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Can targeted defense elicitation improve seaweed aquaculture?

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Abstract
Diseases increasingly threaten aquaculture of kelps and other seaweeds. At the same time, protection concepts that are based upon application of biocides are usually not applicable, as such compounds would be rapidly diluted in the sea, causing ecological damage. An alternative concept could be the application of immune stimulants to prevent and control diseases in farmed seaweeds. We here present a pilot study that investigated the effects of oligoalginate elicitation on juvenile and adult sporophytes of *Saccharina japonica* cultivated in China and on adult sporophytes of *Saccharina latissima* cultivated in Germany. In two consecutive years, treatment with oligoalginate clearly reduced the detachment of *S. japonica* juveniles from their substrate curtains during the nursery stage in greenhouse ponds. Oligoalginate elicitation also decreased the density of endobionts and the number of bacterial cells on sporophytes of *S. latissima* that were cultivated on sea-based rafts. However, the treatment increased the susceptibility of kelp adults to settlement of epibionts (barnacles in Germany and filamentous algal epiphytes in China). In addition, oligoalginate elicitation accelerated the aging of *S. japonica* adults. Based upon these findings, oligoalginate elicitation could be a feasible way to provide "environmentally friendly" protection of kelp juveniles in nurseries. The same treatment causes not only beneficial, but also unwanted effects in adult kelp sporophytes. Therefore, it is not recommended as a treatment after the juvenile stage is completed. Future tests with other elicitors and other cultivated seaweed species may allow for the development of more feasible applications of targeted defense elicitation in seaweed aquaculture.

Introduction

Plants and seaweeds consist, in part, of specific polysaccharides that differ among taxonomic groups and play various

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roles as structural and storage compounds. In addition, some of them—and in particular their oligomeric degradation products—have been shown to activate specific biological responses in plant cells (Courtois 2009; Vera et al. 2011). Also, alginates, which are the main cell wall constituent of kelps and other brown algae, can increase the production of useful metabolites and enhance seed germination, shoot elongation, and root growth in higher plants (Akimoto et al. 1999; Luan et al. 2003; Hu et al. 2004). Since an oligosaccharide isolated from a fungal pathogen of soybean became the first known elicitor of defense responses (Sharp et al. 1984), many other oligosaccharides, derived from fungal and plant cell walls, have also been identified as defense elicitors (Shibuya and Minami 2001). These are perceived by specific pattern recognition receptors which activate innate immune responses (Nurnberger and Kemmerling 2006). Various concepts for the application of plant defense elicitors in plant crop protection have been proposed and some have reached the market (Walters et al. 2008, 2013).

Some seaweed oligosaccharides derived from algal cell walls are also designated as endogenous elicitors, since they can elicit algal immune responses (Potin et al.

1999; Weinberger 2007). For example, oligo-agar-elicited defense responses in the agarophyte *Gracilaria conferta* were characterized by an oxidative burst which led directly to the elimination of agar-degrading epiphytic bacteria (Weinberger et al. 1999; Weinberger and Friedlander 2000). Likewise, Küpper et al. (2001) found that an alginate-derived oligoguluronate could induce a defense response with a rapid, strong oxidative burst and a marked potassium efflux from elicited cells in the kelp *Laminaria digitata*. The same response was also observed in sporophytes of other tested kelp species, including some members of the genus *Laminaria*, *Saccharina latissima*, *Macrocystis pyrifera*, *Lessonia nigrescens*, and *Saccorhiza polyschides*. In all these species, recognition of alginate oligosaccharides was involved in the natural and induced immunity against epiphytic bacteria and endophytes (Küpper et al. 2002).

Seaweed aquaculture is growing worldwide, and the economic production of macroalgae has roughly doubled every decade since the early 1980s (Gachon et al. 2010). In China, cultivation of *Saccharina japonica* has successfully been implemented since the 1950s and contributes 60% of kelp yield and 90% of alginate globally (Wu 1998). During the last decades, attempts to establish commercial aquaculture of kelps have been launched on many other temperate coasts with variable success and also in Germany, where the native *Saccharina latissima* was selected as the target organism (Lüning and Mortensen 2015).

Similar to agricultural crops, economically important seaweeds are susceptible to disease and parasitism. Disease outbreaks may occur not only in natural populations, but also during cultivation, which can result in significant economic losses. In particular, the recent intensification of seaweed cultivation with larger mono-cultures has led to some disastrous disease outbreaks (Gachon et al. 2010), and this is also the case in *Saccharina* aquaculture. For example, certain bacteria of the genera *Pseudoalteromonas* and *Vibrio*—often with the ability to degrade alginate—were identified or suspected as pathogens causing red spot disease, rot disease, and rotten hole disease, all of which resulted in the decay of *S. japonica* (Sawabe et al. 1998; Sawabe et al. 2000; Wang et al. 2008). Likewise, green rot disease can cause the destruction and loss of summer juveniles of *S. japonica* in China (Chen et al. 1979, 1981; Wu 1998).

Another problem in *Saccharina* aquaculture can be endo- and epibionts, which compromise yield, appearance, and quality of the kelp product. For example, infection rates of *S. latissima* in the German Baltic Sea by endophytic microalgal pathogens can be as high as 60–100% (Peters and Schaffelke 1996; Bernard et al. 2018). Also epibiotic organisms such as bryozoans or tube-building amphipods and opportunistic algal epiphytes occur abundantly on the blades of kelp and have been identified as a major obstacle on the way to more productive *S. latissima* aquaculture in Europe (Lüning and Mortensen 2015).

