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Environmental effects on growth and fatty acids in three isolates of *Derbesia tenuissima* (Bryopsidales, Chlorophyta)

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ABSTRACT

We quantified the effects of temperature, light and nitrogen availability on the growth and fatty acids (FAs) in three isolates of the green seaweed *Derbesia tenuissima* to portion the environmental and the genotypic (between isolates) components of variation. Growth ranged from 13 to 33% day^{-1} and 27% of the variation was between isolates and 48% of variation was explained by light intensity. The content of total FA (TFA) ranged from 34 to 55 mg g^{-1} dw and 49% of the variation was between isolates, while the TFA was 20% lower in the high light and low nitrogen treatment combination. The proportion of omega-3 polyunsaturated FA (PUFA(n-3)) ranged from 31 to 46% of TFA with a strong interactive effect of isolate and temperature. In two isolates, the proportion of PUFA(n-3) increased by 20% under cultivation at low temperature while in a third isolate temperature had no effect. Increases in PUFA(n-3) occurred with a stable content of TFA and high growth rates, leading to net increases in PUFA(n-3) productivity in two isolates. This research highlights the potential for environmental manipulation and strain selection to further improve the productivity and quality of fatty acids in seaweed.

1. Introduction

Derbesia tenuissima (Bryopsidales, Chlorophyta) is a species of seaweed recently identified as a biomass resource rich in polyunsaturated omega-3 fatty acids (PUFA(n-3)) with applications in functional foods and nutraceuticals. *D. tenuissima* is a species rich in lipids (>12% dry weight (dw)) with a content of total fatty acids (TFAs) greater than 5% dw of which 40% are in the form of valuable PUFA(n-3) [1,2]. It also has a high biomass (15–25 g dw m^{-2} day^{-1}), and consequently, fatty acid productivity (0.8–1.4 g dw m^{-2} day^{-1}) [2,3]. Furthermore, the productivity of biomass and fatty acids in *D. tenuissima* is stable over time and consistency in the production of biomass and fatty acids is key criteria for the successful commercialization of new species for the nutraceutical market [4,5]. Although the general suitability for cultivation and biomass applications has been established in *D. tenuissima*, biomass and fatty acid productivities are likely not yet fully exploited and may be improved through refined culture strategies of environmental manipulation and the selection of strains with advantageous attributes. The basis for these improvements is the identification and quantification of natural variability in growth and the content or composition of fatty acids, and subsequent quantification of the drivers thereof.

The environmental parameters of temperature, light and nutrients are key factors for growth in large, foliose seaweeds [6] and it is predicted that they are also important drivers for growth and the content and composition of fatty acids in filamentous, clonal seaweeds such as *Derbesia*. While there is extensive literature on environmental effects on the content and composition of fatty acids in microalgae [7,8], research on seaweeds is largely restricted to field studies based on broad environmental correlations with fatty acids [9–12] and only a few studies in a limited number of species have experimentally quantified the effects of temperature [13,14] and light [15,16] on fatty acids. Additionally, while the effect of nitrogen starvation can lead to substantial increases in content and composition of PUFA(n-3) in microalgae [17], the quantification of the effects of nitrogen availability on the content and composition of fatty acids in seaweeds is restricted to a few species of *Ulva* [18–20] and *Gracilaria* [21]. Importantly, the direction and degree of these effects for microalgae and macroalgae are species specific [19,21,22] and environmental conditions often have opposing effects on growth and the content and composition of fatty acids [19,22], and so may result in highly specific net changes in fatty acid productivity.

Furthermore, while the content and composition of fatty acids vary considerably between the broad taxonomic groups of red, green and brown seaweeds and at the taxonomic level of families, orders and within a genus [1,23], there is no evidence for genotypic variation of the content and composition of fatty acids within species of seaweed [24]. While strain selection in species and subsequent crop improvements are common in terrestrial plants [25,26], and more recently in microalgae with substantial improvements in growth and the quantity

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and quality of fatty acids [17,22,27], this process is still in its infancy in seaweeds [24]. Strain selection and subsequent improvements in growth, temperature tolerance and the yield of iodine in the Chinese kelp industries have progressed since the 1960s [28], and there is strong support for a genetic component of basic morphological features [29] and growth rates [30] in seaweeds. A limited number of studies have also confirmed the genotypic variation and heritability of the natural products furanones [31] and phlorotannins [32] and the selective breeding of the brown seaweed *Macrocystis* has resulted in increases in total lipids and protein compared to natural populations [33]. Importantly however, genotypic variation in the content and composition of fatty acids, in particular the nutritionally important PUFA(n-3), within seaweeds has not been demonstrated [24]. Although field based studies have quantified variability in the content and composition of fatty acids between individual conspecific individuals from within and between spatially isolated populations of seaweeds [1,10], the distinctions between the effects of local environmental conditions and a genetic component remain speculative.

The quantification and portioning of genetic and environmental components in the variability of growth and the content and composition of fatty acids are the first step to fully exploit the potential of *D. tenuissima* and other target seaweeds. Therefore, we quantified the effects of temperature, light and nitrogen availability as the key environmental factors on the growth and content and composition of fatty acids in three isolates of *D. tenuissima* in controlled growth trials.

2. Materials and methods

2.1. Study organism and biomass collection

D. tenuissima is a filamentous marine macroalga found in temperate to tropical waters including the North Atlantic, Mediterranean and South Pacific [34–36]. It was selected as an oil rich species (>12% dw) with a high content of TFA and a high proportion of PUFA(n-3) [1]. Furthermore, it has high biomass productivities in culture which makes it an interesting species for the production of oil based bioproducts [2,3]. Biomass isolates of this species were collected from an intertidal flat at Rowes Bay, Townsville, Australia (19.14°S, 146.48°E) (isolate 1) and a local pet shop (Pet HQ) in Townsville where it was found as fouling organism in a fish tank (isolate 2). A third biomass sample was collected from the Marine & Aquaculture Research Facility (MARFU) at James Cook University, Townsville where it was identified as a fouling species in cultures of other seaweeds (isolate 3). Biomass of the three isolates was transported to James Cook University, Townsville where cultures were established and scaled up and kept separate as stock cultures under controlled conditions (12:12 h, light:dark cycle, 24 °C) in aerated 2 L culture vessels with weekly changes of autoclaved seawater enriched with F/2 medium (Algaboost F/2 (1000×) silicate free, AusAqua) [37] (~12 mg N L^{-1}).

2.2. Species identification

Strains were identified by comparing the morphology with taxonomic keys [34] and literature [35,36], and by DNA barcoding. This approach compares short DNA sequences from a standardised region of the genome – the 'barcode' – to a library of reference sequences derived from individuals of known identity [38]. Genomic DNA was isolated from fresh tissue samples of each isolate using a Qiagen DNEasy Plant Mini Kit following the manufacturer's instructions and amplified at the DNA barcoding marker rbcL3′ using the primers GrbcLFi [39] and 1385R [40]. Polymerase chain reaction (PCR) amplifications were performed in a 25 μL reaction mixture containing 1.5 U of MyTaq HS DNA polymerase (Bioline), 5× MyTaq reaction buffer, 0.4 μm each primer, and 1 μL of genomic DNA (25–30 ng). Amplifications were performed on a BioRad C1000 Thermal Cycler with a touchdown PCR cycling profile (cycling parameters: 5 min at 94 °C, 30 cycles of 30 s denaturing at 95 °C, 45 s annealing at 56 °C with the annealing temperature decreasing by 0.5 °C each cycle, 60 s extension at 72 °C, and a final extension at 72 °C for 5 min). PCR products were column purified using Sephadex G-25 resin and sequenced in both directions by the Australian Genome Research Facility (Brisbane, Australia). If sequences were unreadable or contaminated a second PCR attempt was made and sequenced. If these sequences were also unreadable or contaminated, then DNA was re-extracted from a fresh sample and further PCRs and sequencing attempts were made. All attempts to generate readable sequences were unsuccessful for isolate 3. Attempts were also made to amplify and sequence all isolates at the alternative barcoding marker *tufA* using a range of primers and PCR conditions, however we were unable to obtain readable sequences for any isolate at this marker.

Sequences were edited using Bioedit [41] and submitted to GenBank under the accession numbers KM998970 for isolate 1 and KM998971 for isolate 2. Sequence similarity searches using a nucleotide BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/blast/) failed to find an exact match. Therefore we identified our isolates by constructing phylogenetic trees using sequences downloaded from GenBank. All publically available *Derbesia*, *Bryopsis* and *Pedobesia* rbcL sequences were downloaded. Duplicate sequences were removed from each dataset and then all remaining sequences were aligned with ours and trimmed to a standard length in MEGA 5.0 [42]. Maximum likelihood (ML) phylogenetic trees were constructed in MEGA using a *Caulerpa filiformis* sequence (GenBank accession: AY004763) as an outgroup. We used the simple Kimura two-parameter model to estimate genetic distance [43] as this is the standard model of molecular evolution used in barcoding studies [38]. The reliability of tree topologies was estimated using bootstrapping (9999 replicates).

