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The use of biorefinery by-products and natural detritus as feed sources for oysters (*Crassostrea gigas*) juveniles

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ABSTRACT

New research is currently underway to explore the potential of macroalgae for the production of biofuels. Marine biofuels in general and macroalgae in particular, offer a number of advantages over terrestrial biofuels including reduced competition for freshwater resources and for land use. Sugars can be extracted from macroalgae and processed into biofuels by anaerobic digestion and fermentation. This process generates significant waste biomass, which, if used, could improve the economic sustainability of the biorefinery sector. Bivalves' aquaculture relies heavily on the production of unicellular algae to feed juvenile individuals and this can represent a bottleneck for the bivalve industry especially in locations where sunlight is limited. Previous research explored the use of macroalgae derived digestate as alternative or integrative feed for juvenile bivalves, exploiting the notion that organic particulate matter (detritus) is an integral part of this animal class natural diet. The prospect of using waste products from the emerging biorefinery industry to solve a bottleneck for aquaculture businesses and, by so doing, improving profitability of both, is an exciting one. In this paper we describe the main nutritional profiles (Protein, Lipid, Carbohydrates and Fatty acids) of the tested diets and investigate the potential for the use of a biorefinery a by-product as replacement option for bivalves' production, by benchmarking it against aquaculture industry standards (live microalgae and commercially available algae paste) and natural detritus constituted by farmed sea urchin digesta. Both the digestate and the natural detritus supported the survival and growth of bivalve spat, especially when used at 50% inclusion rate, over the course of 4-week preliminary trials. Data suggest that a synergistic effect of the nutritional profiles of the diets employed may underpin the observed results.

Statement of relevance: With this study we compare the biochemical composition and suitability as oyster feed of the Single Cell Detritus produced by *S. latissima* enzymatic saccharification and natural detritus produced by sea urchin digestive action, with live microalgae as well as commercial algae paste. A comparison between biochemical composition and suitability as aquaculture feed between biorefinery by-products and natural detritus was, to our knowledge, lacking.

Results indicate that both the digestate and the natural detritus supported the survival and growth of oyster spat, especially when used at 50% inclusion rate, over the course of 4-weeks trial.

1. Introduction

Aquaculture is the fastest food production sector globally and the industry was worth US$144.4 billion in 2012 (FAO, 2014). 90% of the industrial finfish and shellfish aquaculture producers have juvenile or larval life stages that are micro-planktivorous (Duerr et al., 1998) and therefore would greatly benefit from advances in early feeding protocols and products. Hatchery production of bivalves is particularly reliant of constant and cost-effective production of unicellular algae. Consequently, there is a pressing need in the production of bivalve juvenile to develop an inexpensive and reliable feed that alleviates the reliance on live microalgae, a bottleneck of the bivalve industry which constitutes as much as 30% of the overall spat production cost (Coutteau et al., 1994). In addition to the financial aspect of producing microalgae, this process is also highly technical and labour intensive, and the unpredictable growth of microalgae and the susceptibility of the culture to contamination, has spurred interest in the development of more consistent and reliable alternative. At present several species of live microalgae are utilised in the feeding of bivalve juveniles (Spolaore et al., 2006). In traditional outdoor algae production systems it has proven difficult to maintain a monoculture and successful growth is limited to regions with suitable temperature and sufficient sunlight (Persoone, 1980). For these reasons more controlled and consistent systems that could be utilised anywhere with a suitable power source were developed. Photobioreactors of various layouts have been designed to produce highly controlled monocultures of algae for feed and for biofuels. While these designs are often very successful at a laboratory

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scale it has generally been challenging to scale them up to a commercial scale due to the relative decrease in illumination per unit area and therefore an increased energy cost to adequately illuminate the microalgae (Ugwu et al., 2008). It must also be noted that for the mass cultivation of algae, a large area is often needed and this represents a common shortfall in many developed countries and has led scientists to investigate alterative food sources for hatchery bivalves' production. An ideal replacement diet must be nutritionally complete while being easily assimilated and absorbed. It must also exhibit characteristics such as a long shelf life, an appropriate particle size for ingestion and a high retention of its nutrients. Crucially, it must be less expensive to produce than current methods of microalgae production. Currently, there are a number of alternatives to growing live microalgae available to the aquaculture industry such as concentrated preparations of preserved non-viable microalgae (PNVMA), yeasts and bacteria (Knauer and Southgate, 1999). However, these have met with limited success either due to cost of production, their physical properties or their nutritional content. Therefore, the development of a diet to replace unicellular algae has a significant industrial value (Schiener et al., 2015). The role of macrophyte detritus as a food source in many ecosystems is well documented (Charles, 1993; Nagelkerken et al., 2008). It has been long established that bivalves readily absorb Kelp detritus and its associated bacteria, which suggests that it can be an important food source for this animal class (Stuart et al., 1982). In previous studies successes have been observed when utilising a single cell detritus (SCD) feed produced from the degradation of marine macrophytes, (Uchida, 1996; Uchida and Murata, 2002; Perez Camacho et al., 2004). Degradation of macroalgae can be achieved through a multitude of processes involving exposure to a combination of proteolytic, alginolytic and cellulolytic enzymes, pH manipulation and bacteria. The size of the particles available after degradation and processing is below 20 μm, which is analogous with typical dietary phytoplankton species and suggests its usefulness as a nursery feed for molluscs. Early studies (Uchida et al., 1997a; Uchida et al., 1997b) confirmed this and found that SCD from thalli of *L. japonica* degraded using the marine bacteria was a viable food source for *Artemia salina* nauplii and, more recently, SCD from *Porphyra haitanensis* was found to be a successful substitution diet for nursery production of the tropical oyster *Crassostrea belcheri* (Tanyaros and Chuseingjaw, 2014).

