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Optimum conditions for cultivation of the *Trailliella* phase of *Bonnemaisonia hamifera* Hariot (Bonnemaisoniales, Rhodophyta), a candidate species for secondary metabolite production

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Abstract

Red algae of the order Bonnemaisoniales produce secondary metabolites that may be used as preservatives for industrial applications. Whereas species of *Asparagopsis* are cultured on a large scale for this purpose, no similar applications have been attempted for *Bonnemaisonia* species, despite evidence suggesting a similar potential for production of valuable natural products. Optimal conditions for growth of the *Trailliella* phase of *Bonnemaisonia hamifera* were assessed experimentally under controlled conditions. Several factors (temperature, photon irradiance, daylength, aeration, culture medium, concentration of nutrients) were tested. Optimal conditions for biomass production in *Trailliella* are represented by a combination of temperatures of 15–20°C, photon irradiances of 20–30 μmol photons m^{-2} s^{-1} and long daylenghts (16:8 h L:D). Quarter-strength von Stosch medium proved to be the best of those tested; aeration contributed also to a higher biomass production. Any attempts of large-scale cultivation should be performed therefore under similar conditions. The growth responses indicate that strains maintained in long-term culture collections can be successfully used for large-scale production.

Keywords: algal culture; *Bonnemaisonia*; growth; Rhodophyta; *Trailliella*.

Introduction

Bonnemaisonia hamifera Hariot (Bonnemaisoniales) is a red alga distributed on the shores of Europe, eastern Asia, and Pacific and Atlantic North America (Guiry et al. 2005). Japan is generally considered the original centre of distribution of *B. hamifera* (Hariot 1891, Dixon and Irvine 1977, Breeman et al. 1988). This species first appeared in Europe around the beginning of the last century, probably through human agency (Dixon and Irvine 1977, Hardy and Guiry 2003). As typical of the order Bonnemaisoniales (Feldmann and Feldmann 1942, Dixon and Irvine 1977, Guiry 1987), the life history of *B. hamifera* involves an alteration of generations between morphologically different gametophyte and sporophyte phases. The gametophyte is a large, coarsely branched seaweed, up to 20–30 cm tall (Dixon and Irvine 1977, Breeman et al. 1988). The sporophytic phase consists of delicate uniseriate filaments, 2–3 cm tall (Dixon and Irvine 1977, Hardy and Guiry 2003). Due to its radically different morphology, the sporophyte was originally described as a separate species, *Trailliella intricata* Batters (Batters 1896). In general, apart from regulation of life history and geographical distribution (Suneson 1939, Lüning 1979, Knappe 1985, Breeman et al. 1988, Breeman and Guiry 1989), the information available on the biology of the *Trailliella* phase is limited.

The Bonnemaisoniales have long been known to produce a large set of secondary metabolites, in particular halogenated compounds (McConnell and Fenical 1977, Combaut et al. 1978, Jacobsen and Madsen 1978, Woolard et al. 1979, Marshall et al. 1999). For *Bonnemaisonia hamifera* (including the *Trailliella* phase), a complete account of the natural products was given by McConnell and Fenical (1980). Bioactive metabolites of varying chemical nature are produced in many groups of algae and have many functional roles. The functions of compounds of this type include chemical defences against bacteria, fungi and protozoa, anti-grazing deterrents, metal transporting agents, sexual hormones and differentiation effectors (Tringali 1997, Demain and Fang 2000). Secondary halogenated metabolites of Bonnemaisoniales have good antimicrobial activity, which makes them suitable for preservation of several types of products (for example, cosmetics). *Asparagopsis armata* Harvey, a bonnemaisonialean alga common in Atlantic Europe, is currently cultured in some regions of northern Europe for commercial production of secondary metabolites (Lognoné and Dion 2003, Kraan and Barrington 2005). No similar exploitation has been attempted so far for *B. hamifera*, despite the close affinity of the two genera and a similar potential for the production of valuable natural products (McConnell and Fenical 1980). Recently, we have examined the potential of *Bonnemaisonia* and *Traillliella* as sources of natural products suitable for the preservation of cosmetics. Since these algae are generally not abundant on Atlantic shores of Europe, and field collections would likely be contaminated with other organisms, production under controlled conditions is necessary. For such purpose, the growth of a large biomass of alga capable of producing adequate amounts of natural products is a fundamental requirement. We chose to focus on the *Trailliella* phase, which in controlled conditions is generally easier to grow than the gametophyte

Table 1 *Bonnemaisonia hamifera*: summary of experiments performed.

