New Zealand Journal of Marine and Freshwater Research

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/tnzm20

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To cite this article: Leslie T. Sampollo, Lesley L. Rhodes, María Múgica & Andrew G. Jeffs (01 Sep 2024): Assessing new microalgae species as potential feed for bivalve spat in nursery culture, New Zealand Journal of Marine and Freshwater Research, DOI: 10.1080/00288330.2024.2396403

To link to this article: https://doi.org/10.1080/00288330.2024.2396403

NEW ZEALAND JOURNAL OF MARINE AND FRESHWATER RESEARCH
https://doi.org/10.1080/00288330.2024.2396403

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Assessing new microalgae species as potential feed for bivalve spat in nursery culture

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ABSTRACT

Microalgae remains a primary food source for hatchery and nursery culture of juvenile bivalves. However, there is a mismatch between the characteristics of the microalgae currently used in nursery culture and the feeding abilities and nutritional requirements of juveniles of some bivalve species, such as the green-lipped mussel, *Perna canaliculus*. Therefore, there is a need to identify new species of microalgae for this purpose that have favourable cell size, good growth performance under culture conditions and appropriate nutritional content for juvenile bivalves by using these characteristics as primary selection criteria. In this study, the growth performance under standard culture conditions of four microalgae species (*Chrysochromulina camella*, *Gymnodinium simplex*, *Pyramimonas parkeae* and *Stephanodiscus niagarae*) which are of a sufficiently large cell size to meet the needs of juvenile mussels (i.e. 10–20 µm size range) were tested in comparison to three commonly cultured commercial species (*Chaetoceros muelleri*, *Diacronema lutheri* and *Tisochrysis lutea*). Results show that *G. simplex*, *S. niagarae* and the two strains of *P. parkeae* (*P. parkeae* (C) and *P. parkeae* (I)) can achieve similar biomass as the reference species in the intermediate (1 L) culture stages despite the large difference in cell densities. The poor growth of *C. camella* in F/2 media indicates a necessity of determining its appropriate culture conditions before further assessing its suitability as a nursery feed for juvenile bivalves. The proximate analysis of *S. niagarae*, *G. simplex* and *P. parkeae* (I) reveals nutritional profiles conducive to spat development. However, the *P. parkeae* (C) strain contained low carbohydrate content, suggesting inferior nutritional value compared to the *P. parkeae* (I) strain, showing that variants of the same species can have different biochemical composition and suggesting the former might have limited suitability for feeding in nursery culture. The findings indicate that *G. simplex*, *S. niagarae* and *P. parkeae* (C) hold sufficient promise as spat feed in nursery culture to warrant further testing to confirm their efficacy as live microalgal feed for bivalve spat.

ARTICLE HISTORY
Received 12 March 2024
Accepted 20 August 2024

HANDLING EDITOR
Dave Taylor

KEYWORDS
Microalgae; bivalve; nursery culture; biomass; cell size; proximate analysis

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Introduction

Microalgae are unicellular or simple multi-cellular microscopic organisms that often thrive through cell division under favourable conditions (Šoštarič et al. 2009) that are frequently isolated from wild habitats, such as lakes, coastal areas and thermal springs, to be cultivated for various applications including bioremediation, production of biofuel and other bioproducts, or they are used directly as feed sources, such as in aquaculture (Muller-Feuga et al. 2003; Liu et al. 2023). Microalgae provide valuable nutrients (i.e. fatty acids, amino acids, sugars, sterols, vitamins and trace elements) that are captured and utilised by organisms that feed on microalgae, such as filter-feeding bivalves (Brown 1991; Brown et al. 1997; Rivero-Rodríguez et al. 2007; Napiórkowska-Krzebietke 2017). In bivalve aquaculture, microalgae are the most common feed source, usually provided as live-feed or preserved products, such as microalgal paste (De La Peña et al. 2018) or microalgal concentrates (Southgate et al. 2016) during the larval hatchery and juvenile nursery phases of production.

There are few accounts of microalgae species being tested for their potential as bivalve feed (Brown et al. 1997; Lora-Vilchis and Maeda-Martinez 1997), with only about 15 species being used routinely in bivalve aquaculture worldwide (Creswell 2010), with the selection narrowed often further for use with each bivalve species (Ponis et al. 2006). The most widely used species by bivalve hatcheries belong to the genera *Chaetoceros*, *Tetraselmis*, *Diacronema, Tisochrysis*, *Nannochloropsis* and *Thalassiosira* (Creswell 2010). The same species from these genera are also commonly used as feed for juvenile bivalves, commonly known as spat, for nursery culture (Benemann 1992; FAO 2004). However, these species are often suboptimal for nursery spat production, resulting in reduced spat growth (Willer and Aldridge 2019). In larval rearing, the quality and quantity of fatty acids are usually critical, given they play a vital role in larval development (Pettersen et al. 2010; Rodríguez-Pesantes et al. 2022). However, fatty acid provisioning is not so critical for the nutrition of juvenile shellfish, while the supply of carbohydrates and protein plays a more significant role in spat development (Flaak and Epifanio 1978; Enright et al. 1986b; Ponis et al. 2003). Hence, the nutritional profile of existing microalgal feeds widely used in bivalve hatcheries and transferred for use in bivalve nurseries may not match the nutritional requirements of spat, leading to a need to further optimise microalgal feed specifically for nursery culture. This appears to be the case for the green-lipped mussel, *Perna canaliculus*, with research indicating that the dietary supply of carbohydrate and protein is of greater significance than lipid and specific fatty acids for early juveniles (Supono et al., 2021, 2023).

Microalgae species that are routinely used in bivalve hatcheries are mostly small (size range mostly <10 μm in diameter, except for *Tetraselmis* spp.) to best suit the feeding capabilities of trochophore and veliger larvae (Enright et al. 1986a; Brown et al. 1997). Larval settlement and metamorphosis typically involve significant rearrangement of the filter-feeding apparatus in bivalve shellfish and their corresponding filtering abilities (Beninger et al. 1994; Cannuel et al. 2009; Gui, Zamora et al. 2016). Consequently, bivalve spat is capable of capturing a broader range of microalgal cell sizes, often up to 30 μm, depending on the species and size of the spat (Knauer and Southgate 1999). Green-lipped mussel spat up to 2 mm in shell length have high capture rates for cells that measure 10–15 μm in diameter but low capture rates for small (<7 μm) and large algal

cells (>20 μm) (Sanjayasari 2021). The transfer of 15 μm inert particles suspended in seawater into the gills, stomach, hindgut and anus in post-settlement mussel spat (<1 mm shell length) confirms the ability of small spat to capture and consume much large particles compared to their preceding larval stages (Gui, Kaspar et al. 2016). This knowledge of the feeding capabilities of the early juveniles of this species provides valuable starting criteria for identifying potential microalgae that are more suitable as mussel spat feed in terms of both cell size and nutritional composition.

The mismatch in the cell size and nutritional profile used in nursery spat production creates a strong impetus to find new microalgae feed to improve the effectiveness of the nursery production phase. This advancement is crucial to help overcome the massive losses of spat resulting from seeding spat directly onto coastal farms, a problem particularly observed in *Perna* (Skelton and Jeffs 2021; Skelton et al. 2022; South et al. 2022) and *Mytilus* species (Calderwood et al. 2014).

