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Utilising mixotrophically cultured ”designer algae” as Blue Mussel larval feed

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Abstract

The production of blue mussels is currently reliant upon supply of wild larvae, which is a limiting factor to upscaling production. A potential solution to address this limiting factor is to develop a larval hatchery; however, costs associated with microalgal cultivation to feed the larvae account for around 30 % of total costs. Microalgae are classically considered to be photoautotrophic and are cultured under light, in seawater with additional nitrate and phosphate. Many microalgae can be cultured mixotrophically, or heterotrophically, on an organic carbon source which results in an enhanced cell density and altered biochemical profile. Over the course of two feeding trials this study compares larval growth and survival employing current ‘industry standard’ and mixotrophic ‘designer’ microalgal feeds as diets. The ‘designer’ feed employed proved to be as effective as the standard feed and subsequent optimisation demonstrated that a diet tailored towards the development of the larvae performs the most effectively in

terms of larval growth. A sequential diet of mixotrophically cultured microalgae, where the primary component of the diet changed as the larvae increased in size, resulted in the greatest larval growth (174.35 μm) and total fatty acid content (7.20 % dry weight). First order modelling indicates that approximately ten times lower volume of mixotrophically cultivated microalgae was required to produce sufficient larval feed. Thus, mixotrophic culture has the potential to reduce the costs of microalgal cultivation and increase the profitability of a mussel larval hatchery.

Keywords

microalgae, mixotrophy, mussel aquaculture, larvae

1 Introduction

The production of shellfish averaged 13.47 million tonnes globally per year between 2010-2015 (Wijsman et al., 2019), with production of *Mytilus edulis* in Scotland has increasing from approximately 4000 tonnes in 2006 (Munro and Wallace, 2016) to approximately 7000 tonnes in 2018 (Munro et al., 2019). Commercially shellfish aquaculture in Scotland was valued at £9.5 million, of which blue mussels accounted for £7.8 million (Munro et al., 2019). This has stimulated the industry, supported by the Scottish government, to set a target to approximately double production of mussels by 2020 (Alexander et al., 2014). However, a major limiting factor in expansion of mussel aquaculture is larval supply, both in the UK and globally. Current production methods are 'at the mercy of wild supply' as they require the collection of wild spat (Galley et al., 2010), which is spatially and temporally variable (Lazo and Pita, 2012), with only 40 % of all spat collection sites in the UK producing 'sufficient' spat in 2018 (Munro et al., 2019). This variable nature of larval supply has large impacts upon mussel production, with a drop in spat supply resulting in a 15 % drop in production of mussels in Chile between 2011-2012 (Carrasco et al., 2014). There have been calls for the establishment of mussel larval hatcheries for over 20 years (Hickman, 1992) and there are shellfish hatcheries producing *M. galloprovincialis* seed (Kamermans et al., 2013); however, the main species in Scotland is *M. edulis* (Dias et al., 2009) for which, as yet, there is no larval hatchery.

Initially trochophore larvae are lecithotrophic, but later larval stages are planktotrophic and, in nature, only feed on live microalgae (Laing and Millican, 1992). Larvae can consume 30-60 % of their body mass in algae per day (Lutz and Kennish, 1992) and these must be <10 μm in diameter for efficient feeding (Knauer and Southgate, 1999). In addition to ensuring that the feed is within the correct size range, the biochemical profile of the feed is an important consideration. The optimal biochemical profile may change depending on the larval species, for example, in *Magallana gigas* larval culture the optimal fatty acid contents are considered to be: 7-17 % Eicosapentaenoic acid (EPA), 7- 14 % Docosahexaenoic acid (DHA) and 7-14 % total fatty acid (TFA) of the total diet (Rico-Villa et al., 2006), which maximised larval survival and growth, with good settlement success. Microalgal species which are commonly used, such as *Isochrysis galbana, Chaetoceros calcitrans* and *Diacronema lutheri* (formerly *Pavlova lutheri*) have the advantage of being easy to culture and have a good biochemical profile (Robert and Trintignac, 1997), although there is currently no 'standardised diet' in common use. This means comparison between diets is difficult, if not impossible, as different species have altered biochemical profiles, which may influence larval survival and growth (Brown, 1991). In addition some species have their own challenges to culture sufficient biomass, such as *C. calcitrans*, which often has low productivity and a higher likelihood of culture crash compared to other species (Pettersen et al., 2010).

The costs associated with microalgal culture, to produce biomass for commercial use are high. Estimates vary from €9-10 kg^{-1} dry biomass (Slade and Bauen, 2013), down to €3.4 kg^{-1} dry biomass for a facility of 100 hectares of photoautotrophically cultured microalgae in flat panel bioreactors (Ruiz et al., 2016); however, there is a large cost saving associated with economies of scale (Laing and Helm, 1981), with costs reported as high as €900 kg$-$1 (Benemann, 1992) when less than 1000 litres of microalgae is cultured to produce live feed for hatcheries. Previous studies of hatchery operating costs have calculated that the culture of live feed accounts at 30 % of total hatchery operating costs (Coutteau and Sorgeloos, 1992), 90 % of the value of the seed for a larval hatchery in the United States of America (Brake et al., 2001) and 91 % of the net present value of the seed of *Mytilus chilensis* in Chile (Carrasco et al., 2014). One mechanism to reduce these costs may be mixotrophic culture of the microalgal feed (Leonardos and Lucas, 2000). There are a number of advantages to utilising the ability of some microalgae to be cultured with an additional carbon source, termed mixotrophy (Burkholder et al., 2008), including a theo-

retically lower cost (Perez-Garcia et al., 2011), an increased cell density (Kumar et al., 2018), an increase in biomass (Day and Tsavalos, 1996) and the ability to tailor the biochemical profile of the microalgal species (Penhaul Smith et al., 2020). The ability to tailor the biochemical profile gives greater control of the diet and potential for optimisation based on specific biochemical components, rather than by species (Knauer and Southgate, 1999). In this study three mixotrophically cultured species: *Tetraselmis suecica, Phaeodactylum tricornutum* and *Cyclotella cryptica* were selected due to their enhanced cellular density and biomass when cultured mixotrophically, compared to photoautotrophic culture (Penhaul Smith et al., 2020). All three species have been demonstrated to be effective larval feeds (Gladue and Maxey, 1994), with *T. suecica* and *C. cryptica* having been previously reported to enhance settlement in *Tapes philippinarum* (Laing and Millican, 1992). Mixotrophic culture of *T. suecica* on glucose had a significantly increased carbohydrate content, a six times greater cell density and faster growth (7 days to reach stationary phase compared to 14) compared the photoautotrophically culture (Penhaul Smith et al., 2020). *P. tricornutum*, when cultured on glycerol, resulted in shorter cells with significantly greater protein and TFA contents when harvested at stationary phase. *C. cryptica* mixotrophically cultured on glycerol had nearly double the biomass compared to photoautotrophic cultures and a significantly increased carbohydrate content (Penhaul Smith et al., 2020). This study utilises these 'designer microalgae' as a feed for blue mussel larvae. Comparison between a photoautotrophically cultured 'reference' diet and mixotrophically cultured microalgal feeds in Trial 1 was initially undertaken, followed by optimisation of the mixotrophic diet in Trial 2.

2 Materials and methods

2.1 Feeding trials overview

The following study incorporates the results of two feeding trials undertaken between March 2018 and March 2019. While the overall protocol was maintained across the two trials, alterations were made between trials to fertilisation and sampling protocols as noted below.

2.1.1 Fertilisation

Live west coast Scottish mussels were purchased fresh, within 24 hours of delivery, and were kept in seawater at 10 °C for 48 hours before being moved to a fridge at 4 °C overnight. No genetic testing was undertaken, therefore the larval population in these feeding trials was likely to be a complex of *M. edulis* and *M. galloprovincialis*, the two species predominantly present in Scotland (Dias et al., 2009). These were induced to spawn by heat shock using the previously described methodology (Galley et al., 2010). Prior to spawning the mussels were placed in to 0.45 μm filtered seawater for 20 minutes before being placed in the 20 °C tank, which had been filled with 0.45 μm filtered seawater. Larvae were left to develop at 16 °C in 250 ml glass dishes at an approximate concentration of 200 eggs cm^{-2}. Twenty-four hours post-fertilisation the larvae were recovered from the glass dishes used for fertilisation and concentrated on to a 44 μm mesh for subsequent placement in to the feeding trials conical vessels. To enumerate these larvae, 15 ml of the concentrated culture was removed and fixed using three drops of formalin. Of this sample 3 ml was counted using a Sedgewick rafter slide (Pyser-SGI). Following this the larvae were allowed to settle and twenty were photographed using a stereo microscope (Zeiss Stemi 2000-c) and Axiovision (40x64 V4.9.1.0), and shell length measured using the image analysis program Image J(v1.51). All samples removed for subsequent analysis were stored at -20 °C and freeze-dried for a minimum of 24 hours until dry, unless otherwise stated.