2.3. Experimental design

We tested the effects (high vs. low) of water temperature (high: 29 °C, low: 21 °C), nitrogen concentration of the culture medium (high: 12 mg N L^{-1}, low: 3 mg N L^{-1}) and light intensity (high: 100 μmol photons m^{-2} s^{-1}, low: 24 μmol photons m^{-2} s^{-1}) on the specific growth rate (SGR % day^{-1}), internal concentrations of nitrogen and carbon, and the content and composition of fatty acids in three isolates of *D. tenuissima*. As the concentration of phosphorous (P) was kept constant (N:P ratio of 5:1 in initial F/2 media), the N:P ratio was accordingly increased at the high nitrogen treatments. The selected water temperatures represent typical summer and winter seawater conditions at Rowes Bay (Australian Institute of Marine Science) and were controlled in culture chambers (New Brunswick Biological Shakers Innova 44/44R, Eppendorf). The tested nitrogen concentrations were controlled by adding F/8 (3 mg N L^{-1}) to both treatments and an additional 9 mg N L^{-1} in the form of sodium nitrate ($NaNO_3$) to the 'high nitrogen' treatment culture medium. These nitrogen concentrations provided 0.96 mg nitrogen per 80 mL jar in the 'high nitrogen' treatment cultures and therefore provided an excess of nitrogen at an estimated harvest biomass of 25 mg dw^{-1} and an estimated internal nitrogen concentration of 3% of dw biomass, while the 'low nitrogen' treatment cultures had only 0.24 mg N per jar and therefore nitrogen was clearly limiting at the same estimated biomass growth and internal biomass nitrogen concentration. The culture chambers were equipped with photosynthetic lamps (New Brunswick photosynthetic growth lamp, Eppendorf) and culture light intensity was controlled by using clear 200 mL culture jars with semi-transparent lids for the 'high light' treatments (100 μmol photons m^{-2} s^{-1}) and paper wrapped culture jars with light-blocking lids for the 'low light' treatments (24 μmol photons m^{-2} s^{-1}). The effects of temperature, light, nitrogen and isolate were tested with a factorial design where each culture treatment in each growth trial was duplicated to account for methodical variability and also to obtain enough biomass for subsequent biochemical analyses. A sample was then considered

the average of the duplicate treatments and was replicated 3 times in successive and identical growth trials.

2.4. Acclimation and growth trials

Biomass was acclimated in culture chambers in 200 mL plastic jars filled with 80 mL autoclaved seawater and added F/4 medium (~6 mg N L^{-1}) seven days prior to a growth trial. A total of 10 jars for each isolate were stocked with approximately 250 mg fresh weight (fw) biomass per jar at conditions that were similar to the experimental growth trials with a 12:12 h light:dark photoperiod and activated shaking function of the culture chamber (100 rpm) to provide gas exchange (water movement) and prevent biomass from attaching to the culture jars. However, water temperature (24 °C) and nitrogen concentration (F/4–6 mg N L^{-1}) were set intermediate of the specified treatment conditions of the growth trials to account for possible stress responses and therefore bias toward either of the tested treatment conditions. After the acclimation period, the biomass of each isolate was harvested separately and spin-dried for 3 min and considered as 'fresh weight' (fw) biomass. Three random 150 mg fw biomass samples from the fresh weight biomass of each isolate were directly frozen to −80 °C and freeze dried to calculate the average fresh weight to dry weight ratio (fw:dw) of the biomass in each growth trial. For each growth trial, 200 mL plastic jars filled with 80 mL autoclaved seawater and treatment specific nitrogen addition were inoculated with 20 mg ± 1 mg fresh weight biomass and randomly placed in the incubator chambers. Positions of jars were randomly changed every day. After a 7 day culture period, samples were harvested, and stored at −80 °C. The growth trial was replicated 3 times with new and acclimated biomass from the stock cultures.

2.5. Carbon, nitrogen and fatty acid analysis

Frozen samples were freeze-dried and ground to a fine powder of which 10 mg were sent to OEAlabs (www.oealabs.com) for analysis of internal carbon and nitrogen content while another 20 mg was used for the analysis of fatty acids. A direct transesterification method was used to simultaneously extract and esterify the fatty acids to fatty acid methyl esters (FAMEs) for analysis by gas chromatography mass spectrometry (GC–MS; 7890A GC, 5975C MS, DB-23 capillary column with 15 μm cyanopropyl stationary phase, 60 m length and 0.25 mm inner diameter (Agilent Technologies Australia Pty Ltd.)), as described in detail in Gosch et al. [1]. The content of TFA was determined as the sum of all FAMEs with fatty acids being designated as CX:Y(n-z), where X is the total number of carbon, Y is the number of double bonds, and z is the position of the ultimate double bond from the terminal methyl group.

2.6. Growth rates

Growth of biomass was determined as specific growth rate (SGR) with the following equation SGR (% day^{-1}) = 100 × [ln(W_f / W_i)] / t; with W_f and W_i being the final and initial dry weight biomass of the samples and t the culture period (7 days). Although the initial stocking biomass was measured out as fresh weight biomass, it was converted to dry weight on the basis of the fresh weight to dry weight conversion factor estimated for each growth trial and isolate.

2.7. Statistical analysis

Four-way factorial ANOVA and Tukey's honest significant difference (HSD) post-hoc tests (IBM SPSS Statistics version 21) were used to test for the effects of temperature, light, nitrogen and isolate (all fixed factors) on the SGR, C:N ratio, TFA and fatty acid composition (saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), PUFA(n-3) and PUFA(n-6)) in *D. tenuissima* with 3 replicate samples for each treatment combination. Eta-squared (%) $\eta^2 = SS_{factor} / SS_{total} \times 100$; with SS_{factor} and SS_{total} being the sum of squares of a particular factor and the total sum of squares respectively was calculated as a measure of effect size describing the proportion of variation (%) of the total variation of the independent variable explained by a particular factor or factor interaction [44]. The relationships between the SGR and the C:N ratio with the content of TFA and fatty acid composition (SFA, MUFA, PUFA(n-3), PUFA(n-6)) were analysed by correlations (Correlation, IBM SPSS version 21). The variation in average composition of fatty acids (% of TFA) between the three isolates (iso1, iso2, iso3) and the different culture treatment conditions of temperature, nitrogen and light was analysed using non-metric multidimensional scaling (MDS, Primer 6). Groups of samples forming distinct groups based on their composition of fatty acids were visualized as shaded circles. A vector loading bi-plot (Pearson's product correlations) was used to visualize the relative load of individual fatty acids for the samples, with the lengths and directions of the vectors representing the strength and direction of correlations.

3. Results

3.1. Species and isolate identification

The sequences of isolates 1 and 2 formed a distinct, well supported (99% bootstrap support) clade that did not contain any other GenBank samples (Fig. A.1). These sequences fell within a larger, well supported (86% bootstrap support) clade that was distinct from all other sections of the phylogenetic tree and contained *Derbesia marina* and a sequence identified as *Derbesia* sp. 1GWS. These results demonstrated that isolates 1 and 2 are from the genus *Derbesia* and not *Pedobesia*, and more specifically are not the species *D. marina* (Fig. A.1). There were no *D. tenuissima* sequences available in GenBank for the region of the rbcL gene that we sequenced. However, our samples matched morphological descriptions of *D. tenuissima* [34–36] (Fig. A.2, Table A.1) including the general characteristics of cylindrical siphonous filaments without septa, infrequent branching, and elongated chloroplast (5–10 μm) with a single pyrenoid (Fig. A.2, Table A.1). We were not able to obtain readable sequences for isolate 3 and there were differences in filament diameter with isolates 1 and 2 (14–20 μm) having smaller filament diameter than isolate 3 (20–30 μm) (Fig. A.2, Table A.1). However, all remaining morphological characteristics of isolate 3 were the same as isolates 1 and 2, therefore we have also identified it as *D. tenuissima* (Fig. A.2, Table A.1).