When selecting microalgae as bivalve feed, its cultivability is one of the primary considerations, along with the appropriate cell size and nutritional value of the species (Muller-Feuga, Robert et al. 2003; Creswell 2010; Catarina and Xavier 2012; Tahir and Ransangan 2021). Cultivability is vital in live microalgal feed selection because the overall quality and quantity of the microalgae that can be produced and delivered efficiently to the cultured bivalves greatly determine the overall outcomes for the bivalve culture (FAO 2004; Rico-Villa et al. 2006). For example, in bivalve nurseries, the amount of food available to the juvenile bivalves is a key determinant of the success of spat rearing, even though feed quality also plays a significant role (Muller-Feuga, Robert et al. 2003). Microalgae must be produced relatively easily and reliably for practical and economic reasons because live-feed production usually underpins the production efficiency of shellfish hatchery and nursery operations (Robert and Trintignac 1997). The cost of live microalgal production is a significant contributor to the overall production cost for commercial rearing of early stages of bivalves, typically between 30% and 60% of the overall production cost (Coutteau and Sorgeloos 1992). Microalgal species with high nutritional value might not be utilised commercially because of the challenges associated with their large-scale production. For example, the diatom *Chaetoceros calcitrans* is a highly effective larval feed for some shellfish species, but its culture performance becomes unreliable when raised in mass culture systems (Muller-Feuga, Robert, et al. 2003; Kaspar et al. 2014).

A microalgal species must proliferate in an artificial and controlled environment, creating a culture profile that can be applied repeatedly to reliably up-scale the production to meet bivalve feeding requirements (Tahir and Ransangan 2021). Typically, a stock culture of a microalgal strain or species is retained and maintained regularly, and a portion is used to inoculate starter cultures (Creswell 2010). In turn, the starter cultures are used to inoculate intermediate cultures that are used to initiate mass production systems which can be undertaken in different ways, including batch culture, continuous culture and semi-continuous culture (Creswell 2010), with batch culture considered the most straightforward, reliable and flexible method (FAO 1996). The growth performance of the microalgae is determined through the observation of indicators, such as specific growth rate, parent-daughter intercept, maximum cell density achieved and time required to achieve peak density (Lananan et al. 2013). Species with good growth

performance can achieve high cell densities and proliferate within shorter cultivation period when reared under suitable culture conditions (Lananan et al. 2013).

This study aims to apply a selection process for identifying microalgae with the potential to be used for feeding mussel spat in a nursery system. Based on prior knowledge, cell size was used as an initial selection criterion for identifying suitable microalgae from the existing microalgal culture collections in Aotearoa New Zealand and new isolates of wild microalgae. Secondary selection criteria involved assessing the growth performance of individual species compared with the commonly used microalgae in bivalve aquaculture by characterising their population growth in terms of cell density and biomass under standard small-scale laboratory culture conditions. Finally, a proximate analysis was also conducted to provide additional information on the nutritional suitability of the species as spat feed in nursery culture.

Materials and methods

Microalgae

Microalgae within the size range of 10–20 μm were selected for this study, following the previous studies which confirmed that green-lipped mussel (*P. canaliculus*) spat can most effectively capture microalgae of this size range (Gui, Kaspar, et al. 2016; Sanjayasari 2021). To qualify for selection, the maximum cell size measurement (diameter, width, height, and depth) of the microalgae, as determined through cross-referencing with previous studies and actual cell measurement, could not be larger than 20 μm, and there must be at least one cell dimension that measures at least 10 μm. Over 15 microalgae Cawthron Institute Culture Collection of Microalgae (CICCM) in Aotearoa New Zealand were reviewed for their cell dimensions with only qualifying species selected for further appraisal via experimental culture.

Isolation of microalgae

Unialgal cultures were isolated from water samples provided by Cawthron Institute, which were identified as from the commercial scale outdoor microalgal culture ponds at the Cawthron Aquaculture Park (CAP), at Glenduan, near Nelson. Water samples (1 ml) were transferred to a 12-well cell culture plate and observed under an inverted microscope. The estimated cell diameter of observed individual microalgae was measured using the graduated reticule on the inverted microscope to identify which cells to pick, and the actual cell measurement was determined later once a monoalgal culture was established. Microalga cells within the target cell size (10–20 μm) were individually picked out using a modified Pasteur pipette (Rhodes et al. 1994) and transferred to another well containing 1 ml F/2 media in sterile seawater. The transferred cells were left overnight inside a culture cabinet to allow the cells to settle at the bottom of the well. The transferred cells were re-picked and transferred to another well containing F/2 media in sterile seawater. This process of picking and transferring ensures a clean unialgal isolate and was repeated as many times as needed. Successfully isolated cells were transferred to a 250-ml Erlenmeyer flask with 100 ml of sterile seawater with added F/2 media and covered in breathable bung and aluminium foil and kept in a culture cabinet. After

obtaining a stable culture, a 1-ml sample was centrifuged to recover a microalgal pellet. The DNA from the recovered biomass was extracted using a NucleoSpin Tissue kit. The 23S and *rbcL* regions of the recovered DNA were sequenced and the optimal species assignment was determined by comparison with the National Centre for Biotechnology Information (NCBI) genetic sequence database (GenBank), which is the largest repository of sequences for species across the globe (Hotaling et al. 2021).

Cell size measurement

Before initiating a culture set-up, cell dimensions were taken to confirm the cells were within the 10–20 µm cell size target range. Diameter, width, height and depth of the microalgae were measured with a Leica fluorescence microscope by measuring 30 healthy cells. For dinoflagellates, cell width was measured across the cell at the widest point; cell height was measured from apex to antapex, and depth was measured from anterior to posterior of the cell (L. Rhodes, pers. comm.). The cell diameter was measured for spherical cells. Highly motile microalgae were fixed with Lugol's iodine at a concentration of less than 20 µL per 100 ml to prevent cell lysis and to facilitate measuring under the microscope (Rhodes et al. 1994).

Microalgal culture

The growth performance of the candidate microalgae was tested using a simulated batch culture as specified by international guidelines with a slight modification in the culture volumes that were used (FAO 1996). Microalgal cultures progressed from the stock culture to a starter culture in a 250-ml Erlenmeyer flask up to an intermediate culture in a 1-L Schott culture bottle. The performance of each microalga was assessed by measuring the cell density and dry biomass. Culturing at mass scale beyond 1 L volume was reserved for subsequent trials in a commercial shellfish facility. Three microalgae species that are commonly used in commercial shellfish hatcheries were used as reference cultures, i.e. *Tisochrysis lutea*, *Diacronema lutheri* and *Chaetoceros muelleri*. Microalgae were cultured in sterilised seawater with added standard F/2 media (Guillard and Ryther 1962). Microalgae from culture collections that required a different media were tested in two culture media: F/2 media (Guillard and Ryther 1962) and its currently recommended media for culture (GP media) (CICCM [unknown date]) to test its viability in commercially available culture medium represented by the F/2 media (Guillard and Ryther 1962). Culture conditions were maintained throughout the culture period in both experiment stages as standard for microalgae culture: temperature at 17–19°C, photoperiod set to 18 h light: 6 h dark period (FAO 1996), and light intensity between 90 and 110 µmol $m^2 \cdot s^{-1}$ using LED lights.

Starter culture

Three replicates of each microalgae starter culture were prepared in a 250-ml flask, each with a 100-ml total culture volume of sterilised seawater media with 10 ml inoculum from the stock culture that was standardised to a concentration of 10^5 cells·ml^{-1}

(Creswell 2010). Therefore, at day 0 the initial density of all microalgae groups was at 10^4 cells·ml^{-1}. The cell density was determined using the Luminex Muse® Cell Analyser from a 2-ml sample of the culture. Cultures were swirled every 2 days to resuspend cells that had settled at the bottom of the flask. The culture was grown for 12 days with monitoring of cell density every 2 days, and only those cultures exhibiting good growth were scaled up to the intermediate culture.

Intermediate culture

The starter cultures were transferred to a 1-L culture bottle, serving as 10% inoculum of 1 L total culture volume. At this stage, there were no adjustments to the density of the inoculum to simulate the scaling up in a batch culture. All cultures were provided with continuous aeration by delivering 0.22 µm filtered air through a bottle head with a designated air inlet and outlet to minimise the risk of contamination. The culture was run for 8 days with monitoring of cell density every 2 days and cell biomass determined every 4 days.