2.1.2 Larval culture

Fertilisation success changed between trials; therefore, the relevant volume of larvae was removed from the previously concentrated cultures and these were placed in to 44 μm mesh sieves in 15 identical, static, eight litre conical vessels at a concentration of 10 larvae ml^{-1} similar to the one previously described by Kamermans (2008). Each algal diet was tested in triplicate and the diets were assigned to random conicals to ensure independence. The conical vessels were filled with seawater that had been filter sterilised (0.45 μm) and UV treated, with additional aeration after seven days post-fertilisation (0.0038 litres of air second^{-1} through 0.2 μm Whatman HEPA in line filters). Larval culture took place in a temperature controlled room kept at 16 °C for the duration of the study. The seawater in the vessels was changed every Monday, Wednesday and Friday (70 % on Mondays and Fridays and a full water change on Wednesday). During the full water change sieves were lifted and larvae washed in to a 44 μm mesh sieve. These were

then resuspended in 1 litre of seawater and 10 ml removed for counting and shell length determination using the above methodology. The amount of algae fed to the larvae was adjusted to account for the number of surviving larvae. The larvae were fed using live algal feed, after the water change, to the defined FAO *Isochrysis* equivalent ratio, for known larval lengths of *M. gigas* (Helm and Bourne, 2004) (Supplementary material, Table: 1). The microalgal cell volumes were taken to be equal to those previous measured (Dickson and Kirst, 1987; Helm and Bourne, 2004). After 16 days post-fertilisation the larvae were transferred in to 80 μm mesh sieves due to their increased size.

2.1.3 Algal culture

Six different algal species were cultured; three mixotrophically and three photoautotrophically. The cultures grown mixotrophically were bacteria-free strains of: *Phaeodactylum tricornutum* CCAP 1055/1, *Tetraselmis suecica* CCAP 66/60 and *Cyclotella cryptica* CCMP 332. The species cultivated photoautotrophically were: *Chaetoceros calcitrans* CCAP 1010/11, *Diacronema lutheri* CCAP 931/1 and *Isochrysis galbana* CCAP 927/1. Algal cultures were maintained in ten litre carboys with constant aeration, through an air-stone (0.12 litres air second^{-1} via 0.2 μm Whatman HEPA in line filters) at 20 °C under a 12h:12h light:dark cycle (white light, 1.5 x10^{2} μMol photons m^{-2} s^{-1}). The mixotrophic species were stirred using a magnetic paddle at 100 rpm,. All cultures were maintained for 9 days prior to the first larval feed and were maintained for 21 days. Each ten litre carboy was inoculated using a healthy one litre culture which had been previously grown from a master stock culture. The medium utilised for the photoautotrophic species was f/2, with the addition of silica for the diatom species employed (Guillard and Ryther, 1962). Mixotrophically grown cultures were also grown in f/2 supplemented with 0.5 g l^{-1} of yeast extract and either: 5.12 g l^{-1} of glycerol (*P. tricornutum* and *C. cryptica*) or 5 g l^{-1} of glucose (*T. suecica*). Prior to each feed a 50 ml sample was removed, of this 1 ml was used for cell density counting using the haemocytometry. A further 1 ml of sample was used for cell width/ diameter measurements using a coulter counter (Penhaul Smith et al., 2020). The remaining sample was centrifuged (3721 g, Heraeus Multifuge X3FR, 20 minutes), resuspended in 10 ml of ultra-pure water, frozen and freeze-dried. The freeze-dried material was subsequently analysed for fatty acids using a methanolic HCl, direct derivitisation method (Slocombe et al., 2015), modified as previously described in (Penhaul Smith et al., 2020).

2.2 Trial 1

The diets tested were: a photoautotrophically cultivated diet (20:40:40 *Chaetoceros*, *Isochrysis* and *Diacronema*) as a 'reference' diet (a diet previously shown to be effective in a preliminary unpublished feeding trial, conducted by Penhaul Smith in 2017), a single species diet of *T. suecica*, *P. tricornutum* and *C. cryptica* and a mixed diets of the three species, as an equal mix (33:33:33). To ensure that excess organic carbon, present in the mixotrophic media, did not cause a bacterial bloom, all mixotrophic cultures were centrifuged (3721 g, Heraeus Multifuge X3FR, 20 minutes) and resuspended in to filtered seawater (0.2 μm) using 10 ml of seawater for every 50 ml of algae extracted. Adult mussels were purchased on the 18^{th} of May and the trial started on 21^{st} of May. Following spawning, at two days post-fertilisation, 65 ml of the concentrated larvae were used to make a concentration of 8.87 larvae ml^{-1} per conical. A further 25 ml of larvae was centrifuged (3721 g, Heraeus Multifuge X3FR, 20 minutes), frozen and freeze-dried for fatty acid analysis. During the trial conical number eight was damaged so was discarded (*P. tricornutum* alone) and results discounted. Upon the conclusion of the feeding trials each sieve was concentrated to 500 ml. Following this 5 ml was transferred for counting and size measurements, using cut tip 10 ml pipettes. The remainder was further concentrated down to 250 ml, by filtering through a 44 μm sieve and was centrifuged (3721 g, Heraeus Multifuge X3FR, 20 minutes), frozen and freeze-dried.

2.3 Trial 2

Diet optimisation utilising mixotrophically cultivated microalgae was performed to maximise survival and growth of the larvae. The diets were: an equal mix of all three mixotrophic microalgae as a 'reference' diet, a diet containing a 60:20:20 mix of each of the mixotrophically cultivated microalgae and a sequential diet. The sequential diet also contained a 60:20:20 mix of mixotrophic microalgae, with the 60 % component of dietary microalgae changing every week, following the full water change. The order of microalgae utilised was: *P. tricornutum, T. suecica* and *C. cryptica*. Cultures of *P. tricornutum* were cultured semi-continuously with the addition of five litres of medium 18 days post-fertilisation (21 days of culture). In addition to fatty acid analyses, a further 100 ml of culture was removed and analysed for protein, carbohydrate and cell lengths using the previous described methodology (Penhaul Smith et al., 2020). All seawater was filtered through a 0.2 μm filter, to reduce any potential bacterial load prior to use,

and the trial ran for 23 days post-fertilisation. Mussels were purchased on the 27^{th} of February and were maintained at 9 °C and fed for five days prior to spawning using approximately 1 ml of Instant Algae 1800 (Reed Mariculture) per mussel. Spawning took place on the 4^{th} of March and prior to fertilisation 150 ml of embryos were collected for fatty acid analysis. At 2 days post-fertilisation 50 ml of the larvae were required per conical to ensure that each conical had a larval concentration of 12.77 ml^{-1}. The remaining 150 mlof sample was utilised for fatty acid analysis and vacuum filtered onto a 0.45 μm (pore size) ashed, gf/f filter paper to maximise lipid recovery. All samples were frozen and freeze-dried. During the trial larvae were removed using cut-tip pipettes to ensure no size selection. At 23 days post-fertilisation following the removal of 10 ml aliquots for counting, the larvae were concentrated to 160 ml. Of this 10 ml was removed for dry weight measurements, while the remainder was frozen at -20 °C until it could be vacuum filtered on to 0.45 μm (pore size) ashed filters. These were then frozen and freeze-dried for fatty acid analysis.

2.4 Larval fatty acid analysis

Following freeze-drying the larval lipids were extracted using a modified Folch extraction (Folch et al., 1957). Briefly, 4 ml of chloroform: methanol 2:1 (*v/v*) was added to the samples and these were homogenised using an Ultra Turex (Cam lab, Janke & Kunkel GmbH) homogeniser. This homogenate was filtered through Whatman number 1 paper filter. The filter was pre-washed with chloroform: methanol to remove contaminants. Using 3 ml of chloroform: methanol the vials were washed. The initial extraction was modified for samples from Trial 2. The freeze-dried filters were cut in to 4 pieces and placed in to 4 ml of chloroform: methanol (2:1) and sonicated for 5 minutes. To the filtrate 1.5 ml of 0.88 % (*w/v*) potassium chloride solution was added. These were then vortexed and centrifuged (50 g, Eppendorf centrifuge 5810R, 2 minutes). The upper layer containing water and methanol was removed and the lower layer had a stream of nitrogen blown over the sample until dry (N-EVAP system), before being dessicated for a minimum of 45 minutes. Lipid samples were weighted prior to methylation using 50 μl of 23:0 internal standard, 1 ml of toluene, and 2 ml of the methanol sulphuric acid (99:1 *v/v*). The samples were vortexed for 10 seconds, the tube purged with nitrogen and these were left at 50 °C overnight.