Experiment	Testing for differences in	Factors (levels)	Time (days)
1	Growth	Temperature (10, 15, 20 and 25°C) Photon irradiance (5, 15 and 30 μmol photons m^{-2} s^{-1})	60
2	Growth	Nutrient concentration (VS_{20}, VS_{10}, VS_5) Temperature (15 and 20°C) Photon irradiance (15 and 30 μmol photons m^{-2} s^{-1})	50
3	Growth	Temperature (15 and 20°C) Culture medium (VS_5, PES)	60
4	Growth	Temperature (15 and 20°C) Culture medium (VS_5, Carna medium)	60
5	Growth	Aeration (with and without aeration)	50
6	Growth	Daylength (16:8 h L:D, 12:12 h L:D, 8:16 h L:D)	60
7	Reproduction (production of tetraspores)	Content of nitrogen and phosphorus (full and reduced to 10%)	90

phase, and attempted to clarify which combinations of factors best promote the growth in culture. Reproduction and regulation of life history of *Trailliella* have been studied in great detail (Chen et al. 1969, Lüning 1979, Breeman et al. 1988, Breeman and Guiry 1989) but, to date, no studies have assessed the optimal conditions for vegetative growth nor proposed methods for large-scale cultivation. We also assessed whether long-term maintenance in culture has an effect on the reproductive capabilities of the alga. We present here the results of this investigation, based on a number of experiments in which several factors were considered and manipulated. The results provide new and original information on the biology of *Trailliella*, which will be of critical importance for attempts at large-scale cultivation.

Materials and methods

Optimal conditions for the growth were assessed by culture experiments carried out on a strain of *Trailliella* maintained in the algal culture collection of the Martin Ryan Institute, National University of Ireland, Galway (culture no. 1027, origin: Orgella, País Vasco, Spain; leg. W.F. Farnham; voucher specimen: GALW015293). This culture was originally initiated from a single fragment of a field-collected material and is therefore genetically homogeneous. The material has been maintained for about 20 years at 15°C, 16:8 h L:D, 5 μmol photons m^{-2} s^{-1}.

The growth of *Trailliella* was tested in a number of experiments with combinations of several factors. The factors included were: temperature (four levels: 10, 15, 20 and 25°C), photon irradiance (three levels: 5, 15 and 30 μmol photons m^{-2} s^{-1}), daylength (three levels: 16:8 h L:D, 12:12 h L:D and 8:16 h L:D), aeration (two levels: with and without aeration in the culture vessels), culture medium [three levels: von Stosch, Provasoli's enriched seawater (PES) and MRI Carna medium; see details below] and concentration of nutrients [for the von Stosch medium, three levels: full strength (VS_{20}), corresponding to 20 ml of stock solution per litre of sterile seawater; half strength (VS_{10}), corresponding to 10 ml/l; quarter-strength (VS_5), corresponding to 5 ml/l]. Each experiment tested one, two or three factors (Table 1). The normal duration of the experiments was 60 days.

The experiments were carried out in four constant temperature rooms at 10, 15, 20 and 25°C, in which daylengths of 16:8 h L:D were maintained. Wooden boxes, in which the photoperiod regimen was regulated by a digital timer and the air kept in continuous circulation by a fan, were used to obtain the other daylenghts. Photon irradiance was checked using a Licor LI-1400 data logger (Licor Biosciences, Lincoln, USA). In all experiments, all possible precautions were taken in order to avoid, or to reduce to a minimum, the risk of confounding the effect of the factors tested. This was logistically impossible to obtain in the case of temperature and daylength, for which the equipment available did not allow use of more than one room for each temperature and one box for each daylength regime. In this case, effort was made to ensure that any other condition potentially affecting the outcome of the experiments would be identical among different temperature and daylength levels. In the rooms and in the daylength boxes, air was kept in continuous circulation to avoid production of small-scale differences in temperature; temperature measurements were taken periodically to ensure that temperature was uniform in each part of each coldroom and was not significantly different between daylength boxes and the rest of the room. For the experiments involving culture medium, concentration of nutrients and aeration, replicates were placed in an interspersed arrangement, to avoid the potential risk that differences between different factor levels could be confounded by small-scale spatial variability.

The culture media used included a von Stosch medium modified after Guiry and Cunningham (1984), PES (Bold and Wynne 1978) and a medium routinely employed for large scale cultivation of phytoplankton in the shellfish station of the National University of Ireland at Carna, Co. Galway, referred to hereafter as Carna medium. Its composition is as follows: $NaNO_3$–570 g/10 l distilled water; EDTA–45 g/10 l; ALGOFLASH–660 ml/10 l; buffer: Tris (300 g/10 l) and HCl (170 ml/10 l) 250 ml of stock solution/1000 l of seawater. ALGOFLASH is a general multipurpose garden fertiliser 6N.6P.6K. with trace elements (Algoflash America, Leesburg, USA). Details of its composition are as follows: total nitrogen (N) 6% (ammoniacal N 1.2%–nitric N 2.2%–uric N 2.6%); phosphorus (P) soluble in neutral ammonium citrate and water 2.6%; P soluble in water 2.6%; potassium (K) soluble in water

5%; magnesium (Mg) soluble in water 0.6%; copper (Cu) chelated EDTA 0.003%; iron chelated EDTA 0.025%; manganese (Mn) chelated EDTA 0.025%; zinc (Zn) chelated EDTA 0.0125%; boron (B) water soluble 0.016%; molybdenum (Mo) water soluble 0.001%.