Cell biomass monitoring

Dry biomass was sampled every 4 days at day 0, day 4 and day 8 of the intermediate culture. A 20-ml sample of the culture was filtered using a dried (105°C for 1 h or until constant weight was achieved) and pre-weighed glass fibre filter and rinsed with de-ionised water. The filter with the microalgae sample was freeze-dried for 24 h, and the microalgae mass was determined by subtracting the weight of the filter paper.

Proximate analysis of microalgae

At the end of the intermediate culture, the microalgae were harvested for proximate analysis. An initial sample of 50 ml was centrifuged at 4000 rpm for 15 minutes at 17° C. The samples were washed twice by completely resuspending the cell pellets with 20 ml of 0.5 M ammonium formate and centrifuging them again (Guimarães et al. 2021). The recovered samples were lyophilised for 24 h and used for proximate analysis. Three replicate samples of 100 mg of lyophilised microalgal biomass were burnt in a muffle furnace (Nabertherm LT15/11 B410, Germany) at 450°C for 4 h and residual ash was weighed to determine the proportion of ash-free dry weight (AFDW) in relation to total dry weight. To determine the protein content of the lyophilised microalgal biomass, three 50 mg subsamples of biomass were incubated in 0.1 M NaOH for 16 h at 50°C, and then centrifuged at 10,000 rpm at 4°C for 10 min. The protein in each subsample was then quantified by BCA (bicinchoninic acid) method (Smith et al. 1985) using a micro BCA protein assay kit (ThermoFisher Scientific, USA) and read against bovine serum albumin (BSA) standard at 562 nm. The lipid content of the spat was determined for three 300 mg lyophilised subsamples of microalgal biomass with a modified methanol–chloroform solvent extraction method of Bligh and Dyer (1959). The total lipid extracted from each sample was quantified following the removal of the residual solvent using a stream of nitrogen gas following the method of Wang et al. (2014).

The total carbohydrate content of spat was determined for three 100 mg subsamples of lyophilised microalgal biomass that was homogenised in 1 ml of distilled water using a homogeniser (Polytron PT 1200, US). The solution was then centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant was transferred to a 1.5-ml Eppendorf tube and stored at –20°C prior to analysis. The total carbohydrate content of the solutions was determined using the phenol sulphuric acid reagent method (Dubois et al. 1956) and was read against D-glucose standard curve using a Multiscan Sky Microplate Spectrophotometer at 490 nm (Masuko et al. 2005).

Statistical analyses

Two-way analyses of variance (ANOVA) was used to compare each of the response variables (i.e. cell density and cell biomass) of the different microalgal species for each sampling period. Data were assessed for confirmation of compliance with the assumptions for parametric analyses using Q–Q plot, Shapiro–Wilk test, residuals versus means and Levene's test prior to advancing to ANOVA, and where departures were detected, the data were transformed using log transformation and Box–Cox transformation and reassessed.

Results

Isolation

Two microalgae were isolated and successfully cultivated in laboratory conditions: *Stephanodiscus niagarae* (99.8% match, accession number: NC_072630) and *Pyramimonas parkeae* (100% match, accession number: NC_012099). Their estimated cell sizes were within the target cell size range of 10–20 μm.

Cell measurements

A total of five candidate microalgal species initially qualified for the present study, three of which were from Cawthron Institute Culture Collection of Microalgae (CCICM) (*Chrysochromulina camella*, *Gymnodinium simplex*, *Pyramimonas parkeae*) and two were isolated from Cawthron Aquaculture Park (CAP) (*Pyramimonas parkeae* and *Stephanodiscus niagarae*). To differentiate the two *P. parkeae*, the strain from CICCM has a suffix – (C), while the isolate from CAP has a suffix – (I). The reference species (*Chaetoceros muelleri*, *Diacronema lutheri* and *Tisochrysis lutea)* measured less than 10 μm in all cell dimensions, while the candidate species had all measured cell dimensions larger than 10 μm (Figure 1, Table 1).

Starter culture

Among the reference species, the mean cell density of *T. lutea* was significantly higher than that of *C. muelleri* and *D. lutheri* starting from day 4 ($P < 0.0001$, $P < 0.0001$) up to day 12 of culture ($P < 0.0001$, $P < 0.0001$, respectively) (Figure 2). Meanwhile, both *C. muelleri* and *D. lutheri* exhibited similar mean cell densities ($P > 0.05$) in all culture

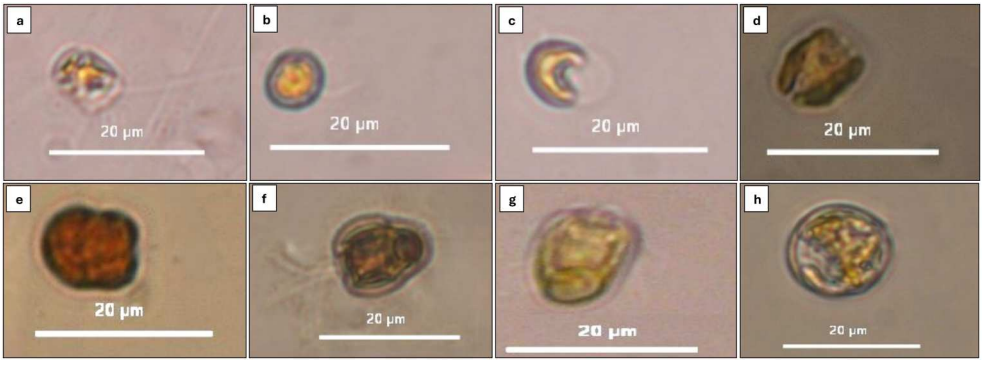

Figure 1. Images of the microalgae at 40× magnification. Reference species obtained from Commonwealth Scientific and Industrial Research Organisation (CSIRO): a = *Chaetoceros muelleri*, b = *Diacronema lutheri*, c = *Tisochrysis lutea*; candidate species from the Cawthron Institute Culture Collection of Microalgae (CICCM): d = *Chrysochromulina camella*, e = *Gymnodinium simplex*, f = *Pyramimonas parkeae* (C), g = *Pyramimonas parkeae* (I), *Stephanodiscus niagarae*.

days. In general, the reference species achieved higher mean cell density than all candidate species from day 8 to day 12 of culture.

Among the five candidate species, *P. parkeae* (I) initially had a significantly higher mean cell density ($P < 0.0001$) at day 2 compared to all other candidate species. At day 12 of culture, *P. parkeae* (C) and *P. parkeae* (I) were at equivalent cell densities of 7.56×10^5 cells·ml^{-1} and 9.26×10^5 cells·ml^{-1}, respectively ($P > 0.05$, Table 2), and both were higher than *C. camella* (GP) (3.53×10^5 cells·ml^{-1}), *G. simplex* (GP) (3.38×10^5 cells·ml^{-1}) and *S. niagarae* (3.77×10^5 cells·ml^{-1}) ($P < 0.0001$). The cell density of *S. niagarae* consistently increased until day 10, when it reached a peak cell density of 3.95×10^5 cells·ml^{-1} before a slight decrease in its cell density by day 12 of culture, 3.77×10^5 cells·ml^{-1} (Table 2). This same trend was observed in *C. camella* (GP), however, the decrease was sharper with a decrease from its peak cell density of 5.97×10^5 cells·ml^{-1} at day 8 of culture down to 3.53×10^5 cells·ml^{-1} at day 12. The cell density of *G. simplex* (GP) was steadily increased from the outset of culture, but it was

Table 1. Cell measurements of microalgae. Reference species obtained from Commonwealth Scientific and Industrial Research Organisation (CSIRO): *Chaetoceros muelleri*, *Diacronema lutheri*, *Tisochrysis lutea* and candidate species from the Cawthron Institute Culture Collection of Microalgae (CICCM): *Chrysochromulina camella*, *Gymnodinium simplex*, *Pyramimonas parkeae* (C) and isolated species: *Pyramimonas parkeae* (I) *Stephanodiscus niagarae*.

