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Natural production of brominated aromatic compounds in the red alga *Ceramium tenuicorne*

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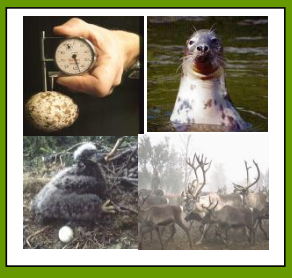

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Summary

High levels of the toxic, brominated substances; hydroxylated polybrominated diphenyl ethers (OH-PBDEs), methoxylated polybrominated diphenyl ethers (MeO-PBDEs), and 2,4,6-tribromophenol (2,4,6-TBP) have been found in Baltic biota with levels fluctuating over seasons. A growing body of evidence is pointing towards filamentous algae as a natural producer of these chemicals. However, conclusive scientific evidence supporting this hypothesis is lacking and why such production occurs has not comprehensively been addressed. This pilot study, carried out with a limited set of replicates reveals a production of 6-OH-BDE47, 6-OH-BDE137, and 2,4,6-TBP in the filamentous alga, *Ceramium tenuicorne*, a common red alga both in the Baltic Sea and in temperate waters. When exposed to grazing or strong light, monocultures of the female marine clone of *C. tenuicorne* tended to produce elevated levels of 2,4,6-TBP compared to undamaged controls. When exposed to increased levels of salinity, monocultures of the female brackish clone of *C. tenuicorne* tended to produce elevated levels of 2,4,6-TBP compared to undamaged controls. Algae collected from the Swedish east coast also tended to produce higher levels of 2,4,6-TBP than algae collected from the west coast. From field collected material, a seasonal variation with a peak of OH-PBDEs, MeO-PBDE, and TBP concentrations in July- August could also be detected.

The results of this study open up for further studies regarding natural production of brominated aromatic compounds by filamentous algae as a response to environmental stress. Increased understanding regarding sources and possible variations in production of brominated aromatic compounds is crucial for successful monitoring and assessment of environmental status.

Background

The filamentous red alga *Ceramium tenuicorne*

Macroalgae constitute the base in many coastal ecosystems and function both as primary producers and as habitat for numerous marine and brackish water organisms (Pihl et al. 1995, Korpinen et al. 2007, Schiel and Lilley 2011). The red algae (Rhodophyta) are a group of eukaryotic macroalgae, containing about 6 000 to 10 000 species, that are widespread all over the world, both in marine and brackish waters. One of the most common red alga in the Baltic Sea is the filamentous species *Ceramium tenuicorne* (Fig. 1 a-c). It grows like an epiphyte on other macroalgae, on rock or in loose lying algal mats (Bergström and Bergström 1999), from a depth of 0-1 m down to around 10 m. It has an isomorphic lifecycle (Fig. 1a), and is thus capable of both sexual and vegetative reproduction. The vegetative propagules are the dominating reproductive strategy for *C. tenuicorne* in the Baltic Sea (Bergström 2003, Bäck and Likolammi 2004).

The ecology of *C. tenuicorne* in the Baltic Sea is rather unknown, however, in the Gulf of Finland, *C. tenuicorne* is reported abundant at 1-3 m depth in the beginning of the summer and in the middle of July, propagules, plants with tetrasporangia and female gametophytes with cystocarps are found as epiphytes on *F. vesiculosus*. In the autumn *C. tenuicorne* is most abundant at 3 m depth, and no male gametophytes are found (Bäck and Likolammi 2004). In the northern Baltic proper *C. tenuicorne* starts to grow in May or June (Eriksson Wiklund et al. 2012) and reach the highest cover in July to

August (Qvarfordt 2006). Propagules are released just after the period of active growth (Kiirikki and Lehvo 1997).

C. tenuicorne is commonly used as a test organism in reproduction (Eklund 1993) and growth inhibition tests (Bruno and Eklund 2003, Eklund 2005). The growth inhibition test became an international standard test in 2010 (ISO 107 10).