After allowing samples to cool a further 2 ml of ultra-pure water and 2 ml hexane: diethyl (1:1 *v/v*) were added. These were vortexed, centrifuged (50 g, Eppendorf centrifuge 5810R, 2 minutes) and the upper layer transferred to a clean glass boiling tube using a Pasteur pipette. To this a further 2 ml $NaHCO_3$ (2 % *w/v*) was added, vortexed, centrifuged and the lower layer discarded using a Pasteur pipette. This was dried using a stream of nitrogen and desiccated for a minimum of 45 minutes. After weighting the samples were resuspended to a concentration of 1 mg ml^{-1}, the entirety of the sample applied to the base of a thin layer chromatography plate, with excess solvent removed with nitrogen gas. The TLC plate was developed in a hexane: diethyl ether: acetic acid (90:10:1) solvent system. Once the solvent front was within 2 cm of the top of the TLC plate it was removed from developing tank and solvents allowed to evaporate (approximately 20 mins). The TLC plate was developed by spraying the plate lightly with dichlorofluorescien stain and the fatty acid methyl esters visualised using UV light. These FAMEs were scraped off plate and placed in a new, clean glass vial. To this vial 2 ml hexane: diethyl ether (1:1 *v/v*) and 1 ml $NaHCO_3$ (2 % *wt/v*) was added, vortexed and centrifuged (50 g, Eppendorf centrifuge 5810R, 2 minutes). This was frozen overnight -20 °C. Following this the upper layer was removed to second clean tube, dried using nitrogen and dessicated for approximately 30 minutes. The FAMEs were weighted and resuspended at a concentration of 1 mg ml^{-1} and stored at -20 °C under nitrogen prior to analysis using GC-FID (Shimadzu 2014).

2.5 Statistic screening and analysis

Larval growth, survival and the microalgal biochemical profiles in Trial 2 were all initially tested for normality (Shapiro-Wilks test). If these data were normally distributed ($P>0.05$) and variances could be considered to be equal (Levene's test $P>0.05$) then ANOVA and *post hoc* Tukey's test was used to elucidate the differences. If these data were not normally distributed ($P<0.05$) a Kruskal- Wallis test and *post hoc* Dunn's non-parametric comparison were utilised (Dinno, 2015). This analysis was conducted in Minitab (V18.0). Larval and feed fatty acid profiles were analysed in Rstudio (v3.6.1). This was done by placed the profiles into a dissimilarity matrix (Euclidean) utilising the 'dist' function in R studio, without transformation, and comparing the differences between groups analysed using ANOSIM. *Post hoc* the drivers of the differences between groups was understood using SIMPER, followed by graphical display using Non-metric Multidimensional Scaling (nMDS) utilising the vegan package for nMDS display (Ok-

sanen et al., 2019). The same method was utilised for the biochemical profile of feeds utilised in Trial 2. All values were displayed to 2 decimal places.

2.6 Costs and volume of media modelling

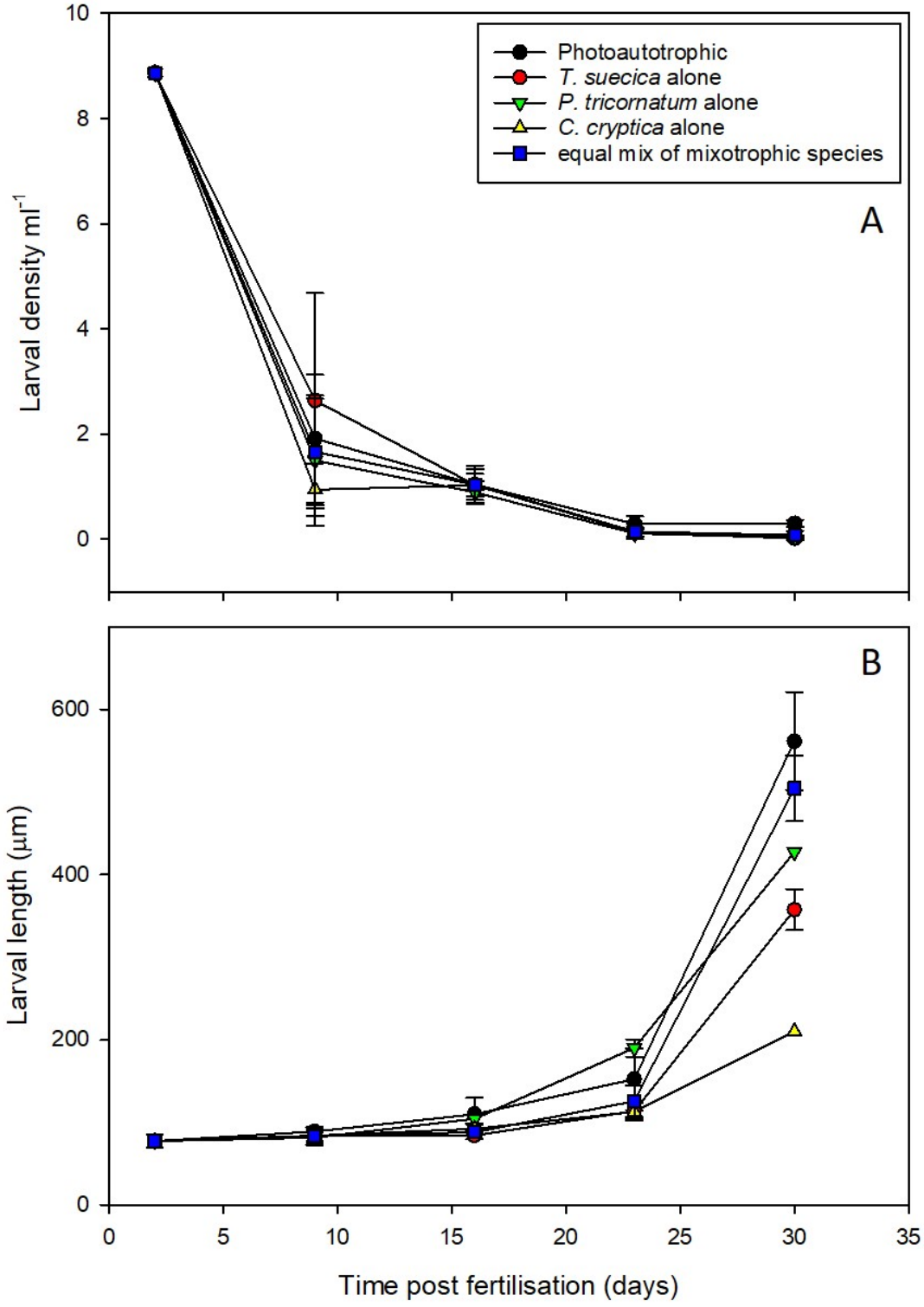
The costs and volume of media required were calculated utilising a first order model (Table: 5). In this model, the price of each reagent grade component, at the lowest price available, was taken from the Sigma-Aldrich website in August 2019 (Merck KGaA, 2019). The average productivities per feeding trial for each microalga were utilised to calculate the amount of media required to cultivate 10^6 larvae assuming that: there was no larval mortality and the same feeding ratios were utilised for the named Trials. The volumes of media required were calculated both as the total volume required and the costs of that media. This modelling purely accounted for the volumes of media required, not the other operational of capital expenditures such as space required for this cultivation, aeration, stirring, harvesting, light input, or maintaining the temperature of culture. All outputs were displayed to 2 decimal places.