	Microalgae	Culture collection code	Size (µm)
Reference species	*C. muelleri*	CS-176	6–9 (D) × 4–8 (d)
	D. lutheri	CS-182	5–8 (D)
	T. lutea	CS-177	5–8 (D)
Candidate species	*C. camella*	CAWP16	9–12 (l) × 9–12 (w)
	G. simplex	CAWD86	10–15 (l) × 10–13 (w)
	P. parkeae (C)	CAWG07	11–16 (l) × 9–14 (w)
	P. parkeae (I)	NC_012099*	13–17 (l) × 8–11 (w)
	S. niagarae	NC_072630*	11–16 (D) × 10–15 (d)

l = cell length, w = cell width, D = cell diameter, d = cell depth; *n* = *30*.
*accession number.

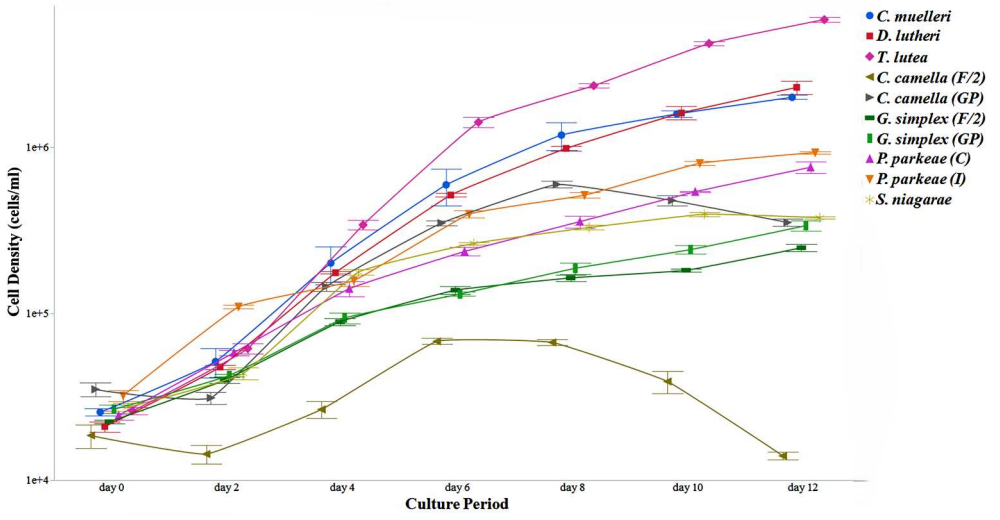

Figure 2. Mean cell density of microalgae in the starter culture (±SE). Reference species: *Chaetoceros muelleri*, *Diacronema lutheri*, *Tisochrysis lutea*; and candidate species from the Cawthron Institute Culture Collection of Microalgae (CICCM): *Chrysochromulina camella*, *Gymnodinium simplex*, *Pyramimonas parkeae* (C) and isolated species: *Pyramimonas parkeae* (I), *Stephanodiscus niagarae*; *C. camella* (GP) = using GP media; *C. camella* (F/2) = using F/2 media; *G. simplex* (GP) = using 50% GP media; *G. simplex* (F/2) = using F/2 media.

consistently lower than other candidate species (e.g. *C. camella* (GP), *P. parkeae* (C), *P. parkeae* (I), *S. niagarae*) at day 6 through day 10 of culture. However by day 12, the cell density of *G. simplex* (GP) was equivalent to *C. camella* (GP) ($P > 0.05$) and *S. niagarae* ($P > 0.05$), which was due to the declining cell densities of *C. camella* (GP) and *S. niagarae* towards the end of the culture period.

To test the growth performance of *C. camella* and *G. simplex* in a commonly used media, their cell densities when cultured in their recommended media were compared to the cell densities achieved in F/2 media in seawater. The mean cell density of *G. simplex* in both 50% GP media and F/2 media was similar on all culture days ($P = 0.10$), with the highest mean cell density observed at 3.38×10^5 cells·ml^{-1} and 2.49×10^5 cells·ml^{-1} at day 12 of culture, respectively, suggesting that *G. simplex* can be cultured in F/2 media. In contrast, *C. camella* (F/2) had consistently lower mean cell density than *C. camella* (GP) ($P < 0.0001$), reaching a peak density of only 6.72×10^4 cells·ml^{-1} at day 8 of culture. This is significantly lower ($P < 0.0001$) than the peak density of *C. camella* (GP) of 5.97×10^5 cells·ml^{-1} over the same culture period.

The mean cell densities reached by cultures of the *G. simplex* (F/2) *P. parkeae* (C), *P. parkeae* (I) and *S. niagarae* at 12 days were sufficient for initiating intermediate cultures, hence, these four species were progressed into testing in intermediate culture. The poor growth performance of *C. camella* in F/2 media suggests that it could currently only grow in its recommended media, which is onerous to prepare, unlike F/2 media which is readily available from commercial suppliers. Therefore, the *C. camella* was not progressed to evaluation in intermediate culture.

Table 2. Mean cell density in starter culture of the reference species and candidate species.

		Mean cell density (cells·ml^{-1}) (± SE)						
	Microalgae	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Reference species	*C. muelleri*	2.56E + 04^{abc} ± 1.34E + 03	5.15E + 04^{bc} ± 1.02E + 04	2.01E + 05^{b} ± 5.13E + 04	5.93E + 05^{b} ± 1.48E + 05	1.18E + 06^{b} ± 2.25E + 05	1.58E + 06^{b} ± 7.52E + 04	1.99E + 06^{b} ± 5.74E + 04
	D. lutheri	2.10E + 04^{bc} ± 1.47E + 03	4.78E + 04^{bc} ± 1.38E + 03	1.76E + 05^{b} ± 3.05E + 03	5.15E + 05^{b} ± 1.19E + 04	9.81E + 05^{b} ± 3.08E + 04	1.60E + 06^{b} ± 1.47E + 05	2.28E + 06^{b} ± 2.08E + 05
	T. lutea	2.61E + 04^{abc} ± 1.37E + 03	6.17E + 04^{b} ± 4.30E + 03	3.41E + 05^{a} ± 2.17E + 04	1.41E + 06^{a} ± 9.75E + 04	2.34E + 06^{a} ± 6.89E + 04	4.19E + 06^{a} ± 1.33E + 05	5.82E + 06^{a} ± 1.78E + 05
Candidate species	*C. camella* (F/2)	1.86E + 04^{c} ± 3.01E + 03	1.44E + 04^{d} ± 1.86E + 03	2.66E + 04^{e} ± 3.06E + 03	6.82E + 04^{f} ± 2.90E + 03	6.72E + 04^{f} ± 2.66E + 03	3.91E + 04^{f} ± 6.01E + 03	1.40E + 04^{e} ± 7.44E + 02
	C. camella (GP)	3.51E + 04^{ab} ± 3.38E + 03	3.12E + 04^{c} ± 2.61E + 03	1.45E + 05^{bc} ± 8.83E + 03	3.50E + 05^{bcd} ± 1.24E + 04	5.97E + 05^{c} ± 2.74E + 04	4.78E + 05^{d} ± 3.24E + 04	3.53E + 05^{d} ± 1.88E + 04
	G. simplex (F/2)	2.24E + 04^{abc} ± 6.20E + 02	3.98E + 04^{bc} ± 1.52E + 03	8.94E + 04^{d} ± 4.53E + 03	1.38E + 05^{e} ± 7.15E + 03	1.64E + 05^{e} ± 7.40E + 03	1.82E + 05^{e} ± 3.67E + 03	2.49E + 05^{d} ± 1.21E + 04
	G. simplex (GP)	2.67E + 04^{abc} ± 1.52E + 03	4.25E + 04^{bc} ± 7.94E + 02	9.41E + 04^{cd} ± 7.12E + 03	1.31E + 05^{e} ± 3.89E + 03	1.87E + 05^{e} ± 1.47E + 04	2.42E + 05^{e} ± 1.43E + 04	3.38E + 05^{d} ± 2.46E + 04
	P. parkeae (C)	2.45E + 04^{abc} ± 1.56E + 03	5.83E + 04^{b} ± 2.19E + 03	1.41E + 05^{bcd} ± 1.48E + 04	2.36E + 05^{d} ± 1.33E + 04	3.57E + 05^{d} ± 2.93E + 04	5.40E + 05^{cd} ± 5.19E + 03	7.56E + 05^{c} ± 5.84E + 04
	P. parkeae (I)	3.22E + 04^{ab} ± 2.31E + 03	1.10E + 05^{a} ± 2.37E + 03	1.58E + 05^{b} ± 1.22E + 04	3.96E + 05^{bc} ± 1.97E + 04	5.14E + 05^{cd} ± 1.74E + 04	8.00E + 05^{c} ± 2.21E + 04	9.26E + 05^{c} ± 1.60E + 04
	S. niagarae	2.75E + 04^{abc} ± 1.58E + 03	4.38E + 04^{bc} ± 3.73E + 03	1.78E + 05^{b} ± 6.56E + 03	2.64E + 05^{cd} ± 4.49E + 03	3.29E + 05^{d} ± 1.07E + 04	3.95E + 05^{d} ± 1.19E + 04	3.77E + 05^{d} ± 5.93E + 03