Most experiments were carried out with disposable plastic dishes containing approximately 30 ml of medium (single vent Bibby Sterilin, Stone, UK). For some experiments, glass dishes containing approximately 300 ml of medium were used. In each experiment, dishes of the same size were used for different levels. The culture medium was replaced periodically in each culture, in order to avoid depletion of nutrients. The frequency of medium replacement depended on the experiment and the size of the cultured specimens, but it was usually 1–2 weeks. Three replicates were used for each condition under examination. Each replicate consisted of a vegetative tip of *Trailliella* obtained by excision from stock cultures (approximately 20 cells in length, with no side branching present). Growing in culture, the specimens tended to produce filamentous clumps with a more or less spherical shape. The sizes of the specimens were estimated by measuring two different variables: 1) dry weight of the specimen at the end of the experiment (measured after drying for three hours at 70°C); 2) mean diameter of the specimen (obtained as mean of three different measurements), measured in mm by observation under a binocular microscope.

A separate experiment was carried out to establish whether the strain of *Trailliella* used, which has been maintained in culture for almost 20 years, was still able to produce tetraspores. We repeated an experiment done previously by Breeman et al. (1988), where the conditions required for reproduction were investigated. For this experiment, the medium used was quarter-strength von Stosch medium modified further by reducing to 10% the original concentration of nitrogen ($NaNO_3$) and phosphorus (Na_2glycerophosphate). Control cultures were grown in normal quarter-strength medium.

Results

Experiment 1: effect of temperature and photon irradiance

The growth of *Trailliella* was tested at four different temperatures (10, 15, 20 and 25°C) and three different light intensities (5, 15 and 30 μmol photons m^{-2} s^{-1}) (daylength: 16:8 h L:D; medium used: quarter-strength von Stosch). Major differences between treatments were observed for both the dry weight and the diameter data. At 10°C, the biomass obtained was clearly lower than at the other temperatures tested (Figure 1); at all temperatures, no evident differences were observed for different photon irradiances. For the diameter of the specimens, no difference between photon irradiances was observed

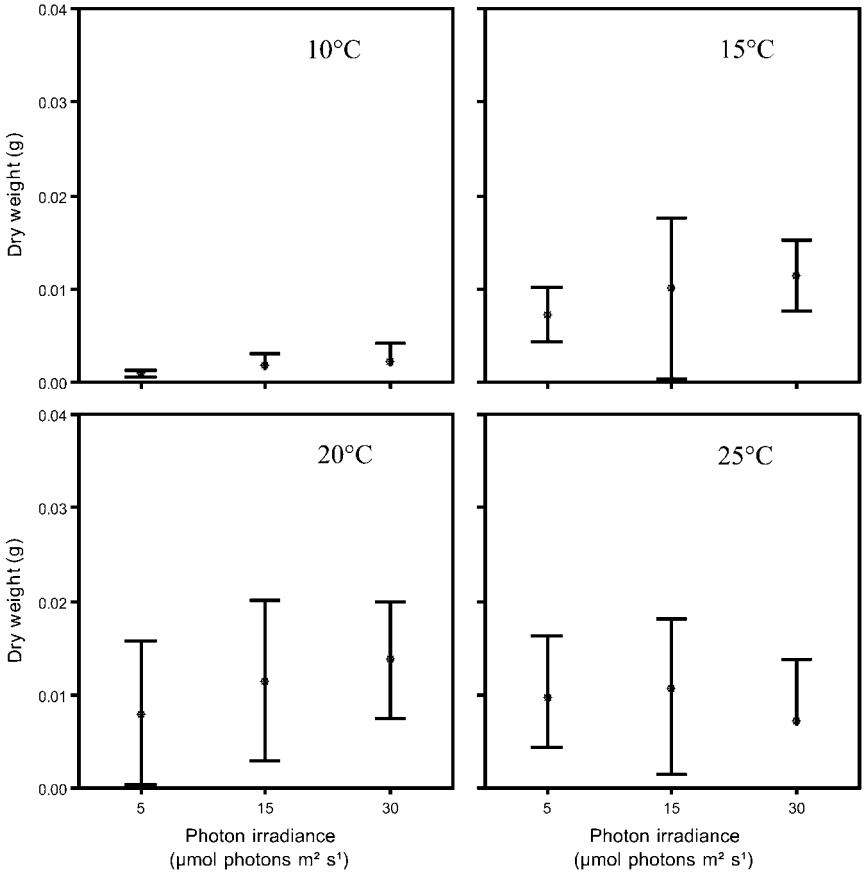


Figure 1 *Bonnemaisonia hamifera:* growth of *Trailliella* phase in different combinations of temperature and photon irradiance. Bars indicate standard deviations (n=3).