3.2. Specific growth rate

The SGR ranged from 12.8 to 33.4% day^{-1} across all treatments and isolates, and 27% of the total variation in SGR can be explained by the type of isolate with isolate 1 and isolate 2 having similar SGR (~27% day^{-1}) while the SGR of isolate 3 was significantly lower at 21% day^{-1} (ANOVA: *Isolate* (η^2 = 27.1%), Tukey's HSD: $p < 0.001$, Table 1, Fig. 1). The most important factor, however, that explained nearly 50% (η^2 = 47.9%) of the variation in SGR was light intensity. Low light intensity significantly reduced the SGR between 27 and 50% in all three isolates (ANOVA: *Light* (η^2 = 47.9%), Table 1, Fig. 1). There was a significant interaction effect between temperature and light intensity (ANOVA: *Temperature* × *Light* (η^2 = 4.0%), Table 1), however, this interaction was relatively weak and the SGR was only slightly higher at the treatment combination of high light intensity and high temperature compared to the treatment combination of high light intensity and low temperature. There was no such temperature effect on the SGR at low light intensity (Table 1, Fig. 1). The addition of nitrogen had no significant effect on growth in any of the three isolates (Table 1, Fig. 1).

3.3. Carbon to nitrogen ratio

The C:N ratio ranged from 6.3:1 to 13.1:1 across all treatments and isolates and the most important factors explaining the variation were

Table 1
Output of 4-way factorial ANOVA testing the effects of temperature (T) (29 °C, 21 °C), light intensity (L) (100 μmol photons m^{-2} s^{-1}, 24 μmol photons m^{-2} s^{-1}) and nitrogen concentrations (12 mg N L^{-2}, 3 mg N L^{-1}) in three isolates of *Derbesia tenuissima* (I) (isolate 1, isolate 2, isolate 3) on the specific growth rate (SGR, % day^{-1}), carbon to nitrogen ratio (C:N ratio), total fatty acids (TFA, mg g^{-1} dw), saturated fatty acids (SFA, % of TFA), monounsaturated fatty acids (MUFA, % of TFA), polyunsaturated fatty acids (PUFA, % of TFA), PUFA(n-3) (% of TFA) and PUFA(n-6) (% of TFA). Reported is the F-statistic (F), the significance level at 0.05 (p) with significant effects in bold and the eta-squared value (η^2, % of variance (Var)) as a measure of effect size.

		SGR			C:N ratio			TFA			SFA			MUFA			PUFA(n-3)			PUFA(n-6)		
Source of variation	df	F	p	η^2	F	p	η^2	F	p	η^2	F	p	η^2	F	p	η^2	F	p	η^2	F	p	η^2
T	1	3.239	0.078	1.0	3.129	0.083	0.5	0.872	0.355	0.2	89.046	**<0.001**	34.1	23.903	**<0.001**	22.8	117.998	**<0.001**	42.1	643.502	**<0.001**	57.8
L	1	154.596	**<0.001**	47.9	151.175	**<0.001**	22.7	86.096	**<0.001**	18.2	71.311	**<0.001**	27.3	2.767	0.103	2.6	33.757	**<0.001**	12.0	24.559	**<0.001**	2.2
N	1	0.434	0.513	0.1	167.120	**<0.001**	25.1	47.332	**<0.001**	10.0	14.119	**<0.001**	5.4	1.827	0.183	1.7	12.340	**0.001**	4.4	0.989	0.325	0.1
I	2	43.787	**<0.001**	27.1	20.979	**<0.001**	6.3	115.253	**<0.001**	48.7	0.419	0.660	0.3	8.400	**0.001**	16.0	2.842	0.068	2.0	163.534	**<0.001**	29.4
T × L	1	12.995	**<0.001**	4.0	4.454	**0.040**	0.7	0.345	0.560	0.1	3.598	0.064	1.4	4.156	**0.047**	4.0	7.589	**0.008**	2.7	2.136	0.150	0.2
T × N	1	0.103	0.750	0.0	0.189	0.665	0.0	0.239	0.627	0.1	0.955	0.333	0.4	1.312	0.258	1.3	0.090	0.765	0.0	0.074	0.786	0.0
T × I	2	0.956	0.391	0.6	2.927	0.063	0.9	0.391	0.678	0.2	11.411	**<0.001**	8.8	0.505	0.607	1.0	20.801	**<0.001**	14.8	21.836	**<0.001**	3.9
L × N	1	0.892	0.350	0.3	106.608	**<0.001**	16.0	19.712	**<0.001**	4.2	1.749	0.192	0.7	0.323	0.573	0.3	4.703	**0.035**	1.7	2.707	0.106	0.2
L × I	2	2.219	0.120	1.4	13.074	**<0.001**	3.9	8.879	**0.001**	3.8	0.108	0.898	0.1	0.267	0.767	0.5	0.047	0.954	0.0	2.646	0.081	0.5
N × I	2	0.225	0.799	0.1	39.451	**<0.001**	11.8	5.732	**0.006**	2.4	0.289	0.750	0.2	0.074	0.928	0.1	0.952	0.393	0.7	2.632	0.082	0.5
T × L × N	1	3.016	0.089	0.9	6.894	**0.012**	1.0	2.389	0.129	0.5	0.560	0.458	0.2	0.174	0.679	0.2	0.332	0.567	0.1	0.470	0.496	0.0
T × L × I	2	1.535	0.226	1.0	0.628	0.538	0.2	0.846	0.436	0.4	0.668	0.518	0.5	0.797	0.456	1.5	1.693	0.195	1.2	0.682	0.510	0.1
T × N × I	2	0.123	0.885	0.1	0.613	0.546	0.2	0.658	0.523	0.3	1.979	0.149	1.5	0.322	0.726	0.6	0.067	0.935	0.0	2.509	0.092	0.5
L × N × I	2	0.157	0.855	0.1	11.172	**<0.001**	3.4	2.245	0.117	0.9	0.758	0.474	0.6	0.280	0.757	0.5	0.976	0.384	0.7	1.019	0.369	0.2
T × L × N × I	2	0.668	0.517	0.4	0.780	0.464	0.2	0.100	0.905	0.0	0.089	0.915	0.1	0.475	0.625	0.9	0.333	0.718	0.2	0.235	0.792	0.0
Residuals	48			14.9			7.2			10.1			18.4			45.8			17.1			4.3

SGR specific growth rate; C:N ratio carbon to nitrogen ratio; TFA total fatty acids; SFA saturated fatty acids; MUFA monounsaturated fatty acids; and PUFA polyunsaturated fatty acids.

nitrogen concentration ($\eta^2 = 25.1\%$, Table 1) and light ($\eta^2 = 22.7\%$, Table 1) with a strong interaction between the two factors (ANOVA: *Light* × *Nitrogen* ($\eta^2 = 16.0\%$), Table 1, Fig. 2) that also differed significantly between isolates (ANOVA: *Light* × *Nitrogen* × *Isolate* ($\eta^2 = 3.4\%$), Table 1). In isolate 1 and isolate 2, there was a significant and strong interaction between light and nitrogen with a higher C:N ratio (11.1:1–13.1:1) at the low nitrogen treatment when grown at high light conditions while at low light conditions, this nitrogen effect was not evident (Fig. 2). This interaction between light and nitrogen on the C:N ratio did not occur in isolate 3 where the C:N ratio was relatively uniform across treatments with a range from 6.6:1 to 8.3:1 (Fig. 2).

3.4. Total fatty acid content

The content of TFA ranged from 34.8 to 54.1 mg g^{-1} dw across all treatments and isolates and the most important factor explaining the variability in the content of TFA was isolate ($\eta^2 = 48.7\%$, Table 1) with isolate 1 (47.2 mg g^{-1} dw) and isolate 2 (48.3 mg g^{-1} dw) having similar contents of TFA, while isolate 3 had a significantly lower content of TFA (38.2 mg g^{-1} dw) (ANOVA: *Isolate*, Tukey's HSD: $p < 0.001$, Table 1, Fig. 3). The parameter with the second strongest effect was light intensity (ANOVA: *Light* ($\eta^2 = 18.2\%$), Table 1) followed by nitrogen (ANOVA: *Nitrogen*, $\eta^2 = 10.0\%$, Table 1, Fig. 3) with a weak interaction between the two factors (ANOVA: *Light* × *Nitrogen* ($\eta^2 = 4.2\%$), Table 1). The content of TFA was reduced by up to 20% at the treatment combination of high light and low nitrogen while at low light there was no reduction in the content of TFA irrespective of the nitrogen concentration (Table 1, Fig. 3). Further, the effects of light and nitrogen were only present in isolate 1 and isolate 2 while isolate 3 had a relatively uniform content of TFA (ANOVA: *Light* × *Isolate* ($\eta^2 = 3.8\%$); ANOVA: *Nitrogen* × *Isolate* ($\eta^2 = 2.4\%$); Table 1, Fig. 3). Temperature had no significant effect on the content of TFA in all isolates and at all treatment combinations (Table 1, Fig. 3). The content of TFA was not correlated with the SGR ($r = 0.071$, $p = 0.554$, $n = 72$; Fig. 5a) but was negatively correlated with the C:N ratio ($r = -0.401$, $p < 0.001$, $n = 72$; Fig. 5b).