Data are expressed as cells·ml^{-1} (± SE). Mean values in the same column with different superscript letters indicate significant difference (two-way ANOVA, Tukey's HSD test, $P < 0.05$). *C. camella* (GP) = using GP media; *C. camella* (F/2) = using F/2 media; *G. simplex* (GP) = using 50% GP media; *G. simplex* (F/2) = using F/2 media. *P. parkeae* (C) = strain from Cawthron Institute Culture Collection, *P. parkeae* (I) = isolated strain.

Intermediate culture

The reference species still exhibited a higher mean cell density ($P < 0.0001$) for all culture days than the candidate species, with *T. lutea*, *D. lutheri* and *C. muelleri* reaching a density of 1.58×10^7 cells·ml^{-1}, 1.25×10^7 cells·ml^{-1} and 8.32×10^6 cells·ml^{-1} at day 8, respectively (Figure 3). Between the four candidate species, the *P. parkeae* (C) and *P. parkeae* (I) had a consistently higher mean cell density than *G. simplex* ($P < 0.0001$) and *S. niagarae* ($P < 0.0001$) from day 2 to day 8 of culture. The mean cell densities of the reference strains (*T. lutea*, *D. lutheri* as well as *C. muelleri*), *P. parkeae* (C) and *P. parkeae* (I) all recorded a higher peak mean cell density in the intermediate culture than in the starter culture. The highest mean cell densities of the reference species

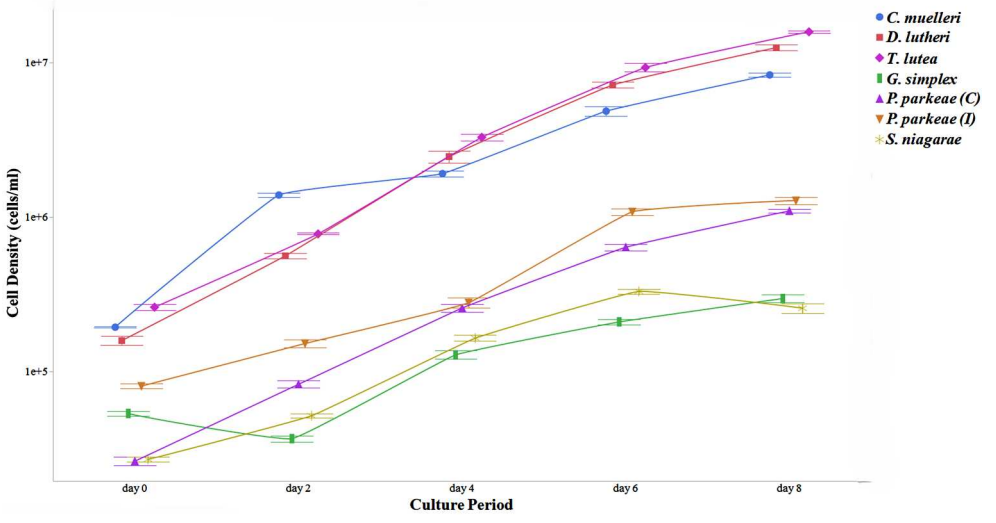

Figure 3. Mean cell density of microalgae in the intermediate culture (±SE). Reference species: *Chaetoceros muelleri*, *Diacronema lutheri*, *Tisochrysis lutea*; and candidate species from the Cawthron Institute Culture Collection of Microalgae (CICCM): *Gymnodinium simplex*, *Pyramimonas parkeae* (C) and isolated species: *Pyramimonas parkeae* (I), *Stephanodiscus niagarae*.

increased from 10^6 cells·ml^{-1} in the starter culture (Table 2) to 10^7 cells·ml^{-1} for *T. lutea* and *D. lutheri* and 8.32×10^6 cells·ml^{-1} for *C. muelleri* in the intermediate culture (Table 3), while *P. parkeae* (C) and *P. parkeae* (I) increased from 7.56×10^5 cells·ml^{-1} and $9.26 \times$

Table 3. Mean cell density in starter culture of the reference species and candidate species.

		Mean cell density (cells·ml^{-1}) (± SE)				
	Microalgae	Day 0	Day 2	Day 4	Day 6	Day 8
Reference species	*C. muelleri*	1.93E + 05^{b} ± 1.28E + 03	1.39E + 06^{a}± 4.07E + 04	1.91E + 06^{b} ± 8.59E + 04	4.85E + 06^{b} ± 3.46E + 05	8.32E + 06^{b} ± 2.58E + 05
	D. lutheri	1.58E + 05^{b} ± 1.02E + 04	5.61E + 05^{c} ± 2.11E + 04	2.46E + 06^{b} ± 2.12E + 05	7.17E + 06^{a} ± 3.10E + 05	1.25E + 07^{a} ± 5.63E + 05
	T. lutea	2.61E + 05^{a} ± 1.17E + 04	7.77E + 05^{b} ± 1.02E + 04	3.28E + 06^{a} ± 1.65E + 05	9.30E + 06^{a} ± 5.96E + 05	1.58E + 07^{a} ± 3.07E + 05
Candidate species	*G. simplex*	5.33E + 04^{d} ± 2.01E + 03	3.67E + 04^{g} ± 1.67E + 03	1.28E + 05^{d} ± 8.02E + 03	2.09E + 05^{f} ± 8.44E + 03	2.97E + 05^{d} ± 1.79E + 04
	P. parkeae (C)	2.62E + 04^{e} ± 1.66E + 03	8.25E + 04^{e} ± 4.52E + 03	2.58E + 05^{c} ± 1.53E + 04	6.37E + 05^{d} ± 3.24E + 04	1.10E + 06^{c} ± 2.83E + 04
	P. parkeae (I)	8.03E + 04^{c} ± 2.96E + 03	1.52E + 05^{d} ± 8.61E + 03	2.79E + 05^{c} ± 2.16E + 04	1.08E + 06^{c} ± 5.30E + 04	1.28E + 06^{c} ± 7.07E + 04
	S. niagarae	2.69E + 04^{e} ± 9.36E + 02	5.16E + 04^{f} ± 1.71E + 03	1.64E + 05^{d} ± 7.34E + 03	3.30E + 05^{e} ± 1.16E + 04	2.56E + 05^{d} 1.85E + 04

Data are expressed as cells·ml^{-1} (± SE). Mean values in the same column with different superscript letters indicate significant difference (two-way ANOVA, Tukey's HSD test, $P < 0.05$). *P. parkeae* (C) = strain from Cawthron Institute Culture Collection, *P. parkeae* (I) = isolated strain.

