Optimising the settlement and hatchery culture of Saccharina latissima (Phaeophyta) by manipulation of growth media and substrate surface condition

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<td>Article Type:</td>
<td>Original Research</td>
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<td>Keywords:</td>
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Optimising the settlement and hatchery culture of *Saccharina latissima* (Phaeophyta) by manipulation of growth media and substrate surface condition

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Abstract

The Phaeophyte macroalgae *Saccharina latissima* is gaining economic importance as an aquaculture crop. To decrease costs associated with the hatchery, the time required for meiospores to develop into sporophytes ready for outplanting must be minimised and survivorship maximised. The settlement and juvenile development of *S. latissima* was examined in a series of experimental manipulations. It was determined that: 1) Meiospore settlement should be conducted in the dark in nutrient enriched media. 2) Continued nutrient enrichment in the hatchery increased growth and survival of the developing sporophytes. 3) It is best to use the diatom inhibitor germanium dioxide
(GeO$_2$) only during settlement and the first week of light exposure, rather than continuously or not at all. This treatment leads to the highest survival rate and sporophyte length. 4) Pre-treating the settlement surface with a commercial yeast extract can increase settlement and early development size, however over-application can be highly detrimental leading to reduced survival, size and patchy growth.

KEYWORDS: *Saccharina latissima*; germanium dioxide; hatchery; settlement; cultivation; nutrient

Introduction

*Saccharina latissima* is a fast-growing Phaeophyte macroalgae which has economic value as an aquaculture crop. It may be grown as a monoculture, or as a component in an integrated multi-trophic aquaculture system where it can benefit from the additional dissolved nutrients released into the water by co-cultured animals such as *Salmo salmar* and *Mytilus edulis* (Wang et al. 2014; Sanderson et al. 2008; Rößner et al. 2014). The lifecycle of *S. latissima* is a heteromorphic alteration of generations: The diploid sporophytes release meiospores which settle and develop into microscopic single sex haploid gametophytes. Following fertilisation, an embryonic sporophyte develops, which continues to grow to adult size over the following months or year (Schiel and Foster 2006).

This lifecycle is manipulated for aquaculture, using the method developed in China for *Saccharina japonica* (FAO 2004). Meiospores are extracted from fertile sporangial tissue, and then allowed to settle onto string or twine on spools within enclosed tanks. During early development *in situ*, the microscopic stages are at risk of being eliminated by grazers or overgrown and outcompeted by fouling organisms. To improve survivorship, the seeded twine is cultured under controlled conditions within a hatchery until sporophytes are 2-10 mm. These are then deployed to a coastal aquaculture site. Ideally, the hatchery stage should maximise meiospore settlement, minimise the sporophyte
developmental time and result in a dense coverage of healthy young sporophytes which have high resilience to the fluctuating environmental parameters following outplanting. This may be achievable through simple manipulation of the hatchery conditions such as light, nutrients, removal of competitors, twine pre-treatment or surface roughness manipulation.

The juvenile stages of *S. latissima* and other kelps are often cultured at irradiances between 20-100 µmol·m⁻²·s⁻¹ (tom Dieck 1993; Hanelt et al. 1997; Shea and Chopin 2007), but few have investigated the effect of light on meiospore settlement. High light or long exposure times can lead to reduced settlement or germination in both *Macrocystis pyrifera* and *Pterygophora californica* (Graham 1996; Cie and Edwards 2008). Meiospore germination is known to occur in both light and darkness (Han et al. 2011; Huovinen et al. 2000) and darkness has been used previously for meiospore settlement in *S. latissima* (Shea and Chopin 2007; Flavin et al. 2013). Nevertheless, the authors are not aware of any reports showing whether darkness is preferable during settlement.

Inorganic nutrients are essential for the growth of phototrophic organisms. Nutrient concentration also influences the behaviour and settlement of meiospores, the development of gametophytes and the growth rate of sporophytes (Reed et al. 1999; Amsler and Neushul 1990; Kinlan et al. 2003; Morelisen et al. 2013; Chapman et al. 1978), with higher concentrations generally favoured. To optimise sporophyte development, sufficient nutrients must be supplied to them within the kelp hatchery, without concentrations becoming inhibitory (Amsler and Neushul 1989) or encouraging the growth of fouling organisms. Nutrient addition may be achieved through either constant or intermittent refreshment of the tanks with nutrient rich seawater or the addition of prepared nutrients (Forbord et al. 2012).