3 Results

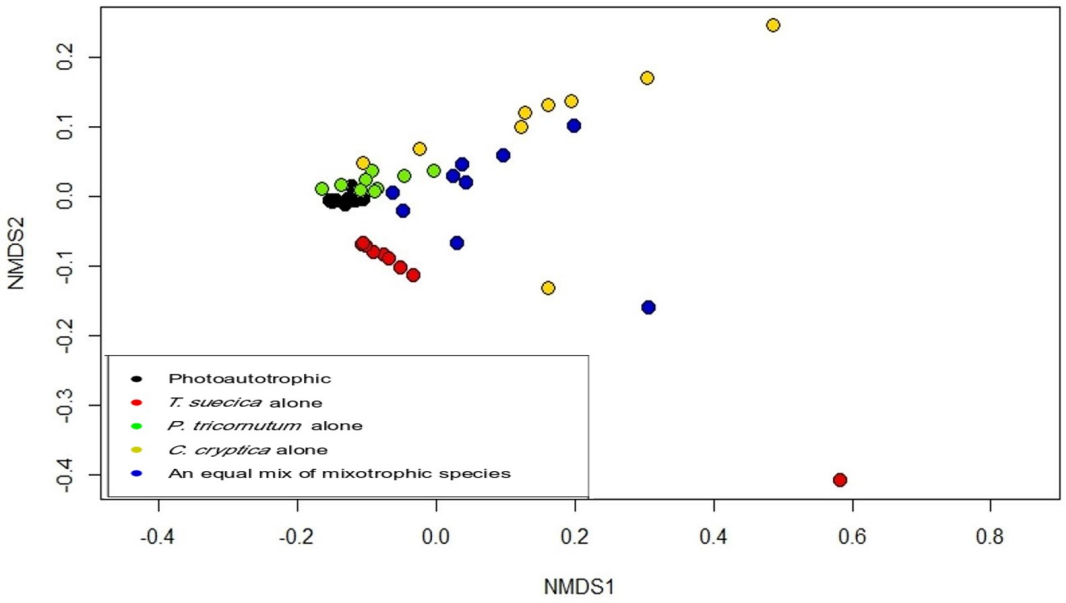
3.1 Trial 1

Across the time that feeding trial 1 was run, the larval survival declined, with the greatest drop in larval survival over the seven days post fertilisation (Fig: 1A). By contrast larvae grew across the trial, with the greatestet in crease in growth during the final seven days post fertilisation (Fig: 1B). Larval survival at the end of the trial were not normally distributed (Kolmogorov-Smirnov, $P<0.05$). At the end of the trial there was a significant difference in the larval survival (Kruskal-Wallis H= 14.73, df= 4, $P<0.05$, Fig: 1A). *Post hoc* Dunn's non-parametric comparison indicated that the photoautotrophically cultivated 'reference' diet resulted in a significantly increased larval survival compared to employing *T.suecica* or *C. cryptica* alone, but not *P. tricornutum* alone or the mixed mixotrophically cultivated diet (median= 4, range= 6, median= 1, range= 1 larvae ml^{-1} for the photoautotrophically cultivated 'reference' and *C. cryptica* alone, respectively). The larval growth at the final sampling point was not normally distributed (Kolmogorov-Smirnov, $P<0.05$). There was a significant difference in larval growth (Kruskal-Wallis, f= 17.61, df= 4, $P<0.05$, Fig: 1B), with using *C. cryptica* alone resulting in a significantly reduced growth

compared to both the mixed species diets (*post Hoc* Dunn's non-parametric comparison, median= 210.08 μm compared to median= 439.39 μm for the mixotrophic mixed diet). Furthermore, the photoautotrophically cultivated 'reference' diet also resulted in greater larval growth compared to *T. suecica* alone, but not the mixed mixotrophically cultivated diet. There were greater differences in the larval fatty acid profile within groupings that between the different diets (ANOSIM, R= -0.001972, P=0.4594, Supplementary data, Table: 2).


Photoautotrophic
T. suecica alone
P. tricornatum alone
C. cryptica alone
equal mix of mixotrophic species
A
B
Larval density ml-1
Larval length (µm)
Time post fertilisation (days)
0
2
4
6
8
10
200
400
600
5
15
20
25
30
35

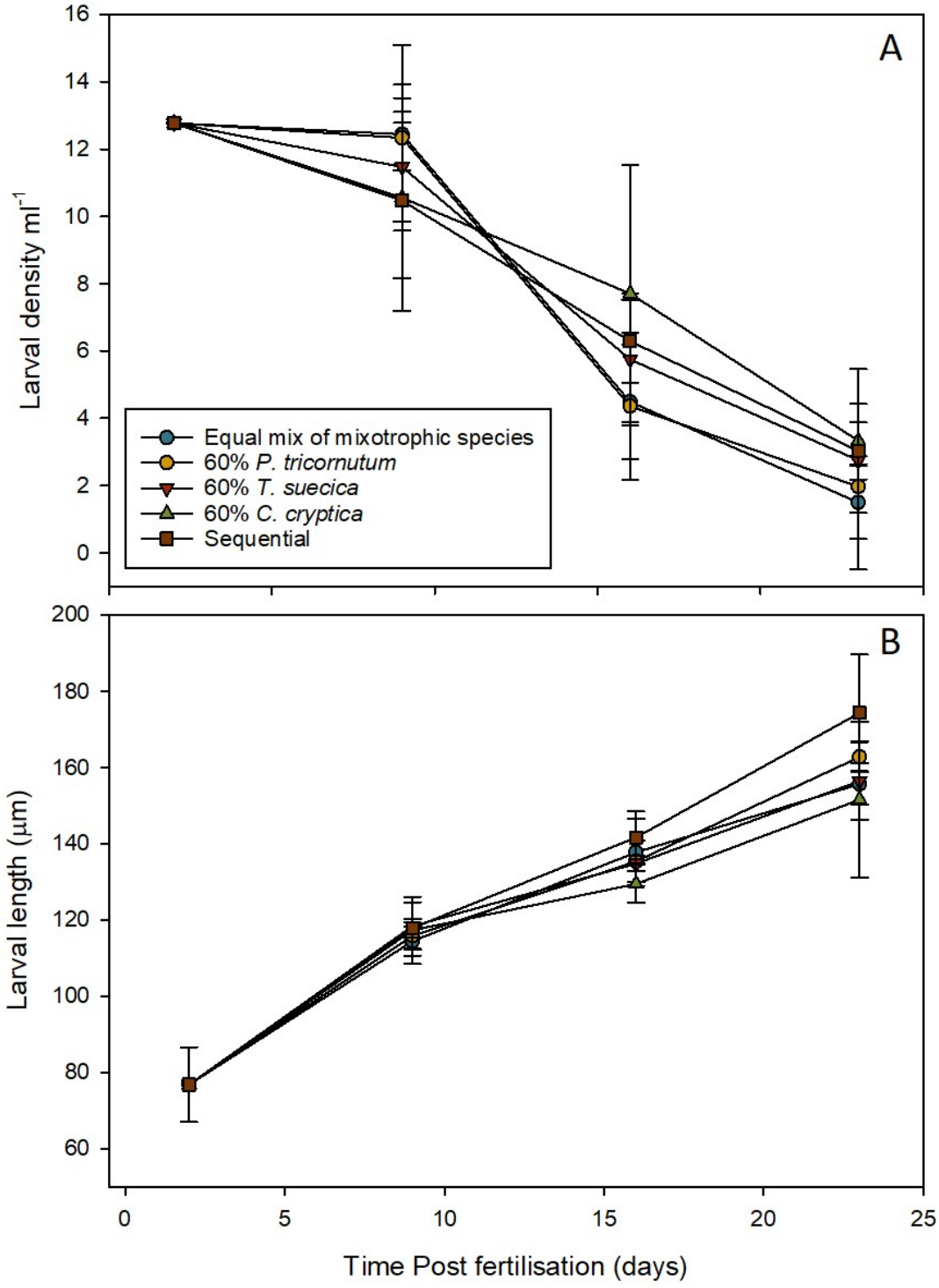
When the mixed diets were combined in to a single overall diet, assuming no preferential feeding by the larvae, and plotted in a dissimilarity matrix compared to the single species feeds, the untransformed feed fatty acid profiles for Trial 1 produced an arch plot, therefore the data was square root transformed (McCune et al., 2002). Once transformed this matrix significantly explains a proportion of the the variation (ANOSIM, R=0.4756, $P<0.05$, Fig: 2). *Post Hoc* SIMPER ($P<0.05$) indicated that the fatty acids primarily leading to this dissimilarity was a reduction in: 16:0, 17:0, 18:1 n-9, 18:2 n-6, 18:3 n-6, 20:3 n-6 and 22:2 in the photoautotrophically cultivated 'reference' diet compared to *T. suecica* alone, while there was a significant increase in: 18:3 n-3, 22:0, 22:1 n-9 and DHA. This increase in 22:0 was consistent when compared to all other diets. Compared to *P. tricornutum* alone, the photoautotrophically cultivated 'reference' diet had a significantly reduced content of 17:1, 24:0, 20:4 n-6 and EPA, and when compared to *C. cryptica* alone, a reduced: 15:0, 16:0, 16:1, 17:1 and 18:0 content. *T. suecica* as a single species diet also displayed a significantly change in fatty acid profile compared to the remaining mixotrophically cultivated feeds, with an increase 16:0, 17:0, 18:0, 18:1 n-9, 18:2 n-6, 18:3 n-6, 18:3 n-3, 20:1 n-9, 20:3 n-3, 20:3 n-6 and 22:2 content compared to *P. tricornutum* alone and an increased 18:1 n-9, 18:2 n-6, 18:3 n-6, 18:3 n-3, 20:1 n-9, 20:3 n-6 and content compared to *C. cryptica* as a single species diet. *P. tricornutum* as a single species diet had a significantly reduced content of 15:0, 16:0, 16:1, 18:0, 20:4 n-6 and DHA compared to *C. cryptica* and a significantly increased content of 20:3 n-3 and 24:0. Finally compared to the mixed mixotrophically cultivated diet, *P. tricornutum* also had a reduced 18:0 content (Table: 1).