Although the use of farmed macroalgae for biofuel production and the potential for modifying their biochemical profile via environmental manipulation dates back to the 1980s (Ryther et al., 1981; Bird and Benson, 1987), recently, the concept has seen an increased interest (Hughes et al., 2012; Kraan, 2013) and it has been significantly developed to improve its economic viability. One further significant improvement in the economic performance of biorefinery could be represented by the use of the process's by-products as valuable feed sources for livestock, including marine bivalves. At laboratory scale, the use of biorefinery by-product has been shown to have potential as bivalve feed, mostly due to the feeding habit of this animal class, which includes particulate organic matter (POM) as a significant component of its natural diet (Mann, 1988; Duggins et al., 1989). Therefore, these digestates, or Single Cell Detritus (SCD), from marine macroalgae, obtained via enzymatic digestion, have the potential to mimic the physical properties and biochemical profiles of natural particulate organic matter and consequently fulfil, at least partially, bivalves' nutritional requirements. Indeed, the elemental composition of macroalgae degraded via enzymatic saccharification and their potential as a replacement for commercially available PNVMA has been recently described (Schiener et al., 2015). With this study, we take this concept further and compare the biochemical composition and suitability as oyster feed of the SCD produced by *S. latissima* enzymatic saccharification used in a previous study (Schiener et al., 2015) with live microalgae as well as commercial algae paste. Importantly, a comparison between biochemical composition and suitability as aquaculture feed between biorefinery by-products and natural detritus is, to our knowledge, still lacking. The reduction of macrophytes to a SCD product through acidic, bacterial, enzymatic and mechanical action can, in fact, be associated to the animal digestive process. It could therefore be hypothesised that the digestive action of a marine grazing herbivore would produce a product of similar composition to that of "artificially" produced detritus. Sea urchins are one of the major consumers of macro-phytobenthos and, as such, possess the potential to significantly contribute to the particulate organic matter fraction in several marine ecosystems, providing an important link in the nutrients fluxes between the benthic and pelagic domains. This study, therefore, assesses the viability of SCD produced via the digestive action of the sea urchin *Paracentrotus lividus* compared to SCD produced by enzymatic saccharification in an anaerobic digester, for the hatchery production of *Crassostrea gigas* spat, by benchmarking these two novel diets against industry standards: live microalgae and commercially available algae paste. This paper describes the growth, survival and biochemical composition (Carbohydrates and Lipids) of juvenile oysters (*C. gigas*) and reports on the biochemical composition of the tested diets (Proteins, Carbohydrates, Lipids and Fatty acids).

2. Materials and methods

2.1. General methods

In this study six diets were trialled in triplicate; a live microalgae diet consisting of a 70:30% by algae cell volume mix of *T. suecica* and *I. galbana* (MA), an algal paste diet (AP) supplied be Reed Mariculture Inc. (Shellfish Diet 1800®), Single Cell Detritus produced by enzymatic saccharification (SCD); natural detritus produced from *Paracentrotus lividus* faeces (UF); 50% MA-SCD and a 50% MA-UF. The oysters were kept in 3 l glass bowls in a static system with an air stone in each bowl to maintain circulation and prevent settling of feed particles. Into each bowl was placed 700 mg of spat (approximately 150 individuals; wet weight 4.6 ± 0.2 mg; shell length 1.96 ± 0.44 mm) on a raised mesh platform, to allow full circulation of water and feed to each individual. Water temperature of the bowls was maintained at 16.3 °C (±0.8 SD) using manipulation of the ambient room temperature. Where required feeds were converted into a liquid form by adding the dry feeds to either ambient seawater or to the respective algae mix, algae paste was diluted with ambient filtered seawater as per supplier instructions. The feed rations were administered in a pulse format of 24 separate feeds of 10 ml once every hour. Daily Feed rations for each treatment were calculated and replenished once per day. The Jebao DP 4 peristaltic pump was used to apply the hourly rations for each replicate. Bowls were cleaned using warm fresh water and complete water change was conducted every three days. Treatments were kept in a temperature controlled room and maintained on a photoperiod of 8 h of daylight and 16 h of darkness. Rations of the live algae mix used to feed the MA, MA-SCD and MA-UF treatments was calculated daily according to published methods (FAO, 2004).