There is a significant threat that the microscopic stages of kelp will be overgrown by the proliferation of benthic diatoms while in the hatchery, leading to patchy survival or their complete eradication (P.
Kerrison unpublished results). To prevent this, germanium dioxide (GeO\textsubscript{2}) treatment has been recommended at concentrations between 0.01-0.50 mL of saturated GeO\textsubscript{2} solution L\textsuperscript{-1} of seawater (Markham and Hagmeier 1982; Shea and Chopin 2007). GeO\textsubscript{2} interferes with the formation of the diatom’s silica frustule inhibiting their growth (Lewin 1966), but also has an inhibitory effect on the growth of Phaeophyte macroalgae (Markham and Hagmeier 1982). The mechanism for this toxicity is currently unknown but two hypotheses have been suggested. Firstly, silica deposition has been documented in \textit{S. japonica} and may have a protective function (Mizuta and Yasui 2012). Therefore, Phaeophyte inhibition may have the same physiological basis as in diatoms. However, silica is not normally considered an essential element for the Phaeophyceae and can be omitted from culture media with no apparent deleterious effects (McLachlan et al. 1971). Secondly, Tarahovskaya \textit{et al.} (2012) showed that GeO\textsubscript{2} interferes with growth and development in the Phaeophyte \textit{Fucus vesiculosus} leading to morphological abnormalities. The authors suggest that this action maybe due to Ge substituting for Boron within various complexes, which is known to have deleterious effects in land plants (McIlrath and Skok 1966). Regardless of the mechanism, the beneficial inhibition of diatoms by GeO\textsubscript{2} is balanced against its potential to negatively impact macroalgal development. Determining the ideal GeO\textsubscript{2} application method is important to optimising hatchery growth.

When a surface is immersed in seawater it immediately begins to absorb macromolecules, such as proteins and polysaccharide, which adhere reversibility to the surface (Lejars et al. 2012). This is known as surface conditioning and creates a complex chemical topography altering the surface characteristics, and in turn, the settlement of organisms (Thome et al. 2012). Pre-treatment of the settlement surface with certain organic compounds, i.e. polylysine and D-glucose, has been shown to significantly increase settlement in a number of algal species (Lee et al. 2008; Santelices and Aedo 1999). Such compounds are of commercial interest, by facilitating settlement within the hatcheries of aquaculture species. The commercially available yeast extract Marmite© is a complex mixture of 11.8 % carbohydrate, 23.4 % ash and 28 % protein including 1.9% lysine (www.Foodcomp.dk). This
chemical complexity, combined with its low price and high availability as a commercial condiment may make it an effective pre-treatment to increase hatchery settlement of meiospores by conditioning the settlement surface.

The effect of substrate roughness on the settlement of the spores of fouling macroalgae, usually *Ulva* spp., has been extensively studied with the aim to develop more effective antifouling coatings. Surface irregularities increase the surface area and so the number of attachment sites (Fletcher and Callow 1992). This can lead to a tenfold increase in settlement per unit area within specific microenvironments (Callow et al. 2002; Fletcher and Callow 1992). Conversely, specific microtopographical features can reduce or deter fouling, with a 86 % decrease in *Ulva* settlement observed on a Shark scale biomimetic topography (Carman et al. 2006). It is unclear whether surface roughness affects the settlement and attachment of kelp meiospores. Exposure to a high velocity flow or spray, has often been used before to determine attachment tenacity (Finlay et al. 2002; Cassé et al. 2007).

The aim of this study is to determine whether the settlement and early development of *S. latissima* can be optimised through simple manipulation of the hatchery environment. Through a series of experiments we investigate:

1. How settlement is influenced by light or darkness, nutrient enrichment the presence of germanium dioxide, yeast extract and surface roughness.
2. How these factors influence the survival and development rate of *S. latissima* juvenile stages.
3. From these results, we determine the hatchery conditions which result in the shortest development time and the largest final size.
Materials and Methods

Three experiments were designed to test the effect of five variables on *S. latissima* development during the hatchery phase: light/dark, nutrients, germanium dioxide (GeO$_2$), a pre-treatment with a yeast extract and roughness (Fig. 1). In addition, a flume was utilised to examine whether attachment success was influenced.

For each experiment, five fertile specimens of *S. latissima* were collected at low tide. The sporangial areas were cut from the thalli, rinsed with Tyndallised seawater (Kawachi and Noël 2005) and then wiped firmly until dry, using laboratory tissues (Kimtech, UK), to remove epiphytes. This was repeated 4-5 times. The material was then cut into 1-2 cm$^2$ pieces and left overnight in a refrigerator at 4 °C. The following morning, these were placed in 8.5 °C Tyndallised seawater (salinity 33 psu) enriched with F/2 without silicate (herein referred to as F/2) and incubated in the dark for one hour to induce meiospore release. The media was agitated every 15 minutes to encourage the process (Gordon and Brawley 2004). The resultant solution was passed through a 50 µm filter and kept in motion using a magnetic mixer while the meiospore concentration was determined using a Sedgwick Rafter counting chamber.