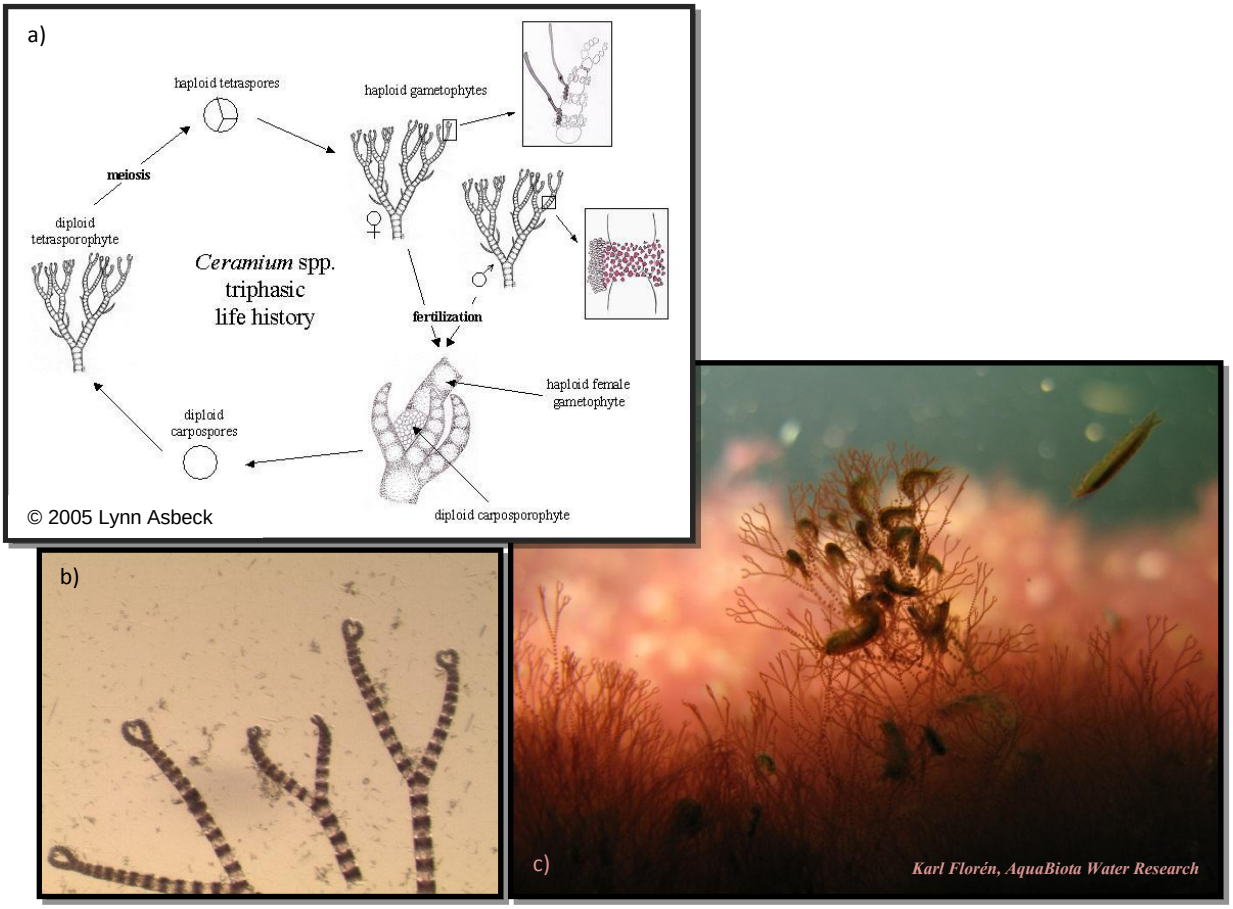

Figure 1. a) The isomorphic lifecycle of *C. tenuicorne* (www.mbari.org), **b)** microscope picture of the apical structures, **c)** *C. tenuicorne* with *Gammarus* spp.

Natural production of organohalogens

Organohalogens are organic compounds containing one or more halogen element, e.g. fluorine, chlorine, bromine, iodine or mixtures of these. More than 4000 natural organohalogen compounds have been identified in the environment, and it has been shown that the marine environment is the single largest natural producer of these compounds (Gribble 2003).

Brominated flame retardants and natural derivates

Polybrominated diphenyl ethers (PBDEs) are commercially synthesized and widely used in various products to prevent fire. Unlike PBDEs, neither hydroxylated polybrominated diphenyl ethers (OH-PBDEs) nor methoxylated polybrominated diphenyl ethers (MeO-PBDEs) are per se synthesized flame retardants or used in industrial processes, but OH-PBDEs can be formed as metabolites of PBDEs (Malmberg et al. 2005). However, both MeO-PBDEs and OH-PBDEs are also natural products, assumedly produced by for example sponges (Handayani et al. 1997) and by filamentous algae such as *Cladophora glomerata* and *C. tenuicorne* (Malmvärn et al. 2008, Löfstrand 2011).

