 ± 11	22 ± 6
	EC P	15 ± 3	$P < 0.01$	69 ± 6	$P < 0.01$	4 ± 4	0	54 ± 18
	EC H_2O_2	40 ± 4	$P < 0.01$	36 ± 4	$P < 0.01$	33 ± 2	8 ± 3	15 ± 1
St3 + As	EC control	81 ± 2		6 ± 1		36 ± 2	5 ± 1	36 ± 11
	EC N	77 ± 2	NS	6 ± 2	NS	32.6 ± 0.4	20 ± 10	31 ± 4
	EC P	36 ± 3	$P < 0.01$	55 ± 5	$P < 0.01$	35 ± 2	0	43 ± 9
	EC H_2O_2	64 ± 5	$P < 0.01$	18 ± 2	$P < 0.01$	32.6 ± 0.1	13 ± 10	34 ± 3

Arsenosugars in Ectocarpales collected in their natural habitats

All water fractions were measured with HPLC-ICP-MS, and a *Pylaiella* sample studied with HPLC coupled simultaneously to ESIMS and ICP-MS to identify the main AsSugars. A similar pattern was found for all samples and the other AsSugars were identified by retention times of the ESIMS-identified peaks of the *Pylaiella* sample. The peak at R_t 14 min was iAs, which was confirmed both with spiking experiments as well as analyses with HG-ICP-MS (not shown). The small unidentified peak at

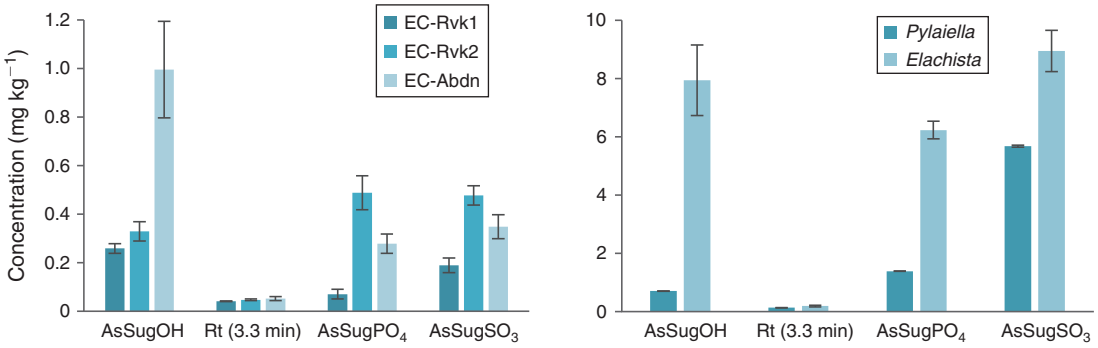


Fig. 7. Quantities of AsSugars in filamentous brown algae found in nature and their AsSugar profile with identified species for (a) *Ectocarpus* from Aberdeen Beach (EC Abdn) and *Ectocarpus* from Iceland (EC Rvk) (collected on separate days); (b) *Pylaiella* and *Elachista*. More details in Table S12.