**Experiment one (E1)**

To test the effects of light on settlement, 100,000 meiospores were distributed into Petri dishes containing 20 mL F/2 and a glass microscope slide (76x25 mm). The slides were pre-cleaned with: a 24 hour soak in 5 % Deco90, followed by 24 hours in 10 % hydrochloric acid, then rinsed thoroughly in ultra-high purity water and finally dried at 40 °C. These dishes were incubated at 8.5 °C for 48 hours without agitation to allow settlement under two conditions: darkness or illuminated by blue fluorescent lighting at 15-25 µmol·m$^{-2}$·s$^{-1}$ 12:12 L:D (n=5; Fig 1). At the end of this period, each slide was placed into a fresh Petri dish with 20 mL of F/2 to rinse away unattached meiospores.
Epifluorescent microscopy was used to determine the meiospore settlement density (cells·mm$^{-2}$).

This employed an Axioskop 2 microscope, a UV light source and filter set 09 (Zeiss, Germany).

**Experiment two (E2)**

100,000 meiospores were distributed into Petri dishes containing 20 mL of F/2 and a pre-cleaned glass slide. These were allowed to settle for 48 hours in the dark at 8.5 °C without agitation. Seven variations were made to test the effects of: nutrients, GeO$_2$, pre-treatment with a yeast extract and surface roughness (n=5; see Fig 1):

1. With nutrients (F/2). Settlement in F/2.

2. No nutrients (NW): Settlement in seawater only.

3. With nutrients and diatom inhibitor (F/2+GeO$_2$): Settlement in F/2 media containing 0.56 mg·L$^{-1}$ of germanium dioxide (0.125 mL of a saturated solution·L$^{-1}$).

4. With nutrients and surface pre-treatment (F/2+PT): The slides were thinly coated in the yeast extract (1.6±0.5 mg·cm$^{-2}$; Marmite®, Unilever plc, UK). This was allowed to dry for 2 hours in a laminar flow cabinet before settlement in F/2.

5. With nutrients and light roughness (F/2 + L rgh): Slide roughened using sandpaper with a mean particle size of 100 µm. Settlement in F/2.


7. With nutrients and coarse roughness (F/2 + C rgh). Roughened using sandpaper with a mean particle size of 400 µm. Settlement in F/2.

Following the 48 hours settlement phase, slides were transferred to new Petri dishes of fresh media to rinse away unattached meiospores. These were kept in low light at <12 °C for up to two hours. One set of samples were directly examined by fluorescent microscopy to the determine settlement...
density. The second set were secured in the test section of a 3.5 m fibreglass flume (Macleod 2013), and exposed to a turbulent flow velocity of 1 m·s⁻² for five minutes. Following this, the slides were removed and examined using fluorescent microscopy.

A separate set of samples were transferred into borosilicate basins containing 150 mL of the respective media with gentle bubbling of 0.45 µm filtered air (Whatman, UK). These were illuminated by blue fluorescent lighting at 15-25 µmol·m⁻²·s⁻¹ 12:12 L:D for a further three weeks with weekly media and basin refreshments. At the end of the experiment, all slides were examined using fluorescent microscopy. Counts were made and the longest dimension of the five largest S. latissima developmental stages recorded. From these, the survival rate (%) of the settled meiospores, germination rate (%) and mean size was calculated. These slides were then exposed to the flume under the conditions described above and re-analysed using fluorescent microscopy.

Experiment three (E3)

100,000 meiospores were distributed into borosilicate basins containing 150 mL of F/2 + 0.125 mL GeO₂·L⁻¹ and a glass slide with Kuralon twine (φ 2.5 mm) wound ten times around and secured using cyanoacrylate glue. These were then cleaned using the procedure for the slides. Seven combinations were examined: seawater with and without: F/2, GeO₂, or slide pre-treatment with (20.7±2.4 mg·cm⁻²) yeast extract (Marmite®, Unilever plc, UK; n=5; Fig 1/2).

These were settled for 48 hours in the dark at 8.5 °C, then transferred into fresh borosilicate basins containing the respective media with gentle bubbling. These were cultured for six weeks with weekly media and basin refreshment. For the first week, illumination was by blue fluorescent lighting at 15-25 µmol·m⁻²·s⁻¹ 12:12 L:D. This was then increased to 30-50 µmol·m⁻²·s⁻¹ to encourage growth.
After four weeks, fluorescent microscopy was used to make counts, measure size and estimate % cover. Slides were photographed through a stereomicroscope and measurements were made of the ten largest sporophytes using ImageJ 1.45s (National Institutes of Health, USA). Incubations then continued for a further two weeks, in which all treatments were refreshed with F/2 media (Fig 1c). After six weeks the experiment was ended and measurements were repeated. In addition, cover was estimated and then all sporophytes were scraped from the twine, dried and weighed.