3.5. Fatty acid composition

The average proportion of SFA was 32.2% of TFA ± 0.6 SE across all treatments and isolates, and ranged from 27.7 to 37.9% of TFA. The most abundant SFA was C16:0 which ranged from 21.5 to 29.3% of TFA. Although temperature alone explained 34.1% (η^2, Table 1) of the variation in SFA, the effect of temperature was largely restricted to isolate 1 and isolate 2 where the proportion of SFA was approximately 15% lower in the low temperature treatments, while in isolate 3, temperature had no significant effect on the proportion of SFA (ANOVA: *Temperature* × *Isolate* ($\eta^2 = 8.8\%$), Table 1, Fig. 4). The second largest effect on the proportion of SFA was light in all three isolates and, irrespective of the temperature, the proportion of SFA was significantly higher (~10%) at the high light treatments (ANOVA: *Light* ($\eta^2 = 27.3\%$), Table 1, Fig. 4). In addition there was a small, but significant, positive effect of nitrogen on the proportion of SFA (ANOVA: *Nitrogen* ($\eta^2 = 5.4\%$), Table 1, Fig. 4). The proportion of SFA was weakly correlated with the SGR ($r = 0.332$, $p = 0.004$, $n = 72$; Fig. 5c) and more strongly correlated with the C:N ratio ($r = 0.517$, $p < 0.001$, $n = 72$; Fig. 5d).

The proportion of MUFA was 11.0% of TFA ± 0.3 SE across all treatments and isolates and ranged from 8.6 to 14.1% of TFA. The most abundant MUFA was C18:1(n-9) (3.6%–7.2% of TFA). Overall, temperature had a strong effect (ANOVA: *Temperature* ($\eta^2 = 22.8\%$), Table 1, Fig. 4) on the variability of the proportion of MUFA and the increases in the proportion of MUFA at the low temperature treatments were larger at high light (23%) compared to low light (10%) (ANOVA: *Temperature* × *Light* ($\eta^2 = 4.0\%$), Table 1, Fig. 4). The second most important source of variation was the type of isolate (ANOVA: *Isolate* ($\eta^2 = 16.0\%$), Table 1) with isolate 1 (11.3% of TFA) and isolate 3 (11.7% of TFA) having similar proportions of MUFA while the proportion of MUFA in isolate 2 (10.0% of TFA)

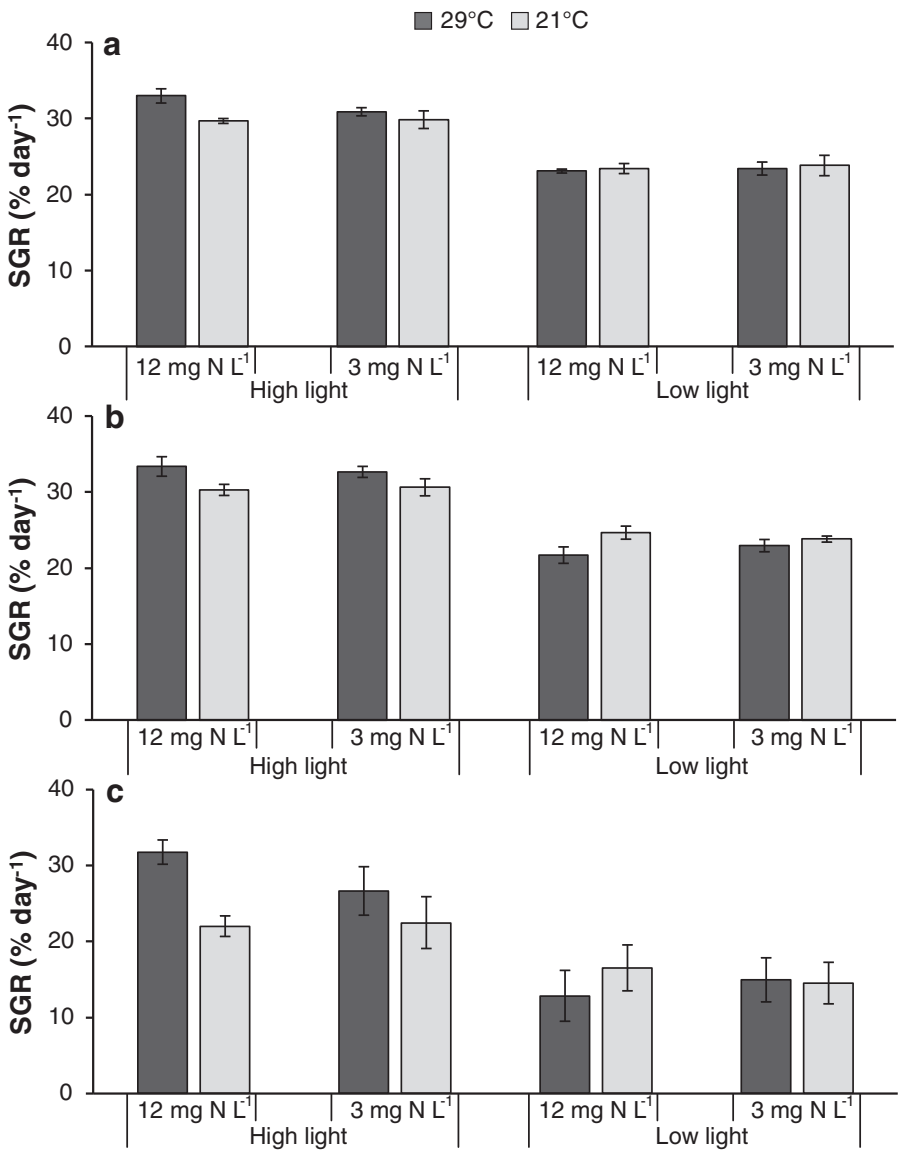

Fig. 1. Mean specific growth rate (SGR) (% day^{-1} ± SE, n = 3) in three isolates of *Derbesia tenuissima* (**a** isolate 1, **b** isolate 2, **c** isolate 3) grown at different temperatures (29 °C, 21 °C), different nitrogen concentrations (12 mg N L^{-1}, 3 mg N L^{-1}) and different light conditions (100 μmol photons m^{-2} s^{-1}, 24 μmol photons m^{-2} s^{-1}).

was significantly lower. The proportion of MUFA was only weakly correlated with the SGR ($r = -0.237$, $p = 0.045$, $n = 72$) and there was no correlation with the C:N ratio ($r = 0.071$, $p = 0.556$, $n = 72$).

The proportion of PUFA(n-3) was high with 40.0% of TFA ± 0.9 SE across all treatments and isolates and ranged from 31.3% to 46.3% of TFA. The most abundant PUFA(n-3) was C18:3(n-3) (17.3–25.6% of TFA) followed by C16:3(n-3) (9.9%–16.1% of TFA). Although temperature had a strong overall effect ($\eta^2 = 42.1\%$) on the variability in the proportion of PUFA(n-3), this effect was restricted to isolate 1 and isolate 2 where the proportion of PUFA(n-3) was over 20% higher at the low temperature treatments, while in isolate 3 temperature had no significant effect on the proportion of PUFA(n-3) (ANOVA: *Temperature* × *Isolate* ($\eta^2 = 14.8\%$), Table 1, Fig. 4). This effect of temperature on the most abundant individual PUFA(n-3) (C16:3(n-3), C18:3(n-3)) was also restricted to isolate 1 and isolate 2 while the proportion of C20:5(n-3) increased in all three isolates at low temperature as demonstrated by MDS (Fig. 6). There was also a significant but weak interaction between the effects of temperature and light (ANOVA: *Temperature* × *Light* ($\eta^2 = 2.7\%$), Table 1) and the lowest proportion of PUFA(n-3) was found for the high temperature and high light treatment combination (Fig. 4). The proportion of PUFA(n-3) was only weakly negatively correlated with the SGR ($r = -0.273$, $p = 0.020$, $n = 72$; Fig. 5e) but there was a stronger negative correlation with the C:N ratio ($r = -0.488$, $p < 0.001$, $n = 72$; Fig. 5f).