In the naturally occurring MeO-PBDEs and OH-PBDEs, the OH- or MeO- group is located in the *ortho* position relative to the diphenyl ether bond (Marsh et al., 2003, Malmvärn et al. 2005, Athanasiadou et al. 2008). On the contrary, the hydroxylated metabolites of anthropogenic PBDEs have the OH-group mostly in a meta and/or para position relative to the diphenyl ether bond (Malmberg et al. 2005, Marsh et al. 2006). Studies have shown the possibility of demethylation of MeO-PBDEs to OH-PBDEs (Wan et al. 2009). However, in the Baltic Sea algae, this conversion of MeO-PBDEs to OH-PBDEs is unlikely to play a major role, since the concentrations of OH-PBDEs in algae and blue mussels during the summer months are much higher than concentrations of MeO-PBDEs (Malmvärn et al. 2008; Löfstrand et al. 2011).

2,4,6-tribromophenol

2,4,6-tribromophenol (2,4,6-TBP) is produced in large quantities as a wood preservative and reactive flame retardant intermediate. However, many bromophenols have been shown to be of natural origin (Flodin and Whitfield 1999).

Biological effects

MeO-PBDEs are lipophilic and hence stored in fatty tissues. In contrast to OH-PBDEs, the toxicity of MeO-PBDEs is low. High levels of OH-PBDEs and MeO-PBDEs have been found in Baltic biota, e.g. in cyanobacteria (Malmvärn et al. 2008), macroalgae and mussels (Malmvärn et al. 2005; Löfstrand et al. 2011), fish (Haglund et al. 1997, 2010, Marsh et al. 2004) and seals (Haglund et al. 1997, Routti et al. 2009). Exposure to brominated compounds, such as 2,4,6-TBP, has been demonstrated to lead to lethal as well as various nonlethal malformations in zebrafish embryos (Kammann et al. 2006). Olsen et al. (2001) discovered that bromophenols showed estrogen-like activities. Further, the OH-PBDE congener, 6-OH-BDE47 has been demonstrated to cause acute toxic effects in developing and adult zebrafish via disruption of the oxidative phosphorylation (van Boxtel et al. 2008). Several other effects have also been linked to OH-PBDEs, such as potential to disrupt the endocrine system (Legler 2008) like anti-estrogenic effects (Hamers et al. 2008).

Temporal trends

A seasonal variation in MeO- and OH-PBDE concentrations has been documented in filamentous algae (mainly *C. tenuicorne* and *C. glomerata*) and blue mussels (*Mytilus edulis*) adjacent to Askö Island in the Stockholm archipelago (Löfstrand 2011). Based on a limited sampling (four samples from three months), the results of the study indicated that the algae probably are the main source of the compounds, which then transfers to the mussels directly via feeding on algal particulates or indirectly via the water when the algae decompose. The highest concentrations of OH- and MeO-PBDEs in algae and mussels at Askö were found in the end of June, while the PBDE concentrations were low and more or less constant over time in blue mussels from the same area. This supports the theory of natural production of OH- and MeO-PBDEs.

Plant production of secondary metabolites as part of a chemical defence system

Generally, studies on plant production of secondary metabolites have demonstrated that these substances can play a defensive role in plant protection against grazing (Pavia and Toth 2000a, Gribble, 2003). In the red alga *Odonthalia corymbifera* the diphenylmethane is a potent antifeedant against abalone and sea urchins (Kurata et al. 1997). The secretion of bromophenols from brown algae has limit grazing by the gastropod *Turbo cornutus*, and bromophenols thereby act as a chemical defence against environmental stress (Shibata 2006). In *C. tenuicorne,* indication of a similar defence

strategy has been observed as the species is the least preferred fresh algae among six Baltic Sea species by the isopod *Idotea baltica*. However, after limiting the possibilities of activation of a chemical defence (e.g. removal and freezing of plant parts), the preference of the plant increased significantly (Jormalainen et al., 2001).

An induced defense meachanism in the plant can also protect against strong UV-radiation (Pavia and Toth 2000b, Swanson and Druehl 2002, Toshiyuki et al. 2006). Previous results indicating a peak in OH- and MeO-PBDEs production in the Baltic Sea during the summer (Löfstrand et al. 2011) suggests that increased sunlight radiation may trigger a production of these substances in the Baltic. Changes in salinity are an additional environmental parameter likely to trigger stress responses in plants, especially as many Baltic species are already at, or close to, their salinity tolerance limits (Jaanus et al., 2011, Müren et al. 2005).

Aim of study

The aim of the present study is to investigate whether OH-, MeO-PBDE and 2,4,6-TBP production in *C. tenuicorne* occur as a response to grazing, strong light intensity, or variations in salinity. This will be investigated in relation to differences in sex and ploidy level. Possible seasonal variation in the levels of OH-, MeO-PBDE and 2,4,6-TBP in Baltic *C. tenuicorne* will be observed and evaluated in relation to whether these levels are linked to different life stages of the algae.