NMDS2
NMDS1
-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
Photoautotrophic
T. suecica alone
P. tricornutum alone
C. cryptica alone
An equal mix of mixotrophic species

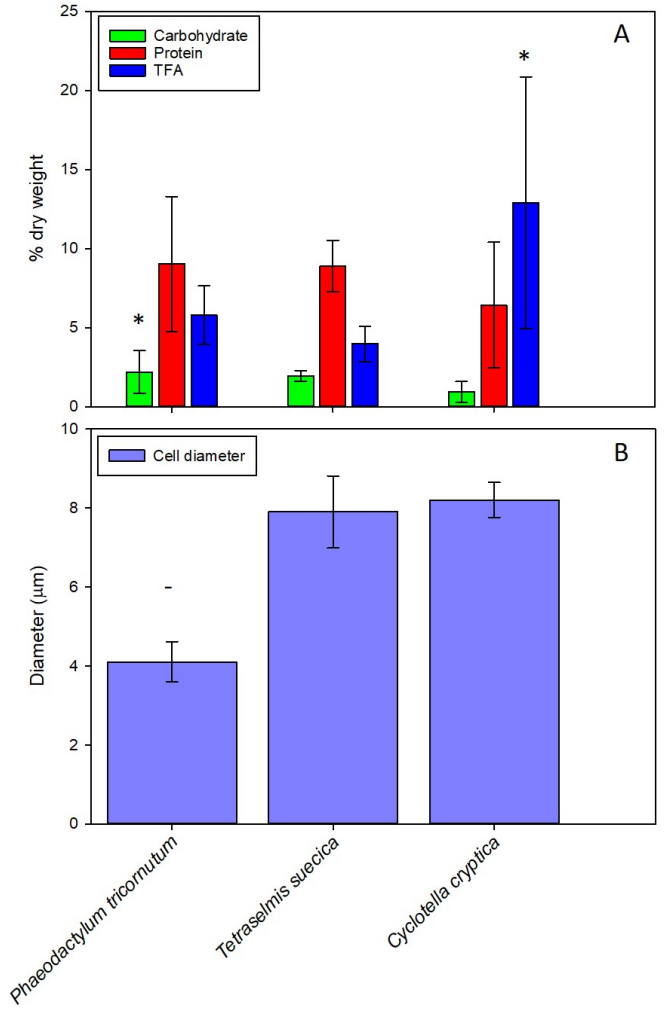
Species	C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1 n-9	C18:2 n-6	18:3 n-6	C18:3 n-3	C20:0	C20:1 n-9	C20:2	20:3 n-6	C20:3 n-3	C20:4 n-6	EPA	C22:1 n-9	C22:0	C22:2	C24:0	DHA	TFA
C. calcitrans	0.61	0.034	1.073	1.036	0.063	0.14	0.058	0.065	0.0069	0.0030	0.0098	0.0037	0.053	0.0031	0.00080	0.0069	0.0046	0.33	0.0060	0.0043	0	0.00093	0.028	4.22
D. lutheri	1.10	0.017	0.83	0.19	0.012	0.020	0.10	1.37	0.26	0.0019	0.28	0.0079	0.18	0.045	0.0076	0.0095	0.0085	0.075	0.0050	0.045	0.00074	0	0.46	5.58
I. galbana	0.70	0.079	1.18	1.49	0.0036	0.0076	0.035	0.13	0.083	0.036	0.023	0	0.059	0.0092	0.0012	0.017	0	0.75	0.0012	0	0.0016	0	0.61	5.73
T. suecica	0.076	0.0063	2.21	0.043	0.086	0.062	0.036	1.13	0.46	0.068	0.93	0	0.14	0.0031	0.10	0.020	0.00069	0.17	0	0	0.0043	0	0	6.022
C. cryptica	4.013	0.14	1.32	3.30	0.045	0.21	0.039	0.40	0.087	0.0061	0.080	0.0022	0.054	0.013	0.0023	0.0020	0.0026	0.60	0	0.0056	0.00056	0	0.26	11.082
P. tricornutum	3.19	0.11	1.97	3.12	0.082	0.63	0.028	0.95	0.19	0.030	0.17	0.0057	0.066	0.0074	0.0029	0.068	0.015	2.26	0.0029	0.00059	0.0087	0.14	0.11	13.62
Standard deviation																								
C. calcitrans	0.45	0.021	0.60	0.48	0.043	0.054	0.036	0.070	0.0083	0.0090	0.014	0.0073	0.16	0.0061	0.0024	0.021	0.014	0.17	0.013	0.0068	0	0.0028	0.024	1.85
D. lutheri	0.42	0.0086	0.14	0.34	0.022	0.049	0.041	0.60	0.16	0.0056	0.13	0.0094	0.14	0.038	0.0075	0.0086	0.0066	0.13	0.0062	0.024	0.0022	0	0.25	1.28
I. galbana	0.32	0.089	0.50	0.84	0.0075	0.0099	0.020	0.059	0.033	0.020	0.010	0	0.16	0.0057	0.0036	0.013	0	0.44	0.0035	0	0.0031	0	0.38	2.73
T. suecica	0.017	0.0050	0.40	0.011	0.044	0.044	0.0078	0.53	0.27	0.025	0.25	0	0.12	0.0051	0.027	0.017	0.0021	0.064	0	0	0.0054	0	0	1.50
C. cryptica	1.86	0.081	0.73	2.79	0.058	0.25	0.025	1.06	0.21	0.0054	0.20	0.0050	0.16	0.033	0.0069	0.0059	0.0078	0.58	0	0.017	0.0017	0	0.36	5.81
P. tricornutum	1.94	0.10	1.49	2.27	0.13	0.39	0.028	0.78	0.20	0.036	0.090	0.0063	0.15	0.0098	0.0046	0.059	0.017	0.96	0.0066	0.0018	0.024	0.097	0.069	7.36

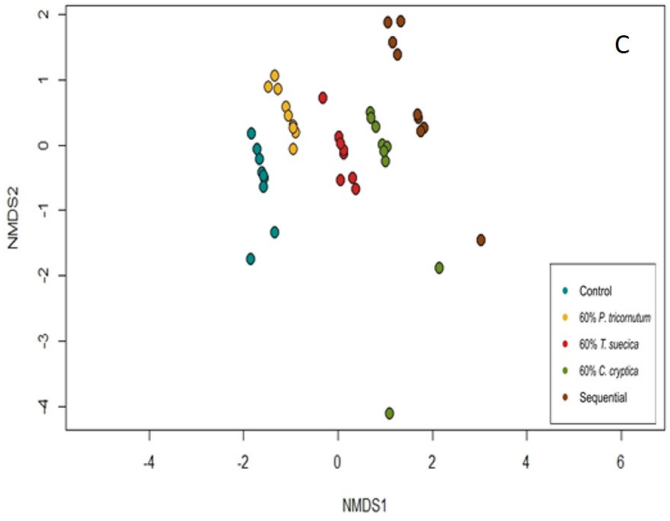
3.2 Trial 2

Across the period of the feeding trial, larval survival appeared to be highest initially in diets with a greater content of small width/ diameter microalgal species, followed by survival rates declining more rapidly in these feeds, compared to those with a higher proportion of larger microalgal species (Fig: 3A). Growth rates appeared initially to be high, followed by a decline later in the trial (Fig: 3B). The larval survival at the end of the study was normally distributed (Kolmogorov-Smirnov, $P>0.05$) and variances could be considered to be equal (Levene's test, $F= 1.56$, $P>0.05$). There was no significant difference in the survival between diets (ANOVA, $f= 2.33$, $df= 4$, $P>0.05$). Larval growth at the end of the trial was normally distributed (Kolmogorov-Smirnov, $P>0.05$) and variances could not be considered to be equal (Levene's test, $f= 3.47$, $P<0.05$). The ratio of maximum: minimum variance was less than three, allowing use of ANOVA (Dean et al., 1999). At the end of the trial the sequential diet had a significantly greater larval length compared to all diets, except the 60 % *P. tricornutum* diet (ANOVA, *Post Hoc* Tukey's test, $f= 6.32$, $df= 4$, $P<0.05$, Fig: 3A). The dissimilarity matrix significantly explaining only a some of the variation in the larval fatty acid profiles (ANOSIM, $R= 0.26$, $P<0.05$, Fig: 3B, Supplementary data, Table: 3). *Post Hoc* SIMPER shows that this variation is primarily driven by a significant increase in the TFA (mean= 6.44, s.d= 2.19 for 60 % *C. cryptica*, compared to mean= 2.31, s.d= 2.20 %DW for 60 % *T. suecica*) and C20:2 contents of both the 60 % *C. cryptica* and sequential diets, which were significantly greater compared to all other diets.