Rations of the Shellfish Diet 1800® for the algae paste treatments were calculated based on the manufacturer guidelines. Rations of both the SCD and UF diets were calculated based on a 40% of oyster live weight per week in diet dry weight, in a way that the ration for these diets matched the ration of both live microalgae and algae paste (FAO, 2004). Randomly picked 80 individuals from each of the replicates were weighed to determine individual wet weight and were measured using calipers to determine shell length. A mortality count was also undertaken on the same amount of individuals per replicate. Oysters were considered to be dead when presenting open shells or showed no dark coloration or mantle movement when observed under dissecting microscope.

2.2. Preparation of the diets

The UF feed was produced from the faeces of *Paracentrotus lividus* fed to satiation with *S. latissima* fronds. The faeces were collected soon

after production in an effort to minimize nutrient leeching. The wet faeces was sieved through a 200 μm mesh to remove large uneaten particles and broken urchin spines, it was then allowed to settle in tall 1 l measuring cylinder and the supernatant was siphoned off. The faeces were transferred to a shallow tray and allowed to air dry at room temperature (21 °C), any remaining spine fragments were removed during this process by hand while the faeces was still moist. As soon as the faeces had dried sufficiently to be scraped from the tray as a paste it was freeze dried to remove moisture. The dried faeces were then ground to a fine powder using a pestle and mortar and stored in a desiccator. Using a fume-hood to minimize dust inhalation the fine powder was sieved using a 20 μm test sieve to ensure all particles were below 20 μm and could be ingested by the spat.

Live algae diet was a 70:30 mix of *Tetraselmis suecica* and *Isochrysis galbana* grown in sterile 20 l carboys with the addition of f/2 medium. Algae Paste used was the Shellfish Diet 1800® purchased from Reed Mariculture Inc. four days prior to the start of the trial.

To produce the SCD diet fronds of *Saccharina latissima* were treated using cellulosic and hemocellulosic enzyme blends provided by Novozymes, Denmark (Schiener et al., 2015). Approximately 13.00 ± 0.002 g of dried seaweed was added to 250 ml Duran glass bottles with 100 ml of deionised water. The pH of the solution was adjusted to 5.2 with 10% HCl and the bottles autoclaved at 121 °C for 15 min. Once cooled to 45 °C in a water bath, enzymes were added at 10% NS 22086 (w w-1) and 1.2% NS 22119 (w w-1). Bottles were placed in an orbital shaker (New Brunswick Scientific, Innova 4230) at 200 rpm and incubated at 45 °C for 2 days. Following this, the digested seaweed was centrifuged for 10 min at 3.200*g* and residue was washed with equal volumes of deionised water before re-centrifugation. Washed solids were frozen at −20 °C and vacuum freeze dried to remove all moisture. The dried matter was then mechanically ground using a pestle and mortar to reduce particles size and sieved through a 20 μm mesh.

2.3. Biochemical analysis

Each of the six diets was processed into a dry powder by centrifugation at approximately 5000 rpm for 10 min, supernatant was drained and the remaining pellet was freeze dried and ground into a fine powder. The MA-SCD and MA-UF dried diets were made by combining the respective dried powders at a 1:1 ratio based on weight.

The lipid fraction of diets and oysters was extracted using procedures described by Folch (Folch et al., 1957). In brief, samples were homogenized in the chloroform/methanol using a tissue disrupter (Ultra Turax™, IKA Werke Gmbh & Co. KG, Staufen, Germany), and 1 ml 0.88% KCl was added and the homogenates mixed before centrifugation at 600*g* for 5 min (Jouan C412, Pegasus Scientific Inc., Rockville, USA). The upper aqueous phase was aspirated and the solvent evaporated under a stream of oxygen-free nitrogen (OFN). Lipid content was determined gravimetrically after desiccation overnight. The total lipid extracts were re-dissolved at a concentration of 10 mg/ml in chloroform/methanol (2:1, v/v) plus BHT. Fatty acid compositions of total lipid were determined by gas chromatography according to standard protocols (Christie, 2003). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transesterification at 50 °C for 16 h with extraction and purification by thin layer chromatography as described previously (Ackman, 1980). The FAME were separated and quantified by gas–liquid chromatography using a GC 8000™ series EL 980 GLC (Fisons instruments) equipped with a 30 m × 0.32 mm i.d., 0.25 μm capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column injection. Hydrogen was used as carrier gas and temperature programming was from 50 to 150 °C at 40 °C min^{-1} and then to 230 °C at 2.0 °C min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data were collected and processed using Chromcard for Windows (version 1.19), and FAME quantified through a comparison with a heptadecanoic acid (17:0) internal standard.