10^5 cells·ml^{-1}, respectively, in the starter culture to 10^6 cells·ml^{-1} in intermediate culture. Despite being the same species, there is a difference in the growth behaviour of *P. parkeae* (C) and *P. parkeae* (I) strains. For example, *P. parkeae* (I) reached 10^6 cells·ml^{-1} at day 6 of culture, which was a higher cell density than *P. parkeae* (C) (i.e. 6.37×10^5 cells·ml^{-1}, $P < 0.0001$) and *P. parkeae* (C) only reached 10^6 cells·ml^{-1} at day 8 of culture. Furthermore, there was only a slight increase in the peak cell density of *G. simplex* from 2.49×10^5 cells·ml^{-1} in starter culture at day 12 to 2.97×10^5 cells·ml^{-1} in the intermediate culture at day 8. However, a slight decrease was observed in the peak cell density of *S. niagarae* of 3.95×10^5 cells·ml^{-1} at day 10 in the starter culture compared to 3.30×10^5 cells·ml^{-1} at day 6 in the intermediate culture. As with the starter culture, the same slight decrease in the mean cell density was observed in *S. niagarae* after reaching its peak cell density of 3.30×10^5 cells·ml^{-1} at day 6 of culture decreasing to 2.56×10^5 cells·ml^{-1} at day 8 of culture.

There was no difference in the biomass of all microalgae (Table 4) at all sampling times during the intermediate culture (Figure 4) despite the significant differences in the cell density between reference species (e.g. *C. muelleri*, *D. lutheri*, *T. lutea*) and the candidate species (e.g. *G. simplex*, *P. parkeae* (C), *P. parkeae* (I), *S. niagarae*) (Table 3).

Proximate analysis

The lipid content of *C. muelleri*, *D. lutheri*, *T. lutea* exhibited a high degree of similarity, with values of 254.8, 293.6, 231.1 mg·g^{-1}, respectively (Table 5). Among all the candidate species, only *S. niagarae* demonstrated a lipid content comparable to the reference strains at 262.2 mg·g^{-1}. Meanwhile, *P. parkeae* (C) and *P. parkeae* (I) presented similar lipid content (175.1, 194.0 mg·g^{-1}, respectively) which were lower than reference strains. *Gymnodinium simplex* showed the lowest lipid concentration of all the studied microalgae at 142.1 mg·g^{-1}. The carbohydrate content of *C. muelleri* (199.7 mg·g^{-1}) and *T. lutea* (171.3 mg·g^{-1}) was found to have comparable levels. However, *D. lutheri* exhibited a much lower carbohydrate concentration at only 67.4 mg·g^{-1} which was relatively similar to the carbohydrate content of *G. simplex* (22.3 mg·g^{-1}), *P. parkeae* (I) (35.5 mg·g^{-1}) and *S. niagarae* (95.5 mg·g^{-1}). Notably, the carbohydrate content of *P. parkeae* (C) (12.2 mg·g^{-1}) is much lower than *P. parkeae* (I) (35.5 mg·g^{-1}). The protein concentrations among all species had a range of 103.6–198.2 mg·g^{-1}. The protein: lipid: carbohydrate ratio for both *C. muelleri* and *T. lutea* was similar at 1:2:2

Table 4. Mean cell dry biomass of microalgae in intermediate culture.

		Mean dry biomass (mg·ml^{-1}) (±SE)		
	Microalgae	Day 0	Day 4	Day 8
Reference species	*C. muelleri*	0.03 ± 0.02^a	0.13 ± 0.01^a	0.33 ± 0.08^a
	D. lutheri	0.05 ± 0.01^a	0.15 ± 0.06^a	0.39 ± 0.11^a
	T. lutea	0.03 ± 0.00^a	0.08 ± 0.01^a	0.24 ± 0.02^a
Candidate species	*G. simplex*	0.03 ± 0.01^a	0.04 ± 0.01^a	0.10 ± 0.01^a
	P. parkeae (C)	0.02 ± 0.01^a	0.14 ± 0.01^a	0.26 ± 0.01^a
	P. parkeae (I)	0.04 ± 0.04^a	0.22 ± 0.03^a	0.34 ± 0.01^a
	S. niagarae	0.04 ± 0.02^a	0.17 ± 0.01^a	0.13 ± 0.02^a

Data are expressed as mg·ml^{-1} (± SE). Mean values in the same column with different superscript letters indicate significant difference (two-way ANOVA, Tukey's HSD test, $P < 0.05$). *P. parkeae* (C) = strain from Cawthron Institute Culture Collection, *P. parkeae* (I) = isolated strain.

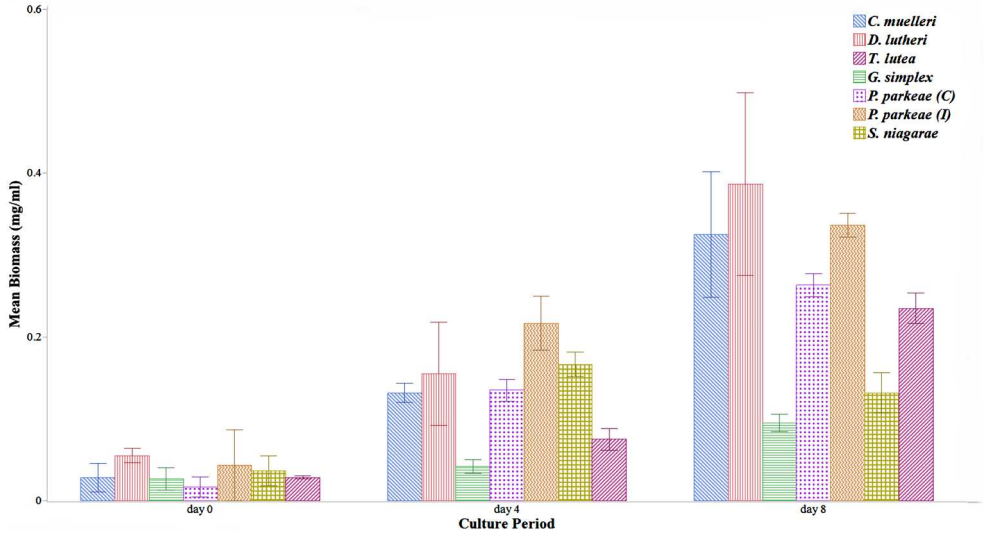

Figure 4. Mean microalgal biomass in the intermediate culture (±SE). Reference species: *Chaetoceros muelleri*, *Diacronema lutheri*, *Tisochrysis lutea*; and candidate species from the Cawthron Institute Culture Collection of Microalgae (CICCM): *Gymnodinium simplex*, *Pyramimonas parkeae* (C) and isolated species: *Pyramimonas parkeae* (I), *Stephanodiscus niagarae*.

while *D. lutheri* had a 2:4:1 P:L:C ratio (Table 5). Both *S. niagarae* and *P. parkeae* (I) were characterised by proportionately more lipid compared to protein and carbohydrate, i.e. P:L:C ratios of 2:3:1 and 3:5:1, respectively. The P:L:C ratio of *P. parkeae* (C) was characterised by low carbohydrate content compared with the protein and lipid (11:14:1).