A
B
Larval density ml-1
Larval length (µm)
Time Post fertilisation (days)
Equal mix of mixotrophic species
60% P. tricornutum
60% T. suecica
60% C. cryptica
Sequential
0
2
4
6
8
10
12
14
16
60
80
100
120
140
160
180
200
0
5
10
15
20
25

Between microalgal species there were significant changes in the biochemical profile (Table: 2 and supplementary material, Table: 4 and 5). Carbohydrate and protein content were normally distributed (Kolmogorov-Smirnov $P>0.05$) and variances could be considered to be equal (Levene's test $P>0.05$, $F=2.6$ and 2.02 respectively) while the TFA and cell diameters were not normally distributed (Kolmogorov-Smirnov $P<0.05$). *P. tricornutum* had a significantly greater carbohydrate content compared to *C. cryptica* (ANOVA and *post hoc* Tukey's test, $F=5.16$, $df=2$, $P<0.05$). There were no significant differences between the protein contents of any of the species utilised in this trial (ANOVA, $F=1.57$, $df=2$, $P>0.05$). *T. suecica* contained significantly less TFA compared to *C. cryptica* (Kruskal-Wallis and *post hoc* Dunn's non-parametric comparison, $H=10.87$, $df=2$, $P<0.05$). *P. tricornutum* had a significantly lower cell length compared to the cell diameters of *T. suecica* and *C. cryptica* (Kruskal-Wallis and *post hoc* Dunn's non-parametric comparison, $H=17.51$, $df=2$, $P<0.05$). When the mixed diets were combined in to a single overall diet, assuming no preferential feeding by the larvae, analysed using a dissimilarity matrix and plotted graphically (Fig: 4C) there were greater differences between the average biochemical composition of the diets than within the diets (ANOSIM, $R=0.61$, $P<0.05$). *Post Hoc* SIMPER shows there are significant differences in the cell diameter with the 60 % *P. tricornutum* diet have a significantly lower mean cell length compared to the diets containing 60 % *T. suecica*, *C. cryptica* and the sequential diet, while the 60 % *T. suecica* diet contained significantly less EPA compared to the *C. cryptica* diet (mean= 0.034, s.d= 0.014 compared to mean= 0.073, s.d= 0.036 pg).


A
Carbohydrate
Protein
TFA
% dry weight
25
20
15
10
5
0
*
*
B
Cell diameter
Diameter (µm)
10
8
6
4
2
0
Phaeodactylum tricornutum
Tetraselmis suecica
Cyclotella cryptica
Species


C
NMDS2
2
1
0
-1
-2
-3
-4
-4
-2
0
2
4
6
NMDS1
Control
60% P. tricornutum
60% T. suecica
60% C. cryptica
Sequential

Species	Cell Diameter/ length (µm)	Carbohydrate (% DW)	Protein (% DW)	TFA (% DW)	14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1 n-9	18:2 n-6	18:3 n-6	18:3 n-3	20:0	20:1 n-9	20:2	20:3 n-6	20:3 n-3	20:4 n-6	EPA	22:1 n-9	22:2	24:0	DHA
P. tricornutum	4.11	2.19	9.00	5.79	1.87	0.54	1.41	0.76	0.32	1.22	0.13	0.26	0.20	0.18	0.18	1.47	0.078	0.0047	0.50	0.012	0.012	2.28	0.78	0.0015	0.064	0.11
T. suecica	7.91	1.94	8.89	3.97	0.89	0.35	1.87	0.71	0.11	0.14	0.69	0.78	0.29	0.20	0.87	0.31	0.72	0.0039	1.47	0.041	0.015	0.20	0.041	0.0019	0.0039	0.0019
C. cryptica	8.21	0.92	6.42	12.90	2.68	0.28	1.14	3.024	0.47	0.44	0.041	0.095	0.12	0.89	0.17	0.25	0.39	0.31	0.99	0.0087	0.23	1.29	0	0.0030	0.0040	0.079
Standard deviation																										
P. tricornutum	0.51	1.34	4.28	1.86	1.87	1.51	2.64	0.53	0.33	2.98	0.23	0.21	0.13	0.25	0.21	4.40	0.23	0.0095	1.47	0.013	0.030	2.66	2.34	0.0044	0.059	0.23
T. suecica	0.91	0.34	1.61	1.12	1.61	0.68	1.46	1.61	0.27	0.36	1.72	0.75	0.16	0.33	0.57	0.79	1.081	0.0076	3.29	0.035	0.026	0.15	0.12	0.0060	0.012	0.0058
C. cryptica	0.44	0.66	3.98	7.96	2.30	0.26	1.00	3.049	0.56	0.59	0.046	0.056	0.26	1.81	0.35	0.64	0.89	0.83	2.36	0.017	0.45	1.14	0	0.0089	0.0084	0.12

microalgal feed culture conditions	Diet ratio	Cell Diameter/ width (µm)	Carbohydrate (% DW)	Protein (% DW)	TFA (% DW)	C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1 n-9	C18:2 n-6	C18:3 n-6	C18:3 n-3	C20:0	C20:1 n-9	C20:2	C20:3 n-6	C20:3 n-3	C20:4 n-6	EPA	C22:1 n-9	C22:2	C24:0	DHA
T. suecica: *P. tricornutum*: *C. cryptica*																											
Mixotrophic 'reference' from Trial 1	1 (33:33:33)	6.67	0.075	0.59	0.96	0.14	0.032	0.064	0.096	0.015	0.019	0.013	0.087	0.0091	0.12	0.048	0.016	0.018	0.091	0.096	0.0024	0.036	0.052	0.0048	0.00014	0.00089	0.0058
Mixotrophic	2 (20:60:20)	5.68	0.068	0.45	0.68	0.10	0.023	0.049	0.065	0.012	0.020	0.0089	0.063	0.0075	0.075	0.030	0.019	0.011	0.055	0.061	0.0016	0.022	0.049	0.0076	0.000094	0.0012	0.0044
Mixotrophic	3 (60:20:20)	7.19	0.073	0.50	0.75	0.10	0.025	0.068	0.069	0.011	0.014	0.019	0.023	0.0099	0.077	0.043	0.015	0.023	0.055	0.082	0.0020	0.022	0.034	0.0040	0.00013	0.00064	0.0036
Mixotrophic	4 (20:20:60)	7.32	0.087	0.84	1.48	0.23	0.050	0.077	0.16	0.023	0.025	0.011	0.085	0.010	0.22	0.072	0.015	0.019	0.17	0.15	0.0037	0.064	0.073	0.0029	0.00018	0.00085	0.0097
Mixotrophic	5 sequential change	6.67	0.080	0.72	0.93	0.16	0.029	0.067	0.094	0.014	0.023	0.020	0.016	0.0082	0.074	0.034	0.019	0.014	0.066	0.14	0.0020	0.022	0.053	0.0076	0.0000094	0.00087	0.0043
Standard deviation																											
Mixotrophic 'reference' from Trial 1	1 (33:33:33)	0.48	0.036	0.54	0.74	0.097	0.046	0.036	0.088	0.014	0.016	0.023	0.013	0.011	0.29	0.10	0.026	0.021	0.25	0.17	0.0050	0.098	0.022	0.014	0.00026	0.0010	0.0097
Mixotrophic	2 (20:60:20)	0.48	0.035	0.34	0.43	0.056	0.029	0.031	0.054	0.0099	0.026	0.014	0.010	0.0073	0.18	0.061	0.041	0.013	0.15	0.10	0.0031	0.060	0.025	0.023	0.00016	0.0013	0.0059
Mixotrophic	3 (60:20:20)	0.63	0.031	0.32	0.45	0.080	0.031	0.044	0.071	0.013	0.013	0.043	0.020	0.0092	0.18	0.065	0.025	0.028	0.15	0.12	0.0032	0.060	0.014	0.012	0.00026	0.00073	0.0060
Mixotrophic	4 (20:20:60)	0.43	0.050	0.99	1.42	0.16	0.085	0.045	0.14	0.022	0.020	0.015	0.011	0.018	0.54	0.18	0.023	0.029	0.45	0.32	0.0090	0.18	0.036	0.0086	0.00044	0.0014	0.018
Mixotrophic	5 sequential change	1.17	0.051	1.081	0.56	0.14	0.029	0.043	0.10	0.012	0.025	0.043	0.014	0.010	0.18	0.060	0.041	0.016	0.15	0.32	0.0030	0.060	0.022	0.023	0.00016	0.0013	0.0059