Carbohydrate content of the diets was measured using a Uvikon™ 860 spectrophotometer and compared to a calibration curve generated from known quantities of glucose standard. Between 2.7 and 14.5 mg of whole, freeze dried oyster was used in each replicate. Between 2.8 and 8.4 mg of dried, powdered diet was used in each replicate. Each treatment was analysed in triplicate. Solutions made up of 2.5 ml deionised water, 1 ml of 5% phenol solution and 8 ml of concentrated sulphuric acid in the necessary order and at the necessary time in the procedure. The absorbance of each solution was read at 520 nm against a blank standard. From the calibration curve the mg of glucose for each replicate can be determined and converted into total carbohydrate using the following formula:

$$\%\text{total carbohydrate} = (\text{mg of glucose in sample}/\text{sample weight (g)}) \times 100$$

Protein of the diets was measured using the Kjeldahl analysis on a Tecator Kjeltec according to Lynch and Barbano (1999). Between 71.9 and 276.9 mg of dried, powdered diet was used for each replicate, all samples were analysed in duplicate. Two copper Kjeltabs and 5 ml of concentrated sulphuric acid was added to each replicate before placing the tubes into a digestion block at 420 °C for 1 h. 20 ml of deionised water was then added before allowing the mixed solution to distil using a Kjeltec™ 2300 analyser (FOSS).

2.4. Statistical analysis

All analyses were carried out using the statistical package of Minitab 15.0 (Minitab Ltd., UK). Normality and homogeneity of variance were ere confirmed using Kolmogorov–Smirnov test and improved where necessary by either log or reciprocal transformations. Differences were tested using one-way ANOVA, followed by the Tukey's multiple comparison test to assess where significant differences occurred. The non-parametric multivariate analysis ANOSIM (analysis of similarities) was used to identify significant differences in the diets fatty acids profiles. SIMPER (similarity percentage) test was used to identify which FAs were primarily responsible for the observed differences (Carboni et al., 2013). Data were untransformed and Euclidian distance was used as the metric. In all cases, significant differences were determined at $p < 0.05$.

Table 1
Nutritional reserves, size (shell length) and survival of the oysters spat at the end of the 4 weeks experimental period (mean ± sd; n = 3). Superscripts indicate statistically significant differences.

	Initial	MA	AP	SCD	UF	MA-SCD	MA-UF
Lipid (% tissue weight)	0.53 ± 0.28^{c}	1.49 ± 0.32^{a}	0.41 ± 0.12^{c}	0.38 ± 0.08^{c}	0.52 ± 0.12^{c}	1.19 ± 0.33^{b}	0.68 ± 0.19^{c}
Carbohydrates (% tissue weight)	0.71 ± 0.17^{c}	2.66 ± 0.60^{a}	1.59 ± 0.32^{b}	1.65 ± 0.32^{b}	1.64 ± 0.39^{b}	1.90 ± 0.53^{b}	1.83 ± 0.57^{b}
Shell length (mm)	1.96 ± 0.44^{b}	3.80 ± 0.36^{a}	3.05 ± 0.72^{a}	3.00 ± 0.53^{a}	2.93 ± 0.65^{a}	3.51 ± 0.28^{a}	4.16 ± 0.46^{a}
Survival (%)		97.7 ± 3.13	95.36 ± 5.15	95.85 ± 4.80	93.98 ± 6.16	93.91 ± 5.22	94.83 ± 4.80

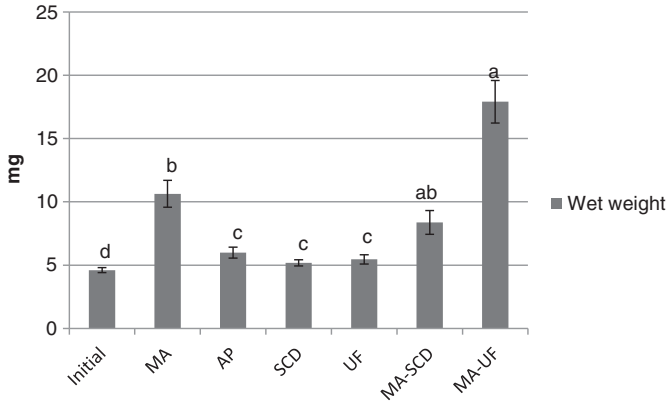

Fig. 1. Average individual wet weight (mg) at the end of the four weeks feeding trial (mean ± sd; n = 6). Superscripts indicate statistically significant differences.

3. Results

3.1. Oyster growth, survival and nutritional reserves

The feeding trial showed that the oysters in all treatments have significantly grown during the trial period ($p < 0.05$) and that survival was generally high with no difference across treatments (Table 1). However, the biofuel residue (SCD), the detritus produced from sea urchin faeces (UF) and commercial algae paste (AP) were only marginally capable of supporting oyster spat growth when fed on their own. Conversely, when both SCD and UF were used as 50% live algae substitute, significant faster growth was observed (Fig. 1). This confirms the nutritional value of these residues as potential bivalve diet supplement or partial replacement but not as standalone diets. Indeed, by the end of the four weeks feeding trial, oysters fed the MA-UF diet had a significantly higher mean individual weight compared to all other diets, including live microalgae (Fig. 1), suggesting that the nutritional profile and/or the digestibility of the UF supplement should be further investigated as it appears to provide a growth advantage. Although shell length at the end of the trial was significantly higher than at the beginning, no significant differences were observed between the treatments (Table 1).

