



Bromoform, mycosporine-like amino acids and phycobiliprotein content and stability in *Asparagopsis armata* during long-term indoor cultivation

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Received: 5 August 2021 / Revised and accepted: 31 January 2022 / Published online: 16 March 2022
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Abstract

As the commercial use of seaweed for natural product extraction calls for abundant, uniform biomass, this study focused on the production and the variability of the harvested biomass of valuable compounds produced by a commercially relevant red algal species, *Asparagopsis armata*. Its tetrasporophyte stage was retrieved from two Irish localities and grown for over 2 years in indoor cultures at 13 °C and 17 °C, and bromoform, mycosporine-like amino acids and phycobiliprotein contents were monitored over time. Growth rates at different temperatures were specific to isolates, and one isolate failed to grow at 17 °C. All compounds of interest were detected by the end of the 2-year cultivation period, and most of them were produced at an exponential rate at 13 °C but not at the higher temperature. At 13 °C, bromoform reached concentrations of $10.00 \pm 0.55 \text{ mg g}^{-1}$, total mycosporine-like amino acids of $2.65 \pm 0.10 \text{ mg g}^{-1}$, phycoerythrin of $11.46 \pm 0.35 \text{ mg g}^{-1}$ and phycocyanin of $72.13 \pm 1.74 \text{ mg g}^{-1}$ in Irish isolates. The observed variability in compound content was statistically significant but not large enough to impede commercial utilization. Bromoform content in cultivated samples was almost 6-fold higher than in field-collected samples though natural bromoform variability remains to be elucidated. Our findings suggest that the tetrasporophytic phase of *A. armata* is a suitable candidate for indoor cultivation; abundant and homogeneous biomass composition can be obtained which can be further optimized by growth temperature.

Keywords Aquaculture · Cultivation · Falkenbergia · Invasive species · Ireland · Optimization · Tetrasporophyte

Introduction

Seaweeds (marine macroalgae) are sources of bioactive compounds with multiple commercial applications, ranging from food to pharmaceuticals as well as agriculture (Stengel and Connan 2015; Wang et al. 2019). Although over 30,000 marine compounds have already been characterized (Chuan et al. 2021), except in a few species, their commercial exploitation is currently restricted due to, among other things, limitations in the supply of biomass. Natural

seaweed populations vary greatly in metabolite composition, and hence also often the associated bioactivity, both geographically and temporally (Stengel and Connan 2015). In addition, harvesting of wild populations should only be considered if detailed knowledge of the sustainability of such activity is available, as target or associated species may become diminished and non-reversible ecosystem decline may occur. However, broad geographical and seasonal variation in both content and activity of bioactive compounds actually presents a highly valuable opportunity for culturing opportunities, reflecting the richness and plasticity of wild populations, which can be exploited in indoor cultivation systems.

Strain domestication of marine biota in aquaculture is considered an important development towards the sustainable production of biomass (Hafting et al. 2015). However, domestication of seaweeds remains underdeveloped compared to that of terrestrial crops, and only two genera—*Saccharina japonica* (Areschoug) C. E. Lane, C. Mayes, Druehl and G. W. Saunders and several species of *Euclima*

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J. Agardh—provide the majority of the world's seaweed mariculture production, concentrated in nine East and Southeast Asian countries (FAO 2020; Costa-Pierce and Chopin 2021). The expansion of seaweed farming depends on domestication of wild strains, which in turn requires a good understanding of the life cycle of the species to control reproduction and propagation. In this regard, optimal growth and stable chemical composition are essential attributes for commercial biomass production.

Invasive seaweed species are of special interest as biotechnological targets (Balboa et al. 2015; Epstein and Smale 2017; Máximo et al. 2018; Albuquerque et al. 2019); they are considered specialists at producing highly variable secondary metabolites to enable their success in host ecosystems, to avoid grazing and to increase competitiveness, and they display higher growth rates. The red seaweed *Asparagopsis armata* Harvey (Rhodophyta) is an invasive seaweed introduced into many temperate areas worldwide, arriving in Ireland in 1939 (de Valéra 1942). Since then, it has spread along the Irish coast (*pers. obs.*). The species has proven to be a promising source of metabolites with antibacterial (Paul et al. 2006; Pinteus et al. 2015), antiviral (Haslin et al. 2001; Bouhlal et al. 2013), antioxidant and antitumor potential (Pinteus et al. 2018). Also, it has potential as a source of ultraviolet (UV) screening and antioxidant compounds, namely mycosporine-like amino acids (MAAs) (Dunlap and Yamamoto 1995; Bandaranayake 1998) and accessory pigments widely used as colour additives, such as phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (APC) (Roda-Serrat et al. 2018). Lately, both species in the genus *Asparagopsis* have been in the media hotspot because of the reported *in vitro* (Kinley et al. 2016; Machado et al. 2016) and *in vivo* (Roque et al. 2019; Stefenoni et al. 2021) anti-methanogenic capacity of their halogenated compounds. Given that bromoform is also toxic for human consumption, bromoform accumulation by cattle and any potential negative impacts on animal tissues and milk production require further investigation. Biomass processing and preservation protocols need to be improved but, principally, sourcing proposed quantities to achieve an impact remains a challenge.

Although removing 'naturally' occurring biomass of invasive species would reduce the negative impact these have on native ecosystems, this activity might also be associated with ecological risks. There is evidence that dispersal of invasive species is enhanced when removed manually (Critchley et al. 1986) or when intentionally cultured in the open sea (Fletcher and Farrell 1998), including in Ireland (Kraan and Barrington 2005). Additionally, damage to the native ecosystem may be induced by the physical removal of the species, e.g. through changes in incident irradiance that might favour species altering their abundance and misidentification of native species. Therefore, on-land cultivation techniques provide ecologically safe options, with the benefit

of high-level control of environmental parameters, which are species- and even life stage-specific, to optimize the yield of the targeted compound(s). Some disadvantages are related to high implementation costs and the difficulty of growing particular species or life stages indoors.