Discussion

Cell density

Several factors affect the growth performance of microalgae, including salinity, light, pH, temperature and culture medium (FAO 1996). However, for microalgae grown in similar environmental conditions, their cell size influences the maximal cell density (Banse

Table 5. Proximate compositions of the microalgae species assessed in this study.

		Mean proximate composition (mg·g^{-1} ash-free dry weight) (±SE)			
	Microalgae	Protein	Lipid	Carbohydrates	P:L:C ratio
Reference species	*C. muelleri*	109.4 ± 2.48	254.8 ± 8.09	199.7 ± 11.84	1: 2: 2
	D. lutheri	170.5 ± 6.96	293.6 ± 7.95	67.4 ± 4.43	2: 4: 1
	T. lutea	107.8 ± 1.36	231.1 ± 9.41	171.3 ± 17.04	1: 2: 2
Candidate Species	*G. simplex*	175.2 ± 7.05	142.1 ± 12.00	22.3 ± 4.35	8: 6: 1
	P. parkeae (C)	138.7 ± 4.69	175.1 ± 2.33	12.2 ± 6.64	11: 14: 1
	P. parkeae (I)	103.6 ± 4.64	194.0 ± 9.23	35.5 ± 1.45	3: 5: 1
	S. niagarae	198.2 ± 2.12	262.2 ± 8.45	95.5 ± 2.99	2: 3: 1

Data are expressed as mean mg·g^{-1} AFDW (± SE) where $n = 3$.
P:L:C ratio: Protein: Lipid: Carbohydrate ratio.
P. parkeae (C) = strain from Cawthron Institute Culture Collection, *P. parkeae* (I) = isolated strain.

1976). Generally, microalgae with larger cell sizes achieve lower maximum cell density than smaller algal cells under culture conditions (Banse 1976; Agusti et al. 1987; Borowitzka 1992; Nielsen 2006). This is observed in the lower peak cell densities of candidate species (e.g. *C. camella*, *G. simplex*, *P. parkeae* (C), *P. parkeae* (I), *S. niagarae*) compared to peak cell densities for the much smaller reference species (e.g. *C. muelleri*, *D. lutheri*, *T. lutea*) in both starter and intermediate cultures. The same observation has been reported previously where the cell densities reached by cultures of *Chlorella minutussima* at the end of a 21-day culture period had higher cell density compared to *Scenedesmus* sp., which has 20 times more cell volume than *C. minutussima* (Klin et al. 2018). Similarly, the maximum cell densities of *Dunaliella* sp. (9–10 µm cell size) exhibited lower cell density than *Isochrysis* sp. (2–3 µm cell size) when cultured under identical conditions (Lananan et al. 2013).

The influence of cell size on the cell density reached in cultures of the microalgae can be due to several factors, such as its overall cell shape (Nielsen 2006) and the package effect which consists of a change in photosynthetic parameters related to the characteristics of the algal cell (Finkel and Irwin 2000; Malerba et al. 2018). Small microalgae cells have spherical and spheroidal shapes (Niklas 2000). However, as size increases, retaining such a shape would be a disadvantage because due to greatly decreasing efficiency of the diffusion pathways of substances from the surface to the interior of the microalgal cells (Nielsen 2006). Therefore, larger cells benefit if they are flattened to avoid limitations caused by prolonged diffusion pathways (Nielsen 2006).

The package effect slows the growth of cultures of larger cells, where the chlorophyll-specific absorption becomes less effective with cell size at a constant pigment concentration (Morel and Bricaud 1981; Finkel and Irwin 2000). This is largely attributed to the self-shading among pigments (Finkel and Irwin 2000; Malerba et al. 2018). Self-shading occurs in microalgae suspensions when high microalgal biomass at the surface increases its light absorption and reduces the light penetration into the interior of the culture vessel (Agusti et al. 1987; Ruiz-Marin et al. 2010), but it can also occur internally among cell pigments (Malerba et al. 2018). As an evolutionary response to minimise the package effect, the chlorophyll-*a* concentration per unit mass tends to decrease as cell size increases (Agusti 1991; Finkel and Irwin 2000; Malerba et al. 2018). The relationship between chlorophyll content and cell density is complex, but several studies have reported a link between the chlorophyll content and the cell density (Fabregas et al. 1985; Zhao et al. 2019; Tunali et al. 2020).

The cell densities of both *P. parkeae* (C) and *P. parkeae* (I) were higher when scaled up to the intermediate culture, although these did not reach the cell densities of the reference species. This cell density of *P. parkeae* (both strains) in the intermediate culture reached sufficient cell densities for commercially used microalgae, which are typically between 10^6 and 10^7 cells·ml^{-1} (Lananan et al. 2013). This indicates the two strains of *P. parkeae* should be considered further for mass culture, because the ability to reach these higher cell densities would enable production in large volumes (Lananan et al. 2013).

In contrast to *P. parkeae*, the cell densities of *G. simplex* and *S. niagarae* in the intermediate culture did not show an improvement beyond that observed for their starter cultures. This could either indicate that these two species have already reached their peak density or the changes in the culture conditions, especially active aeration in a larger

volume, are unsuitable for greater growth in these two species. In the starter culture set-up, a large head space and small volume of seawater media allow sufficient gas exchange (L. Rhodes, pers. comm.), and the swirling of the flask facilitates the mixing of nutrients and resuspension of microalgae. In contrast, aeration is introduced in the intermediate culture set-up to prevent the microalgae from settling and increase their exposure to light, while also facilitating sufficient gas exchange and nutrient mixing throughout the larger volume of seawater media (Brand et al. 2013; Lee et al. 2013). However, some microalgae cannot tolerate agitation, such as from active aeration (Sobczuk et al. 2006). This sensitivity to gas bubbles is reported in the culture of *Dunaliella* species, which can require carboxy-methylcellulose and agar included in the culture media to help protect the microalgal cells from physical damage (Silva et al. 1987). Similarly, the diatom *Phaeodactylum tricornutum* and the red algal *Porphyridium cruentum* can only tolerate limited aeration and require the addition of Pluronic F68 to culture media to protect their cells from being ruptured by gas bubbles (Sobczuk et al. 2006).

It is possible the 8-day culture period for the intermediate culture could have been insufficient for *G. simplex* and *S. niagarae* to reach maximum cell density if they have slower rates of cell division. However, commercial scale microalgae production cycles, especially for batch cultures, typically run within 3–7 days of culture (FAO 1996; Muller-Feuga et al. 2003; Creswell 2010). Therefore, extending the culture period to achieve higher cell density is less practical in a commercial setting as it will substantially reduce overall microalgal production from large-scale culture infrastructure which is costly to operate. Therefore, while microalgal cell density is only one determinant of growth performance, the shorter time required for microalgal species in culture to achieve sufficient cell density is essential in commercial and industrial applications (Lananan et al. 2013). Improvements in the growth performance of *G. simplex* and *S. niagarae* may be achieved through adjusting culture conditions such as nutrients and light quality, through further studies.

The experimental results indicated that F/2 media is not a suitable culture media for *C. camella* given its superior performance in GP media. The F/2 media (Guillard and Ryther 1962) is the most widely used nutrient media for microalgal culture, and pre-mixed formulations are commercially available (Creswell 2010), lessening the preparation time and labour. The ability of microalgae to proliferate in commonly used culture media, such as F/2 media, simplifies the culture process since hatcheries and nurseries usually cultivate more than one species (Muller-Feuga et al. 2003). However, *C. camella* can be grown under laboratory conditions, as evidenced by the growth observed in the GP media, and it is possible that simple additions to F/2 media might be sufficient to better meet the needs of *C. camella* and further testing of such adjustments may be warranted if the species shows promise as effective feed for juvenile bivalves.