Methods

Production of brominated aromatic compounds as a response to grazing, high light intensity, and salinity *(Laboratory study)*

As part of the experimental set-up, marine and brackish monocultures of *C. tenuicorne* were cultivated in growth chambers at the Department of Applied Environmental Science at Stockholm University during October and November, 2011. The marine clone originates from the Oslo fjord (20 - 25 ‰) and was isolated by Dr. Jan Rueness at the University of Oslo about 30 years ago. The brackish water clone originates from the Baltic Sea, approximately 100 km south of Stockholm (7 ‰) and was isolated by Dr. Britta Eklund at Stockholm University in 1995.

Water and jars were autoclaved and nutrients were added according to the standard test (ISO 107 10). The water was changed every fourth day. The algae was first cultivated in petri dishes, and then moved to larger glass jars. Algal material was grown in growth chamber with a light regime of 14:10 h light and darkness at a light intensity of 35 μmol^{-1} s^{-1} and a temperature of 22 ± 1 °C, corresponding to the normal situation in August when the species is most abundant (pers. comm. Eklund, B 2012). Marine and brackish water clones were grown in natural seawater, at a salinity of 20 ‰ and 7 ‰, respectively, levels reported to provide optimum growth rates (Eklund 2005).

Treatments

The algae (female, male or diploid plants from the east- or the west coast) were subjected to, an undamaged control treatment, high or low salinity (females, brackish/east coast clones), strong light intensity (females, west coast/marine clone), or grazing (females, west coast clones). For more details, see Table 1. The experiment was running for three weeks. To inflict damage to the female west coast algae in the herbivore treatment, specimens of *Idotea sp.* were added to the jars at the

first and last treatment. To maximise and standardize the amounts of damage, cutting with scissors (5 clips/algae/occasion) was conducted every third day. The plants in the strong light treatment were placed in a light intensity of 120 $\mu mol^{-1} s^{-1}$. To achieve the lower light intensity of 35 μmol^{-1} s^{-1} at same place these jars were covered with cloths. For variations in salinity, the brackish water clones were successively transferred to low or high salinity, 5 ‰ and 9 ‰ respectively (control 7 ‰). To find a lower and higher stress salinity grade, compared to the control, the salinity grades were chosen from the maximum growth rate curves provided in Eklund (2005).

Table 1. Number of C. tenuicorne and treatments conducted during the laboratory experiment.

	East coast, marine clone			West coast, brackish clone		
Treatment	**Female**	**Male**	**Diploid**	**Female**	**Male**	**Diploid**
5 ‰	3	0	1	0	0	0
9 ‰	3	0	0	0	0	0
Strong light	0	0	0	3	0	0
Grazing	0	0	0	3	0	0
Control	3	1	1	3	1	1
Total	9	1	2	9	1	1

Seasonal variation in production of brominated aromatic compounds *(Field study)*

Material of *C. tenuicorne* was collected weekly during mid-June to September 2011, from a location outside Nämdö in the Stockholm archipelago. All algae were collected from a depth of 0-1 meters and frozen immediately after collection. The water temperature, general status and abundance of the algae were noted at every sampling occasion. Algal material was investigated in the laboratory with a magnifying glass to distinguish different sexes and life stages.

Chemical analysis

Analysis of cultivated algae (Laboratory study)

The method was a slightly modified version of the method described by Jensen et al. (2009). The solvent volumes in the following description are suitable for a 1 g sample wet weight.

The sample was placed in a test tube and acetone (1 mL) was added, the algae were then gently mashed using a glass rod before centrifuging the sample. The liquid phase was transferred to a new test tube. The sample was then homogenized in a solution of 2-propanol (2.5 mL) and *n*-hexane/diethyl ether (3:1 v/v, 1 mL). The sample was placed in an ultra sonic bath for 30 min before centrifugation. The liquid phase was isolated and pooled with the acetone phase. The homogenization procedure was then repeated with a solvent mixture of 2-propanol (1 mL) and *n*-hexane/diethyl ether (3:1 v/v, 4 mL), after centrifugation the liquid phase was pooled with the previous fractions. Finally the remaining sample was vortex-mixed with *n*-hexane (1.5 mL), which was pooled with the three previous fractions after centrifugation. The total extract was then washed twice with hydrochloric acid (0.2 M) in aqueous sodium chloride (0.9% w/v) (5+2 mL).