The tetrasporophyte phase of *A. armata* (former *Falkenbergia* stage) has enormous potential as a source of bioactive compounds (Paul et al. 2006; Figueroa et al. 2008; Mata et al. 2011). Here, isolates from two localities on the Irish west coast were maintained for 2 years in indoor culture, with the aim of investigating the temporal production of commercially interesting compounds (MAAs: shinorine and palythine; phycobiliproteins: PE, PC and APC; and bromoform) at two temperatures. Genetic characterization of the isolates was performed to detect unique haplotypes at the locations. Given that compound production is a pivotal aspect of cultivation, we also estimated growth rates at each experimental temperature. To evaluate the tetrasporophytic stage of *A. armata* as a candidate for long-term, on-land cultivation, we studied the following characteristics: (1) importance of geographically distinct isolates regarding growth and metabolite content; (2) growth rates and compound production enhancement by culture conditions and through time, (3) consistency and stability of metabolite content in the harvested biomass as a prerequisite for industrial long-term (>2 years) cultivation where the physiological state of the species is not compromised.

Material and methods

Algal material and culture conditions

Isolates were obtained from two locations: Finavarra (Co. Clare, Ireland) (53° 09' 09.8" N 9° 07' 11.6" W) on 11/7/2017; and Lettermore (Co. Galway, Ireland) (53° 17' 16.1" N 9° 39' 31.6" W) on 23/07/2017. From each population, approx. five individual 'pompoms' '*Falkenbergia*' stages were taken to the laboratory within 1 h, cleaned of epiphytes and treated against diatom fouling using germanium dioxide (GeO₂) for 1 day in 10-mL Petri dishes. Subsequently, they were transferred to 2-L flasks with sterile seawater supplemented with 20 mL L⁻¹ of Provasoli-enriched media (PES; Provasoli 1957). *Asparagopsis armata*, as all Florideophyceae, has a tri-phasic heteromorphic life cycle, where gametophytes alternate with carposporophytes (parasitic to gametophytes) and tetrasporophytes. Cultures were established using tetrasporophytes given that gametophytes failed to grow in laboratory conditions (*pers. obs.*, data not shown). After 2 months, cultures were transferred to 20-L tanks (translucid, 29 cm diameter, 32.6 cm depth) with aeration provided by air pumps, connected to a tube placed in the middle of the bucket. Culture media were changed monthly.

Two sets of cultures ($n=1$ per set) with an initial biomass density of 5 g FW L^{-1} were established under $15 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ provided by Sylvania Activa 36W/172 (Feilo Sylvania, Germany) and a photoperiod of 12:12 (L:D) in walk-in culture chambers at $13 \text{ }^\circ\text{C}$ and $17 \text{ }^\circ\text{C}$ with the same aeration and monthly seawater enrichment (PES). The lower experimental temperature ($13 \text{ }^\circ\text{C}$) corresponded to the in situ water temperature at the time of sampling and the upper ($17 \text{ }^\circ\text{C}$) to the maximum photosynthetic efficiency (α) of tetrasporophytes from *A. armata* from the Mediterranean Sea (Zanolla et al. 2015), as well as maximum summer temperatures recorded locally in western Ireland (Guihéneuf et al. 2018). Each month, approximately a third of the biomass was removed using a strainer from the 20-L tanks to avoid overcrowding and self-shading and keep the ideal density for optimal growth for this life stage and species at 5 g FW L^{-1} (Mata et al. 2006). This fresh biomass was measured by removing water excess with lab paper, and then placed in a scale.

Molecular methods and analyses

DNA was extracted from three tetrasporophytes from each location and the mitochondrial *cox2-3* spacer was sequenced following protocols detailed in Sherwood (2008). Newly generated and mined sequences of the *cox2-3* spacer from *A. armata* from NCBI were treated as in Zanolla et al. (2018). Briefly, the alignment was assembled from forward and reverse reads using BioEdit (Hall 1999) with available sequences. Genealogical networks were computed using the median-joining algorithm in Network v4.5.1.6 (<http://www.fluxus-technology.com>; Bandelt et al. 1999) to identify unique haplotypes at locations.

Measurements of growth and chemical composition

Growth rate was assessed by determining the difference of total biomass weight over 5 consecutive months and then using the following formula: $\text{Growth (\% day}^{-1}\text{)} = (\text{Ln final FW} - \text{Ln initial FW}) / \text{Time (days)} \times 100$ for each isolate at $13 \text{ }^\circ\text{C}$ and $17 \text{ }^\circ\text{C}$. We studied individual, daily and monthly variations of shinorine, palythine, phycobiliproteins (phycoerythrin-PE, phycocyanin-PC and allophycocyanin-APC) and bromoform content. Monthly variation was monitored over a 2-year period. Every month, for both temperatures and geographical isolates, three replicates of approx. 3 g dry weight (DW) were taken for chemical analysis from the bulk culture vessel. Preliminary analysis of these data portrayed unexpected variability in spite of stable culture conditions. To investigate the underlying cause of this phenomenon, we assessed daily and individual variation in compound production. For the former, three replicates were sampled every 6 h

for a 30-h period. For the latter, we picked four individual pompons, large enough to be split into three analytical replicates. All material for chemical analyses was frozen for 24 h, then freeze-dried (Labconco Freezezone, USA) and ground, as recommended for bromoform, pigment and MAA detection (Hagerthey et al. 2006; Vucko et al. 2017; Guihéneuf et al. 2018; respectively). All material was stored at $-20 \text{ }^\circ\text{C}$ until analysis. Phycobiliproteins and MAAs were measured within 2 months, and bromoform after 2 years.