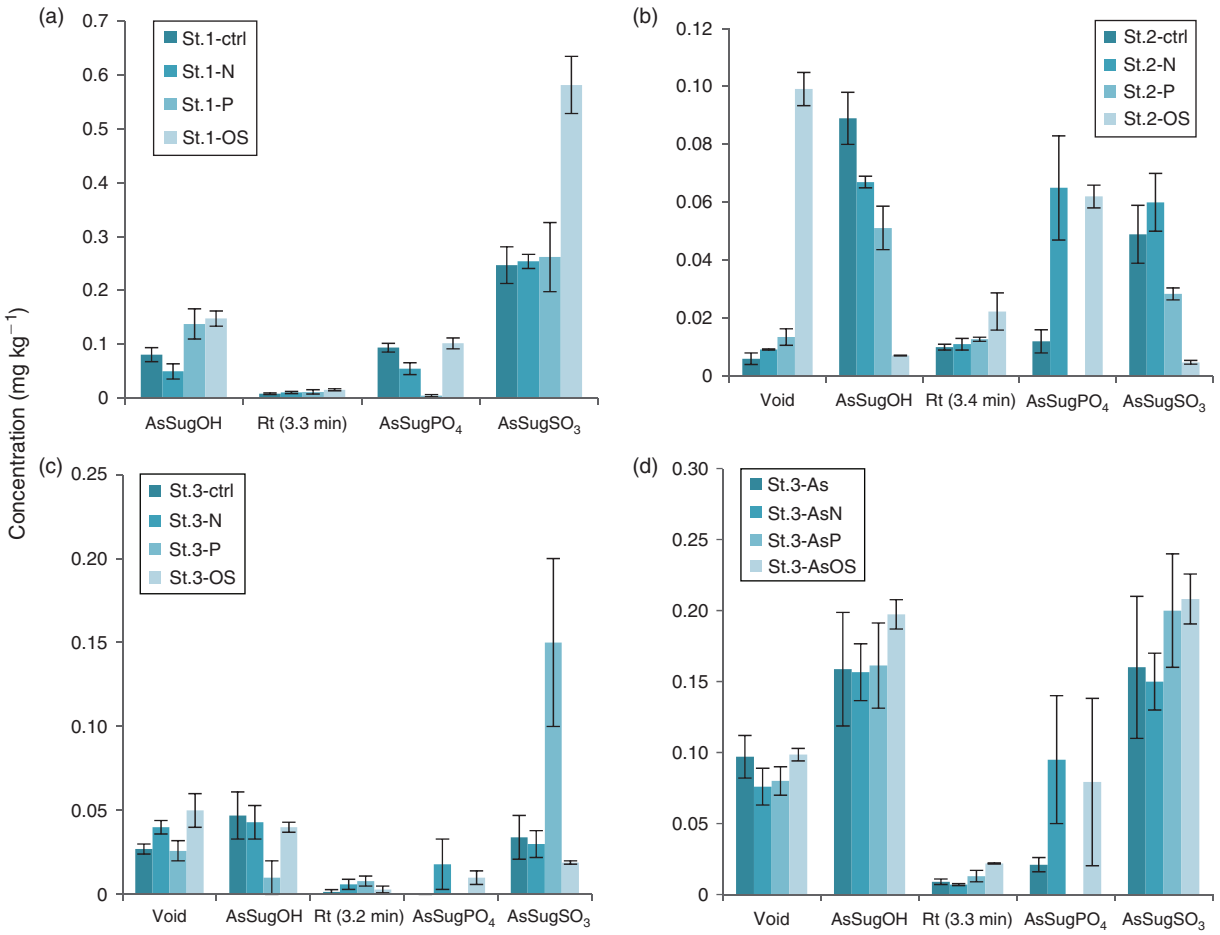


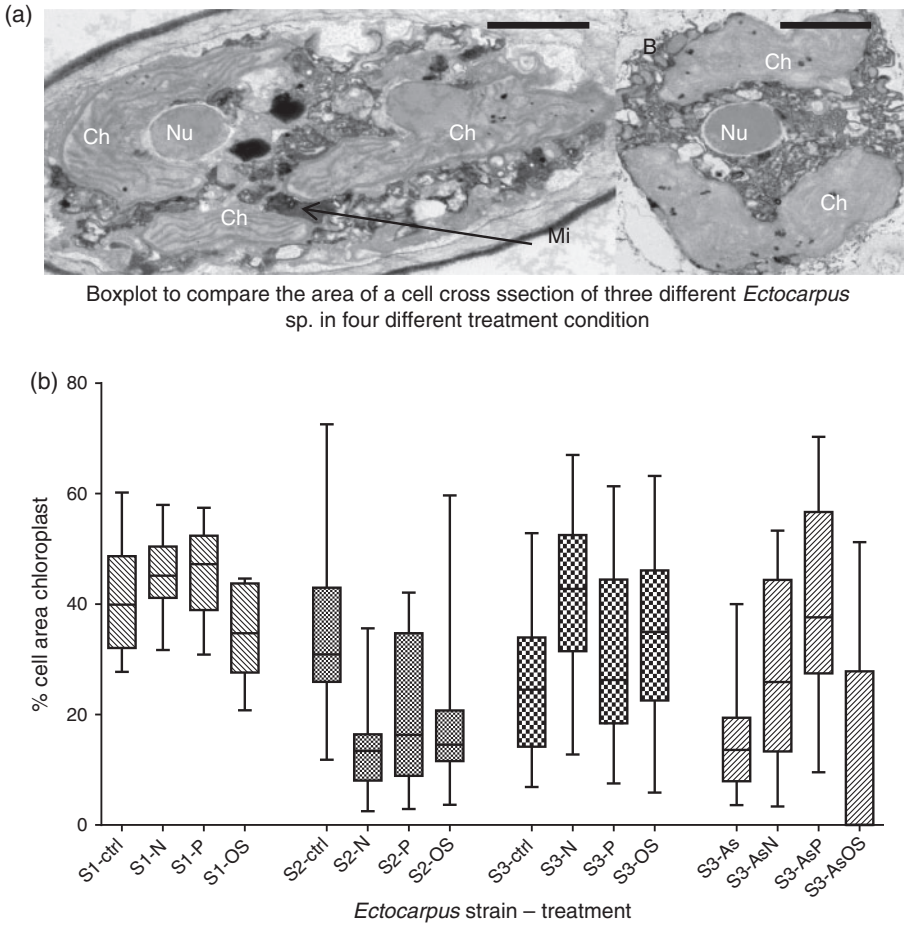
Fig. 8. Quantification of the water-soluble arsenic species found in the different cultures: (a) St1; (b) St2; (c) St3; and (d) St3 under arsenic-enriched conditions. Dimethylarsinic acid (DMA) elutes at 3.3 min.

R_t ~3.3 min is thought to be DMA based on earlier studies on seaweed using the same method (Fig. S12).[48] This was a minor peak and its identification was not pursued further. Quantification and the AsSugar profile in the Ectocarpales can be seen in Fig. 7.

Arsenosugars in Ectocarpus *cultures*

The arsenosugars in the *Ectocarpus* cultures were mainly found to be the same as in the Ectocarpales in nature. AsSugarOH, AsSugarSO_3, AsSugarPO_4 and DMA were identified in all cultures (Fig. 7).

The St1 (*E. crouaniorum*) culture under oxidative stress conditions yielded the highest totAs in the water fraction owing to an increase in AsSugarSO_3 (Fig. 8a). However, for St2 (*E. fasciculatus*), the opposite pattern was noted, where AsSugarSO_3 was found at very low concentrations in the H_2O_2 culture compared with the other three conditions (Fig. 8b). A noticeable pattern was seen for AsSugarPO_4, which was present in all the



Boxplot to compare the area of a cell cross ssection of three different *Ectocarpus* sp. in four different treatment condition

Fig. 9. (a) Electron micrographs of *Ectocarpus* grown in different culture media. The chloroplasts (Ch), nucleus (Nu) and mitochondria (Mi) are all visible. The chloroplast in the control culture (a) are visibly smaller than the chloroplasts present in the *Ectocarpus* grown in low-nitrate media (b). Scale bars: 2 µm. (c) Box plot depicting the changes in chloroplasts for the different *Ectocarpus* cultures dependent on treatments.

strains in nature and in all cultures except in the low-phosphate EC cultures. AsSugarPO$_4$, the only AsSugar that contains phosphate, is fairly abundant (13–20 % of AsSugars for EC in nature, ranging from 5 to 30 % of AsSugars for the cultures but constituting only 0–1 % of total AsSugars under low-phosphate conditions). *Ectocarpus* may have conserved the phosphate for other purposes.

The AsSugar concentrations in the cultures are lower than in field-collected *Ectocarpus*, apart from strain St1 (Fig. 8a), which had similar concentrations. This strain coped best with growing in cultures, because its live cells were more dispersed throughout the culture compared with the other strains where the live cells were clustered in the middle.