Epibiosis is a complex process that depends not only on the presence and abundance of potential settlers, the hydrodynamic conditions, and the host's direct capacity to fend off settlers (Wahl 1997), but also to a large degree on the mediating role of microbes. Microorganisms that form biofilms on non-living substrata may often deter or attract larvae of animal settlers, and the release and attachment of spores of algal epiphytes are also often controlled by bacteria (Wahl et al. 2012). Also, the surfaces of kelps and other seaweeds are usually covered by diverse microbial communities (Egan et al. 2013), and increasing evidence suggests that these may play a similar role for epibiosis as biofilms on non-living surfaces for fouling (Wahl et al. 2012; Egan et al. 2013). In this light, specific microorganisms could threaten kelp aquaculture, not only as pathogens or endophytes, but also as attractors of epibionts. In addition, micro-epibionts may also compromise the availability of nutrients, light, gases, or chemicals to their algal hosts (Wahl et al. 2012).

Given the increased risk of disease outbreaks in seaweed aquaculture due to intensification (Gachon et al. 2010) and global change (Campbell et al. 2011), effective counter-measures need to be developed. However, approaches of targeted application of biocides that have been successful in terrestrial agriculture cannot be used in most of cases of aquaculture. Rapid dilution in the sea would make the use of such toxic compounds uneconomic and, at the same time, they could threaten coastal environments. A potential alternative could be the targeted activation of the natural resistance of kelps and other seaweeds through application of oligosaccharide defense elicitors. These molecular signals are typically perceived at concentrations in the micromolar range (Weinberger et al. 2001), but only by specific taxonomic groups of organisms (Potin et al. 1999; Weinberger 2007). Meanwhile, they are considered as non-toxic and easily degradable, which should limit the potential environmental impact of their application. However, field tests of oligosaccharide elicitors in seaweed aquaculture have not been reported so far.

The objective of our study was to test the feasibility of immune stimulation with oligosaccharide elicitors as a tool in seaweed aquaculture. Considering the established elicitor function of oligoalginate in kelps, our target organisms were *S. japonica* in China and *S. latissima* in Germany. Our main hypothesis was that oligoalginate elicitation could help to provide healthier and stronger kelps with high quality and to mitigate the disease occurrence and epibiosis in field cultivation.

Materials and methods

Preparation of oligoalginate elicitors

Oligoalginate was prepared from two different alginates. Only oligoalginates that are rich in guluronic acid are elicitor-active (Küpper et al. 2001) and alginate A was therefore extracted from *Laminaria hyperborea* stipes that generally contain a high proportion of guluronic acid (Haug et al. 1974). The stipes were collected in 2010 on the island of Helgoland (54° 10′ 49.18″ N 7° 53′ 20.20″ E), freeze dried, milled, and extracted with sodium carbonate, following the procedure of Haug et al. (1974). However, any bulk application of oligoalginate elicitors on a larger scale would require an inexpensive source. For this reason, a commercial standard alginate B (Carl Roth, Karlsruhe/Germany, product no. 9180.1, charge no. 06217203, prepared from *Macrocystis*)—expected to be less rich in guluronic acid than alginate A—was also used. Oligoalginates were prepared from both alginates by partial acid hydrolysis, following Haug et al. (1974): Alginate was suspended at a concentration of 10 g L^{-1} in HCl (0.3 M) and incubated for 2 h at 100 °C. After cooling to room temperature, non-hydrolyzed alginate was removed by precipitation (1400 x g, 15 min) and the remaining supernatant was neutralized to pH 7 with NaOH and dried in vacuo. The content of reducing sugars in the resulting oligosaccharides A and B was determined following Kidby and Davison (1973) and used to calculate molarities.

Oxidative burst elicitation was used to determine the capacity of both oligoalginates to trigger defense responses in *Saccharina* spp. (for details, see online supplementary material). Both oligoalginates elicited oxidative bursts in *Saccharina*. Minimum concentrations of 10 μmol oligoalginate A or 30 μmol oligoalginate B were required to trigger detectable responses in *S. japonica* or *S. latissima*, and increasing concentrations resulted in increasing response strengths (Fig. S1). Based on these observations, *S. latissima* was treated in all following experiments with 50 μmol L^{-1} oligoalginate A, while *S. japonica* was treated with 50 μmol L^{-1} oligoalginate B.

Kelp cultivation

Saccharina japonica juveniles were cultivated on palm substratum curtains in the nursery of Weihai Changqing Ocean Science & Technology Co., Ltd., Rongcheng, China (37° 17′ 24.62″ N 122° 55′ 53.24″ E). The juveniles were grown in a greenhouse pond under natural light (40–200 μmol photons m^{-2} s^{-1}), at 8–10 °C, in natural seawater supplemented with 3 mg L^{-1} NO$_3^-$-N and 0.3 mg L^{-1} PO$_4^{3-}$-P.