Chemical analyses

Mycosporine-like amino acids were extracted and analysed according to Karsten et al. (2009) and adapted as in Guihéneuf et al. (2018). Identification of MAAs was obtained by co-chromatography with in-house (NUI Galway) purified standards of shinorine, palythine, asterina-330 and porphyra-334 as detailed in Guihéneuf et al. (2018). Phycobiliproteins were extracted from 30 mg of biomass using 2 mL of 0.1 M phosphate buffer ($\text{pH}6.80$) and left overnight at $4 \text{ }^\circ\text{C}$ with continuous stirring. After centrifugation at $8000 \times g$ for 5 min (at $4 \text{ }^\circ\text{C}$), the supernatant was analysed using a Cary UV50 Spectrophotometer and CaryWIN software (Varian Inc., USA). Absorbance was measured at wavelengths $455, 564, 592, 615, 618, 645$ and 652 nm . PE and PC contents were calculated using Beer and Eshel formulas (Beer and Eshel 1985) and APC using Sudhakar et al. (2015). The bromoform content was determined by GC-MS analysis following a method previously developed and validated by Cawthron Institute, as described in detail by Romanazzi et al. (2021). Briefly, freeze-dried seaweed samples were extracted twice with methanol and the extracts were combined and further diluted before GC-MS analysis. Analysis was performed on an Agilent 7890B gas chromatograph equipped with a single quadrupole mass analyser (Agilent 5977A). The GC was fitted with an Agilent 19091N-133 HP-INNOWAX silica capillary column ($30 \text{ m} \times 0.25 \text{ mm i.d.}, 0.25 \mu\text{m}$ film thickness) and the injector was maintained at $180 \text{ }^\circ\text{C}$ and operated in split-less mode with a split vent time of 0.5 min and an injection volume of $1 \mu\text{L}$. The GC oven temperature was programmed to start at $40 \text{ }^\circ\text{C}$, and then was ramped up to $250 \text{ }^\circ\text{C}$ at $16 \text{ }^\circ\text{C min}^{-1}$; hydrogen was used as the carrier gas, with a flow rate of 1.5 mL min^{-1} . The ion source electrode was operated in positive mode and the mass analyser was set up in SIM mode with a dwell time of 50 ms for all signals. MassHunter software was used for data analysis. A bromoform calibration curve for calibration and quantitation of bromoform was generated by serial dilution of a bromoform reference standard obtained from Sigma-Aldrich (USA).

Photosynthetic measurements

We used photosynthesis-irradiance (P-E) curves to characterize the photosynthetic performance of Finavarra isolates at 13 °C at the end of the cultivation period, given it was the best growing isolate and growth rates are decisive aspects of cultivation. Four replicates, consisting of 0.04 g fresh weight (FW) each, and belonging to a single individual, were then transferred to the reaction chamber of a Clark-type oxygen electrode system (Hansatech Instruments, UK), in 2 mL sterile seawater at 13 °C to determine photosynthetic performance. Changes in dissolved oxygen concentration were used to estimate the dark respiration and photosynthetic rates under 10 levels of increasing irradiance, ranging from 0 to 475 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. This maximum irradiance was chosen to avoid photoinhibition (Zanolla et al. 2015). Specific details of P-E curve measurements and photosynthetic parameter calculation followed Zanolla et al. (2015). The following parameters were estimated: maximum net photosynthetic rate (NPR_{max}), light compensation point (I_c), photosynthetic efficiency (α), light saturation point (I_k) and dark respiration. These parameters, taken together, are good indicators of the physiological state of the samples (P_{max} , α , DR) and help to refine irradiance culture conditions (I_k , I_c).

Data analyses

Monthly MAA and phycobiliprotein contents were plotted using all replicates from each harvesting event ($n=3$) to determine the best fitting model (linear, exponential, none, indicated using trend lines in the figures) of their content over time. R values from these adjustments were used as

indicators of significance. The variances (s^2) of the replicates within 1 month were used to assess the homogeneity of the harvested biomass in the relevant compounds. To uncover optimal temperature (13 °C vs 17 °C) for MAAs content in Finavarra isolates, we performed a t student test. Daily and individual variations in MAAs and phycobiliproteins were analysed using one-way ANOVA, with ‘hour of sampling’ or ‘individual’ as fixed factor. Bromoform content was analysed every 2 months. Bromoform levels are presented as mean \pm sd and are compared among locations (for the first year only, at 13 °C) and among temperatures (13 vs 17 °C, only for Finavarra isolates, 2 years) using two-way ANOVAs with ‘time and location’ and ‘time and temperature’, respectively, as fixed factors. Normality and homoscedasticity were checked prior to the ANOVAs, as implemented in the software used. No transformation of the data was needed. Significance was set at 0.05 for all tests, which were performed in Kaleidagraph (Synergy Software, Version 4.0).

Results

Molecular analyses

All replicates retrieved from Finavarra and Lettermore belonged to lineage 1 of *A. armata* (as in Dijoux et al. 2014, phylogenetic tree not shown) and more than one haplotype was found at Finavarra. All samples from Lettermore, on the other hand, belonged to same haplotype (in blue in Fig. 1). Most individuals, except one (in red in Fig. 1), were identical to those from the Mediterranean Sea (Fig. 1).



Fig. 1 Haplotype diversity in samples from Finavarra (Fin, $n=3$) and Lettermore (Let, $n=2$). AA, *Asparagopsis armata*; FR, *Falkenbergia rufolanosa*

Growth rates

The isolate from Lettermore failed to grow at 17 °C, and growth at 13 °C was lower than for the Finavarra isolate ($14.8 \pm 1.7 \text{ \% day}^{-1}$ vs $18.4 \pm 4.2 \text{ \% day}^{-1}$, respectively). Temperature had a drastic effect on the growth rate in Finavarra isolates; rates of $1.6 \pm 0.5 \text{ \% day}^{-1}$ were measured at 17 °C, almost 10 times lower than at 13 °C. Cultures based on the Lettermore isolates were maintained for 1 year only, and those from Finavarra for more than 2 years.