Changes in morphological structures dependent on environmental conditions

To obtain information about the morphological structure of the *Ectocarpus* under the different environmental conditions applied, the *Ectocarpus* cultures were prepared and analysed using TEM microscopy (Fig. 9a). This was done in order to see if the AsLps could have an effect on morphological structure, i.e. whether the incorporation of AsLps into the cell membrane would lead to a physical difference in the cell membrane or the mitochondria noticeable by TEM. This was not found to be the case as no sign of different cell membrane structure or difference in mitochondria was observed. However, there was an effect on the chloroplast size. St3 (*E. siliculosus*) had a significantly larger chloroplast size in the cell for low-nitrate and oxidative stress conditions (Table S16). Under the enriched arsenic conditions, the chloroplasts were larger for both low-nitrate and low-phosphate conditions when compared with control.

St2 (*E. fasciculatus*), however, demonstrated a completely different response with regard to chloroplast size, where the three treatments showed a reduction in chloroplast size compared with control conditions, although only statistically significant for low-nitrate conditions ($P = 0.004$) (Fig. 9b). This could be a different response to stress, or possibly the cultures could not cope as well with stress and the reduced chloroplast size indicates cell death. St1 (*E. crouaniorum*) showed no statistical difference for chloroplast size for the three different treatments compared with control conditions. This could be due to the fact that St1 (*E. crouaniorum*) coped better at growing under reduced nutrient conditions compared with the other two strains. No other organelles showed a noticeable visible change in size when comparing culture conditions.

Conclusions

Filamentous algae from nature and grown in laboratory cultures showed a similar distribution with regard to AsHCs and AsPLs,

although some distinctive differences indicate genetic or habitat influence. AsHC360 was identified in all algae samples.

Owing to the similarity of arsenate to phosphate, it was expected that algae under low-phosphate conditions might use non-phosphate-containing AsLps such as AsHCs to replace phospholipids to conserve phosphate for ATP. Here, we demonstrated the opposite, where no increase in AsHC was observed but instead an increase in AsPLs, suggesting that a deficiency in phosphate has a direct positive effect on the biosynthesis of AsPLs. It might be hypothesised that under low-phosphate conditions, the cell membrane transporters for phosphate transport more arsenate to the mitochondria, were most of the phosphate is needed. The higher arsenate concentration at the site of phospholipid synthesis would then result in a higher AsPL concentration at low-phosphate status. The absence of $AsSugPO_4$ confirms this and suggests that if phosphate is still bound to any organoarsenicals, it is preferentially bound as AsPL. Whether $AsSugPO_4$ is a degradation product of AsPLs or an intermediate needs further investigation.

If the arsenic concentration is increased, then the excess arsenic is transformed into AsHCs, mainly AsHC360. Whether this could be an indication of this polar lipid being used to replace phospholipids or some kind of detoxification needs to be studied in more detail. The synthesis of AsHCs or AsPLs is not only dependent on nutritional status and oxidative stress, but may also be dose-dependent as shown in the two concentrations of arsenic tested.

It was observed that the different treatments resulted in morphological change, in particular to chloroplast size, most likely linked to stress. This was supported by the absence of changes in the strain that coped best with stress. The stress conditions also affected the AsLp profiles. Whether these morphological and AsLp changes could be related to each other needs to be addressed in a further study. The present findings with *Ectocarpus* sp. show that there appear to be species- or strain-specific traits affecting markers of stress and arsenolipid profiles.

Supplementary material

Contained in the Supplementary material are: a table of instrumental parameters applied in the study; microscopic imaging of cultures and field-collected algae; a figure depicting the visible difference from oxidative stress compared with control conditions; ahylogenetic tree of the three cultured strains and data on the observed senescence of the cultures; all quantification data, including tables with mass balance of all algae; tables with the quantification of AsLps and AsSugars of all the algae; ESIMS data (graphs) and tables with identified peaks (MS data); information on fragmentation patterns for cultures and field-collected Ectocarpales (MS-MS data); a figure showing *Ectocarpus* sample subjected to acid and base hydrolysis; and TEM imaging data.

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