microalgal feed culture conditions	Diet ratio	Carbohydrates	Protein	TFA	C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1 n-9	C18:2 n-6	C18:3 n-6	C18:3 n-3	C20:0	C20:1 n-9	C20:2	C20:3 n-6	C20:3 n-3	C20:4 n-6	EPA	C22:1 n-9	C22:2	C24:0	DHA
T. suecica: *P. tricornutum*: *C. cryptica*																										
Mixotrophic 'reference' from Trial 1	1 (33:33:33)	0.018	0.087	0.12	0.018	0.0037	0.015	0.0081	0.0025	0.0030	0.0097	0.0059	0.0027	0.0057	0.0061	0.0016	0.0051	0.0025	0.013	0.00025	0.0011	0.0043	0.00039	0.000022	0.00010	0.00025
Mixotrophic	2 (20:60:20)	0.027	0.12	0.16	0.022	0.0054	0.024	0.0081	0.0033	0.0042	0.017	0.010	0.0042	0.0044	0.0097	0.0024	0.0087	0.0016	0.019	0.00038	0.00082	0.0040	0.00062	0.000032	0.00010	0.00016
Mixotrophic	3 (60:20:20)	0.013	0.059	0.076	0.012	0.0023	0.00094	0.0055	0.0017	0.0021	0.0059	0.0038	0.0018	0.0036	0.0038	0.0011	0.0031	0.0015	0.0080	0.00016	0.00069	0.0036	0.00033	0.000014	0.00012	0.00019
Mixotrophic	4 (20:20:60)	0.014	0.081	0.12	0.021	0.0036	0.011	0.011	0.0027	0.0028	0.0060	0.0038	0.0022	0.0094	0.0050	0.0014	0.0037	0.0046	0.013	0.00022	0.0019	0.0053	0.00024	0.000022	0.00008	0.00041
Mixotrophic	5 sequential change	0.015	0.079	0.092	0.015	0.0025	0.011	0.0074	0.0018	0.0021	0.0060	0.0042	0.0021	0.0036	0.0051	0.0012	0.0036	0.0021	0.012	0.00021	0.00072	0.0041	0.00039	0.000021	0.00013	0.00018
Standard deviation																										
T. suecica: *P. tricornutum*: *C. cryptica*																										
Mixotrophic 'reference' from Trial 1	1 (33:33:33)	0.015	0.075	0.12	0.027	0.0063	0.015	0.014	0.0047	0.0058	0.021	0.0070	0.0031	0.0092	0.0065	0.0049	0.0082	0.0051	0.029	0.00034	0.0022	0.0038	0.0011	0.000067	0.00018	0.00038
Mixotrophic	2 (20:60:20)	0.022	0.094	0.19	0.039	0.0099	0.025	0.017	0.0069	0.0090	0.039	0.012	0.0043	0.0073	0.0094	0.0073	0.013	0.0031	0.040	0.00048	0.0016	0.0037	0.0018	0.000094	0.00022	0.00025
Mixotrophic	3 (60:20:20)	0.011	0.050	0.079	0.017	0.0040	0.0097	0.0089	0.0031	0.0039	0.013	0.0044	0.0020	0.0057	0.0040	0.0035	0.0050	0.0031	0.018	0.00021	0.0014	0.0030	0.00094	0.000041	0.00018	0.00027
Mixotrophic	4 (20:20:60)	0.011	0.084	0.10	0.027	0.0052	0.012	0.016	0.0042	0.0048	0.013	0.0044	0.0030	0.015	0.0064	0.0042	0.0066	0.0092	0.029	0.00032	0.0038	0.0049	0.00067	0.000066	0.00015	0.00064
Mixotrophic	5 sequential change	0.012	0.083	0.092	0.021	0.0038	0.0097	0.011	0.0030	0.0038	0.013	0.0042	0.0019	0.0057	0.0044	0.0031	0.0050	0.0037	0.029	0.00023	0.0014	0.0035	0.00094	0.000054	0.00025	0.00026

3.3 Costs of microalgal media

The volumes and costs of media required to cultivate 10^6 larvae were variable, dependant upon culture conditions, species composition and method of culture (Table: 5).

Media type	Diet ratio	Total volume required (L)	Total cost of medium (£)
Chaetocerus: *I. galbana*: *D. lutheri*			
Photoautotrophic 'reference'	1 (20:40:40)	115	1.22
T. suecica: *P. tricornutum*: *C. cryptica*			
Mixotrophic	2 (100:0:0)	14.4	1.54
Mixotrophic	3 (0:100:0)	44.5	3.60
Mixotrophic	4 (0:0:100)	46	3.72
Mixotrophic	5 (33:33:33)	34.6	3.07
T. suecica: *P. tricornutum*: *C. cryptica*			
Mixotrophic 'reference' from Trial 1	1 (33:33:33)	26.1	2.32
Mixotrophic	2 (20:60:20)	29.5	2.54
Mixotrophic	3 (60:20:20)	22.5	2.17
Mixotrophic	4 (20:20:60)	27.2	2.34
Mixotrophic	5 sequential change	25.8	2.30

4 Discussion

Mixotrophically cultured microalgal feeds, when compared to an optimised photoautotrophic 'reference' diet, performed as well as the photoautotrophically cultivated diet. Furthermore, a sequential diet which utilises algae of an increasing size as feed over the time of larval culture, gave the greatest larval growth,

except when compared to a diet with 60 % *P. tricornutum* and the greatest larval TFA content, which was may increase the likelihood of successful settlement. At the scale tested mixotrophic culture of microalgae is a potential mechanism for reducing culturing costs in a hatchery setting and develop a more profitable model for a blue mussel larval hatchery.

During Trial 1, mixed diets outperformed single species diets, while mixotrophically cultured microalgae utilised in mixed diets performed as well, in terms of larval growth and survival, as the photoautotrophically cultivated 'reference' diet (Fig: 1). This is in agreement with previous studies (Galley et al., 2010), although during trial 1 there was a lower larval survival than the highest reported by Galley et al. (2010). It is unknown if these survival are lower in all circumstances as larval survival across the Galley et al. (2010) study were not reported. Furthermore, it is difficult to compare between feeding trials, even conducted within a study, unless the trials are conducted concurrently, due to the genetic variation between broodstock and gametes.

Subsequent optimisation indicated that the best performing mixed mixotrophically cultivated diets, in terms of larval growth, were those which included a greater proportion of *P. tricornutum* initially, while the diet which maximised the EPA content of the larvae contained a greater proportion of *C. cryptica* at the end of the culture period. A mixed species diet has previously resulted in nutrient deficiencies in individual algal species (Brown, 1991; Galley et al., 2010). Mixed feeds outperformed diets of *C. cryptica* during Trial 2, potentially due to the larger cell diameter of *C. cryptica* (Dickson and Kirst, 1987) reducing the number of larvae which could ingest this feed (Lutz and Kennish, 1992). By contrast, the single species diets of *T. suecica* and *P. tricornutum* performed as well as the mixed species photoautotrophically cultivated diet, which has not been observed in previous studies, where the use of *P. tricornutum* was considered to result in lower larval growth and survival in comparison to utilising *I. galbana, C. calcitrans* or *D. lutheri* (Brown, 1991; Lutz and Kennish, 1992). Larvae reached 260 μm in length [considered to be the minimum length for successful settlement (Sprung, 1984)] after approximately 23 days during Trial 1; however, larvae may delay metamorphosis and continue to increase in length until metamorphosis occurs. Delayed metamorphosis has been demonstrated to increase result in increased larval mortality (Sprung, 1984), therefore the observed larval mortality at the 30 days post-fertilisation may have been influenced by the inability of many larvae to settle upon the sieves, or the successful settlement of larvae onto the

sieves removing, larvae from the water column and thus 'skewing' the mortality observed. As a result, for Trial 2 the length of the trial was shortened to reduce this mortality and potential effects upon survival. Changing the amount of *T. suecica, P. tricornutum* or *C. cryptica* in the subsequent trial did not influence larval survival significantly; however, the sequential diet and 60 % *P. tricornutum* diets resulted in the greatest larval growth at the endpoint of the trial, compared to diets with an equal mix of all three species, 60 % *T. suecica* and 60 % *C. cryptica*. One of the main differences between the diets employed was the cell diameter (Fig: 4C). Smaller cells are closer to the optimal cell length for ingestion by the larvae [3-5 μm, Lutz and Kennish (1992)], and a diet with more, smaller cells, may ensure that even smaller larvae can feed (Knauer and Southgate, 1999). The highest larval lipid content was observed in the those larvae fed the sequential and 60 % *C. cryptica* diets, with an increased EPA content in the *C. cryptica* diets. The larval content of energy stores, such as fatty acids, has been linked to an increased likelihood of settlement success (Leonardos and Lucas, 2000) and an optimal dietary content of 7-17 % EPA has been linked to increased larval survival and growth in *M. gigas* (Rico-Villa et al., 2006). Tailoring the diet to the larval species is critical as there is evidence that *M. gigas* larvae displayed preferential feeding depending upon age of larvae and diet provided (Rosa and Padilla, 2020). Further work should aim to understand if mussel larvae display preferential feeding as this may result in efficiency improvements and therefore cost savings in the development of a larval hatchery.