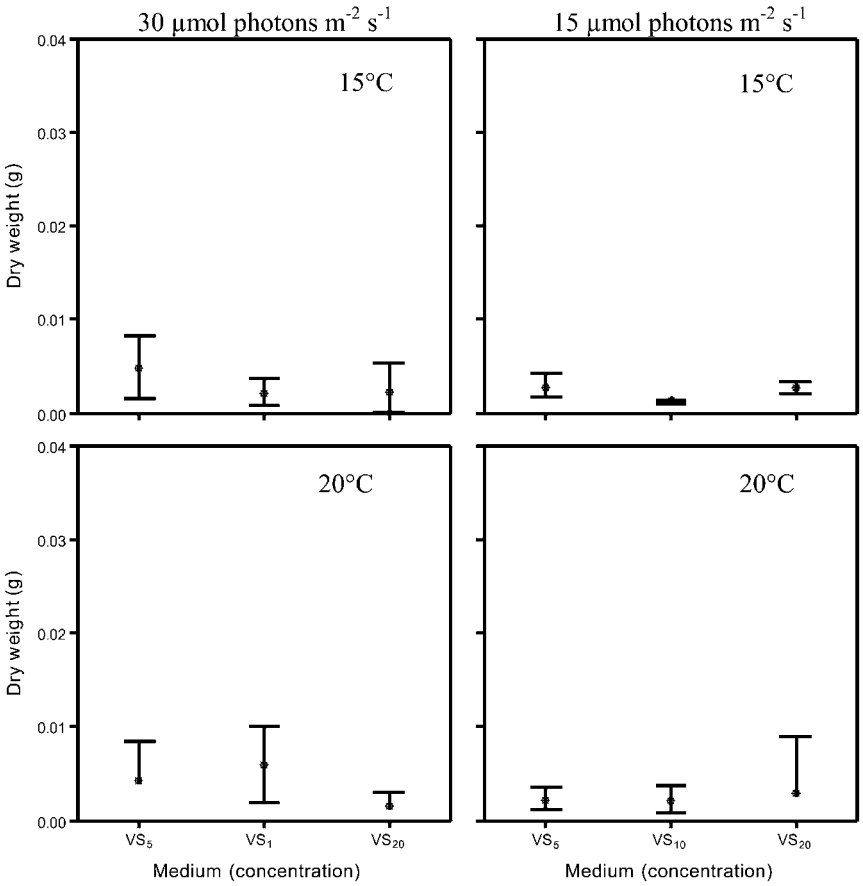


Figure 2 *Bonnemaisonia hamifera*: growth of *Trailliella* phase in different combinations of temperature, photon irradiance and different concentrations of von Stosch medium.
Bars indicate standard deviations (n=3).

at 10 and 15°C, whereas at 20 and 25°C specimens grown in lower photon irradiances (5 µmol photons m^{-2} s^{-1}) had a somewhat larger diameter than specimens grown at higher photon irradiances (15 and 30 µmol photons m^{-2} s^{-1}). The difference in the results between dry weight and diameter was due to the fact that, at the lowest irradiance, specimens grown at 20 and 25°C showed a less dense habit, with thinner and longer branches; the biomass, however, was not higher than in specimens grown in other combinations, which had a denser and more compact branching habit.

Experiment 2: effect of nutrient concentration, temperature and photon irradiance

The growth of *Trailliella* was tested under three different concentrations of von Stosch medium (VS_{20}, VS_{10}, VS_5), two temperatures (15 and 20°C) and two photon irradiances (15 and 30 µmol photons m^{-2} s^{-1}). Daylength was 16:8 h L:D. Similar results were obtained for the dry weight and diameter data. No evident difference was observed between the two temperatures tested. However, differences for medium concentration and photon irradiance were noticed. Whereas no clear difference between photon irradiances occurred for VS_{20}, a photon irradiance of 30 µmol photons m^{-2} s^{-1} was more favourable for VS_{10} and VS_5 (Figure 2). In general, a von Stosch concentration of 5 and 10 ml/l (VS_5 and VS_{10}, respectively), a temperature of 20°C and a photon irradiance of 30 µmol photons m^{-2} s^{-1} were the combinations that promoted the highest biomass production (Figure 2).