Oysters' carbohydrate and lipid content at the end of the trial period is given in Table 1. Data show that individuals in every treatment accumulated nutrients reserves during the trial period, suggesting that efficient feeding was achieved with the employed experimental system. No difference in lipids and carbohydrates content were observed between

Table 2
Biochemical composition of the six tested diets (mean ± sd; n = 6). Superscripts indicate statistically significant differences.

Diets	MA	AP	SCD	UF	MA-SCD	MA-UF
Proteins (% of dw)	8.11 ± 0.73^{d}	21.80 ± 0.14^{b}	30.45 ± 0.40^{a}	9.33 ± 0.06^{c}	19.15 ± 0.60^{b}	8.47 ± 0.29^{d}
Carbohydrates (% dw)	5.63 ± 0.85^{d}	9.90 ± 1.19^{c}	39.34 ± 2.60^{a}	16.77 ± 0.51^{d}	22.63 ± 2.64^{b}	10.26 ± 0.72^{c}
Carbohydrates/Protein	0.70 ± 0.12	0.46 ± 0.06	1.26 ± 0.10	1.79 ± 0.08	1.19 ± 0.16	1.23 ± 0.08
Lipids (% of dw)	5.48 ± 0.71^{b}	12.56 ± 0.16^{a}	6.07 ± 0.40^{b}	3.46 ± 0.84^{b}	5.63 ± 0.49^{b}	4.02 ± 1.10^{b}
Fatty Acids (% of total lipids)						
14:0	10.96 ± 0.76^{a}	8.98 ± 0.27^{b}	4.80 ± 0.08^{d}	7.63 ± 0.11^{c}	8.22 ± 1.01^{bc}	10.40 ± 0.86^{a}
iso 15:0	0.21 ± 0.03^{e}	0.38 ± 0.02^{d}	1.61 ± 0.03^{b}	2.70 ± 0.05^{a}	0.93 ± 0.06^{c}	0.93 ± 0.16^{c}
15:0	0.24 ± 0.01^{e}	0.76 ± 0.02^{b}	0.72 ± 0.01^{c}	1.81 ± 0.16^{a}	0.49 ± 0.03^{d}	0.63 ± 0.10^{c}
16:0	11.55 ± 0.88^{f}	13.49 ± 0.74^{e}	20.24 ± 0.24^{b}	34.48 ± 0.65^{a}	15.67 ± 0.32^{d}	18.19 ± 0.45^{c}
18:0	0.32 ± 0.14^{e}	0.46 ± 0.04^{e}	2.94 ± 0.04^{a}	2.32 ± 0.19^{b}	1.57 ± 0.12^{c}	0.97 ± 0.06^{d}
20:0	nd	nd	0.51 ± 0.02^{b}	0.65 ± 0.01^{a}	nd	0.20 ± 0.02^{c}
Total saturated	23.29 ± 1.65^{d}	24.31 ± 0.64^{d}	31.21 ± 0.33^{b}	49.81 ± 0.65^{a}	27.39 ± 0.95^{c}	31.41 ± 0.88^{b}
16:1n-9 + DMA	5.11 ± 0.18^{c}	6.57 ± 0.53^{b}	8.81 ± 0.22^{a}	6.57 ± 0.19^{b}	7.10 ± 0.25^{b}	5.41 ± 0.31^{c}
16:1n-7	1.86 ± 0.10^{f}	12.53 ± 0.29^{a}	11.19 ± 0.22^{b}	9.79 ± 0.22^{c}	6.35 ± 0.43^{d}	4.27 ± 0.17^{e}
18:1n-9	10.85 ± 1.50^{c}	5.67 ± 0.31^{d}	26.66 ± 0.48^{a}	12.93 ± 0.18^{c}	18.32 ± 0.56^{b}	11.51 ± 1.45^{c}
18:1n-7	2.69 ± 0.11^{c}	0.95 ± 0.05^{e}	2.36 ± 0.05^{d}	4.92 ± 0.13^{a}	2.47 ± 0.05^{d}	3.41 ± 0.18^{b}
20:1n-9	0.78 ± 0.09^{a}	0.18 ± 0.01^{d}	0.21 ± 0.14^{d}	0.26 ± 0.11^{d}	0.43 ± 0.03^{c}	0.55 ± 0.02^{b}
Total monounsaturated	21.68 ± 1.28^{d}	26.10 ± 1.13^{c}	49.31 ± 0.45^{a}	34.85 ± 1.10^{b}	34.84 ± 1.06^{b}	25.41 ± 1.04^{c}
18:2n-6	3.17 ± 0.18^{d}	4.39 ± 0.03^{c}	6.50 ± 0.13^{a}	2.58 ± 0.24^{d}	4.84 ± 0.14^{b}	3.02 ± 0.17^{d}
18:3n-6	0.09 ± 0.01^{d}	1.09 ± 0.04^{a}	0.34 ± 0.01^{b}	0.11 ± 0.01^{d}	0.22 ± 0.01^{c}	0.09 ± 0.00^{d}
20:4n-6	0.12 ± 0.01^{d}	0.60 ± 0.03^{c}	1.69 ± 0.05^{a}	1.65 ± 0.07^{a}	1.01 ± 0.08^{b}	0.61 ± 0.06^{c}
22:5n-6	1.52 ± 0.05^{b}	2.22 ± 0.14^{a}	nd	nd	0.78 ± 0.09^{d}	1.10 ± 0.05^{c}
Total n-6 PUFA	5.36 ± 0.10^{d}	8.43 ± 0.19^{b}	8.67 ± 0.14^{a}	4.83 ± 0.18^{e}	7.16 ± 0.19^{c}	6.16 ± 1.81^{d}
18:3n-3	12.26 ± 1.43^{a}	4.19 ± 0.14^{d}	2.55 ± 0.07^{e}	2.53 ± 0.05^{e}	7.43 ± 0.61^{c}	9.44 ± 1.16^{b}
18:4n-3	11.32 ± 0.25^{a}	8.90 ± 0.87^{b}	1.64 ± 0.06^{d}	1.05 ± 0.03^{e}	6.60 ± 0.42^{c}	8.38 ± 0.22^{b}
18:5n-3	5.06 ± 0.41^{a}	0.74 ± 0.09^{d}	nd	0.32 ± 0.04^{e}	2.67 ± 0.17^{c}	3.82 ± 0.24^{b}
20:5n-3	3.12 ± 0.10^{b}	13.84 ± 1.29^{a}	1.20 ± 0.03^{f}	1.72 ± 0.14^{e}	2.19 ± 0.09^{d}	2.66 ± 0.013^{c}
22:6n-3	8.16 ± 0.33^{a}	5.50 ± 0.63^{b}	nd	0.21 ± 0.02^{d}	4.29 ± 0.33^{c}	6.07 ± 0.25^{b}
Total n-3 PUFA	40.62 ± 2.36^{a}	33.63 ± 3.04^{b}	5.54 ± 0.15^{d}	5.97 ± 0.28^{d}	23.59 ± 1.24^{c}	29.92 ± 2.43^{b}
16;2	0.82 ± 0.15^{b}	2.20 ± 0.08^{a}	0.23 ± 0.01^{d}	0.17 ± 0.01^{e}	0.55 ± 0.05^{c}	0.65 ± 0.10^{bc}
16;3	0.33 ± 0.43^{c}	1.55 ± 1.48^{a}	1.16 ± 0.04^{b}	0.47 ± 0.04^{d}	0.69 ± 0.05^{c}	0.18 ± 0.02^{e}
16;4	5.90 ± 0.21^{a}	0.81 ± 0.15^{d}	nd	nd	2.76 ± 0.26^{c}	3.94 ± 0.19^{b}
15:0 DMA	0.53 ± 0.03^{e}	0.79 ± 0.07^{d}	1.04 ± 0.11^{b}	1.89 ± 0.65^{a}	0.84 ± 0.04^{c}	0.76 ± 0.23^{cd}
16:0 DMA	1.47 ± 0.08^{c}	2.18 ± 0.15^{b}	2.84 ± 0.05^{a}	2.03 ± 0.03^{b}	2.18 ± 0.14^{b}	1.58 ± 0.13^{c}
Total PUFA	53.02 ± 2.88^{a}	46.62 ± 1.98^{b}	15.60 ± 0.25^{d}	11.43 ± 0.38^{e}	34.75 ± 1.47^{c}	40.85 ± 1.89^{b}