Chemical analyses

Mycosporine-like amino acids

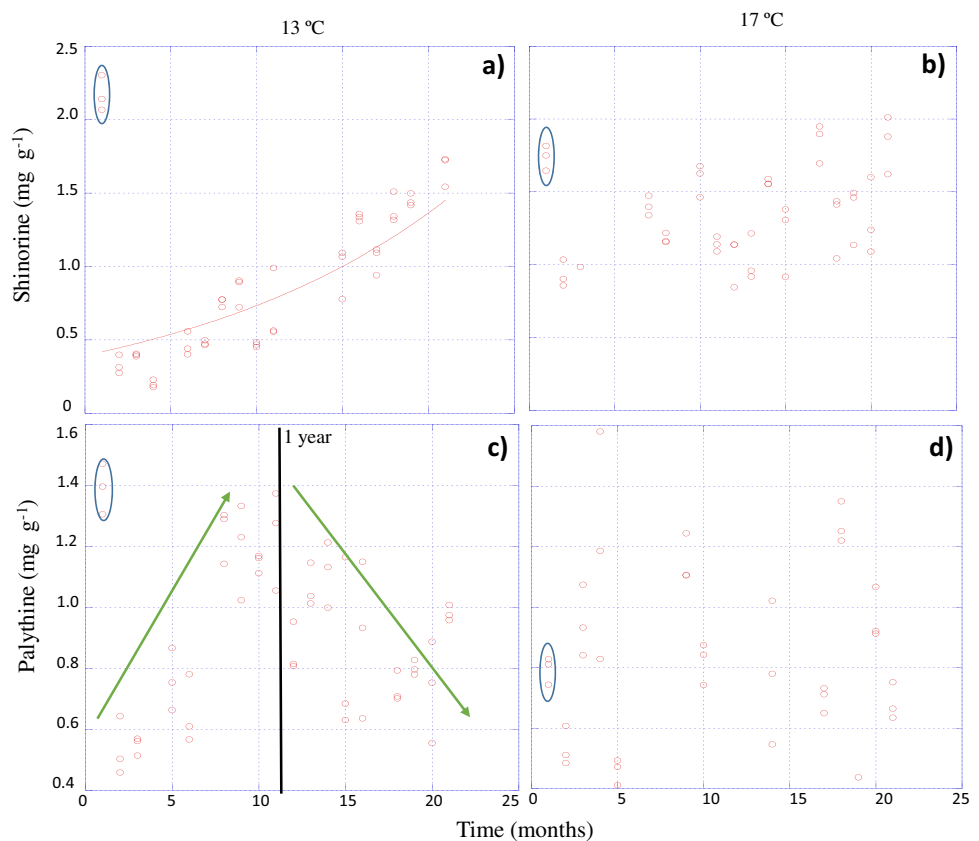
Our quantification method allowed us to detect palythine, shinorine and traces of asterine 330, though the latter could not be quantified. Total MAA content in Finavarra samples grown at 13 °C was $1.93 \pm 0.82 \text{ mg g}^{-1}$ DW and $2.13 \pm 1.07 \text{ mg g}^{-1}$ DW at 17 °C. Lettermore samples grown at 13 °C yielded average values of MAA of $2.64 \pm 0.77 \text{ mg g}^{-1}$ DW throughout the study. Shinorine content was always higher than palythine at all temperatures (Fig. 2). The samples retrieved directly from the field before cultivation were too small to analyse but, at 13 °C in Finavarra isolates, shinorine content decreased from the concentrations determined after

1 month and then increased exponentially (Fig. 2a, Supp. Table 2). Overall, higher content in shinorine was found at 13 °C (Supp. Table 1). At 17 °C, however, initial (month 1) values were surpassed after 6 months, but concentrations did not show any apparent trend (Fig. 2a, b, Supp. Table 2). With respect to biomass homogeneity, at 13 °C, Finavarra isolate variance was always below 0.01, and even decreased with time (Supp. Fig. 1a). At 17 °C, the variation in shinorine was almost three times greater than that at 13 °C, with higher values observed by the 18th month in culture (Supp. Fig. 1a).

Palythine contents from Finavarra isolates also decreased from initial values (Fig. 2c, d), but were lower at 17 °C (Fig. 2d; Supp. Table 1). While there was a linear increase during the first 12 months of cultivation, at 13 °C, no clear trend was observed during year 2 (Fig. 2c). At 17 °C, palythine production was inconsistent (Fig. 2d). As for compound production uniformity, variance of the content of the MAAs increased by the end of the first year and then declined with time under 13 °C (Supp. Fig. 1b). Contrary to shinorine, palythine production under 17 °C was more uniform, remaining below 0.006 during the 2 years of cultivation (Supp. Fig. 1b).

To address the observed variability in MAA content at 13 °C in Finavarra isolates over time—which could suggest underlying variation between individuals or a dependency

Fig. 2 Shinorine and palythine content of Finavarra isolates at 13 °C and 17 °C throughout the 2-year cultivation period. Circles highlight ‘initial’ content of each MAA determined after 1 month of cultivation when sufficient biomass for analysis was produced. Superimposed trend lines indicate the best fit, determined by adjusting all replicates ($n=3$ per month) to linear or exponential models (see text)



of the time of day the biomass was harvested—MAAs were also analysed in individual tetrasporophytes and in samples collected throughout a 24-h period. Variation between individuals (Supp. Fig. 2a) and among times of collection in shinorine, palythine or total MAA content was low and not significant (Supp. Fig. 2b, Supp. Table 1).

Shinorine in Lettermore isolates showed a similar trend to Finavarra isolates cultivated at the same temperature and for the same period (year 1) (Fig. 3a). However, 37 % more of that compound was detected in the Lettermore isolates (Supp. Table 1). Initial values dropped at the beginning of the trial, but then levels increased exponentially (Supp. Table 2). In terms of uniformity of biomass, variance was below 0.015 and decreased after 5 months in culture (Supp. Fig. 1g). Palythine content surpassed initial values by month 2, and while there was a maximum around month 6, no clear trend was observed (Fig. 3b). Overall values of palythine determined for Lettermore isolates were approx. 40 % higher than those of Finavarra (Supp. Table 1). Sample variance remained below 0.014, being almost 0 at the end of year 1 (Supp. Fig. 1h).

Phycobiliproteins

Phycocerythrin was the most abundant pigment recovered in all samples (Figs. 4, 5). Contrary to what was observed for MAAs, levels of all phycobiliproteins increased over time and were higher than the ‘initial’ values determined after 1 month of cultivation (Fig. 4). Phycocerythrin in samples from Finavarra increased exponentially (Supp. Table 2) under both temperatures tested. Variation in phycocerythrin content was very small during the first months of the cultivation period but increased with time, and this trend was more obvious at 17 °C (Supp. Fig. 1c). Phycocyanin in Finavarra isolates also increased exponentially at 13 °C, while 35 % lower levels were detected after culture at 17 °C for 2 years. There was no obvious trend for phycocyanin levels in samples cultivated at 17 °C (Fig. 4, Supp. Table 2). Similar variation in the levels was detected at both temperatures. Allophycocyanin was the least abundant pigment (Fig. 4). Unlike the other two compounds, allophycocyanin levels did not show a marked trend at either temperature and remained remarkably constant during the culturing trial (Fig. 4).

Individual and daily variations of phycobiliproteins in Finavarra isolates grown at 13 °C were analysed (Supp. Fig. 3), and one-way ANOVA results demonstrated variation to be significant only among individuals, but not when daily variations were examined (Supp. Fig. 3b, Supp. Table 1).