The average proportion of PUFA(n-6) was 15.3% of TFA ± 0.6 SE across all treatments and isolates, and ranged from 11.2% to 20.1% of TFA. Similar to PUFA(n-3), the overall variability in the proportion of PUFA(n-6) was mainly affected by the culture temperature ($\eta^2 = 57.8\%$, Table 1), however, in an opposing direction with a higher proportion of PUFA(n-6) at the high temperature treatments. The variability in the proportion of PUFA(n-6) was also affected by the type of isolates ($\eta^2 = 29.4\%$) with isolate 2 (17.0% of TFA) having the highest proportion of PUFA(n-6) followed by isolate 1 (15.7% of TFA) and isolate 3 (13.2% of TFA). Furthermore, the effect of temperature on the proportion of PUFA(n-6) was the strongest in isolates 1 and 2 with a 32–33% difference between the temperature treatments, while in isolate 3 there was only a 23% difference in the proportion of PUFA(n-6) (ANOVA: *Temperature* × *Isolate* ($\eta^2 = 3.9\%$), Table 1). This impact of temperature in the isolates was also evident on individual PUFA(n-6) as demonstrated by MDS (Fig. 6). The proportion of PUFA(n-6) was only weakly correlated with the SGR ($r = 0.240$, $p = 0.042$, $n = 72$) and there was no correlation with the C:N ratio ($r = 0.150$, $p = 0.207$, $n = 72$).

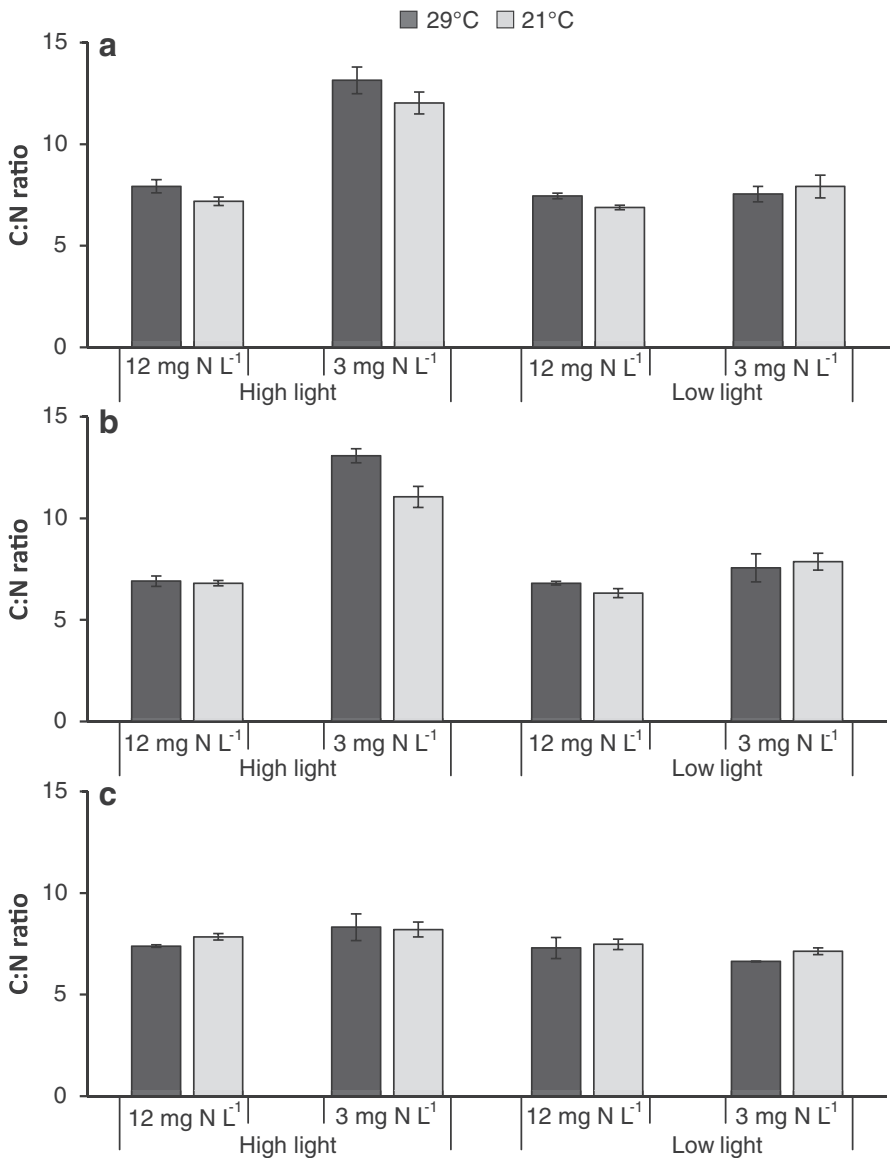

Fig. 2. Mean of the carbon to nitrogen ratio (C:N ratio) (ratio ± SE, n = 3) in three isolates of *Derbesia tenuissima* (**a** isolate 1, **b** isolate 2, **c** isolate 3) grown at different temperatures (29 °C, 21 °C), different nitrogen concentrations (12 mg N L^{-1}, 3 mg N L^{-1}) and different light conditions (100 μmol photons m^{-2} s^{-1}, 24 μmol photons m^{-2} s^{-1}).

4. Discussion

This study confirms the suitability of the green seaweed *D. tenuissima* as a biomass feedstock for the production of functional foods and nutraceuticals because of its high growth rate and high proportion of PUFA(n-3). While its cultivation has been demonstrated at a large scale, the full growth potential has not been exploited. Here we demonstrate that the growth and the proportion of PUFA(n-3) can be further increased through environmental manipulations and also careful strain selection. The growth and the proportion of PUFA(n-3) were different between isolates and in two isolates substantial improvements in the proportion of PUFA(n-3) were achieved at low water temperature treatments. Importantly, these increases in the proportion of PUFA(n-3) were achieved in the presence of high contents of TFA and high growth rates leading to net increases in the productivity of PUFA(n-3).

4.1. Species identification and morphology

Although analysis of DNA sequences was inconclusive, the morphological features of the filaments, including the size and shape of the chloroplasts and the presence of a single pyrenoid in each chloroplast, strongly suggest that all three isolates are *D. tenuissima* [34,35]. However, unlike in the literature, where filament diameter commonly ranges from 25 to 80 μm [34,35], the filaments in our study measured only between 14 μm (isolate 1, isolate 2) and 30 μm (isolate 3) and are therefore the smallest filament sizes reported for *D. tenuissima*. Many of the larger filament sizes previously reported are field-collected samples from the North Atlantic and Southern Australia with filament diameter of up to 80 μm [34] while Mediterranean isolates had smaller filament diameter (30–50 μm) [35] that were even smaller in culture (30 μm) [36]. Variation in morphology within species has been reported for many seaweeds including *Asparagopsis taxiformis* [45], *Caulerpa taxifolia* [46] and *Dictyota dichotoma* [47] and was attributed to both environmental plasticity and differences between genotypes. Therefore, we propose that the observed variation in the diameter of filaments in *D. tenuissima* reported in our study and the disparity with the previously reported diameter of filaments are within the natural variation of this species. Further, it is likely that the variation in the diameter of filaments between the isolates from our study is a genotypic characteristic rather than a phenotypic expression as the filament size differences between the isolates persisted under identical culture conditions over several months.