Total PUFA includes n-6; n-3; 16;2; 16;3 and 16;4. Values below 0.5% for all tested diets are not included in this table. Mean ± SD; n = 6. nd: not detected.

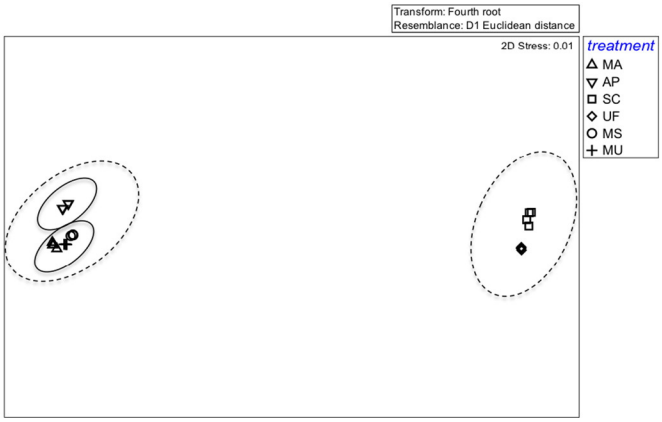

Fig. 2. nMDS plot of the fatty acid profile of the six tested diets. Sample statistic (Global R) = 0.956; Significance level of sample statistic = 0.01%; Number of permutations = 9999 (Random sample from a large number); Number of permuted statistics greater than or equal to Global R = 0.

the oysters fed the detritus based diets and commercial algae paste. However, oysters fed MA had a significantly higher nutritional content ($p < 0.001$), indicating the higher long-term suitability of this diets as oyster feed.