4.1 Mixotrophy within a hatchery setting

The production cost of £1.48 10^6 larvae^{-1} by employing photoautotrophic cultivation of microalgae is lower than the costs of using mixotrophic cultivation, both in terms of the total cost of media and the total cost of carboys. The cost of producing three mixotrophically cultivated species in this study was comparable to the costs of the production of *T. suecica* alone at a cell density of 1 x10^6 cells ml^{-1} in a 200 litre photobioreactor for use in a larval hatchery (£1.70 100 litres^{-1}, or £6.85 100 litres^{-1} accounting for inflation) (Laing and Helm, 1981). However, this price does not include the costs of light, or temperature control and as a result are likely to be greater than the larger scale culture. Future cost savings may be large, with upscale of the photobioreactor system from 20 litres to 200 resulting in a production costs being reduced by approximately 80 % due to the increased efficiency of the system utilised (Laing

and Helm, 1981). The addition of an organic carbon source to the media for mixotrophic cultivation is the major driver of the increased cost of cultivation, with a feed based on a mix of species cultivated on mixotrophic media costing approximately three times more than when photoautotrophically cultivated microalgae were used to produce 10^6 larvae in most cases (Table: 5). The volumes of media required were reduced between mixotrophic and photoautotrophic cultivation, with a mixed, mixotrophic, sequential diet requiring four times less media, or half the number of carboys to produce 10^6 larvae than the photoautotrophic 'reference' diet. This is due to the increased cell density in mixotrophic cultivation of microalgae. This volume reduction suggests that, while at this scale costs may be greater to produce sufficient microalgae to utilise as feed, the reduced space required to cultivate sufficient microalgae may be an important factor at larger scales. This is due to the increase infrastructure requirements to cultivate sufficient microalgae, which may be 86 % of first year costs to build a larval hatchery (Jeffs and Hooker, 2000). Furthermore, the cost of an additional organic carbon source may be mitigated through utilisation of waste products for microalgal culture. For example, crude glycerol has been shown to be an appropriate carbon source for the heterotrophic thraustochytrid *Aurantiochytrium* sp. strain TC20 (Chang et al., 2013). Crude glycerol is a waste product from biodiesel production and its cost is 10 % of that of pure glycerol (Johnson and Taconi, 2007).

Future work is necessary to explore the implications of production methods such as bag culture for photoautotrophic production of *I. galbana* and *D. lutheri*, which trades culture productivity for a lower initial cost and an altered space and infrastructure requirement (Moheimani, 2013). Furthermore, the costs associated with maintaining the temperature and light required for culture was not modelled, which will also represent a significant energy cost (Perez-Garcia et al., 2011). The temperature and light levels were maintained across feeding Trials, so this cost will affect each of the costs equally in this instance and may be lower when a lower volume of medium is required, depending on the type of production method used (Laing and Helm, 1981). Type of production system, both for larval culture (Kamermans, 2008) and microalgal culture are highly variable dependant upon the cultivation system and the harvesting method employed with variation of between AU$0.28 to AU$0.88 kg^{-1} of DW depending upon the flocculant utilised for commercial scale microalgal cultures (Cui et al., 2019). The increased organic carbon content increases the expertise required for microalgal culture, due to the requirement for axenic technique to prevent microalgal culture crash (Morales-Sánchez and Martinez-Rodriguez, 2015). Downstream pro-

cessing is potentially expensive if centrifugation alone is utilised (Grima et al., 2003). Techniques such as the addition of a flocculant to algal feeds to increase harvest efficiency may be viable, but will necessitate additional study as flocculants within algal feeds may have adverse effects upon larval survival (Heasman et al., 2000). Culture 'stability' is a potentially limiting factor in many hatchery systems (Day et al., 1991). There is an enhanced cost in maintaining axenic mixotrophic cultures, in terms of staffing expertise, culture maintenance and culture system, to prevent culture 'crash' and a loss of that batch of microalgae to contamination (Day et al., 1991). Understanding the likelihood of a culture 'crash' in the systems utilised is critical to quantifying these costs.

There are some limitations to this model for assessing the costs associated with the development of a larval hatchery, some of which are inherent within the model assumptions. Total survival of all larvae is not realistic, with variable survival both between diets and feeding Trials in this study, but also there may be a reduction in survival during metamorphosis and spat production (Supono et al., 2020). This was not accounted for in the model because both the microalgal and larval culture systems required to produce 10^6 are likely to be different from the conical system used in these feeding trials (Kamermans et al., 2013). The model does not account for the costs of staffing, infrastructure, light, temperature, harvesting (of larvae or microalgae) or microalgal culture 'stability'. This is due to the alterations depending upon the size and type of system utilised and variable levels of automation available, which make life cycle assessments and economic viability beyond the scope of this study to assess (Parsons et al., 2020). As mixotrophically cultivated microalgae can be as effective in a mixed diet as the photoautotrophically cultured 'reference' diet in terms of larval growth and survival and results in a reduced volume of media required compared to photoautotrophic culture of microalgae, this suggests an area of potential cost savings for the development of a larval hatchery.

4.2 Conclusions

There is a growing need to develop a larval hatchery for blue mussels, both in Scotland and globally to address inconsistent spat recovery harvested from the wild. To do so the costs of microalgal production need to be reduced as microalgal production represents 90 % of the costs of producing spat. To achieve

this mixotrophically cultured microalgae were utilised as feeds for mussel larvae across two feeding trials and the effectiveness of different diets tested. Mixed species microalgal diets outperformed single species diets when utilised as blue mussel larval feeds, both in terms of larval survival and growth, although larval survival was not high for any diet utilised in this study. When a mixed species diet is utilised, the photoautotrophic 'reference' diet performed as well as a mixed diet of mixotrophic microalgal species, in terms of larval survival and growth. Subsequent mixotrophically cultured diet optimisation suggests that a diet which changes the primary component, from smaller to larger microalgal species across the culture period maximises larval growth and results in larvae with a greater TFA content, compared to all other treatments.

Figure captions

Fig: 1. Mean larval density (A) and mean shell length (B) across cultivation period of Trial 1. All samples are ± standard deviation. Colours indicate the diet of that conical: black circles: photoautotrophically cultivated 'reference' diet, red circles: *T. suecica* alone, green triangle: *P. tricornutum* alone, yellow triangle: *C. cryptica* alone, blue square: equal mix of mixotrophic species. End point larval density n=48, larval growth n= 42.

Table: 1. Mean fatty acid profiles of the microalgal species utilised as feed in Trial 1 (%DW, n= 54).

Fig: 2. NMDS of the fatty acid profiles of the average diet in Trial 1. Colours indicate the diet of that conical: black circles: photoautotrophically cultivated 'reference' diet, red circles: *T. suecica* alone, green triangle: *P. tricornutum* alone, yellow triangle: *C. cryptica* alone, blue square: equal mix of mixotrophically cultivated species. n= 54.

Fig: 3. Mean larval density (A) and mean shell length (B) across the time of cultivation in Trial 2. All values are ± standard deviation. Colours indicate the diet of that conical: blue circles: mixotrophically cultivated 'reference' diet, yellow circles: 60 % *P. tricornutum*, red triangle: 60 % *T. suecica*, green triangle: 60 % *C. cryptica*, brown square: sequential diet). End point larval density n= 45, larval growth n= 145.

Table: 2. Biochemical and fatty acid profiles of the microalgal feed species utilised in Trial 2 (% DW, n= 27).

Table: 3. Biochemical and fatty acid profiles of the diets utilised in Trial 2 (% DW, n= 27).

Table: 4. Mean biochemical and fatty acid profiles of the diet in Trial 2 (mg, n= 27).

Fig: 4. Biochemical profile of the feeds for Trial 3. Bulk biochemistry (A), cell diameters (B) and NMDS of the fatty acid profile (C) All samples are $\pm$ standard deviation. * indicates that there was a significantly greater component compared to at least one other species, - indicates that this species had a significantly lower component compared to at least on other species. C: Each point represents a diet and time of feeding conical. Colours indicate the diet: blue circles: mixotrophically cultivated 'reference' diet, yellow circles: 60 % *P. tricornutum*, red triangle: 60 % *T. suecica*, green triangle: 60 % *C. cryptica*, brown square: sequential diet). n= 27.

Table: 5. Predicted costs and volumes of media required to produce 10^6 larvae, utilising the named diets.

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Conflict of Interest and Ethical statement

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors. Conflict of Interest: The authors declare that they have no

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