Minitab v15 (Minitab Inc) and Excel 2010 (Microsoft) were used for all statistical analyses. ANOVA (AN), nested ANOVA (nAN) and 2 way ANOVA (2wAN) were used where the Anderson-Darling test for normality (Anderson and Darling 1952) was satisfied. Where nAN or AN were not appropriate, pseudo-replicated data was averaged and a Mann-Whitney U tests (MW) was used.

**Human and Animal Rights**

No humans or animals were used or harming in the following experimentation.

**RESULTS**

**Experiment 1 (E1)**

Settlement during 48 hours in darkness was significantly greater by 170 % than when illuminated under a 12:12 hour light cycle (AN: p<0.0001; Fig 3a).

**Experiment 2 (E2)**

Settlement in non-enriched seawater was 40 % lower (AN: p<0.05) than in F/2 (Fig 3b). Settlement on the pre-treated surface was increased by 16 % (AN: p<0.05). Neither the presence of GeO₂ or a roughened surface affected settlement (p>0.05).
Exposure to the flume significantly reduced the number of meiospores in all conditions by 44 - 70 % (2WAN: p<0.0001). Following flume exposure, only the meiospore density in non-enriched seawater was significantly different to the standard, being 50 % lower (AN: p<0.01).

After three weeks of culturing, the survival of the settled meiospores was 71 % lower in the absence of nutrient media (AN: p<0.0001; Table 1). Survival was boosted 66, 38 and 72 %, respectively by the pre-treatment (AN: p<0.005), 200 and 400 µm particle roughened slides (AN: p<0.05 and MW: p<0.05, respectively). Germination success was significantly reduced in non-enriched seawater (MW: p<0.001) and in the GeO$_2$ treatment (nAN: p<0.05) by 15 and 5 %, respectively.

Mean size was not affected by the surface roughness (p>0.05) but was significantly reduced in non-enriched seawater (Table 1; MW: p<0.005) and by the presence of GeO$_2$ (nAN: p<0.0001) by 63 and 40 %, respectively. This was seen as a shift in the size distribution (Fig 3). Yeast extract pre-treatment increased mean size by 28 % (AN: p<0.0001).

Flume exposure after three weeks, did not lead to any significant change in the number or size of _S. latissima_ (p>0.05).

**Experiment 3 (E3)**

**Effect of yeast extract surface pre-treatment**

After four weeks of cultivation in non-enriched seawater, the pre-treatment led to a 70 % reduction in the counts of _S. latissima_ (BvC; MW: p<0.05). A non-significant reduction was seen with pretreatment in F/2 media (CvF; p<0.05). The % cover was not significantly affected (p>0.05), neither was the size of the sporophytes by the absence of nutrient media. In F/2, yeast extract led to sporophytes which were 17 % smaller than the control (EvF; nAN: p<0.0001). After six weeks, basins which initially had no nutrient media had 49 % lower counts of _S. latissima_ then when pre-
treated (BvC; nAN: p<0.05), while their size was not affected (p<0.05). However, when always grown in F/2, the sporophytes were 37 % larger with the pre-treatment (EvF nAN: p<0.001), while % cover and final dry weight were not affected (p>0.05).

Overall, the yeast extract led to reductions in the counts of *S. latissima* and slowed development in the first four weeks. After six weeks, counts were still lower, but larger sporophytes with less consistent coverage had been able to develop, leading to a similar end point biomass.

**Effect of Germanium dioxide**

Counts and % cover after four weeks were not affected by the presence of GeO$_2$ (p>0.05). The size of *S. latissima* grown without nutrient media was also not affected. Those cultured in F/2 with 9 day GeO$_2$ exposure were 17 % larger (EvG; nAN: p<0.0001) than those without GeO$_2$.

After six weeks, slides initially grown without nutrient media (A-C) still had no difference in their counts (p>0.05), but were 52 % smaller without a 2 day GeO$_2$ exposure (AvB; nAN: p<0.0001). Those always grown in F/2 showed no difference in the final dry weight or their size due to GeO$_2$ (p>0.05) but had a slightly higher % cover after 9 days exposure rather than 2 days (EvG; AN: p<0.05).

Overall, a short period of GeO$_2$ lead to increased growth rate and a more even distribution, even though the final biomass achieved was not affected.