Adult sporophytes of *S. japonica* were cultivated at the sea-based cultivation facility of Weihai Changqing Ocean Science & Technology Co., Ltd. in Sanggou Bay, China (37° 09′ 19.71″ N 122° 50′ 50.35″ E), on horizontal ropes directly below the sea surface. *Saccharina latissima* sporophytes were cultivated at the sea-based cultivation facility of Coastal Research and Management (CRM) in the Kiel Fjord, Germany (54° 23′ 20.10″ N 10° 10′ 53.50″ E), on vertical ropes in a water depth of 2–3 m.

Oligoalginate treatments

Saccharina japonica juveniles were treated with oligoalginate B from 15 September to 15 October in 2012 and 2013. After the first application, treatments with oligoalginate B were repeated in weekly, bi-weekly, and four-weekly time intervals, which altogether resulted in five, three, and two treatments, respectively. The applied oligoalginate concentrations were 0 (control), 10, or 50 μmol L^{-1}. Oligoalginate powder was diluted in 21 L of seawater in plastic basins and the substratum curtains with juveniles were immersed into this solution for 30 s, and then immersed in fresh seawater to remove residual oligoalginate solution and placed back into the cultivation ponds. Five replicate substratum curtains were designated for each treatment in 2012 and three in 2013. In 2012, the effects of oligoalginate treatment were evaluated directly post application. In 2013, the persistence of oligoalginate effects on the treated juveniles was assessed. Ropes bearing the treated juveniles were transferred from the greenhouse to field cultivation rafts at Sanggou Bay (for details, see above) and cultivated for 2 weeks before the oligoalginate effects were assessed.

For the treatment of adult sporophytes, single individuals ($n = 10$ of *S. japonica* and $n = 16$ of *S. latissima*) were selected haphazardly in the rope cultivation systems in China and in Germany and labeled with cable ties. The period of experimental elicitation started at both sites in April 2012 and ended in July 2012. During this period, the labeled kelps were treated either in bi-weekly or in four-weekly time intervals with oligoalginate. The oligoalginate concentrations applied were 0 (control) or 50 μmol L^{-1} (oligoalginate B in the case of *S. japonica*, oligoalginate A in the case of *S. latissima*). Oligoalginate powder was diluted in 25 L of seawater in plastic buckets onboard a boat and the sporophytes were individually immersed into the solution for 30 s. Some algal individuals were lost during the treatment period due to wave action, and the number of replicates at the end of the treatment period was between 3 and 9 in the case of *S. japonica* and 8 and 15 in the case of *S. latissima*.

Assessment of oligoalginate effects

Density and growth of juveniles The density was determined by counting the total number of juveniles on sections of curtain substratum which were 5 cm long. One section was

evaluated per curtain in 2012 and three sections were evaluated per curtain in 2013 and treated as replicates ($n = 5$ in 2012, $n = 9$ in 2013). The total length of all counted juveniles was determined with a ruler.

Growth, decay, epibiosis, and endobiosis of adults The growth rates of *S. latissima* and *S. japonica* adults were determined using the punch-hole method (Kain 1976). Every 2 weeks, a 2-mm-diameter hole was punched into the thallus at a distance of 15 cm above the blade insertion and distances between successive holes were measured in order to determine growth increments. At the end of the cultivation period, *S. japonica* was partially affected by apical thallus decay and fouling, and a relative scale of affected blade areas was estimated from 0 to 100%. At the same time, *S. latissima* was subject to barnacle fouling and infested by algal endophytes. The total number of barnacles settled on individual algal thalli was counted and divided by the total blade surface area, which was measured with a ruler. The relative areas of blades affected by endophytes were visible due to de-colorations and estimated on a scale ranging from 0 to 100%.

Oligoalginate effects on the deterrence of barnacle larvae Cypris larvae of *Amphibalanus improvisus* were hatched in the laboratory at 23 °C following Nasrolahi et al. (2012) and used to test the effects on barnacle settlement of *S. latissima* surface extracts originating from elicited and un-elicited algal individuals. To do this, 12 thallus discs, of 5-cm diameter, were punched out of each of four large *S. latissima* sporophytes. Each group of 12 was haphazardly split into two groups of 6, of which one was dipped for 30 s into seawater containing 50 µmol L^{-1} oligoalginate (treatment), while the second group was dipped into sterile filtered (mesh size 0.2 µm) seawater (control). After this elicitation treatment, all discs were cultivated individually for 3 days in Erlenmeyer flasks containing 200 mL of seawater at 15 °C under weak artificial light (cool white, 40 µmol photons m^{-2} s^{-1}). Surface-associated compounds were then extracted from all thallus discs by dipping them for 5 s into 2-propanol. This extraction method did not harm the plasma membranes of epidermal cells (see Fig. S2, online supplement), and therefore only the algal outer cell wall and its surface were extracted. The resulting extracts were condensed in a rotary evaporator and impregnated on the polystyrene surface of 6-well plates at onefold natural concentration (based on surface area). To achieve this, 2 mL of 2-propanol containing the desired amount of extract was pipetted into each well and the solvent was evaporated in a lyophilizer. Each well was filled with 20 mL of sterile filtered seawater and 10 freshly hatched cypris larvae were added. The well plates were incubated at 23 °C and monitored daily for 6 days for settled cypris larvae.