Separation and isolation of phenolic compounds was conducted as described for the collected samples. Neutral compounds were not analyzed in the cultivated samples due to the low amounts of neutral analytes expected to occur in such small samples. The column used to clean-up the phenolic

fraction of the collected samples was omitted in this case due to the small size of the samples, but the derivatization and sulfuric acid treatment were conducted accordingly.

Analysis of collected samples (Field study)

The samples (roughly 10 g each) were extracted using cold solvent extraction with 2-propanol, diethyl ether and *n*-hexane according to Jensen et al. 2003. The neutral compounds (methoxylated compounds) in the extract were separated from the phenolic substances using pH partitioning with potassium hydroxide in 50% ethanol. The phenolic fraction was cleaned up using column chromatography (silica gel column) according to Jensen et al. (2009) before derivatization with diazomethane and treatment with sulfuric acid according to Hovander et al. (2000). The neutral fraction was treated with sulfuric acid and further cleaned up using column chromatography. The column used had two layers with 50% silica gel/sulfuric acid (3:1) on top and 50% pure activated silica gel at the bottom, dichloromethane was used as mobile phase.

Instrumentation

All samples were analysed on a Varian gas chromatograph triple quadrupole mass spectrometer in negative ionization electron capture (ECNI) mode, using helium as carrier gas and methane as buffer gas. The analyses were conducted using single ion monitoring (SIM) of the bromide ions m/z 79 and 81.

Quality control

Recoveries and procedural blanks

2'-OH-BDE28, added to the samples before extraction, was used as surrogate standard (SS) in all analyses (both field collected and lab cultivated). The recovery of the SS was calculated against a volumetric standard (VS) (BDE138), which was added to the samples prior to the instrumental analysis. The average recovery was 97±21% (n=34), all samples were recovery corrected. Procedural blanks were analyzed in parallel to all samples. Out of the quantified substances only 2,4,6-TBP was accounted for in the blanks. The amount of 2,4,6-TBP in the blanks were on average 1.7±2.3% of the amount found in the respective sample. All samples were blank subtracted.

Background levels in the water used for cultivation

The water used to cultivate the algae was also analyzed, for the west coast clones and east coast clones respectively. This was done in order to detect possible contribution of the compounds of interest from the water. As natural sea water was used, trace amounts of brominated compound originating from marine primary producer may be present. In the west coast water only 2,4,6-TBP could be quantified with confidence. The concentration of this compound was 0.36 ng/L. 2,4- and 2,6-dibromophenol (DBP) were however also identified. In the east coast water the pattern was quite different, 6-OH-BDE47 had the highest concentration with 0.42 ng/L, 2,4,6-TBP had a concentration of 0.18 ng/L, and 2'-OH-BDE68 had a concentration of 0.09 ng/L. 2,4- and 2,6-DBP, 6-OH-BDE90, 6-OH-BDE99 and 2-OH-BDE123 were also identified. The pattern in the water, in particular the east coast water, differed rather substantially from the pattern in the algae. Some of the analytes that were identified in the water were not detected in a single alga sample, e.g. 2-OH-BDE123. While 6-OH-BDE137, which was present in 12 out of the 13 east coast algal samples, was not identified in the water. Furthermore, the concentration of the predominant analyte, 2,4,6-TBP, was three orders of magnitude smaller in the west coast water (ng/g) than in the west coast algae (based on the average wet weight concentration in ng/g). The corresponding comparison for the east coast sample showed

an average of four orders of magnitude difference between the water and algae. Moreover, the total amount of 2,4,6-TBP in the water, used for each cultivation, could only account for the total amount in the algae, in four cases.

Statistics

The low amount of algal material resulted in only three replicates for all female clones, except for algae subjected to the grazing treatment, where only two replicates were analyzed. Differences in production of brominated aromatic compounds in response to our experimental treatments are presented by means with standard erros calculated in Statistica 10.

Results and discussion

This study provides scientific evidence of a production of brominated aromatic compounds in the filamentous red alga *C. tenuicorne*, proposedly induced by grazing, strong light intensity, and increasing levels of salinity. In field collected *C. tenuicorne* a seasonal variation in concentrations was observed with a peak in July-August for most congeners.