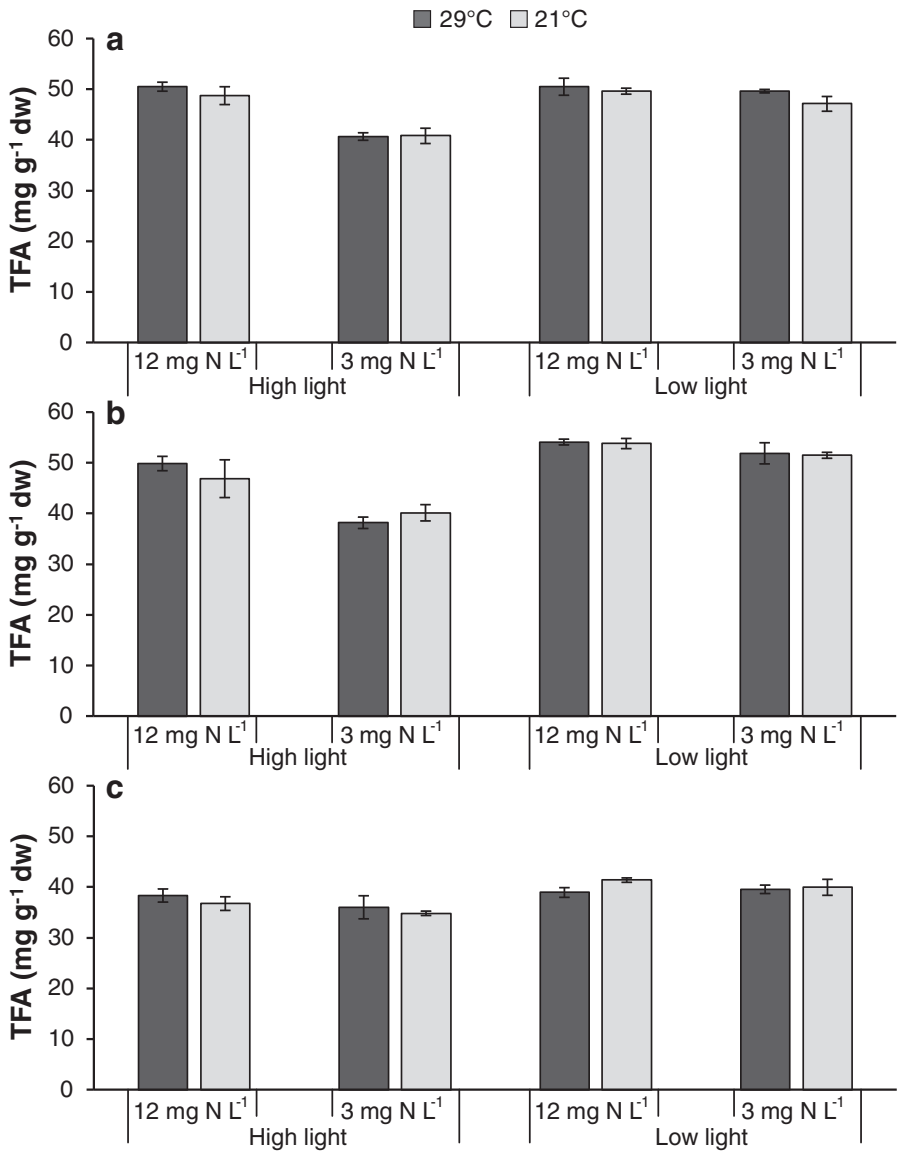

Fig. 3. Mean total fatty acid content (TFA) (mg g^{-1} dw ± SE, n = 3) in three isolates of *Derbesia tenuissima* (**a** isolate 1, **b** isolate 2, **c** isolate 3) grown at different temperatures (29 °C, 21 °C), different nitrogen concentrations (12 mg N L^{-1}, 3 mg N L^{-1}) and different light conditions (100 μmol photons m^{-2} s^{-1}, 24 μmol photons m^{-2} s^{-1}).

4.2. Growth and internal nitrogen

Overall, the SGR (referred to as growth hereafter) of *D. tenuissima* ranged from 13 to 33% day^{-1} and approximately 27% of that variation was due to differences in growth between isolates. While isolate 1 and isolate 2 had similar average growth rates of approximately 27% day^{-1}, isolate 3 only had an average SGR of 21% day^{-1}. Since the isolates were previously kept under identical culture conditions, it is likely that the observed variation in growth is due to genotypic differences between the isolates. Similar variation in growth between different isolates has been reported for the green seaweed *Ulva ohnoi* and the possibility of genotypic variation as the driver for this variation in growth was suggested for strains from different locations [30]. Further, natural selection for high growth and its heritability has been experimentally demonstrated in the brown seaweed *Fucus vesiculosus* [48]. High growth rates are characteristic for opportunistic and invasive species of plants where faster growth rates are favoured over other traits such as herbivory defence [49]. Growth rates in seaweed are also typically influenced by the cell size or filament morphology, where a larger surface area to volume ratio is associated with higher growth rates through more efficient photosynthesis and increased nutrient uptake as demonstrated in many species of seaweed [50]. Therefore, the variation in growth rates found for isolates of *D. tenuissima* in this study can be a direct result of differences in the size of filaments that in itself can be a genotypic trait of a broader survival strategy.

In addition to the variation between isolates, growth was also strongly affected by the environmental treatments and in particular light intensity which explained approximately 50% of the total variation in growth. The strong effect of light on the growth in this species has been demonstrated previously in large-scale outdoor cultures under non-limiting conditions, where light intensity was either controlled by stocking density [2] or varied seasonally [3]. Notably, the addition of extra nitrogen had no effect on the growth in any of the three isolates which suggests that nitrogen was in no case a limiting factor for growth. The C:N ratio, as an indicator for nitrogen limitation for growth [51], was uniformly low across most treatments with an approximate ratio of 7:1 and was similar to outdoor cultures of *D. tenuissima* under non-limiting conditions [2,3]. However, at the high light and low nitrogen treatment combinations a higher C:N ratio of over 13:1 was measured in isolates 1 and 2, which indicates that the available nitrogen supported the high growth but at the cost of internal nitrogen depletion. This internal depletion of nitrogen was not observed in isolate 3 which suggests that

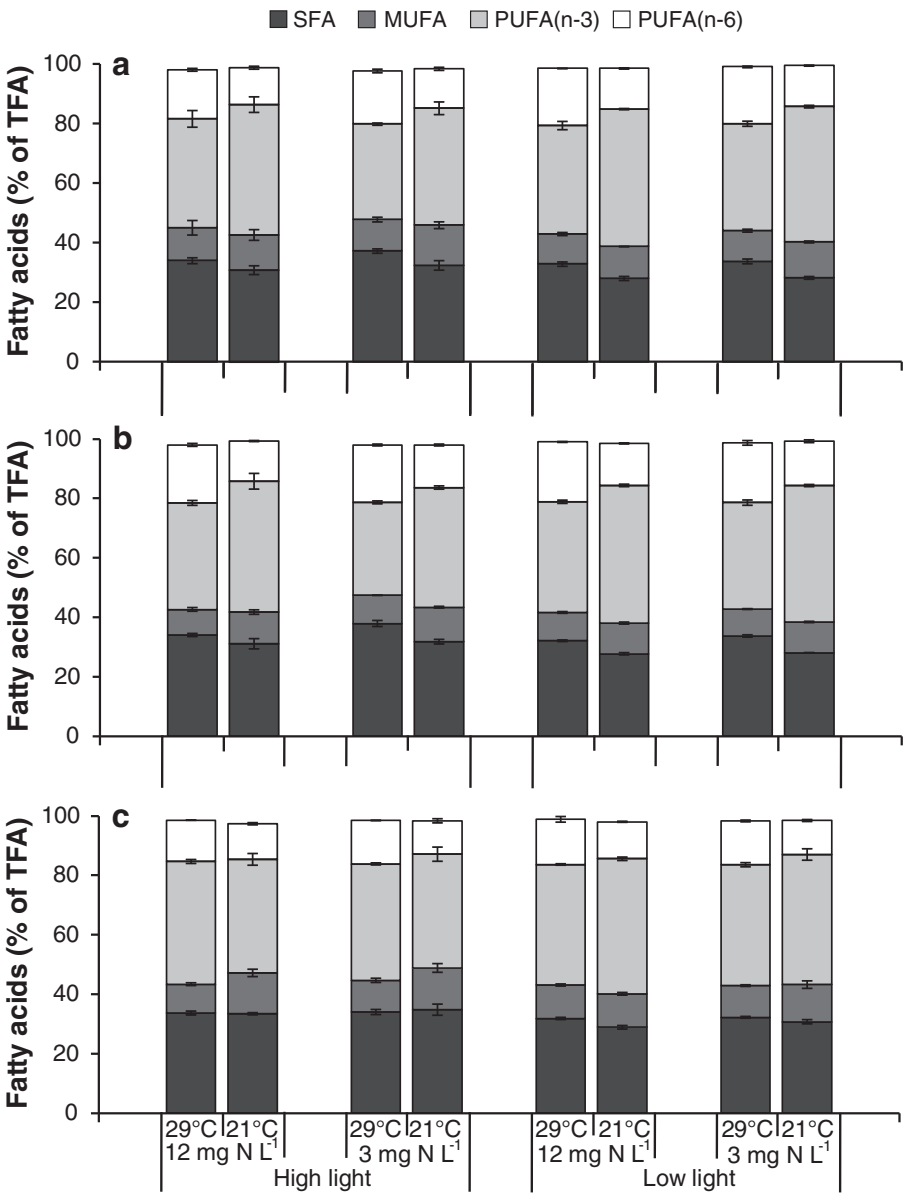

Fig. 4. Mean composition (% of TFA ± SE, n = 3) of the broad fatty acid groups (*SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, PUFA(n-3)/(n-6) polyunsaturated omega-3/omega-6 fatty acids) in three isolates of *Derbesia tenuissima* (**a** isolate 1, **b** isolate 2, **c** isolate 3) grown at different temperatures (29 °C, 21 °C), different nitrogen concentrations (12 mg N L^{-1}, 3 mg N L^{-1}) and different light conditions (100 μmol photons m^{-2} s^{-1}, 24 μmol photons m^{-2} s^{-1}).

the full growth potential of this isolate was not realized possibly due to suboptimal conditions of light and temperature or the limitation of another factor such as phosphorous. Although the N:P ratio was not measured in this study, it is possible that phosphorous became a limiting factor at the high nitrogen treatment conditions and therefore not all available nitrogen was utilised for growth.