Lettermore samples yielded lower levels of all pigments (Fig. 5), but they increased at an exponential rate over time (Supp. Table 2). Phycocerythrin content was similar to that presented by Finavarra samples at 13 °C, while phycocyanin and allophycocyanin were 50 % lower in Lettermore

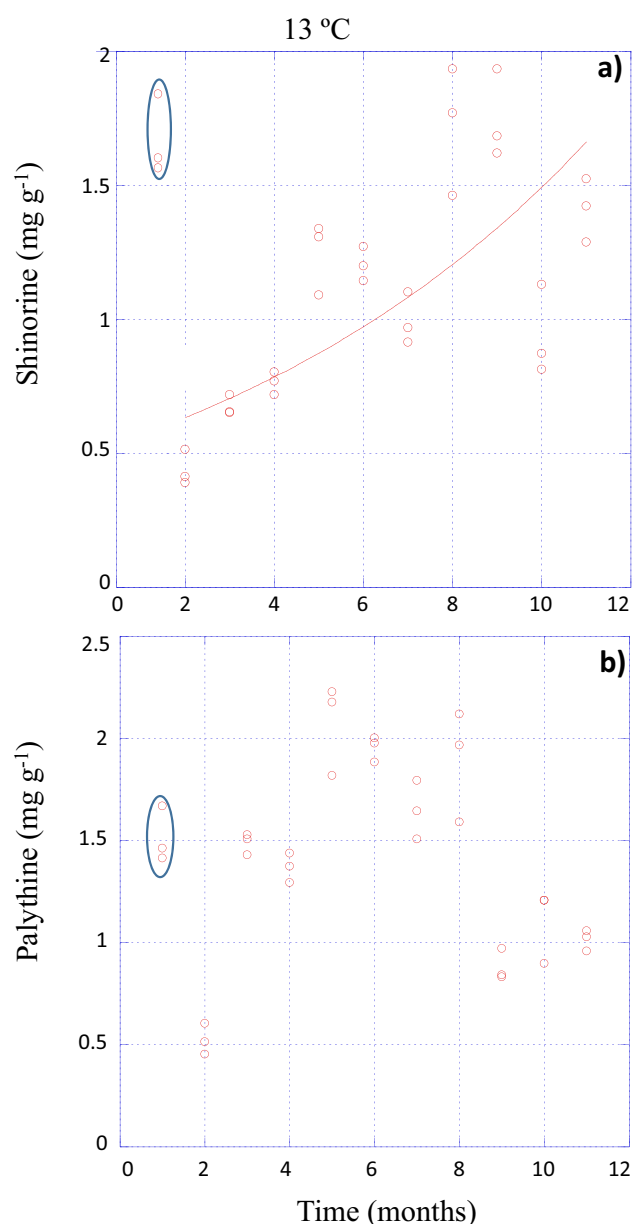


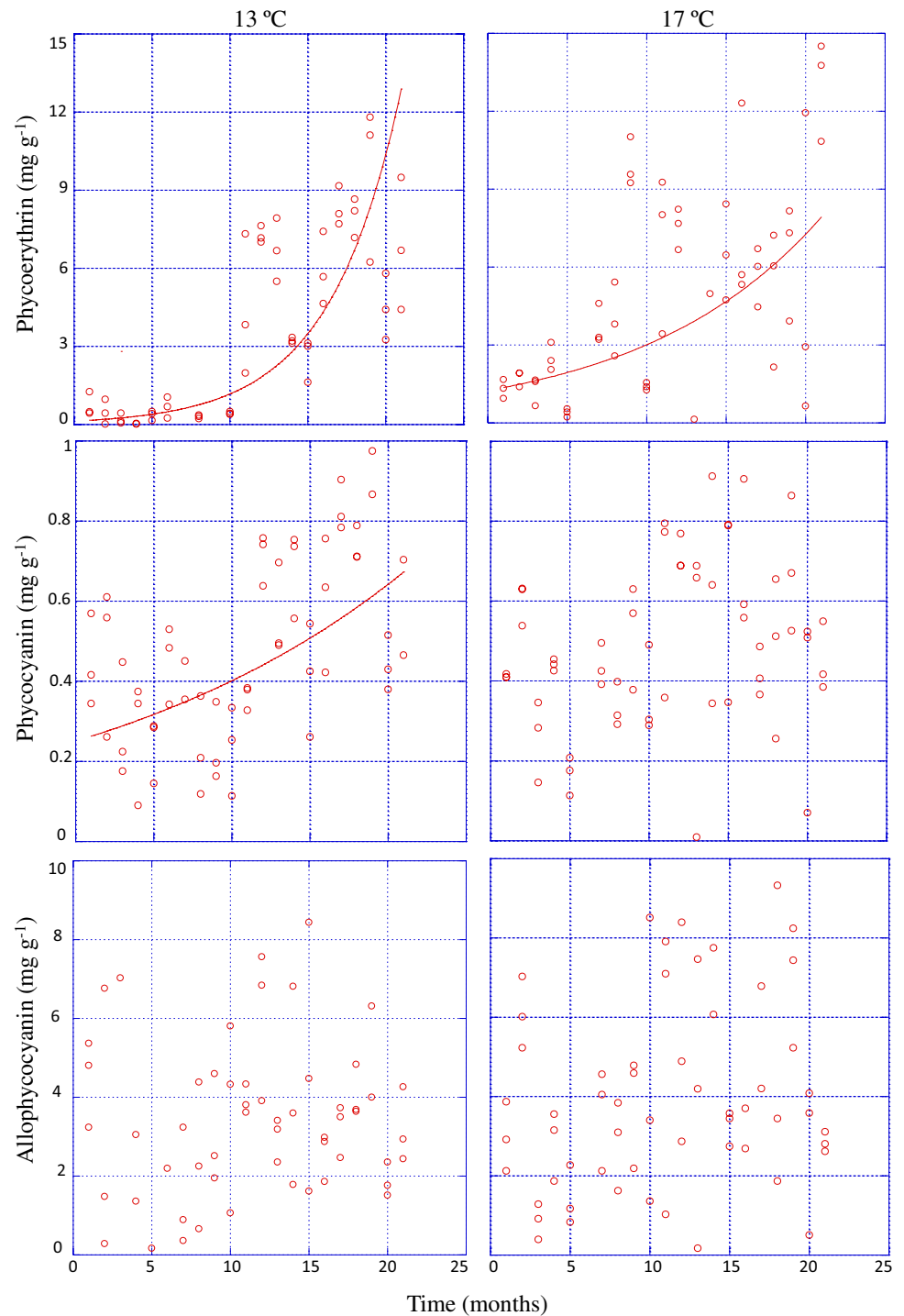
Fig. 3. Shinorine (a) and palythine (b) production of Lettermore isolates at 13 °C throughout the 12-month cultivation period. Circles highlight ‘initial’ content of each MAA determined after 1 month of cultivation when sufficient biomass for analysis was produced. Superimposed trend lines indicate the best fit, determined by adjusting all replicates ($n=3$ per month) to linear or exponential models (see text)

samples during the year. Initial values were exceeded by month 3 (Fig. 5). Variation, interestingly, increased with time only in the case of phycocerythrin (Supp. Fig. 1).