Experiment 3: effect of temperature and culture medium (von Stosch medium and Provasoli's enriched seawater)

The growth of *Trailliella* was tested at two temperatures (15 and 20°C) and in two different culture media, VS_5 and PES; for PES, three different concentrations were used: quarter-strength (5 ml of stock solution per litre of sterile seawater, PES_1), half strength (10 ml/l, $PES_{1/2}$) and full strength (20 ml/l, $PES_{1/4}$). Photon irradiance was 20 µmol photons m^{-2} s^{-1}; daylength was 16:8 h L:D.

At both temperatures, differences between the culture media tested were observed for both dry weight and diameter. The growth of *Trailliella* was generally better in VS_5 than in any concentration of PES (Figure 3).

Experiment 4: effect of temperature and culture medium (von Stosch and Carna medium)

The growth of *Trailliella* was tested at two temperatures (15 and 20°C) and in two different culture media, VS_5 and Carna medium. Photon irradiance was 20 µm photons m^{-2} s^{-1}; daylength was 16:8 h L:D. Growth in VS_5 was clearly better than in Carna medium at both temperatures tested (Figure 4); both dry weight and diameter were higher in VS_5.

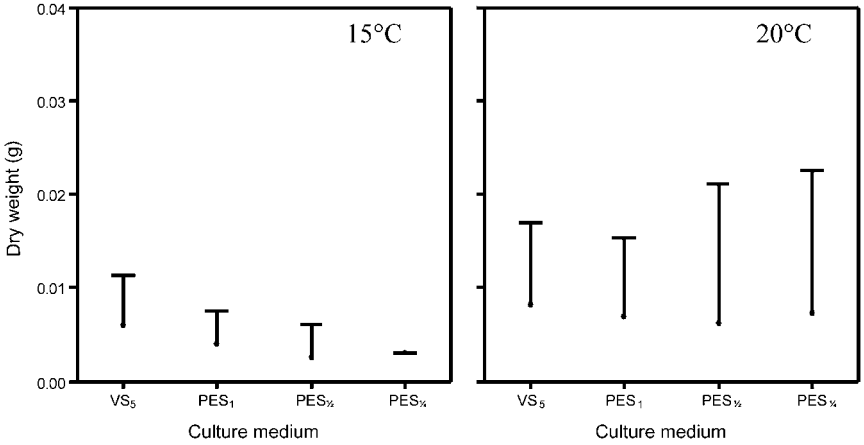


Figure 3 *Bonnemaisonia hamifera*: growth of *Trailliella* phase in different combinations of temperature and culture medium (VS_5 and diffferent concentrations of PES).
Bars indicate standard deviations (n=3).

Experiment 5: effect of aeration

The growth of *Trailliella* was tested with and without aeration. Temperature was 15°C; photon irradiance was 20 μmol photons m^{-2} s^{-1}; daylength was 16:8 h L:D.

Aeration had a clear effect on the growth of *Trailliella*. The dry weight of the alga was higher when aeration was provided (Figure 5); conversely, the diameter of the specimens was higher when aeration was not provided. Aeration had a marked effect on the morphology of the thallus; specimens grown with aeration formed smaller tufts, but they had a much denser branching, resulting in a significantly higher dry weight.

Experiment 6: effect of daylength

The growth of *Trailliella* was tested at three different daylengths (16:8 h L:D, 12:12 h L:D and 8:16 h L:D). The temperature was 15°C and the medium used was VS_5. Two different experiments were carried out in these conditions: in one, photon irradiance was 20 μmol photons m^{-2} s^{-1} for all daylengths; in the other, photon irradiance was correspondingly adjusted at different daylenghts in order to obtain the same photon dose in all conditions.

When the photon irradiance was not adjusted, a marked effect of daylength on the growth of *Trailliella* was observed on both dry weight and diameter of the thallus. The growth of the alga was clearly better in 16:8 h L:D than in the other photoperiodic regimes (Figure 6). When the photon irradiance was adjusted, so that thalli received the same energy in 24 h, no evident difference between daylengths was noted (Figure 7).

Experiment 7: effect of nitrogen and phosphorus content on reproduction

In this experiment, *Trailliella* was grown at 15°C, 20 μmol photons m^{-2} s^{-1}, 8:16 h L:D, in VS_5 with reduced content of nitrogen and phosphorus, as described above; controls with normal content of nitrogen and phosphorus were also grown. All specimens grown in reduced nitrogen and phosphorus became reproductive. The specimens began to show production of tetrasporangia after 21 days. In the following weeks the tetrasporangia became segmented and were released; tetraspores were released and showed a high germination rate, producing viable thalli of *Bonnemaisonia* (Figures 8–14). No production of tetraspores was detected in the controls.