**Effect of Nutrient media**

Nutrient media had the most impact on the growth of *S. latissima*. After both four and six weeks, macroscopic growth was only evident in conditions containing F/2 and were 2 orders of magnitude larger than without it (Figure 5; MW: p<0.00001). In addition after 4 weeks, counts were unequivocally higher in nutrient media incubations; 69 % with a 2 day GeO$_2$ exposure (BvE; nAN: p<0.05).
p<0.005), 48 % without GeO₂ (AvD; nAN: p<0.001) and 185 % with the pre-treatment (CvF; nAN: p<0.0001).

Nutrient media introduction to A, B and C after four weeks led to a large increase in the counts by week six (Figure 5; Table 2); These were 217 and 205 % in the pre-treatment (C) and GeO₂ condition (B), but only 87 % in the non-GeO₂ condition (A). The size change followed a similar pattern with increases of 522, 308 and 78 % for C, B and A respectively.
DISCUSSION

LIGHT

In E1, settlement of *S. latissima* meiospores was 170% higher over 48 hours of darkness rather than under a 12:12 light cycle, and so darkness is recommended to maximise settlement. Dark settlement has been used before in other *S. latissima* experimentation, although no explanation was given (Shea and Chopin 2007). It may be that settlement is stimulated by darkness, as has been observed in *Ulva clathrata* (P Kerrison unpub. results). Then again, it may also be that extended periods of darkness force settlement; Swimming behaviour increases the dispersal capacity of meiospores and is fuelled by both lipid reserves and possibly also photosynthesis (Reed et al. 1999). As in other kelps, *S. latissima* meiospores contain a chloroplast and so may use photosynthesis to maintain swimming in the light. It has been observed that *Pterygophora californica* lipid reserves become depleted during dark periods (Reed et al. 1999), and that in both *P. californica* and *Macrocystis pyrifera*, 48 hours of darkness sharply decreases the rate of swimming (Reed et al. 1992).

Light exposure can also reduce settlement competency through photodamage. High irradiance levels (>300 µmol·m⁻²·s⁻¹) are known to reduce the settlement and/or germination success of *P. californica* and *M. pyrifera* (Cie and Edwards 2008; Graham 1996). Even exposure to only 75 µmol·m⁻²·s⁻¹ for 12 hours has been shown to decrease settlement in *P. californica* and germination success in *M. pyrifera* (Cie and Edwards 2008). These facts, combined with the well described germination of kelp meiospores in darkness (Huovinen et al. 2000; Han et al. 2011; Reed et al. 1992), helps to explain why continuous darkness leads to more successful settlement of *S. latissima* meiospores.

NUTRIENTS

Nutrient enrichment consistently increased settlement in both E2 and E3, similar to findings in *Pterygophora californica* (Amsler and Neushul 1990). This is thought to be an adaptation to facilitate...
settlement in suitable benthic microhabitats (Amsler and Neushul 1989). It may also be that a certain meiospore nutrient quotient is required before successful settlement is achieved, so higher nutrient concentrations lead to greater settlement. Spore release and settlement in nutrient media has been recommended for the hatchery cultivation of kelp by Flavin et al. (2013).

But, very high concentrations can be inhibitory, deterring settlement and causing negative chemotaxis in kelp meiospores (Amsler and Neushul 1989). Conversely, insufficient nutrients can slow or stall the development of microscopic sporophytes and gametophytes (Hoffman and Santelices 1982; Kinlan et al. 2003; Reed et al. 1991). This appears to be the case in E2, where nutrient enrichment lead to increased survivorship, increased germination and more growth observed after three weeks. Similarly, in the fourth week of the E3, increased sporophyte density and faster development was seen in the presence of nutrient enrichment. In fact, the benefit of F/2 media on development was so great that all enriched samples had sporophytes visible to the naked eye (ca 1 mm), while all non-enriched samples were still microscopic (0.02 mm). This agrees with previous investigations where the fertilised hatchery growth of Undaria pinnatifida for ca 6 weeks, lead to faster development of adult sporophytes following outplanting at a cultivation site (Gao et al. 2013). However, it should be noted that there was a confounding effect of temperature control in that study.