4.3. Total fatty acid content and fatty acid composition

The content of TFA in *D. tenuissima* ranged between 35 and 54 mg g^{-1} dw with an average fatty acid composition of 32% SFA, 11% MUFA and 40% PUFA(n-3) which is within the range of earlier reports for this species [1,2]. Approximately 17–26% of the PUFA(n-3) were in the form of C18:3(n-3) (α-linolenic acid, ALA) which is beneficial for cardiovascular health and has potential applications in the redistribution of body fat [52,53] and therefore is an important fatty acid for applications in health and nutrition. However, there was substantial variation in the content and composition of fatty acids across treatments and isolates. The content of TFA was approximately 20% lower in isolate 3 compared to the other isolates and this difference between isolates explained nearly 50% of the total variation in the content of TFA. In a similar manner, the proportion of the fatty acid groups (SFA, MUFA, PUFA) also differed between isolates but to a smaller degree than the content of TFA, and the environmental conditions had a larger impact on the composition of fatty acids. Variation in the content and composition of fatty acids has been demonstrated for genotypes of terrestrial oil crops such as rapeseed [25] and soybean [54] and also for various species of microalgae [17,22,27], with significant increases in the content and also composition of fatty acids through selective breeding and strain selection [55]. In seaweed, however, this branch of research is still in its infancy but first improvements of the total lipid content by 20% in the commercially important kelp *Macrocystis* through selective breeding have been reported [33] and mark a first step in the domestication of seaweeds for oil-based bioproducts. Of particular importance, however, is the quality of the oil and specifically a high proportion of PUFA(n-3) which have applications in nutraceuticals, functional foods and as a

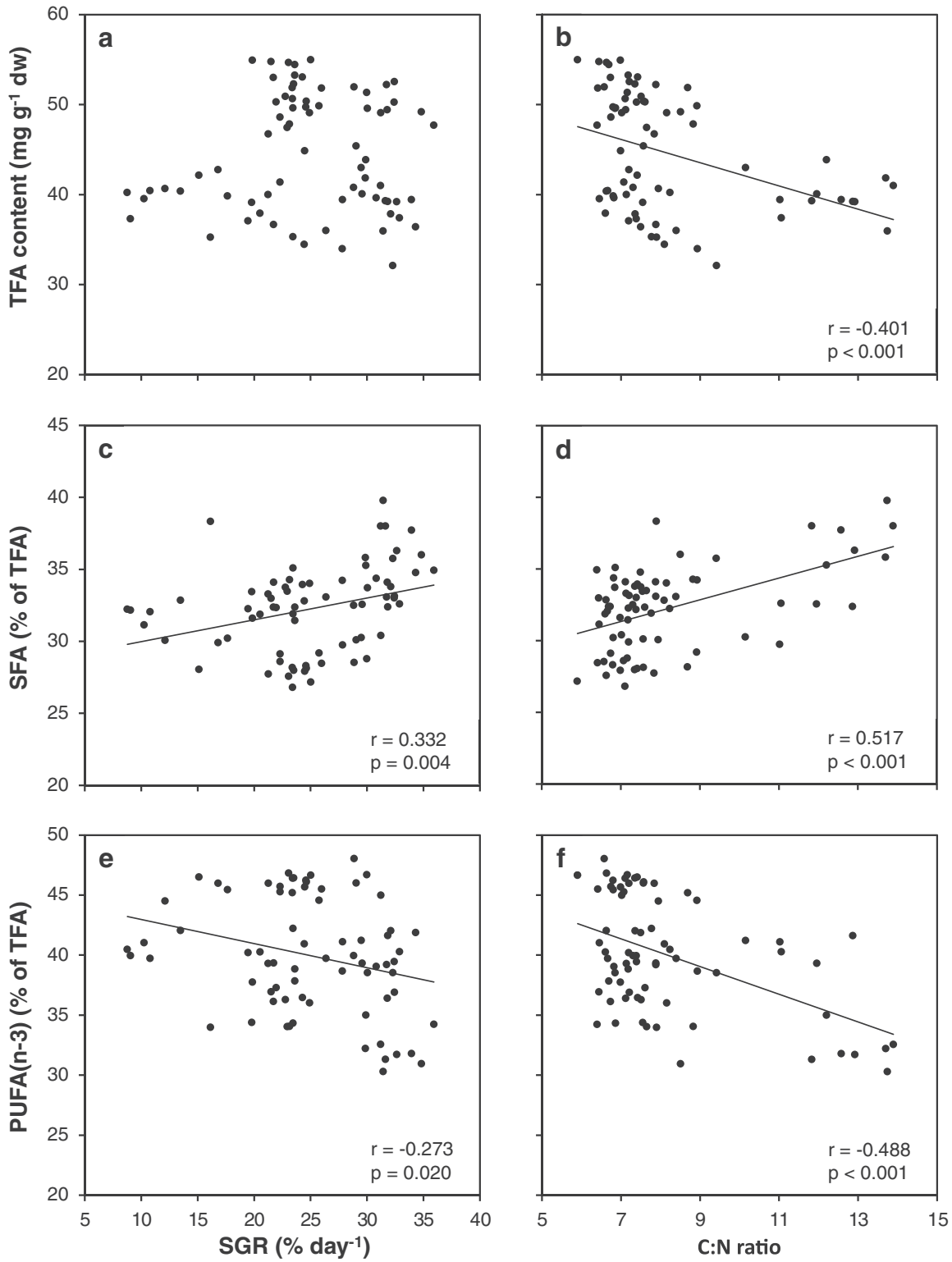

Fig. 5. Correlations (n = 72) of the total fatty acid content (TFA) (mg g^{-1} dw), saturated fatty acids (SFA) (% of TFA) and polyunsaturated omega-3 fatty acids (PUFA(n-3)) (% of TFA) with the specific growth rate (SGR) (% day^{-1}) and the carbon to nitrogen ratio (C:N ratio).

fish oil replacement [56]. The genotypic variation of PUFA(n-3) has previously not been demonstrated for seaweeds [24]. Here we provide the first strong support that part of the variability of fatty acids in seaweed is genotypic, as evidenced by the portioning of the source of the variation in TFA and fatty acid composition between the isolates and the environmental treatment conditions. This has important implications for future strain selection and subsequent selective breeding for improvements in fatty acid quality and in particular increases in the content and composition of PUFA(n-3).

As well as the opportunity for strain selection, improvements in the content and composition of fatty acids can be achieved by exploiting the natural variability driven by environmental factors such as temperature, light and nutrient availability through modified culture and harvest strategies. For the content of TFA, the effect of the environment was