Data analyses All data were analyzed using the NCSS2007 software package (NCSS LLC, Kaysville, UT/USA). D'Agostino tests of skewness, kurtosis, and omnibus normality were used to verify that data were normally distributed ($p < 0.05$), while heteroscedasticity was tested with the modified Levene equal variance test for non-normal data distributions ($p < 0.05$). Differences among data sets that were normally distributed and homoscedastic were tested for statistical significance, using factorial ANOVA and Tukey-Kramer multiple comparisons post hoc tests ($p < 0.05$). In some cases, normal distribution of data sets was not given. Those data were rank transformed, which always resulted in normally distributed and homoscedastic data distributions, and analyzed with the Scheirer-Ray-Hare test, a non-parametric alternative of two-way ANOVA (Dytham 2003), and Dunn's post hoc test ($p < 0.05$). The mean densities and average sizes of *S. japonica* juveniles differed between the two experimental years, 2012 and 2013 (see "Results" section). To facilitate comparisons of treatment effects between years, intra-annual data were related to the annual mean.

To test the effect of oligoalginate elicitation on the modulation of settlement of cypris larvae by *S. latissima*, surface extracts of treated and untreated thallus discs which originated from the same algal individual were compared (see above). Data obtained with extracts from discs that came from the same algal individual and underwent the same treatment were treated as sub-replicates and averaged. Subsequently, mean data obtained with extracts that came from the same individual, but were prepared after different treatments, were statistically treated as connected samples. Differences between data obtained with extracts coming from elicited and un-elicited algal individuals and solvent controls were therefore analyzed with the Friedman test and the sign post hoc test.

Data availability

The data underlying figures and tables shown in this publication are freely accessible at the PANGAEA repository (https://doi.org/10.1594/PANGAEA.896664).

Results

Averaged over all palm substratum rope sections that were evaluated the mean density of juveniles at the end of the nursery experiment was 11.5 cm^{-1} (± 6.0, $n = 45$) in 2012 and significantly higher (32.9 ± 18.7 cm^{-1}; $n = 59$) in 2013 (Welch-corrected t test, $p < 0.0001$). At the same time, the average thallus length was 4.6 (SD ± 1.1) mm in 2012 and significantly smaller (2.3 ± 0.7 mm) in 2013 (Welch-corrected t test, $p < 0.0001$). To facilitate comparisons of treatment effects between years, intra-annual data were related to the annual mean. Treatments of *S. japonica* juveniles at different

time intervals resulted in significantly different juvenile densities (Fig. 1A, Table 1). However, these differences varied with the presence or absence of oligoalginate during treatments, as indicated by the highly significant interactive effect of interval and treatment (Table 1). Application of seawater without oligoalginate (i.e., the control) caused an increasing reduction of juvenile densities when the treatment frequency increased. Obviously, the treatment procedure—removing the substrate curtain from the pond, immersing it into the treatment solution, rinsing it and putting it back into the pond—caused a loss of juveniles. The highest juvenile densities were nonetheless observed after weekly applications of 50 µmol L^{-1} oligoalginate; thus, the presence of the oligoalginate counteracted the loss of juveniles as caused by the treatment procedure. However, this was only observed after weekly and not after bi-weekly or monthly applications of the elicitor. No significant differences were observed between the years 2012 and 2013, although juvenile densities were evaluated before exposure in the sea in 2012 and 2 weeks after this exposure in 2013. Obviously, weekly elicitation in the greenhouse resulted in higher densities on the cultivation rafts. ANOVA also detected a significant overall effect of the treatment interval (Table 1, $p < 0.044$). However, the Tukey test failed to identify significant differences between single treatments ($p < 0.05$). Weekly treatment of S. japonica with a reduced oligoalginate concentration of 10 µmol L^{-1} did not increase the density of juveniles (Fig. S3 and Table S1 in online supplement; this was only tested in 2012).

In contrast, the length of juveniles was not only affected by treatment interval and presence or absence of oligoalginate, but also by the year in which the experiment

Table 1 Density and length of *Saccharina japonica* juveniles after 1 month in the nursery with oligoalginate applications in the September/October of 2 consecutive years. In 2012, the effects were evaluated directly after greenhouse cultivation ($n = 5$), and in 2013, they were evaluated after greenhouse cultivation, plus 2 weeks of growth on a sea-based cultivation raft ($n = 9$). The effects of year, presence or absence of oligoalginate during the treatment, and time intervals between applications were analyzed in a fully factorial ANOVA. Italics indicate statistically significant effects

Source	DF	SS	MS	F ratio	p value
Density					
Year (A)	1	0.281	0.281	1170	0.283
Interval (B)	2	1579	0.789	3282	*0.043*
Treatment (C)	1	0.172	0.172	0.716	0.401
A*B	2	0.259	0.130	0.539	0.586
A*C	1	0.428	0.428	1779	0.187
B*C	2	5347	2674	11.121	*< 0.001*
A*B*C	2	1339	0.669	2785	0.069
Error	71	17.069	0.241		
Length					
Year (A)	1	0.051	0.051	0.880	0.351
Interval (B)	2	0.672	0.336	5833	*0.005*
Treatment (C)	1	0.309	0.309	5352	*0.024*
A*B	2	0.372	0.186	3231	*0.045*
A*C	1	0.020	0.020	0.355	0.553
B*C	2	0.231	0.115	2003	0.142
A*B*C	2	0.992	0.496	8607	*< 0.001*
Error	71	4091	0.058		

was conducted, as all three factors interacted significantly (Table 1). In both years, relatively small thalli were