In E3, the introduction of F/2 to the nutrient depleted conditions A, B and C for the final two weeks, greatly enhanced growth, so that the number and size of sporophytes increased 2-3 and 2-6 times respectively. After 6 weeks, these had reached a similar developmental stage as the continuous F/2 treatments (D-G) after only 4 weeks. This shows the importance of constant or continuing nutrient enrichment within the kelp hatchery to enhance the growth of the developing sporophyte, and so minimise development time. A similar enhancement has been shown by nutrient enrichment through M. edulis hatchery co-culture (Rößner et al. 2014).
It is interesting to see that the counts mm⁻² in A, B and C at 4 weeks, increased following the nutrient enrichment. Given the complex 3D surface of twine, small structures such as meiospores and unbranched gametophytes are easily missed, even using fluorescent microscopy, and so the counts at week 4 are likely to be underestimated. Therefore the increased counts at week 6, maybe partly explained by the growth of missed meiospores into larger more obvious visible structures. However, the counts for A and B were even larger than was seen in D-G at a similar developmental stage (week 4). It is therefore also possible that growth with low nutrients, stimulated increased branching in the developing gametophytes, resulting in larger multi-branched females which gave rise to increased numbers of embryonic sporophytes. This suggests that counterintuitively, a low nutrient period, increases the final sporophyte density, although the cost is an increased development time. The re-supply of nutrients may also lead to more rapid sporophyte development as has been documented in four kelp species (Carney 2011), as the juveniles maybe ‘primed’ to take advantage of any increase in nutrients.

GERMANIUM DIOXIDE

Shea and Chopin (2006) show that a dose of 0.1 - 0.5 mL of saturated GeO₂·L⁻¹, applied after 8 days, eliminated almost all diatoms and allowed optimal S. latissima sporophyte development over a 40 day experiment. We tested a dose within this range (0.25 mL·L⁻¹) and found that its continuous use over 3 weeks lead to a 40 % decline in growth. This agrees with other reports on the inhibition of growth and slowed development seen in the presence of GeO₂ (Markham and Hagmeier 1982; Mizuta and Yasui 2012). Therefore, we wanted to test whether exposure for only a few days at the start of the cultivation period was more favourable, similar to the method of Lüning (1982), who applied a dose of 2 mL·L⁻¹ over the first four days.
In E2, it was found that settlement was not influenced by GeO₂ and so it can be safely used during this period with no ill effects. Further to this, in E3, it was found that a 9 day treatment of GeO₂ resulted in larger sporophytes than only 2 days. No significant differences in size were observed after six weeks, however, cover was higher after the 9 day treatment. It therefore appears that exposure for 9 days leads to the most favourable results, and should eliminate the threat posed by overgrowth by benthic diatoms during the early development of *S. latissima* meiospores.

**YEAST EXTRACT**

Conditioning of the settlement surface with yeast extract was hypothesised to be beneficial to the settlement of algal spores due to its complex composition and the presence of potential settlement cues (Santelices and Aedo 1999; Lee et al. 2008), while its current commercial value as a condiment would make it easily and cheaply available for hatchery use. As expected, it significantly increased spore settlement in E2, and in addition at the end of three weeks, both survivorship and size were significantly enhanced relative to the control. This was unexpected as the amount of yeast extract applied to the slide was very low and appeared to dissolve quickly into the media, which would be lost when the media was exchanged at the end of the settlement period. However, it appears that some beneficial component/s was either absorbed by the meiospores during the settlement period, or remained within the slide boundary layer despite the transfer into fresh media. While the identity of this component/s is currently unknown, possible candidates are vitamins such as B12 and trace metals such as iron. The presence of these components on the slide surface may have also boosted settlement (Amsler and Neushul 1990).

Another possibility is that juvenile *S. latissima* are partially heterotrophic and are making use of the abundant amino acids and complex carbohydrates present in the extract (www.Foodcomp.dk); or that bacterial populations that heterotrophically consumed the yeast extract and releasing a
component beneficial to *S. latissima*. Despite the cause being unknown, the results from E2 show that this pre-treatment is beneficial and cost-effective, however E3 did not agree.

In E3, a thicker layer (20.7±2.4 mg·cm$^{-2}$) was applied to the twine than was used on the slide (1.6.7±0.5 mg·cm$^{-2}$) to ensure an even coverage. However, this 13 fold increase in dosage led to a negative effect on the development of *S. latissima* meiospores, with a reduction in sporophyte counts and size after 4 weeks. Much of the applied extract is assumed to have soaked into the structure of the twine and so would have taken far longer to dissolve into the media than when applied to the glass slides in E2. This could have resulted in inhibitory concentration (Amsler and Neushul 1989) of certain nutrients at the surface of the twine, so reducing settlement and survival. In addition, the high organic matter content, may have led to a bloom of heterotrophic bacteria on the twine surface smothered the developing meiospores and gametophytes. Such bacterial overgrowth has been observed if the media is not exchanged, two days following the introduction of spore solution (P Kerrison unpub results). This is thought to be due to labile organics released from the adult sporangia along with the meiospores.