Bromoform content

As field-derived materials used to start the cultures had insufficient biomass to test bromoform concentrations, new materials obtained from Finavarra were analysed in October 2018, and

Fig. 4 Phycoerythrin, phyco-cyanin and allophycocyanin content in Finavarra isolates grown at 13 °C and 17 °C over a 2-year cultivation period. Superimposed trend lines indicate the best fit, determined by adjusting all replicates ($n=3$ per month) to linear or exponential models (see text)



revealed concentrations of $0.21 \pm 0.01 \text{ mg g}^{-1} \text{ DW}$. Concentrations in Finavarra cultures varied from 3.83 ± 0.45 to $10.01 \pm 0.55 \text{ mg g}^{-1} \text{ DW}$ at 13 °C and from 0.19 ± 0.01 to $13.27 \pm 0.25 \text{ mg g}^{-1} \text{ DW}$ at 17 °C. Lettermore samples yielded a range of 2.76 ± 0.45 to $7.51 \pm 0.55 \text{ mg g}^{-1} \text{ DW}$ (Fig. 6). Both isolates responded differently to 13 °C during the first year of cultivation; effects of time or isolate origin alone were not significant, but their interaction was (Supp. Table 1). A reduction

in bromoform content occurred in Lettermore samples by month 8, and then levels remained constant ($5.85 \pm 0.99 \text{ mg g}^{-1} \text{ DW}$). The only significant decrease in concentrations in Finavarra isolates occurred between months 5 and 8, and levels remained similar for the rest of the year (Supp. Table 1). By the end of the 12th month of cultivation, bromoform content was higher in Lettermore samples compared to Finavarra (Supp. Table 1). As with the other metabolites investigated,

Fig. 5 Phycoerythrin, phyco-cyanin and allophycocyanin content over time in Lettermore isolates grown at 13 °C throughout 1 year of culture. Superimposed trend lines indicate the best fit, determined by adjusting all replicates ($n=3$ per month) to linear or exponential models (see text)

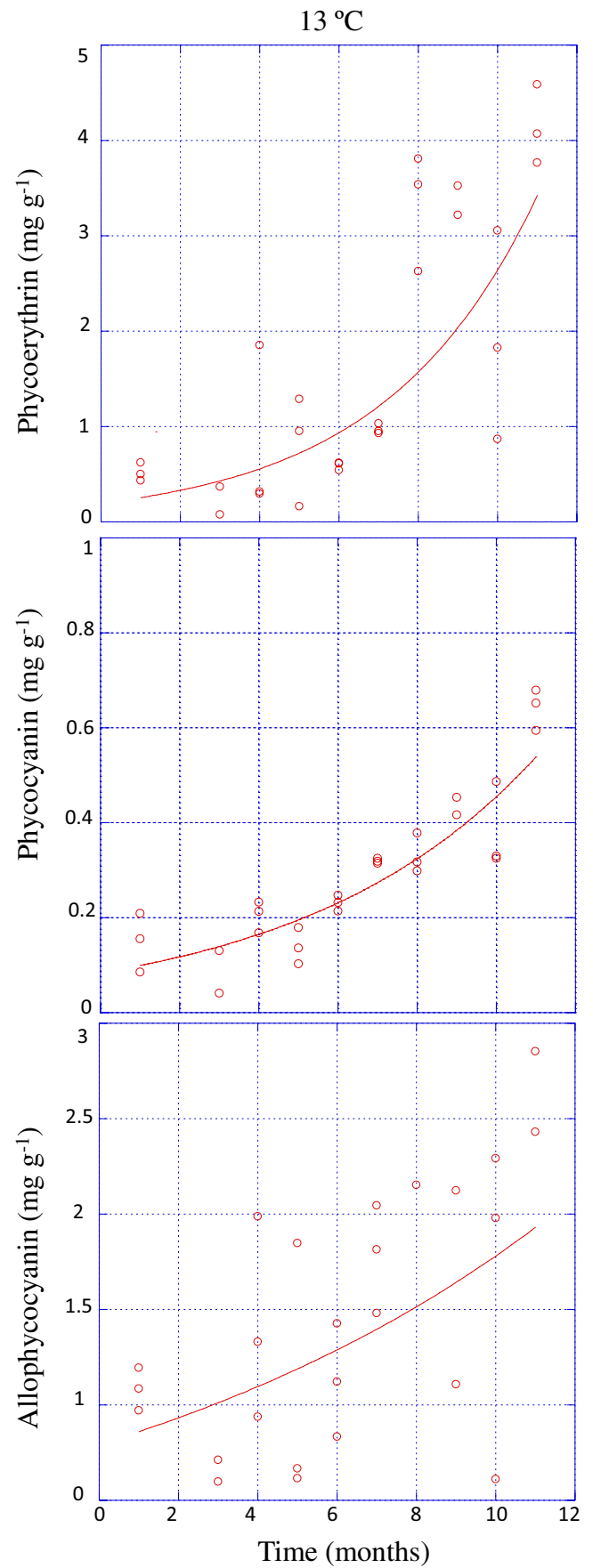
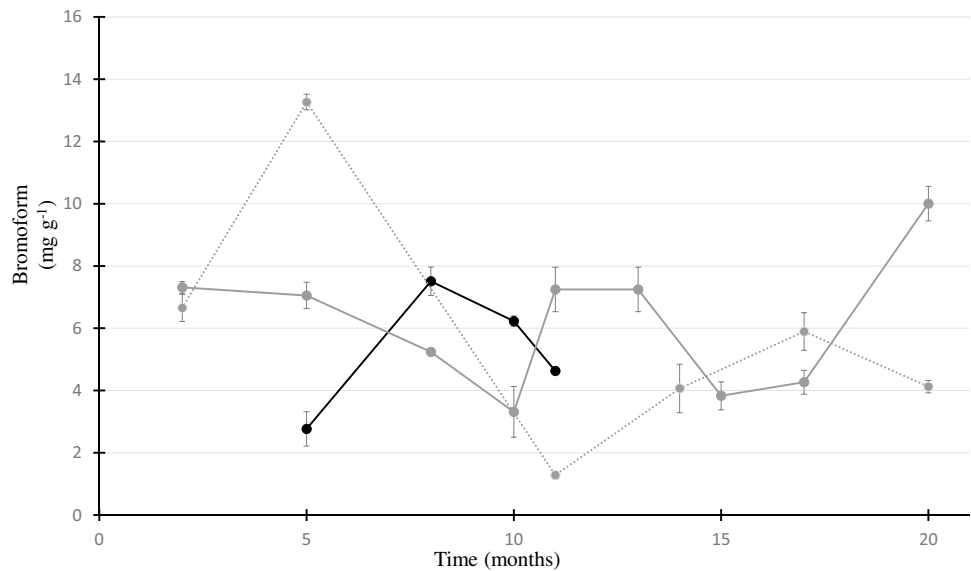