3.2. Biochemical composition of the diets

Table 2 shows the protein, carbohydrate, lipid and fatty acids content of all tested diets. Significant difference between protein content of the diets was observed ($p < 0.001$). The protein content of the Single Cell Detritus produced by enzymatic saccharification (SCD), was significantly higher (30.45 ± 0.40%) than any other diet. The second highest protein content was measured in the commercial algae paste (AP) diet (21.80 ± 0.14%) and in the MA-SCD diet (19.15 ± 0.60%), while no significant difference were observed between the remaining three diets. Ideal dietary protein content for juvenile bivalves has been estimated to be between 13% for *R. decussatus* (Albentosa et al., 1996) and 20% for *C. virginica* (Flaak and Epifano, 1978), although we can assume the requirement for *C. gigas* is closer to the latter. The protein content of the two best performing diets, MA-UF and MA had, however, the two lowest protein content of any diet.

The carbohydrate content of the MA-UF diet (10.39 ± 0.44%) and the AP diet (10.788 ± 0.94%) were not significantly different. The remaining diets showed significant differences ($p < 0.05$). More specifically, the detritus diets and their relative 50% mix with live microalgae had the highest carbohydrates content compared to commercial algae paste and live microalgae. In particular, the detritus produced from anaerobic digestion (SCD) contained almost 8 times the amount of carbohydrates than MA.

Lipid content of the MA, SCD the MA-SCD diet and MA-UF diets did not differ significantly. Lipid content of the AP diet, instead, was significantly higher than all the other diets ($p < 0.001$). The total effect of lipid content of a diet on the growth of *C. gigas* spat has been found to be relatively insignificant (Langdon and Waldock, 1981). This is consistent with the results presented here as the higher lipid content of the AP diet was not matched by animal growth performances. Fatty acids profiles of all the tested diets are presented in the non-Metric Multidimensional Scaling plot (Fig. 2). From this one-way Anosim analysis of the dietary fatty acid profiles, it is clear that the detrital diets (SCD and UF) presented a very distinct profile from the live microalgae and algae paste diet. (MA and AP) Interestingly, however, when the former were mixed with live microalgae their fatty acid profile was tightly clustering with the MA diet. The simper analysis showed that the main fatty acid responsible for the observed difference between MA and AP was 16:1n-7, which on its own contributed for over 20% of the profiles differences, while n-3 and n-6 fatty acids only minimally contributed to the difference. On the contrary the main fatty acids contributing to the differences between detrital diets and AP and MA were of the n-3 group, mainly EPA and DHA.

Significant differences between diets were observed in the main fatty acids groups: saturated, monounsaturated, n-6 polyunsaturated and n-3 polyunsaturated (Fig. 3). Saturated fatty acids were observed to be in significantly higher amount in the UF diet (49.41 ± 0.77%) than all other treatments ($p < 0.001$). Monounsaturated fatty acid (MUFA) content was highest ($p < 0.001$) in the SCD treatment (49.659 ± 0.32%), but there was no significant difference between the MA-SCD diet (34.48 ± 1.66%) and the UF diet (35.758 ± 0.32%). There was also no significant difference between the MA/UF and the AP diet. The n-6 PUFAs content was significantly different between the diets ($p < 0.01$) and AP and SCD showed the highest amounts. Finally, 3-n PUFAs were significantly higher in the MA diet compared to all others ($p < 0.001$). Eicosapentaenoic acid (20:5n-3, EPA), Docosahexaenoic acid (22:6n-3, DHA) and Arachidonic acid (ARA, 20:4n-6) and their respective ratios are considered particularly important in animal physiology and, in many marine species, are considered to be essential fatty acids (EFAs) that need to be provided by the diet (Knauer and Southgate, 1999; Tocher, 2003). Fig. 4 shows the relative abundance of these important compounds in the tested diets. The EPA content was significantly different across each diet ($p < 0.01$) with the AP diet showing a considerably higher content than any other tested diet. The MA diet had significantly higher levels of DHA compared to the other diets ($p < 0.01$). The UF diet contained a comparatively small amount DHA, while the SCD diet did not contain any (Fig. 4). Juvenile *Cerastoderma edule* growth did not change when fed a diet containing high levels of EPA and DHA when ARA was instead deficient; this indicates that EPA and DHA may be the most crucial EFAs for juvenile bivalve growth (Reis Batista et al., 2014). Importantly, bivalves do possess some ability to elongate and desaturate precursor fatty acids such as 18:3n-3 into EPA and DHA, if only at low levels (Da Costa et al., 2015). This in turn