Cell biomass

Biomass production in microalgal cultures is influenced primarily by the cell size or biovolume and cell density that can be achieved in culture (Janta et al. 2013; Lim et al. 2022). Biomass production is typically the primary consideration when evaluating microalgae

for culture (Muller-Feuga et al. 2003) as the cell biomass typically carries the biochemical constituents, such as protein, carbohydrate and lipid, in proportion to biomass the yield of which typically determines the value of the microalgae (Hossain 2019). It was previously hypothesised that species with larger cells will attain less dense populations with lower biomass than for small cell species (Agusti et al. 1987). However, this was not the case in the present study wherein there was no difference in the biomass yield of the large cell species (e.g. *G. simplex*, *P. parkeae* (C), *P. parkeae* (I), *S. niagarae*) and the small cell species (e.g. *T. lutea*, *D. lutheri* and *C. muelleri*). Other studies have found that smaller cells can have lower biomass yield despite achieving higher cell densities (i.e. *Chlorella* sp., *Nannochloropsis limnetica* sp., versus *Carteria* sp., *Monoraphidium* sp. and *Scenedesmus* sp.) (Janta et al. 2013). Overall, the result of this study shows that *G. simplex*, *P. parkeae* (C), *P. parkeae* (I) and *S. niagarae* can deliver the same biomass yield as the reference species and, therefore, hold potential as feed for bivalve spat.

Proximate content

The nutritional value of microalgae is affected by many factors such as the capability of bivalves to capture, ingest and digest the microalgae (Lora-Vilchis and Maeda-Martinez 1997; Reitan 2011), but their proximate composition (e.g. protein, lipids, carbohydrates, etc.), and the presence of toxic compounds largely determines their nutritional value for early stages of bivalves (Catarina and Xavier 2012). It is unclear whether the total lipid content of microalgae directly affects its nutritional value although this is highly likely (Knauer and Southgate 1999), but the fatty acid composition of the microalgae plays a particularly important role in larval development (Whyte et al. 1989; Brown et al. 1997; Rivero-Rodríguez et al. 2007; Pettersen et al. 2010) due to the limited ability of bivalves to synthesise highly unsaturated fatty acids (HUFA) and sterols de novo (Muller-Feuga et al. 2003). The ability of bivalves to synthesise HUFA may be life-stage specific and varies from species-to-species, but even when it is present, it may not be enough to support growth (Knauer and Southgate 1999). However, fatty acid composition appears to be less critical for the nutrition of juvenile bivalves (Flaak and Epifanio 1978; Enright et al. 1986b; Ponis et al. 2003). The overall nutritional requirement in juvenile mussel is not well established, but previous studies suggest that carbohydrate plays a significant role in the growth performance of juvenile mussels, in the presence of adequate dietary protein and lipid (Enright et al. 1986b; Pérez Camacho et al. 1998; Ponis et al. 2003).

Carbohydrates are the primary source of energy in early stages of bivalves (Holland and Hannant 1974), which are also indicated by the utilisation of glycogen (stored carbohydrates) by juvenile bivalves during periods of starvation (Laing 1993; Albentosa et al. 2007; Sim-Smith and Jeffs 2011; Supono et al. 2021). Adequate dietary supply of carbohydrate can also provide a protein-sparing action, allowing proteins to be utilised for growth instead of being catabolised to provide an energy source (Enright et al. 1986b). In most bivalves, nutritional energy assimilated from food is primarily directed toward covering the cost of metabolism, and any excess is distributed toward somatic growth (tissue or shell), accumulating energy stores as glycogen, and reproductive maturation (Fearman et al. 2009).

Protein is normally the most abundant proximate fraction of microalgae, although this proportion varies among species of microalgae (Martínez-Fernández et al. 2006; Fernández-Reiriz et al. 2015). This was not observed for the microalgae species in the current study, except for *G. simplex*, as lipid represented the most abundant proximate content in the other species. There were also previous reports of balanced calculated P:L:C ratio of *C. muelleri*, *D. lutheri* and *T. lutea* at ~1:1:1 P:L:C ratio (Saucedo et al. 2013; Liu et al. 2016), as opposed to a higher protein content of 12:3:1 and 14:9:1 P:L:C ratio of *C. muelleri* and *T. lutea*, respectively (Martínez-Fernández et al. 2006). There are various reasons that affect the biochemical composition of microalgae such as available carbon dioxide and nitrogen, light quality and quantity, as well as culture phase (Brown et al. 1993; Roncarati et al. 2004; Martínez-Fernández et al. 2006; Michelon et al. 2016). For example, the proximate composition of microalgae often varies significantly as they progress through different growth phases in culture, e.g. lipids and carbohydrates are higher while protein is lower in the stationary phase of culture compared to during the exponential phase of culture (Brown et al. 1993; Brown et al. 1997; Huerlimann et al. 2010). Regardless, there is no clear trend between the total protein content of microalgae and their overall value as food for bivalve juveniles (Enright et al. 1986a; Muller-Feuga, Robert et al. 2003; Catarina and Xavier 2012).

Pyramimonas parkeae (C) appears to have a very low carbohydrate content, despite being the same species as *P. parkeae* (I). Strains from the same species may demonstrate different characteristics and chemical composition that can be attributed to their unique responses to differences in environmental conditions (Figueroa Torres et al. 2021). While the carbohydrate content of *P. parkeae* (C) may be low, it may still be useful as a contribution to a diversified diet for juvenile bivalves. The P:L:C ratio of microalgae diets for bivalve spat is generally more balanced ranging from 1:1:1 (Saucedo et al. 2013; Liu et al. 2016) to 4:1:1 P:L:C ratio (Rivero-Rodríguez et al. 2007). Therefore, the proximate analysis underscores the potential of *S. niagarae*, *G. simplex* and *P. parkeae* (I) as viable feed sources for spat rearing due to its relatively balanced P: L:C ratio. A microalgae diet typically comprises of several microalgae species to cater to the comprehensive nutritional requirements during the early stages of bivalve development (Saucedo et al. 2013) Therefore, it might be more beneficial to use a combination of these microalgae to ensure a balanced nutritional intake by mitigating the risks associated with the monodietary limitations. For example, *S. niagarae* can be used together with *G. simplex* to compensate for the lower carbohydrate content of *G. simplex* while the latter supplements the protein content for *S. niagarae*. Nevertheless, the results of the proximate analyses indicate that *S. niagarae*, *G. simplex* and *P. parkeae* (I) hold promising potential as feed for juvenile bivalves and it is important to conduct further research to validate these findings and establish definitive guidelines for their application as spat feed in nursery culture.

Conclusion

The results of this current study indicate that *S. niagarae*, *G. simplex* and *P. parkeae* (I) demonstrate promising characteristics, including appropriately large cell size, sufficient biomass production and acceptable nutritional profile, making them good potential candidates for further appraisal for feeding juvenile bivalves in nursery culture. In contrast, the strain *P. parkeae* (C) appears to be nutritionally inferior to *P. parkeae* (I), particularly

in its carbohydrate content, limiting its suitability as a feed for juvenile bivalves. The microalga *C. camella* was not amenable to culture in standard F/2 media greatly limiting its potential for commercial culture at scale. For the four best performing microalgae species, there is a strong foundation for advancing their experimental testing to confirm their efficacy as feed for juvenile bivalves.

Acknowledgement

We would like to thank Cawthron Institute for providing support during the isolation and acquisition of microalgae, and Dr Aimee van der Reis and Fang Fei Tham for the molecular species identification.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research was supported by a Manaaki New Zealand Fellowship to Leslie Sampollo, together with the Marine Farming Association, and the Ministry for Primary Industries through the Sustainable Food and Fibre Futures fund #SFFF-23044.

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