Fig. 6 Bromoform content of samples from Lettermore grown at 13 °C (black) and Finavarra grown at 13 °C (grey, continuous line) and 17 °C (grey, dotted line) during the culturing period. Values are means \pm *sd* (*n*=3)



bromoform levels differed between Finavarra isolates grown at different temperatures, but also depended on the cultivation time (Supp. Table 1). Overall values indicated that cultivation at 13 °C enhanced concentrations, except for a peak recorded at 17 °C by month 5, when 13.27 ± 0.25 mg g⁻¹ DW was recorded, which represented the highest level of bromoform observed across all samples. A peak occurred by the end of the culturing period at 13 °C, which was lower than that recorded at 17 °C. Regarding uniformity, variance among replicates was smaller in Finavarra samples cultivated at 13 °C (below 0.8 throughout the culturing period). Lettermore isolates displayed even less variance, remaining under 0.6 throughout the year (Supp. Fig. 1).

Photosynthetic measurements

In order to portray the physiological status of the best performing isolate (Finavarra at 13 °C) at month 24, the photosynthetic parameters of maximum net photosynthetic rate, photosynthetic efficiency, light compensation and saturation point and dark respiration were determined, with results presented in Table 1.

Discussion

Genetic analysis revealed that Irish isolates of *A. armata* used in this study were derived from Mediterranean populations, due to the shared haplotype composition of the analysed samples. As was the case with *A. taxiformis* lineage 2 (Zanolla et al. 2018) and also the invasive *Codium fragile* (Suringar) Hariot (Provan et al. 2005), the use of genetic haplotypes allowed the reconstruction of a likely route of the European invasion of *A. armata*: from the Australian/

New Zealand region moving eastwards through the Mediterranean Sea until it reached Ireland.

Our study indicates that the tetrasporophytic phase of *A. armata* represents a suitable candidate for long-term, land-based cultivation systems. *A. armata* tetrasporophytes displayed consistent, continuous vegetative propagation, and no tetrasporangia were detected in either isolate at either temperature. This was advantageous since cultures were very stable; a shift in life stages in *A. armata* typically is a response to narrow changes in the combination of several environmental factors (Guiry and Dawes 1992). Additionally, the physiological state of the algae was healthy after 24 months under the given culture conditions, indicating that even longer cultivation periods are possible. Comparing the physiological performance *A. armata* tetrasporophytes from Finavarra to that of isolates from the Mediterranean Sea (Zanolla et al. 2015) at a similar temperature, Irish isolates had a higher NPR_{max} , I_c , I_k and photosynthetic efficiency. This, related to growth conditions, means that less incident light is required to maintain cultures, thus reducing cultivation costs that might be significant given the amount of energy needed to maintain a stable

Table 1 Photosynthetic parameters (NPR_{max} : maximum net photosynthetic rate; α : photosynthetic efficiency; I_c : light compensation; I_k : saturation point; DR: dark respiration) estimated from P- E curves at 13 °C using samples from Finavarra. Results are means \pm *sd* (*n* = 4)

Photosynthetic parameter	Value (mean \pm <i>sd</i>)
NPR_{max} (mg O ₂ h ⁻¹ gFW ⁻¹)	0.32 \pm 0.05
I_c ($\mu\text{mol photons m}^{-2}\text{s}^{-1}$)	5.69 \pm 0.84
α (mgO ₂ g FW h $\mu\text{mol photons m}^{-2}\text{s}^{-1}$)	0.01 \pm 0.002
I_k	33.64 \pm 3.31
DR (mg O ₂ h ⁻¹ gFW ⁻¹)	-0.11 \pm 0.03

controlled temperature in the culturing rooms. According to the growth rate presented by the Finavarra isolates at 13 °C, using an initial biomass of 5 g L⁻¹, 27.5 g L⁻¹ per month of fresh biomass could be harvested monthly, which indicates good potential for upscaling. Additionally, in haploid-diploid seaweeds, the manipulation of clonal propagation can facilitate genetic selection (Valero et al. 2017). Our results indicate that long-term maintenance under constant conditions had no effect on growth and reproductive responses of the species, which are important considerations for commercial cultivation.

Highly relevant when facing distributional logistics from biomass producers to end users is the bromoform content of biomass following processing, storage and transport. As a volatile compound, bromoform measured in samples varies greatly depending on biomass storage conditions and, additionally, the chosen method of detection. Thus, a direct comparison of bromoform levels reported from isolates gathered in Portugal (0.00126 mg g⁻¹ DW, Muizelaar et al. 2021), Australia (16.7 ± 1.0 mg g⁻¹ DW, Magnusson et al. 2020), Tasmania (1.32 mg g⁻¹ DW, Roque et al. 2019) and Italy (23 mg g⁻¹ DW approx. Mata et al. 2011) is difficult. Here, we provide evidence that bromoform levels depend on growth temperature and also on the origin of the isolates, even on small spatial scale (< 100 km)—this suggests that worldwide differences among populations are likely even greater. In fact, similar results have been found for *A. taxiformis* tetrasporophytes, *A. armata* sibling species (Mata et al. 2017). Differences between pre- and post-harvest treatment also seem to be crucial; previous research has shown that production can be optimized by the addition of H₂O₂ 3 h prior to biomass harvesting (Mata et al. 2011), and increased bromoform content also depended on growth temperature, as in our study.