Production of brominated aromatic compounds in *C. tenuicorne* (laboratory study)

2,4,6-TBP, 6-OH-BDE47 and 6-OH-BDE137 were identified in *C. tenuicorne*, however only 2,4,6-TBP were of high enough concentrations to be quantified with confidence due to small sample sizes. Under optimal conditions regarding light, temperature, and salinity, female west coast clones of *C. tenuicorne* has been reported to grow up to 30 mm in one week (Eklund 2005). However due to the thin filaments the weight does not increase as rapidly and a substantially lower growth rate for both west- and east coast clones (in particular male and diploid plants) were observed during this study. At the termination of the experimental treatments, the low amount of algal material forced us to pool samples, resulting in a small sample size and a limited set of replicates.

In female algae exposed to the strong light treatment (120 $\mu mol^{-1}\ s^{-1}$), levels of 2,4,6-TBP were quantified to a mean of 1.43 ng/g wet weight. This was considerably higher than levels in plants grown as controls with a mean level of 2,4,6-TBP at 0.58 ng/g wet weight. (Fig 2 a). Also female algae exposed to the grazing treatment, with a mean concentration of 2,4,6-TBP at 1.49 ng/g wet weight, had substantially higher than levels in the control plants (Fig 2 a).

Female east coast clones tended to produce higher levels of 2,4,6-TBP with increasing salinity with means levels of 2,4,6-TBP at 1.25, 2.12, and 3.58 ng/g wet weight for 5‰, 7‰, and 9‰, respectively (Fig. 2b). Female east coast clones had almost four times as high levels of 2,4,6-TBP compared to female west coast clones, with mean levels at 0.58 and 2.12 ng/g wet weight, respectively (Fig 2c). 6-OH-BDE137 was detected in all but one east coast clone but not in any of the female west coast clones, while 2,4-DBP and 6-OH-BDE47 were detected in all samples. Due to the limited material we were unable to evaluate possible differences in production of 2,4,6-TBP between sexes and ploidy level.

As it seems, the production of 2,4,6-TBP in *C. teunicorne* is possibly triggered by several types of environmental stress, strong light, grazing, and increasing salinity. This indicates that the production

may be part of a chemical defense system with a multiple aim towards several types of environmental stress. Production of chemical substances in plants as part of a defense strategy against herbivores, pathogens and biofoulers, or solar radiation is well established within the scientific literature (Simms 1992) but has not previously been comprehensively addressed for *C. tenuicorne.*

Conducting the experiments in a controlled laboratory environment using clonal monocultures of *C. tenuicorne*, involved several beneficial aspects. First, it allowed us to control and to largely avoid, unwanted introduction of the compounds of interest to the algae. Furthermore, possible interference from other sources or confounding factors such as life history of the alga could be excluded by using algal clones, cultivated under controlled environmental settings for generations.