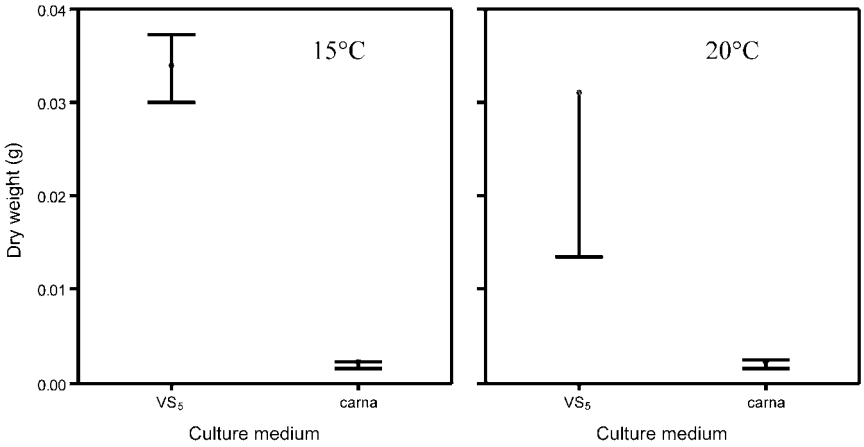


Figure 4 *Bonnemaisonia hamifera*: growth of *Trailliella* phase in different combinations of temperature and culture medium (VS_5 and Carna medium).
Bars indicate standard deviations (n=3).

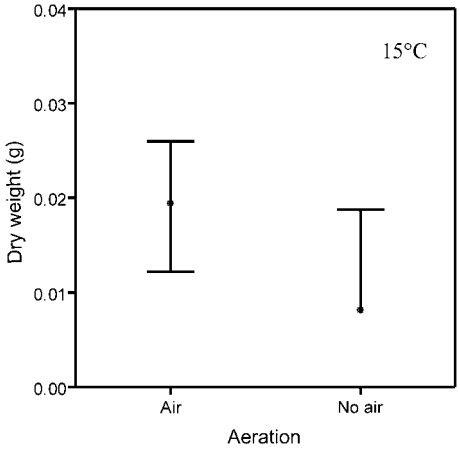


Figure 5 *Bonnemaisonia hamifera*: growth of *Trailliella* phase with and without aeration.
Bars indicate standard deviations (n=3).

Discussion

The results obtained from the experiments are generally clear and straightforward. The fact that the equipment available did not allow appropriate replication for some of the factors tested (temperature and daylength) prevented the possibility of analysing the data by inferential statistics (e.g., ANOVAs). However, clear and obvious differences between different factor levels were observed. In general, identical results were obtained for the two variables used, dry weight and diameter. However, a few exceptions were encountered. In these cases, since the aim of the study was to optimise the biomass production, the results obtained for dry weight were considered. The results indicate that the optimal conditions for biomass production in *Trailliella* are represented by a combination of temperatures of 15–20°C, photon irradiances of 20–30 μmol photons m^{-2} s^{-1} and long daylenghts (16:8 h L:D). Quarter-strength von Stosch medium, routinely used in our laboratory (VS_5; Guiry and Cunningham 1984), appears to be the best among the media used; aeration of the culture vessels is also a factor contributing to a higher biomass production. For large-scale production of *Trailliella*, cultivation in aerated vessels at the temperatures and photon irradiances mentioned above would therefore be the method with the highest probability of success. In fact, a preliminary cultivation trial using these growth conditions in a 1000-l plastic tank provided fairly encouraging results, with an increase in biomass of approximately 505% in 9 days (Nash unpublished data). The type of medium, however, may represent a potential limitation for large-scale cultivation. For cultivation of algae under controlled conditions, several elements (in particular nitrogen and phosphorus) can be supplied in different chemical forms; since some compounds are more expensive than others, choice of fertiliser will obviously be an important factor for commercial cultivation. In VS_5, phosphorus is supplied to the alga in an organic form ($Na_2\beta$-glycerophosphate), which is considerably more expensive than inorganic phosphoric compounds (although this is partly compensated by the fact that the form with the lowest concentration of nutrients, VS_5, is as effective as, or more effective than, VS_{10} and VS_{20}). The use of this medium may therefore represent a cost-disadvantage for large-scale cultivation.