Regarding storage, Stefenoni et al. (2021) could not detect bromoform in freeze-dried and ground biomass after 4 months when stored at 4 °C, which contrasts with results of this study, where the compound was detectable even after storage for almost 4 years (initial samples were collected in August 2017 and were analysed in May 2021). These differences may, at least partially, be explained by the preservation and storage processes and final storage temperature (4 °C vs -18 °C), the different freezing temperatures prior to freeze-drying (-40, -25 or -20 °C vs -18 °C), the stored life stage (gametophytes vs tetrasporophytes) or different analytical methods used for bromoform determination. Biomass produced during the first year of cultivation and stored for 4 years rendered similar bromoform levels to samples collected during the following year, indicating that *Asparagopsis* can be stored over several years, with bromoform retained in the biomass under appropriate storage conditions.

The importance of isolate selection and culture conditions in *Asparagopsis armata*

An important outcome of our experiments was that isolate origin appeared to be critical. Lettermore isolates responded differently to temperature conditions: they grew more slowly and contained lower levels of MAAs and accessory pigments than Finavarra isolates under equivalent conditions. Also, MAA concentrations in Irish isolates following our extraction and detection protocol were higher than in isolates from southern Portugal (Figueroa et al. 2008). Their phycoerythrin content, when cultured at 13 °C, was similar to those of other commercial species, *Pyropia* ssp. (ex *Porphyra*) and *Spirulina* sp. (Osório et al. 2020), but 40 % higher when grown at 17 °C. Regarding phycocyanin, strikingly high levels were found in Finavarra isolates grown at 13 °C (13 times higher than *Pyropia* ssp. and 3 times higher than *Spirulina* sp.; Osório et al. 2020), and somewhat higher levels, in samples grown at 17 °C. Chemical diversity, on a geographical scale, may be due to both genetic differentiation of locally adapted strains, or phenotypically acclimated individuals exposed to combinations of environmental variables (Stengel et al. 2011; Hafting et al. 2015). Several detailed profiling studies have documented the natural chemical diversity within and between populations, further demonstrating the richness of wild raw material derived from different natural populations. For example, genetically different strains of *Porphyra yezoensis* Ueda from different locations may exhibit distinct adaptive features to local environments with regard to phycobiliprotein and chlorophyll contents (Zhang et al. 2012). For the Irish *Asparagopsis* strains, genetic differences were detected among isolates; while Lettermore-derived cultures seemed to be represented by only one haplotype, samples from Finavarra contained at least two, in spite of the amount of original material being the same. Additionally, *Asparagopsis* shows consistently strong phenotypic variation among clones in terms of growth rates (Monro and Poore 2004) and thermal photosynthetic performance among lineages and species (Zanolla et al. 2015). In this present study, we were able to detect such phenotypic variation in both growth and metabolite production, which might be the result of genetic differences among localities. This indicates that there is potential for compound composition to be optimized by modifying culture conditions using pure or mixed isolates. While less variation in shinorine, palythine, bromoform, allophycocyanin and phycocyanin was observed for Lettermore isolates in pure cultures (one haplotype detected), higher overall production values could benefit from the contribution of several genetic variants. Contrary to the case of *Gracilaria chilensis* C.J. Bird, McLachlan and E.C. Oliveira, where artificial and natural population levels are

mostly maintained by thallus fragmentation, and clonal lineages display notable differences in morphology and growth rates when cultured under the same conditions (Santelices and Varela 1993), our cultures showed little, though some statistically significant, variation between harvests.

Long-term compound production in *Asparagopsis armata*: repercussions for industry applications

While the higher temperature treatment enhanced levels of some compounds for our *A. armata* isolates, the drastic reduction in growth rate coupled with the higher variability of the bulk biomass indicates that culture of these isolates is more reliable at 13 °C. This study demonstrates that bromoform, pigments and mycosporine-like amino acids are retained at high levels in cultivation for over 2 years using PES-enriched media at 20 mL L⁻¹, white light at 15 μmol photons m⁻² s⁻¹ and a photoperiod of 12:12 (L:D). However, even though experimental conditions were consistent over time, we found significant changes in quantity and variance of the compounds. No consistent effect of harvest time of day, or individual variation-based culture heterogeneity (given that possibly there is more than one haplotype), could be detected, though >24-h sampling periods might be necessary to uncover cycles of production in MAA that went unnoticed in this study. While the lack of diurnal variation enables more flexible harvesting times, future work is needed to determine how pigment, MAA or bromoform production may be associated with short-term fluctuations in daily growth rates which increase shading and, in turn, pigment production.

Conclusions

1. Irish isolates of *A. armata* represent a potentially valuable source for phycobiliproteins (especially phycocyanin) and MAA and exhibit high bromoform content in long-term indoor cultivation.
2. Industry needs stable product composition and high biomass yields to incorporate macroalgae as a source of bioactive compounds (with multiple high-value applications, other than animal feed); this can be achieved for *A. armata* tetrasporophytes through indoor cultivation.
3. Considering its invasive characteristics, land-based cultivation of *A. armata* is a recommended option in locations where the species is not native, due to the potential negative ecological impacts on native ecosystems.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10811-022-02706-1>.

Acknowledgements The authors appreciate Claudia Cara Ortega's assistance with culture maintenance. Funding from the Marine Institute Ireland ('A National Marine Biodiscovery Laboratory', PBA/MB/16/01) and the AXA Research Fund (2019-AXA-THEME1-021, 'Valorisation potential of invasive seaweed species in Ireland') is gratefully acknowledged.

Author contribution Marianela Zanolla—experimental design, sample and data analysis and manuscript elaboration

Donato Romanazzi—bromoform content analysis, manuscript elaboration

Johan Svenson—funding, bromoform content analysis, manuscript elaboration

Alison Sherwood—genetic delineation of the cultures, manuscript elaboration

Dagmar B. Stengel—funding, provision of analytical and culture facilities, experimental and manuscript design and elaboration

Funding Open Access funding provided by the IREL Consortium. This work was supported by Marine Institute Ireland ('A National Marine Biodiscovery Laboratory', PBA/MB/16/01) and the AXA Research Fund (2019-AXA-THEME1-021, 'Valorisation potential of invasive seaweed species in Ireland').

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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