After 6 weeks the E3 yeast pre-treatment, lead to a more sparse and patchy sporophyte population. While dry weight biomass was found to be not different between conditions, the sporophytes were largest where the pre-treatment had been applied (F). This is not thought to be due to the pre-treatment *per se*, but due to the reduction in density its over-application caused. By reducing competition, it allowed the remaining sporophytes to grow faster achieving a larger size after 6 weeks (Reed et al. 1991; Steen and Scrosati 2004). Such a patchy distribution needs to be avoided for best success of the outplanted twines, however, and the faster growth between week 4 and 6 on the lowered density twines that received the pre-treatment reveals that the settlement density used in these study are likely to be too high. To minimise the hatchery time, the optimal density of meiospores should be settled. This will be a trade-off between limiting the interspecific competition.
between compatriots, slowing overall growth, and ensuring good coverage of sporophytes on the lines. It is also likely that a high density of recruits will be more resilient and likely to success compared to a low density, where disturbance is more likely to lead to partial crop failure. The effect of settlement density on development time and outplanting success warrants future experimentation.

ROUGHNESS

Surface roughness and topography is known to be an important factors affecting the settlement of many benthic organisms, including macroalgae (Kohler et al. 1999; Bers and Wynne 2004). The roughening of the glass slide had no effect on the settlement of *S. latissima* and no preference for settlement within particular features was observed (data not shown). In the green alga *Ulva* spp., zoospore settle preferentially on particular topographic features; aggregating in crevices, depressions and at the intersection of dissimilar features (Schumacher et al. 2007; Long et al. 2010). The lack of an effect of roughness on settlement density or distribution here (data not shown) indicates that *S. latissima* meiospores do not show topographic selectivity at the roughness scale used in this study. Further to this, variation in roughness, did not affect the removal rate of meiospores when exposed to high flow within the flume. This is probably because boundary layer characteristics were not substantially different between the different roughnesses examined.

After three weeks of cultivation, survivorship was substantially boosted on the roughest surfaces by 38-72%. This may simply be because the rougher slides had a higher surface area, allowing more sporophytes to develop with less competition for space. Additionally, surface roughness may have affected holdfast development, as is seen in the red macroalgae *Polysiphonia* sp (Woods and Fletcher unpublished). Roughened areas are thought to improve the attachment strength in developing juvenile, due to the increased attachment area for the holdfast and the physical locking of holdfast rhizoids into microscopic crevices (Milligan and DeWreede 2000; Morrison et al. 2009).
Therefore, the improved survival seen may be a consequence of less accidental detachment due to generally stronger attachment in the developing juveniles.

CONCLUSIONS

This study provides information on how the settlement of *S. latissima* meiospores and their development into juvenile sporophytes is affected by light, nutrients, GeO$_2$, surface conditioning with a yeast extract and surface roughness. Through these simple manipulations of the hatchery conditions, faster growth, increased survival and consistent coverage is achievable on seeded twine of *S. latissima*. This study has shown that:

1. Meiospore settlement should be conducted in darkness to maximise settlement.
2. Nutrient enrichment should be used throughout the hatchery phase as this will improve settlement, survival, germination and maximise growth.
3. GeO$_2$ should be used for the first 9 days as this will inhibit early competition from diatoms, so leading to greater growth and highly consistent coverage.
4. A rougher surface also improves survival, which is thought to be due to improved attachment of developing sporophytes.
5. The use of yeast extract surface pre-treatment shows promise and can improve both survival and growth. However, it is not recommended until further research has been conducted to determine the correct dosage.

While this study examined only *S. latissima*, it is highly likely that these results will be applicable to the hatchery cultivation of other kelp species such as *Laminaria digitata*.

ACKNOWLEDGEMENTS
Funding for this work was provided by the European Commission Community Research and Development Information Service (CORDIS) Seventh Framework Programme (FP7) project - Advanced Textiles for Open Sea Biomass Cultivation (AT~SEA) grant no. 280860. Special thanks is given to Liridon Hoxha for assistance during the experimentation.

CONFLICT OF INTEREST
None declared.

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FAO (2004) Cultured Aquatic Species Information Programme: Laminaria japonica (Areschoug, 1851). In: Chen J (ed) FAO Fisheries and Aquaculture Department [online]


**Figure 1** Summary of experiments 1-3 (E1-3). In E1, settlement was examined in either the light or dark. In E2, dark settlement was examined in either nutrient media (F/2) or seawater (NW), with or without germanium dioxide (GeO₂) or yeast extract pre-treatment (PT) and with three roughness’s: light, medium or coarse (L, M or C rgh). This was followed by either continued cultivation, or examination with or without flume exposure. In E3, settlement was examined in larger vessels, with several conditions of nutrients, GeO₂ and yeast extract. In all, cultivation was continued for a further 6 weeks, with all cultures grown in F/2 for the last 2 weeks (n=5)
Figure 2 Conditions examined in experiment 3 (A-G). These are combinations of: seawater or F/2 enriched seawater (NW or F/2), pre-treatment with a yeast extract (PT) and application of germanium dioxide for either 2 or 9 days (GeO$_2$ 2d or 9d). Horizontal black arrows compare enriched and non-enriched seawater, light grey vertical lines compare the use of pre-treatment while dark grey lines compare GeO$_2$ application and duration.