Although the experiments were carried out with a strain conserved in culture for many years, our results are concordant with the distribution and the growth of *Trailliella* in nature. *Trailliella* occurs in the lower intertidal zone (mostly in rockpools) and in the subtidal zone (Dixon and Irvine 1977, Hardy and Guiry 2003), in habitats that are more or less shaded; its high growth performances in low photon irradiance are therefore not surprising. Aeration is well known to influence physiological processes in marine algae in several direct and indirect ways (Hurd 2000) and, if other conditions are uniform, growth is normally faster in aerated conditions than in absence of aeration (Gao et al. 1991, De Paula et al. 2001, Huang and Rorrer 2003). Aeration or water motion are routinely supplied to containers in which marine algae are grown for commercial purposes (Buschmann et al. 2004, Gal-Or and Israel 2004, Ryder et al. 2004). The importance of temperature, daylength and other environmental variables in the regulation of life history (and, conse-

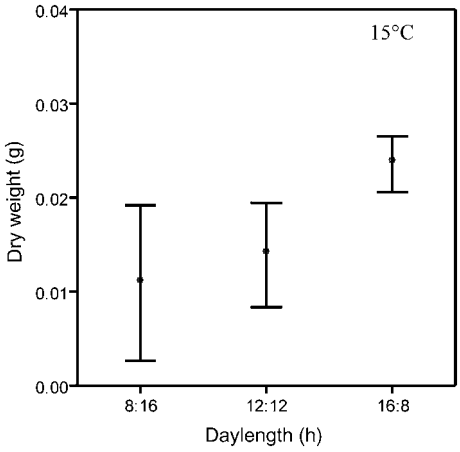


Figure 6 *Bonnemaisonia hamifera*: growth of *Trailliella* phase at three different daylengths; photon flux density was not compensated between different daylengths.
Bars indicate standard deviations (n=3).

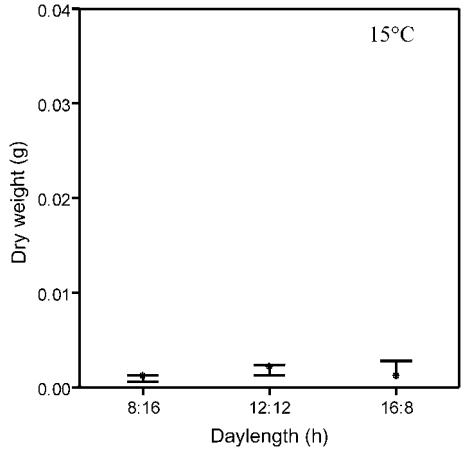
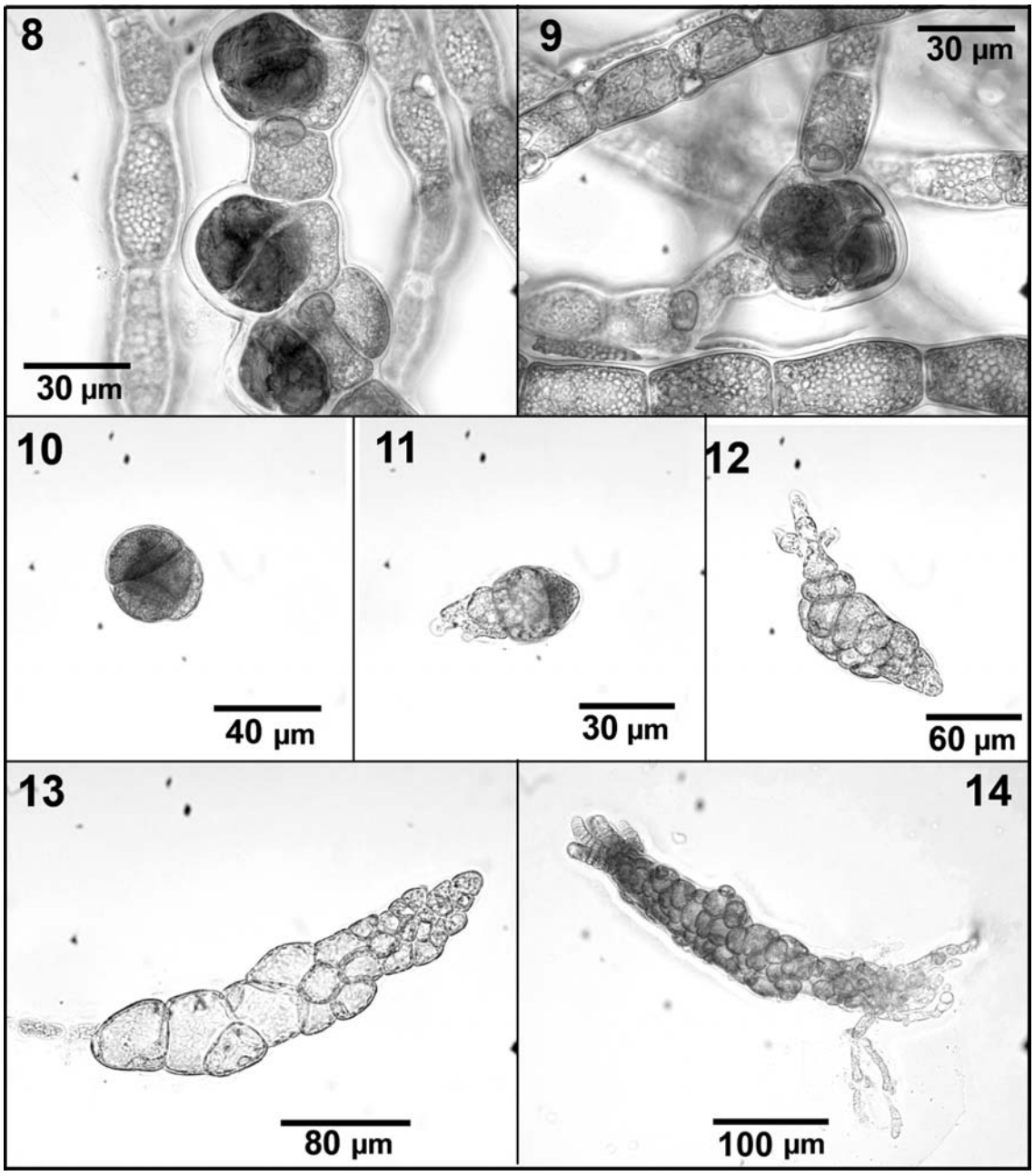