Fig. 1 Effects of oligoalginate applications on (a) density and (b) length of S. japonica juveniles. In 2 successive years, oligoalginate treatments were applied between mid-September and mid-October, at concentrations of 0 (open bars, control) or 50 µmol L^{-1} (hatched bars) at three different time intervals. To allow for statistically valid intra-annual comparisons, data were expressed relative to the annual mean density (A), and annual mean length (B). Different letters or capitals indicate treatments and treatment frequencies that were significantly different (3-way ANOVA and Tukey test, $p < 0.05$, see also Table 1)
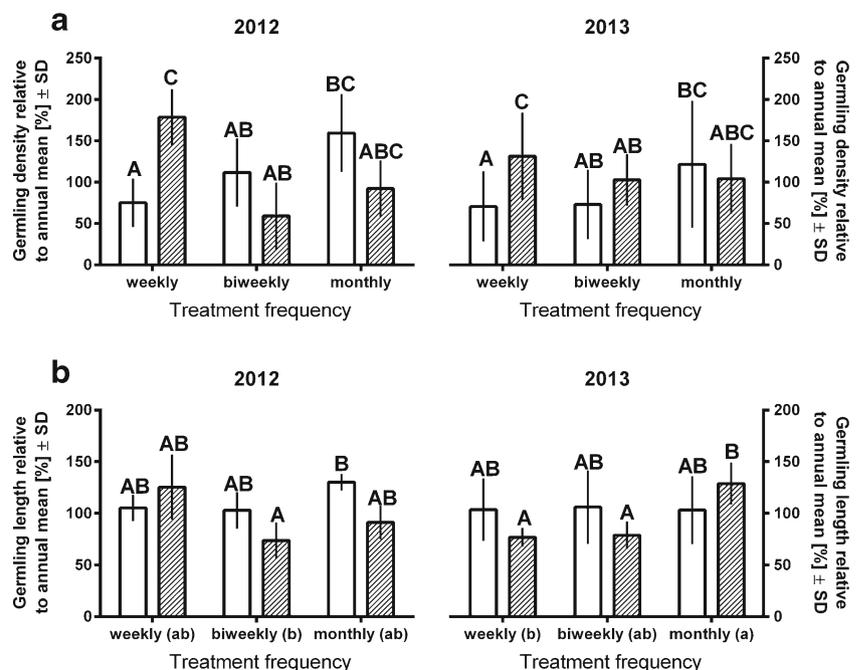
observed after treatment with 50 μmol L^{-1} oligoalginate at bi-weekly intervals (Fig. 1B). In 2012, their length was significantly smaller than that of those juveniles which received monthly control treatments. Further, in 2013, those juveniles elicited with oligoalginate either in bi-weekly or weekly intervals were smaller than juveniles elicited at monthly intervals. A significant interactive effect of treatment and year was also detected (Table 1) and monthly treatments in 2013 resulted in relatively larger juveniles than weekly treatments in 2013 and bi-weekly treatments in 2012 (Fig. 1B). Treatment alone had a significant effect on juvenile length (Table 1) and weekly treatments resulted in significantly smaller juveniles than monthly treatments. Regarding both years and all treatment intervals together, oligoalginate treatment alone also affected the length of juveniles significantly (Table 1), and these were longer than controls after treatment with 50 μmol oligoalginate. In 2012, the effect of weekly applications of 10 μmol L^{-1} of oligoalginate was also tested, but no significant effects on the length of juveniles were found (Tab. S1B, Fig. S3, online supplement).

Dipping adult sporophytic blades of *S. japonica* at bi-weekly or monthly intervals into seawater containing or not containing oligoalginate affected the growth rate differently, as indicated by two-way ANOVA (Table 2, significant interactions of treatment and interval). However, the Tukey test did not detect any significant differences among single treatments at $p < 0.05$ (Fig. 2A). In contrast, irrespective of the treatment interval, *S. japonica* oligoalginate treated showed higher epibiont cover than untreated controls (Fig. 2B, Table 3). Likewise, oligoalginate-treated sporophytes of *S. japonica* exhibited a higher degree of thallus decay at the end of the treatment period than controls and bi-weekly treatments generally resulted in more thallus decay than monthly treatments (Fig. 2C, Table 4).

Similarly as with *S. japonica*, oligoalginate had also no significant effect on the growth of *S. latissima* sporophytes (Fig. 3A, Table 5). However, a bi-weekly treatment with 50 μmol L^{-1} oligoalginate reduced the prevalence of endophytes at the end of the experimental period, which was not observed with the control sporophytes or after monthly treatments (Fig. 3B, Table 6). On the other hand, the barnacle
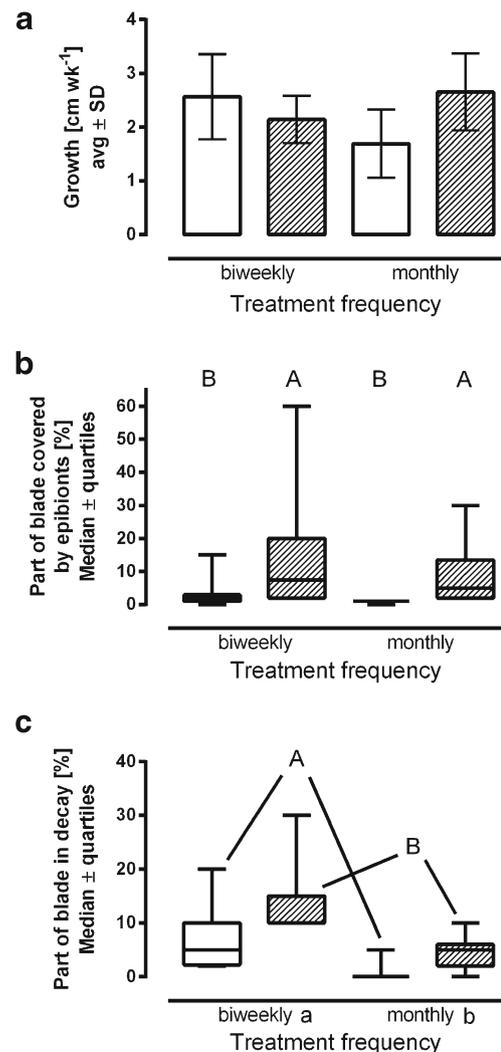