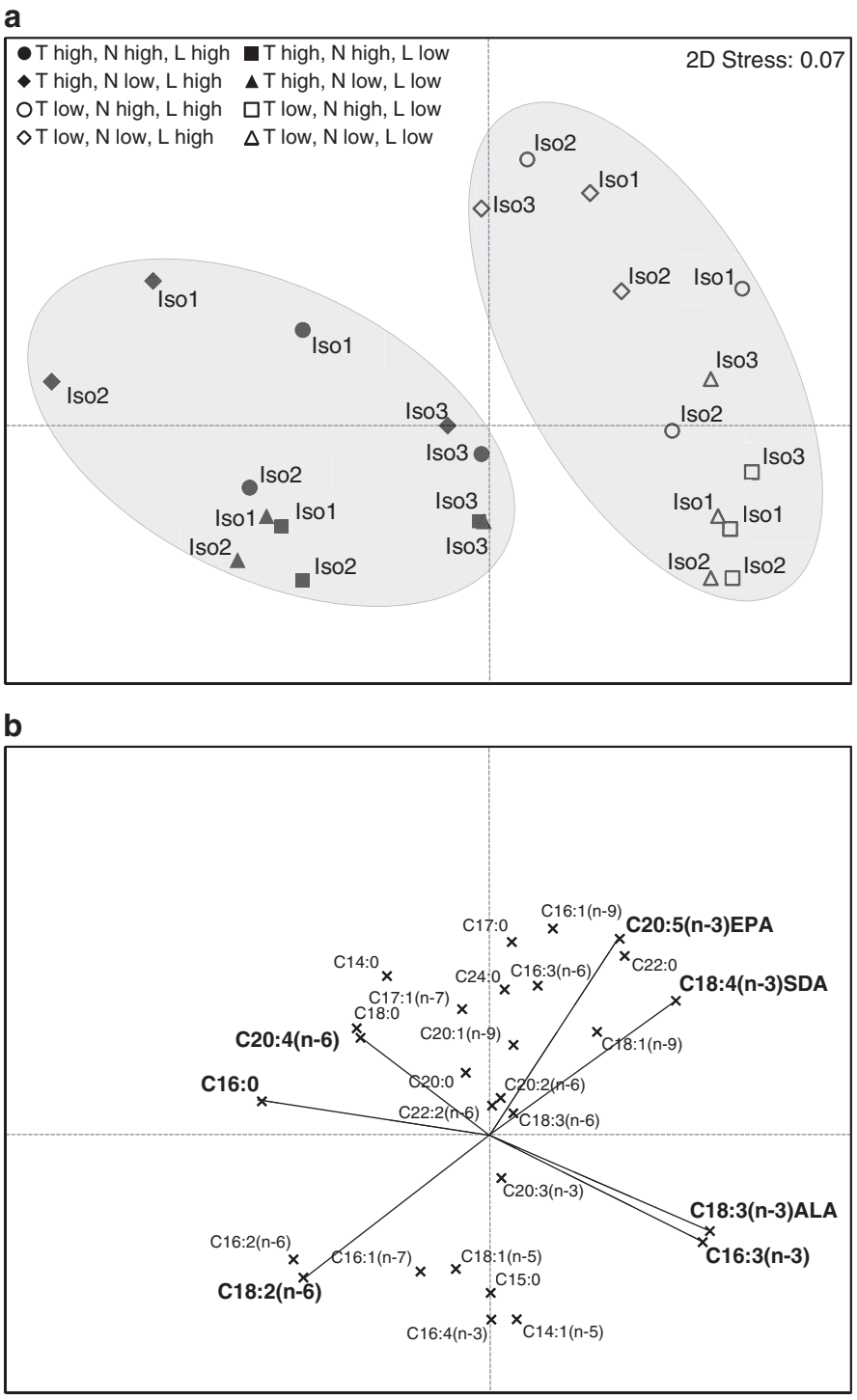

Fig. 6. Multidimensional Scaling (MDS) showing the differences in average fatty acid composition (% of TFA) of three isolates (iso1, iso2, iso3) of *Derbesia tenuissima* grown at different levels (high, low) of temperature (T), nitrogen (N) and light (L) with **a** MDS ordinates and **b** overlayed vector loadings of individual fatty acids. Vectors have only shown for the most abundant fatty acids and fatty acids of particular interest and discussed in this paper.

smaller than that of the genotype but nevertheless remained substantial. There was a strong interaction of the factors light and nitrogen, resulting in a reduction of up to 20% in the content of TFA at the high light and low nitrogen treatment combination. The effect of light on the fatty acids in algae has been investigated previously, and in many microalgae the content of TFA (or total lipids) increases as a result of high light intensity [8], and is generally attributed to increased conversion of photoassimilates into triacylglycerol (TAG) as a means of energy storage [22]. However, for the *Derbesia* strains investigated here, high light intensity does not lead to accumulation of lipids which is supported by a previous study on *D. tenuissima* [2], and in a similar manner several other green seaweeds have higher total lipid contents at low light conditions [15,16]. While high light resulted in a slight general decrease in the content of TFA, a particularly strong reduction only occurred in combination with low nitrogen availability and specifically in the cases where the internal nitrogen was depleted as measured by a high C:N ratio. This is in contrast to many species of microalgae [22,56] where the depletion of nitrogen leads to increases in the content of

TFA (or total lipids) through increased production of TAG. However, there appears to be no general trend as the content of TFA decreases with nitrogen depletion in some species of microalgae [22] and lipid increases induced by nitrogen depletion have only rarely been observed in seaweeds [19]. This inconsistency between species and studies can be related to interactions with other environmental factors such as light [8] or carbon availability [19] and associated heterogeneous responses to nitrogen depletion. Furthermore, nitrogen starvation also results in a reduction of chloroplasts [20,57] with a subsequent reduction in the associated membrane lipids [57] and therefore possible net reductions of total lipids. In green algae specifically, the production of starch, as the major energy storage product, also competes with TAG for the carbon precursor [58,59] and the production of starch can be favoured over TAG under nitrogen depletion [57]. These factors can therefore result in overall net decreases in the content of TFA under nitrogen depletion as occurred in *D. tenuissima*. Temperature had no effect on the content of TFA in this study. Although there are examples where a reduced culture temperature resulted in increased total lipid contents in both microalgae [22] and seaweeds [14], this seems to be a reflection of a narrow range of tested culture temperatures as studies that included multiple temperature treatments often show an optimal temperature that leads to increased total lipid content while temperatures above and below that optimum support lower lipid contents [60,61].

While temperature had no effect on the content of TFA, this parameter had the strongest effect on the composition of fatty acids either as a main effect or in various interactions with light and isolate. In isolates 1 and 2, the proportion of PUFA(n-3) in TFA increased by 20% to 45% at the low temperature treatment with a concomitant decrease in PUFA(n-6) and saturation. Importantly, temperature did not substantially affect the growth or the content of TFA and therefore the overall quality of the biomass as defined by high PUFA(n-3) productivity was improved at low temperature. This temperature effect on the proportion of PUFA(n-3) was not present in isolate 3 where the proportion of PUFA(n-3) remained stable across temperatures at approximately 40% of TFA, emphasizing the significance of appropriate strain selection for different climates to maximise the production of PUFA(n-3). The effect of water temperature on the composition of fatty acids is generally understood as a physiological response to improve membrane fluidity at lower temperatures, as unsaturated fatty acids have a lower melting point than saturated fatty acids [62,63]. However, the increased unsaturation with decreased temperature occurs in many [22,64], but not all species of microalgae [61] investigated to date. This temperature effect on the composition of fatty acids has been investigated experimentally for only a few species of seaweed [13,14], but similar to microalgae there seems to be no general trend as many field based studies also show increased PUFA(n-3) contents during the warmer summer months [10,12] and species-specific interactions between light and growth rates therefore seem likely. Nevertheless, we provide here the first evidence for the temperature dependence of PUFA(n-3) content in *D. tenuissima*, and the observed variation of this temperature effect below the species level has implications for strain selection for different climates.

The composition of fatty acids was also affected by light intensity and there was an increased proportion of SFA at the high light treatment in all three isolates with the lowest overall proportion of PUFA(n-3) at the high light and high temperature treatment combination. Similar effects of light on the fatty acid composition occurred for species of seaweed where the fatty acid profile was more saturated at high light conditions with a lower proportion of PUFA [15,16]. Light intensity can provoke a range of physiological responses including increased production of chloroplasts and associated membrane lipids at low light conditions [22]. Such membrane lipids generally have a more unsaturated composition of fatty acids [65] which increase the fluidity of thylakoid membranes and thereby electron-flow in the chloroplasts [66]. Furthermore, increased rates of oxidation at high light intensity can lead to a breakdown of double bonds and thereby increase the degree of saturation [22]. In addition to the direct influence of light on the composition of fatty acids, it must be emphasized that the composition of fatty acids can also change depending on growth rates. We observed weak correlations between high growth rates and reduced PUFA(n-3) and a higher proportion of SFA, and these highest growth rates were achieved at the high light treatments. There is evidence that growth rate can affect the proportion of fatty acids through proportional changes of lipid classes with a higher production of the structural and more unsaturated glycolipids during periods of high growth [7], reflecting the increased production of organelles and in particular chloroplasts. Many species of microalgae therefore show increased levels of PUFA(n-3) during the exponential growth phase [64]. Growth rate and light intensity therefore have opposing effects on the proportion of PUFA(n-3), which can explain the discrepancies between studies and reported species with either a decrease [15,16] or an increase [15] with light intensity.

4.4. Conclusion

This study confirms the high growth rates, and the high content and proportion of the nutritionally important PUFA(n-3) in *D. tenuissima*. Furthermore, the results of this study clearly demonstrate that there is potential to enhance the biomass growth and content and composition of fatty acids in *D. tenuissima* through environmental manipulations and also careful strain selection. There were substantial differences between the isolates and the selection of a particular isolate of *D. tenuissima* will have important implications for choosing culture strategies because of strong genotype by environment interactions. Isolate 1 and isolate 2 had both higher growth rates and higher contents of TFA, and the proportion of PUFA(n-3) was elevated to nearly 45% of TFA in the low water temperature treatment. Importantly, these increases in the proportion of PUFA(n-3) were achieved in the presence of a stable content of TFA and high growth rates, resulting in a net increase in PUFA(n-3) productivity. Isolate 3, however, had a lower growth rate and a lower content of TFA, but the proportion of the nutritionally important PUFA(n-3) remained stable at around 40% of TFA across treatments. This stability in the composition of fatty acids is beneficial for commercial cultivation for health and food applications where a stable supply of PUFA(n-3) is preferred [4,5]. While it remains to be confirmed that these results are transferable to scaled-up, long-term outdoor cultivation systems, this study provides the theoretical framework for the implementation of strain selection and culture management to improve productivity of selected bioproducts in seaweed at scale.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2015.02.022.

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