Figure 3 Densities of *Saccharina latissima* meiospores after settling for 48 hours onto glass slides under different conditions a) Experiment 1: F/2 media under either: 20-30 µmol·m$^{-2}$·s$^{-1}$ 12:12 L:D (Light) with 0 µmol·m$^{-2}$·s$^{-1}$ (Dark). b) Experiment 2: Comparing dark settlement in F/2 media (F/2), seawater (NW), in F/2 including 0.56 mg·L$^{-1}$ germanium dioxide (GeO$_2$), in F/2 on slides pre-treated with a yeast extract (PT) and in F/2 on slides roughened with three grades of sandpaper: particle size 100-400 µm (L, M and C rgh respectively). Statistical significance: *p<0.05, ****p<0.0001

Figure 4 Frequency distribution of *Saccharina latissima* size in experiment 2. Three weeks following settlement onto glass slides under different conditions. Comparing F/2 media (F/2), non-enriched seawater (NW), including 0.56 mg·L$^{-1}$ germanium dioxide (GeO$_2$), on slides pre-treated with a yeast extract (PT) and on slides roughened with 200 µm particle size sandpaper (Mrgh). Data bins all pseudo-replicates

Figure 5 Variation in size (µm) and density (mm$^{-2}$) of *Saccharina latissima* between incubation week four and six of experiment 3 in each experimental condition (A-G). The week six density for E-G could not be determined and so their horizontal position may not be accurate

Figure 6 Frequency distribution of *Saccharina latissima* size in experiment 3. Six weeks following settlement onto Kuralon twine under different conditions. Comparing culturing in F/2 media (F/2), 2
or 9 day exposure to 0.56 mg·L\(^{-1}\) germanium dioxide (GeO\(_2\) 2/9d) and pre-treatment with a yeast extract (PT). Data bins all pseudo-replicates

Table 1 The characteristics of *Saccharina latissima* three weeks following settlement, in experiment 2. All treatments are compared to the control, which contains only F/2. Statistical significance:

\[ *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 \]

Mann-Whitney U Test (MW), ANOVA (AN), nested ANOVA (nAN)

Table 2 Overview of experiment 3 results on growth of *Saccharina latissima* in conditions A-G. NW-

Natural seawater, GeO\(_2\)- 0.56 mg·L\(^{-1}\) germanium dioxide exposure for 2 or 9 days (2/9d), F/2 – nutrient enrichment with F/2, PT – pre-treatment with yeast extract
Fig 1
Click here to download colour figure: fig 1.docx

a) E1
Settlement

b) E2
Settlement

Cultivation: 3 weeks

C) E3
Settlement

Cultivation: 4 weeks

Cultivation: 2 weeks
Fig 4
Click here to download line figure: fig 4.docx
Fig 5
Click here to download line figure: fig 5.docx
Figure 6 Frequency distribution of *Saccharina latissima* size in experiment 3. Six weeks following settlement onto Kuralon twine under different conditions. Comparing culturing in F/2 media (F/2), 2 or 9 day exposure to 0.56 mg·L⁻¹ germanium dioxide (GeO₂ 2/9d) and pre-treatment with a yeast extract (PT). Data bins all pseudo-replicates.
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<th>Δ vs. control (%)</th>
<th>Germinated (%)</th>
<th>Δ vs. control (%)</th>
<th>Size (µm)</th>
<th>Δ vs. control (%)</th>
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<td>F/2 (control)</td>
<td>16 ±4 ****</td>
<td>92 ±4 ****</td>
<td>23 ±3 ****</td>
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<td>NW</td>
<td>5 ±3 -71 ****AN</td>
<td>79 ±9 WM**</td>
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<td>GeO₂</td>
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<td>PT</td>
<td>26 ±7 +66 ****AN</td>
<td>91 ±2 WM**</td>
<td>29 ±5 +28 WM****AN</td>
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<td>L rgh</td>
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<td></td>
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<td>FOV cover (%)</td>
<td>Size (µm)</td>
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<td>A NW</td>
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<td>5.8 ± 5.4</td>
<td>n/a ± 14</td>
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