Fig. 2 Effects of oligoalginate treatment on (a) growth (values are means ± SD, $n = 9$), (b) part of the thallus blade covered by epibionts, and (c) part of the decaying adult blade of *S. japonica* sporophytes. Oligoalginate treatments were applied over a 1-month period at concentrations of 0 (open bars, control) or 50 μmol L^{-1} (hatched bars) at two time intervals. Different capitals or letters in (b) and (c) indicated significantly different results (2-way ANOVA and Tukey test or Scheirer-Ray-Hare test and Dunn's test, $p < 0.05$, see also Tables 2, 3, and 4)

densities found on those *S. latissima* sporophytes treated with oligoalginate were significantly higher than those on the

Table 2 Growth of *Saccharina japonica* adults on a raft cultivation system with oligoalginate applications. Effects of oligoalginate treatment applied and the time interval between applications, in a fully factorial ANOVA. Italics indicate statistically significant effects

Source	DF	SS	MS	*F* ratio	*p* value
Treatment (A)	1	0.408	0.408	0.88	0.358
Interval (B)	1	0.187	0.187	0.4	0.532
A*B	1	2.735	2.735	5.89	*0.023*
Error	27	13.92			

Table 3 Epiphytism on *Saccharina japonica* adults in field raft conditions, with oligoalginate applications. Effects of oligoalginate treatment applied and the time interval between applications, analyzed by a Scheirer-Ray-Hare test. Italics indicate statistically significant effects

Source	DF	SS	MS	*H*	*p* value
Treatment (A)	1	808.44		12.25	*< 0.001*
Interval (B)	1	101.72		1.54	0.214
A*B	1	29.92		0.453	0.501
Error	27	1782	66		

Table 4 Decay of *Saccharina japonica* adults in field raft conditions, with oligoalginate applications. Effects of oligoalginate treatment applied and the time interval between applications, analyzed by a Scheirer-Ray-Hare test. Italics indicate statistically significant effects

Source	DF	SS	MS	H	p value
Treatment (A)	1	336.64		5.17	*0.023*
Interval (B)	1	611.55		9.39	*0.002*
A*B	1	22.91		0.352	0.553
Error	27	1758.5	65.13		

Table 5 Growth of *Saccharina latissima* adults during open-water cultivation, with oligoalginate applications. Effects of oligoalginate treatment applied and the time interval between applications, analyzed in a fully factorial ANOVA

Source	DF	SS	MS	F ratio	p value
Treatment (A)	1	0.167	0.167	0.33	0.567
Interval (B)	1	0.389	0.389	0.77	0.383
A*B	1	0.0334	0.0334	0.07	0.797
Error	47	22.6499			

controls, and the two groups with different treatment frequencies showed a similar trend (Fig. 3C, Table 7).
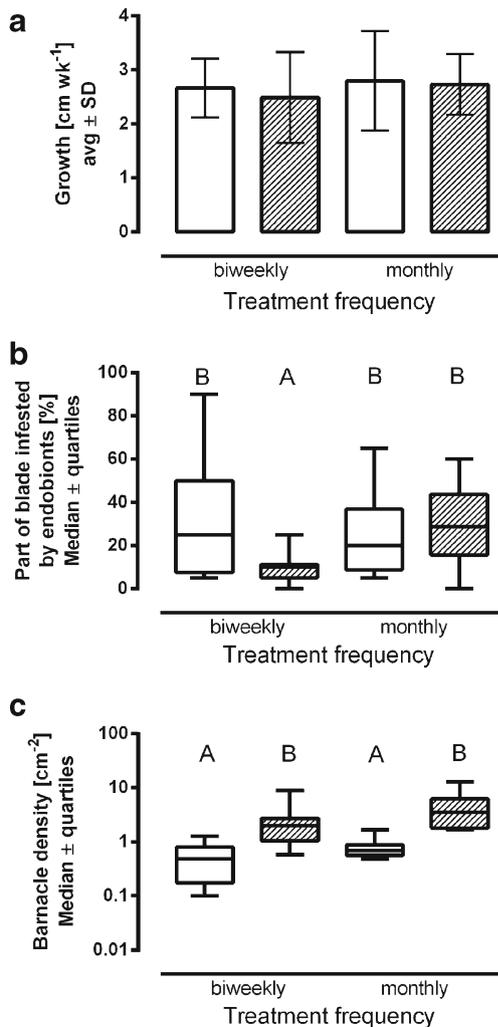