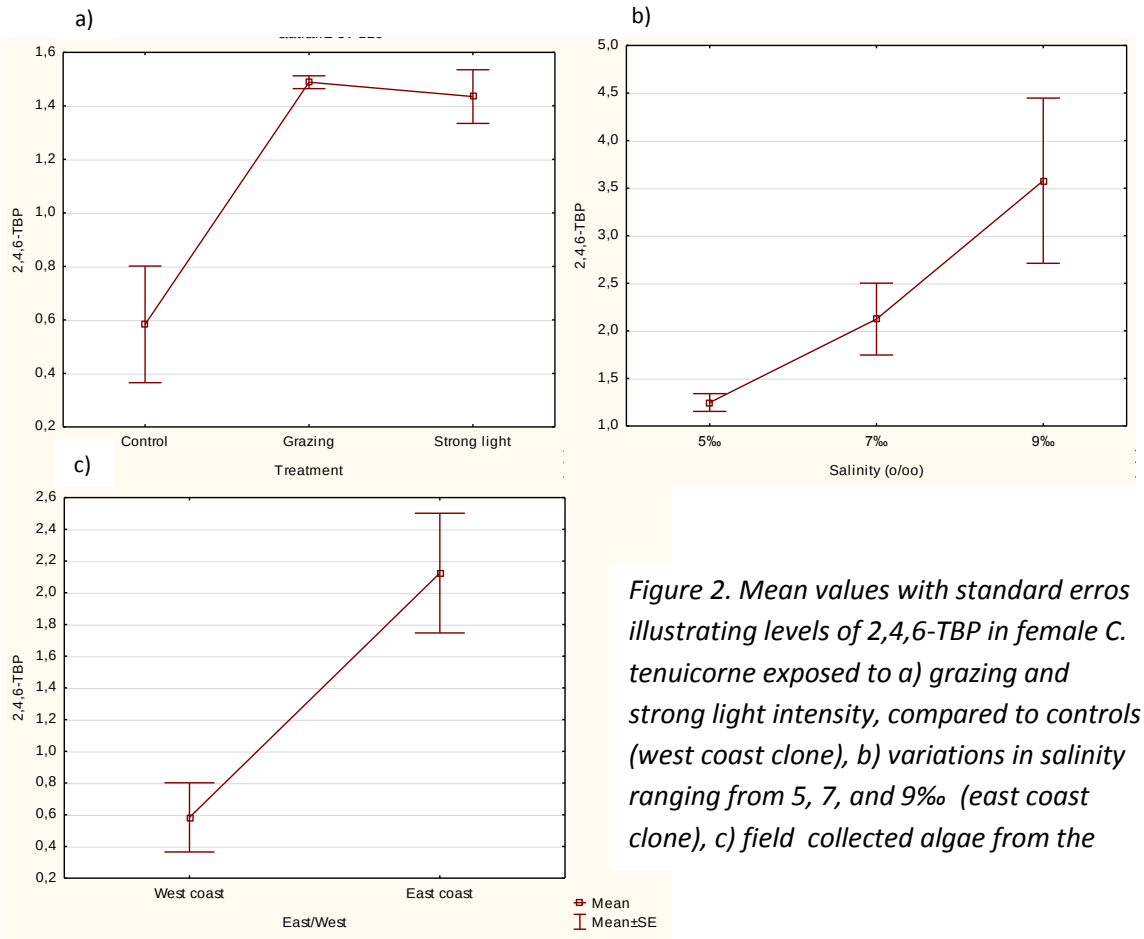

Figure 2. Mean values with standard erros illustrating levels of 2,4,6-TBP in female C. tenuicorne exposed to a) grazing and strong light intensity, compared to controls (west coast clone), b) variations in salinity ranging from 5, 7, and 9‰ (east coast clone), c) field collected algae from the

Some of the analytes did occur in the water used to cultivate the algae. However, the large differences in pattern and concentrations between the water and algae indicate that this will have little or no influence on the results of this study. Most likely an exchange of these compounds occurred between the water and the algae, but as the pK_a values of the analytes (e.g. 2,4,6-TBP) in general, were more than 1 unit below the pH of the respective water, the analytes would have predominantly existed in the water in their ionized form, with low occurrence of absorption through passive diffusion as a result. This would have affected the distribution between the water and the algae in favour of the water, thus further ensuring that the major part of the analytes found in the algae had an endogenous origin.

Seasonal variation in production of brominated aromatic compounds (field samples)

MeO-PBDE, OH-PBDEs, and 2,4,6-TBP levels detected in field collected Baltic *C. tenuicorne*, demonstrate temporal variations in concentrations during the summer months. Most MeO-PBDE and OH-PBDEs congeners peak in July (Fig. 3 a, b) and a similar trend was observed for 2,4,6-TBP where the highest value was observed in August (Fig. 3 c). Also, concentrations of OH-PBDEs were generally higher compared to MeO-PBDEs. These findings are in line with a previous study on *Mytilus edulis* from the Baltic Sea, documenting a peak of concentrations in June (Löfstrand, 2011).

No obvious differences between *C. tenuicorne* life-stages could be observed in the algal material collected before, during and after the peak in concentrations of OH-PBDEs and MeO-PBDEs indicating no support to the hypothesis of a release of brominated substances co-occurring with the release of carposporophytes. Other possible explanations to why the concentrations of brominated compounds peak during the mid of summer are that it is a response to accumulated environmental stress or that the chemicals are released and produced in combination with the death and senescence of the algae.

The peak in production of OH-PBDEs and MeO-PBDEs in July follows the increased activity within the marine biotic community. A distinct change in the abundance and distribution of *C. tenuicorne* was observed in the hydro littoral zone during the summer months. Until the middle of June the algae were growing as large tussocks on primarily *F. vesiculosus*. Up to this point, not many herbivores were seen. At the end of June another filamentous brown alga (unknown) started to grow rapidly and in the first week of July *C. tenuicorne* was scarce. At this time the herbivores were abundant, for example different gastropods, amphipods and isopods. From the middle of July, *C. tenuicorne* started to recolonize and was growing primarily on rocks and boulders. This description is similar to the sampling information given by Löfstrand et al. (2011, supplementary material).

Conclusions

The results of this pilot study reveal a production of 2,4,6-TBP in *C. teunicorne*. Exposing *C. teunicorne* to grazing, strong light treatment, or variations in salinity indicate that the production may be triggered by these factors. In spite of a limited set of replicates, this study provides a strong background material for future research regarding the cause of natural production of brominated aromatic compounds in filamentous algae.