Figure 7 *Bonnemaisonia hamifera*: growth of *Trailliella* phase at three different daylengths; photon flux density was compensated between different daylengths.
Bars indicate standard deviations (n=3).

quently, geographical distribution) has been demonstrated experimentally in the last decades for many marine algae, including members of the Bonnemaisoniales (Breeman et al. 1988, Breeman and Guiry 1989, Guiry and Dawes 1992). The geographical distribution of many species is linked to combinations of temperature and daylenght suitable for growth and reproduction (van den Hoek 1982, Yarish et al. 1984, Breeman 1988, Lüning 1990). In relation to geographic distribution, the results obtained in this study are in general agreement with published information on the phenology of *Trailliella*. Conditions of long days and temperatures of 15–20°C occur in late spring and summer on most shores of the northern Atlantic Ocean. *Trailliella* is usually reported as present year-round and details on distribution and abundance in relation to seasonality are rarely mentioned (e.g., Børgesen 1930, Feldmann 1954, Chen et al. 1969, Sears and Wilce 1975, Hardy and Guiry 2003). It seems, however, that records of this alga are more common in summer than in other seasons (Westbrook 1930, Feldmann 1954), suggesting more favourable conditions for growth in this season. We assume here that a common genetic identity is shared by all populations of *Trailliella* in northern Europe, and that our results can be generalised to all northern European populations. Although an identical morphology of the sporophytic phase has been reported for several species of *Bonnemaisonia* (Dixon and Irvine 1977), the body of information currently available does



Figures 8–14 *Bonnemaisonia hamifera*: production of tetraspores in *Trailliella* phase and germination of gametophyte sporelings. (8) A filament producing tetrasporangia (9) Detail of a tetrasporangium. (10) A discharged tetrasporangium. (11) Sporeling in early phase of germination. (12) Sporeling in a subsequent phase of development. (13, 14) Young gametophyte specimens arisen from development of sporelings.

not provide any evidence indicating the presence of different taxonomic entities (or multiple invasions) in the North Atlantic Ocean; in fact, identical responses have been observed for the strain of *Trailliella* used in this study and strains isolated from Ireland, suggesting genetic homogeneity (Nash unpublished data). Population studies based on molecular data would be very valuable for confirming this theory. To date, however, this type of information is available for very few Bonnemaisoniales (Andreakis et al. 2004, Ní Chualáin et al. 2004), and not for *Bonnemaisonia hamifera*. The common genetic identity of the North Atlantic populations is also indicated by the fact that our experiment on the reproduction, carried out with the same methods used by Breeman et al. (1988), gave results identical to those for the Irish strain used by Breeman et al. (1988). The effect of long-term maintenance and history of environmental conditions on reproductive responses of marine algae have generally received very little attention, although some studies showed that the photoperiodic history may affect the critical reproductive daylength in some species of red algae (Breeman 1993). Overall, the results of our experiments indicate that long-term maintenance in constant conditions has no effect on the growth and reproductive responses of the alga. A useful implication of this is that strains maintained long-term in culture collections can be propagated and used for commercial cultivation.

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