Fig. 3 Effects of oligoalginate on (a) growth (values are means ± SD, n = 6), (b) part of thallus blade occupied by endobionts, and (c) density of barnacles settled onto the adult *S. latissima* sporophytes. Oligoalginate treatments were applied over a 1-month period at concentrations of 0 (open bars, control) or 50 µmol L^{-1} (hatched bars) at three different time intervals. Different capitals or letters in (b) and (c) treatment groups were significantly different (2-way ANOVA and Tukey test or Scheirer-Ray-Hare test and Dunn's test, $p < 0.05$, see also Tables 5, 6, and 7)

To confirm the accelerating effect of oligoalginate elicitation on the settlement of barnacles, an additional experiment was conducted in order to test the attractiveness of surface extracts for cypris larvae from both elicited and un-elicited sporophytes. As shown in Fig. 4, larvae settled equally on substrata that were impregnated with surface extract from un-elicited *S. latissima* and on the solvent control substrata. However, they settled in significantly larger numbers when surface extract from elicited *S. latissima* was tested (Fig. 4, Friedman test ($p = 0.042$) and sign test ($p < 0.05$)), suggesting that the elicitation caused a shift in the composition of the surface extracts towards being less deterrent or towards presence of more attracting chemical compounds.

A second complementary experiment demonstrated that oligoalginate elicitation reduced the number of bacteria on the surface of sea-incubated *S. latissima*, at least temporarily. Compared to their controls, those sporophytes treated with seawater containing 50 µmol L^{-1} oligoalginate exhibited a decreased density of associated microbes even after 24 h (Fig. S4, online supplement).

Discussion

The data presented here indicate that the immune stimulation of cultivated *Saccharina* with an oligoalginate solution may have beneficial, but also undesirable effects. As expected, we observed an elimination of associated bacteria and also a reduced infection with algal endophytes in those *S. latissima* sporophyte thalli which were cultivated on sea-based rafts.

Table 6 Endophytism in *Saccharina latissima* during open-water cultivation, with oligoalginate applications. Effects of oligoalginate treatment applied and the time interval between applications, analyzed by a Scheirer-Ray-Hare test. Italics indicate statistically significant effects

Source	DF	SS	MS	H	p value
Treatment (A)	1	467.4		2.41	0.121
Interval (B)	1	527.6		2.72	0.099
A*B	1	769.0		3.96	*0.047*
Error	47	9126.5	194.2		

Table 7 Settled balanoids on *Saccharina latissima* adults, after open-water cultivation, with oligoalginate applications. Effects of oligoalginate treatment applied and the time interval between applications, analyzed by a Scheirer-Ray-Hare test. Italics indicate statistically significant effects

Source	DF	SS	MS	*H*	*p* value
Treatment (A)	1	5223.0		26.65	*< 0.001*
Interval (B)	1	559.1		2.85	0.091
A*B	1	30.8		0.16	0.692
Error	47	9212	196		

Moreover, fewer juveniles of *S. japonica* dropped off their cultivation substrata when they were treated with oligoalginate during the nursery period. On the other hand, oligoalginate elicitation increased the settlement of epibionts on adult sporophyte thalli of both species and it also increased the symptom of apical blade decay in *S. japonica*.

The detachment of kelp juveniles from their cultivation substrata could be due to multiple impacts. Our experimental manipulation of the substratum curtains was unavoidably associated with certain deleterious mechanical impacts, which probably explains that increasing the frequency of treatments resulted in a reduced density of juveniles. At the same time, loss of juveniles from cultivation substrata is also one of the most frequently observed disease symptoms in *S. japonica* juveniles (see "Introduction" section). It is interesting that weekly applications of oligoalginate, at a concentration of 50 μmol L^{-1}, resulted in an approximate doubling of juvenile densities. This was observed in both of the successive years, suggesting that the treatment either directly increased algal attachment or that it was the algal resistance to microorganisms which caused detachment. Weekly treatments with 10 μmol L^{-1} oligoalginate B had no such effect, which corresponded with the fact that the release of reactive oxygen species was also not observed at this lower concentration.

Elicitation of immune responses is known to often reduce growth in both higher plants and seaweeds, possibly because
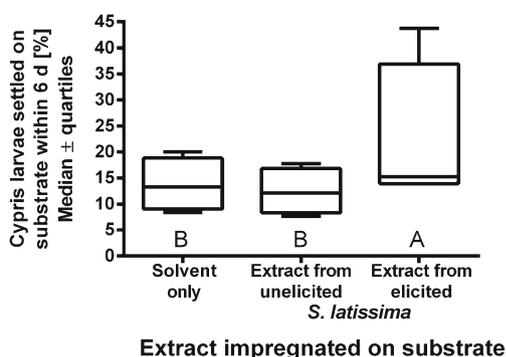

Fig. 4 Cypris larvae of the barnacle *Amphibalanus improvisus* settled within 4 days in 6-well plates impregnated with surface extracts of *S. latissima*. Different capital letters indicated treatments that were significantly different (Friedman test and sign test, *p* < 0.05)

more resources are necessarily allocated into such defense-related processes. For example, 10 and 1 μmol L^{-1} of the elicitor flg22 (an elicitor-active oligopeptide identified in pathogenic bacteria isolated from higher plants) inhibited the growth of female gametophytes of *S. japonica* (Lu et al. 2016). However, in our study, adult sporophytes of both kelp species exhibited no growth reduction after elicitation and, in addition, weekly elicitation of juveniles with 50 μmol L^{-1} oligoalginate solution caused no such reduction, as compared to weekly control treatments.