Insight in which ecological factors that may trigger a production of brominated substances is crucial for predicting future aspects of toxins and environmental impacts. If filamentous algae are responsible for the emissions of brominated aromatic compounds in the Baltic Sea, increased emissions can be expected as the amounts of filamentous algae are increasing in the Baltic Sea. (Bonsdorff et al. 1996). If the production is triggered by environmental stress, the situation becomes even more severe as the Baltic Sea already is subjected to large scale environmental changes such as decreased salinity (Neumann 2010) and future predicted increased solar radiation (Meier et al. 2006).

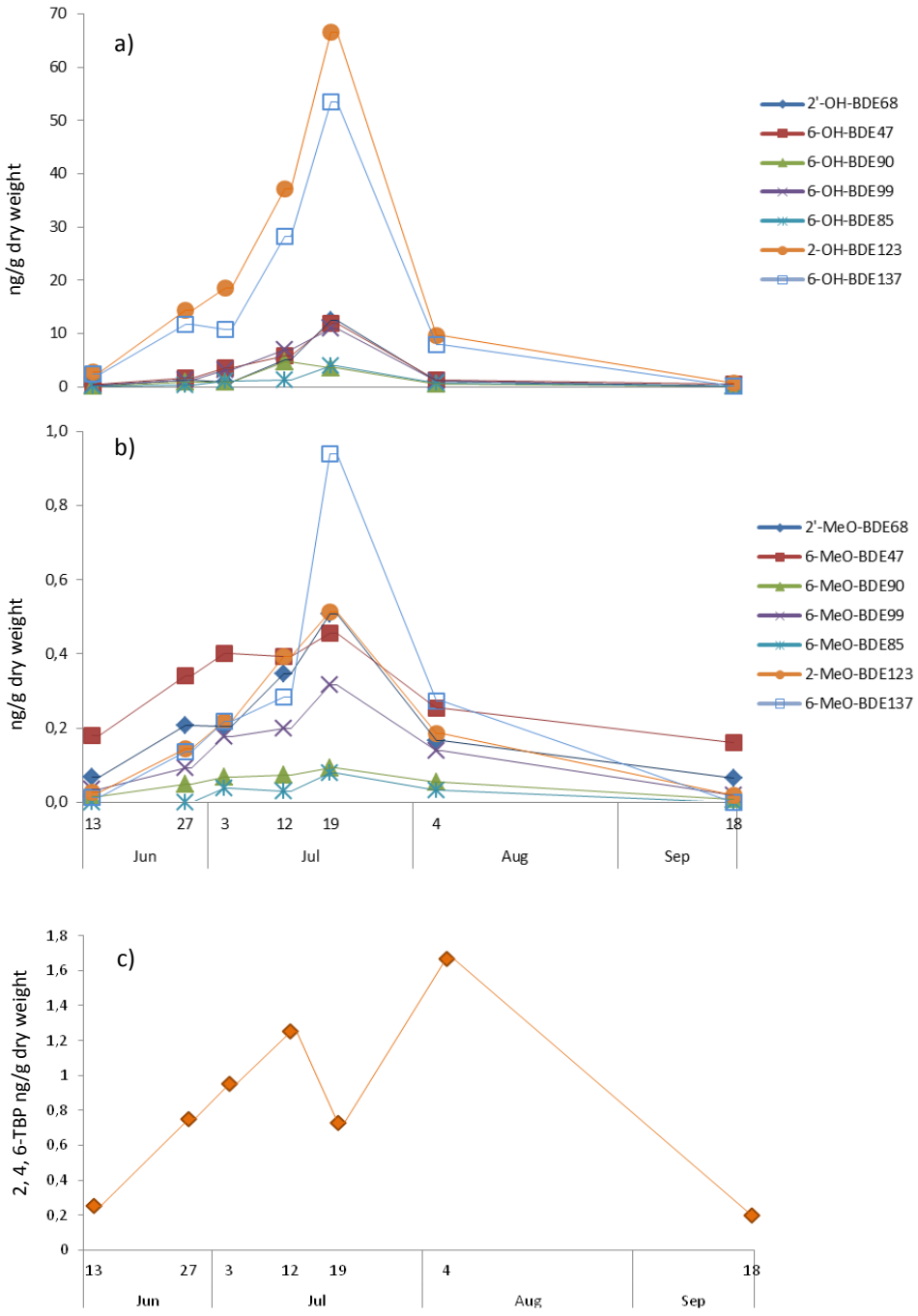

Figure 3. Concentrations of a) OH-PBDEs, b) MeO-PBDEs, and c) 2,4,6-TBP in C. tenuicorne sampled during the summer months.

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