Both the density of algal endophytes (Fig. 2B) and the number of bacterial cells (Fig. S4) on the surface of sea-cultivated sporophytes of *S. latissima* decreased significantly after oligoalginate elicitation. This confirmed for the first time in outdoor experiments the results obtained in earlier laboratory studies on kelps and some other seaweeds, which also reported a reduction and inhibition of algal endophytes and associated bacteria after oligosaccharide elicitation (Weinberger and Friedlander 2000; Küpper et al. 2002). Our results indicated that oligoalginate elicitation effectively protected kelp sporophytes from immediate attack, concomitant with the decreasing number of bacterial cells. However, an elimination of kelp-associated bacteria could not only cause beneficial, but also detrimental effects. If oligoalginate elicitation were to eliminate beneficial microorganisms, then it could be detrimental. Clearly, further work in this area is called for before a commercial treatment protocol can be implemented.

Despite its general suppressing effect on pathogens and other microorganisms, oligoalginate elicitation accelerated the aging of *S. japonica* sporophytes and larger apical thallus parts were subject to decay after such treatment. Excess generation of reactive oxygen species (ROS), which was observed after elicitation of immune responses, can not only be cytotoxic to invading microorganisms, but it could also damage the host itself (Collen and Davison 1999), causing oxidation of lipids, denaturation of proteins, and decomposition of nucleic acids (Van Breusegem and Dat 2006). Moreover, *S. japonica* infected with alginate-degrading bacteria has been shown to respond, within minutes, with rapidly induced production of caspase and other apoptosis markers (Wang et al. 2004), which strongly suggested an involvement of receptors, similar to the hypersensitive defense responses of spermatophytes (Huysmans et al. 2017). An interactive effect of aging and immune receptor-activated apoptosis possibly caused the accelerated aging after oligoalginate elicitation in our study. However, given that certain associated microorganisms provide essential nutrients (e.g., vitamins, fixed nitrogen) to kelps and other seaweeds (Wahl et al. 2012; Egan et al. 2013), the overall performance of the host may also have been weakened and the decay rate increased, if elicitation eliminated such important components of the microbiome.

Oligoalginate elicitation also increased the abundance of algal epibionts on the surface of adult *S. japonica* sporophytes

and an abundance of barnacles on the surface of *S. latissima* sporophytes. Epibiosis is typically a highly dynamic process modulated by chemical settlement cues and deterrents, compounds that may not only originate from the host, but also from the surface-associated microorganisms (Wahl et al. 2012). It was demonstrated that *S. latissima* had a capacity to deter blue mussel larvae from settlement (Dobretsov 1999) and phlorotannins—those phenolic constituents of kelp cell walls—were shown to deter barnacle larvae (Lau and Qian 2000). However, in our study, settlement of barnacle larvae was not inhibited by the surface extracts from *S. latissima*. Much rather, surface extracts from the elicited—but not from the un-elicited (i.e., control)—thalli enhanced the settlement of cypris larvae in a laboratory experiment. This observation not only confirmed the fouling-enhancing effect of oligoalginate elicitation observed on cultivation rafts, but also suggested that the effects could be due to an increased presence of settlement cues on the algal surface and not rather due to elimination of protective microorganisms (Holmstrom et al. 1996; Nasrolahi et al. 2012). However, the chemical nature of these settlement cues is so far unknown. Therefore, currently, it is impossible to decide whether the enhanced presence of the barnacle larvae on the surface of *Saccharina* (after oligoalginate elicitation) was directly due to metabolic changes in the host or due to shifting microbial community composition, with the increased presence of microorganisms which provided settlement cues (Harder et al. 2002).

In conclusion, oligoalginate elicitation clearly had a beneficial effect on *S. japonica* juveniles, reducing their loss from the curtains during their nursery phase. Direct addition of an elicitor into the nursery ponds at weekly intervals may have triggered the same effect, but without the simultaneous loss of juveniles, due to the physical manipulation of the substrate curtains that was observed in our study. Therefore, application of the oligoalginate solution to the *S. japonica* juveniles could help provide healthier and stronger juveniles for field cultivation in an environmentally friendly manner. Oligoalginate elicitation also decreased the density of endobionts in the adult sporophyte thalli of *S. latissima* when it was applied, at least in bi-weekly intervals. However, bi-weekly and even monthly applications caused a severe increase in the susceptibility of the adult kelp sporophytes, of both *Saccharina* species, to epibiont settlements. Moreover, such treatments also accelerated the aging of the *S. japonica* adults. Conversely, in light of this, treatment of selected kelps with oligoalginate solution appeared to be not as applicable in field cultivation for the prevention of diseases in *Saccharina*. Nonetheless, different results may possibly be obtained with adults if other elicitors or combinations of different elicitors were tested in the future. Such research could also allow for the development of more pin-pointed treatments that target specific pathogens more selectively. In a broader perspective, targeted defense elicitation could be a feasible and sustainable protection concept primarily for pond cultivation, at the nursery stage enabling the production of "improved" kelp juveniles which would be more vigorous and robust to prevailing diseases.

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