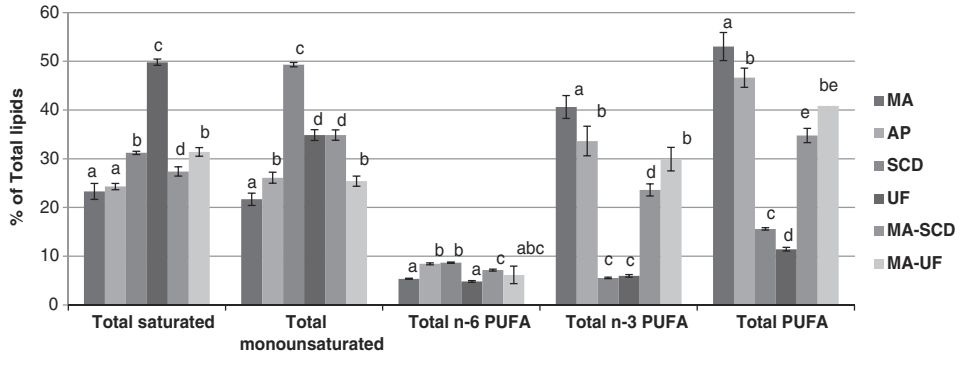

Fig. 3. Abundance of the five main fatty acid groups from the experimental diets (mean ± sd; n = 6). Superscripts indicate statistically significant differences.

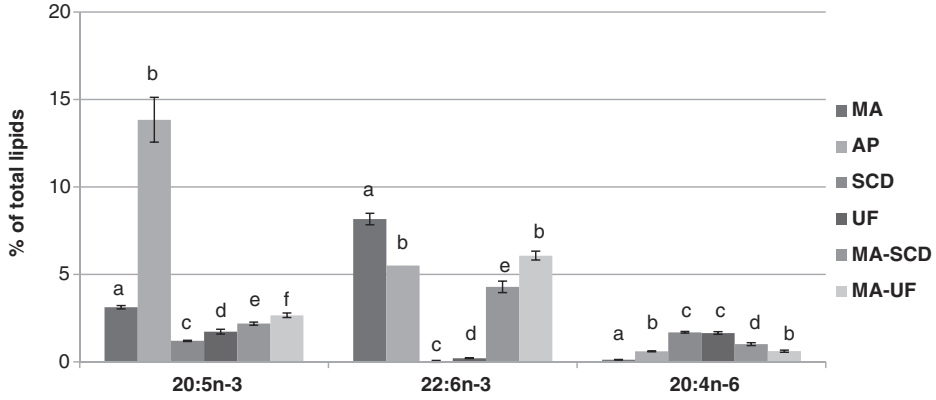

Fig. 4. Abundance of the main essential fatty acids (EPA, DHA and ARA) from the experimental diets (mean ± sd; n = 6). Superscripts indicate statistically significant differences.

indicates that high levels of EPA and DHA may not be as important in marine bivalves as they are in marine carnivorous fish.

4. Discussion and conclusion

Both the digestate and the natural detritus supported the survival and growth of bivalve spat, especially when used at 50% inclusion rate, over the course of this 4-weeks preliminary trial. Despite these promising results, however, it is important to notice that the growth rate achieved by the juvenile oysters fed MA-UF was only half of that commonly observed under commercial conditions (*pers. obs.*) using commercial upwelling systems. This, in combination with the oysters' nutritional reserves, strongly indicates that further research into these new potential feed replacements should be conducted using commercial protocols before these results could up-taken by the industry. This is particularly important considering that the use of static tanks with a low volume (2–4 l) can lead to an increased growth of bacteria which can contribute to the nutrition of the animals (Laing, 1987). The effect of bacterial proliferation is not yet clear. In some circumstances the bacteria caused clumping which inhibited ingestion (Langdon, 1983). However, clumping effect has also been found to serve as an undefined food source with bacteria contributing significantly to the metabolic nitrogen requirement of *C. virginica* in closed systems (Langdon and Newell, 1990).

The protein content of the two best performing diets, MA-UF and MA has shown the two lowest content of any diet. This seems, therefore, to suggest that a protein content of approximately 9% was sufficient under the trial conditions employed here. Nonetheless, full amino acid profile would have provided more clarity for the interpretation of these results. It is also worth noting that the interaction of protein with other nutritional elements and the amino acid profiles of the diets was not analysed in this study and may have been an important factor (Utting, 1986).

The biochemical analysis showed that the detritus produced from anaerobic digestion (SCD) and the natural detritus (UF) contained almost 8 times the amount of carbohydrates than MA. Carbohydrate is mainly utilised as an energy source by juvenile bivalves and acts to balance the utilization of protein and lipid for biosynthesis and growth against catabolism for energy (Whyte et al., 1989). It has been found that ingestion of carbohydrate is closely correlated with growth in *C. gigas* spat (Brown et al., 1998), however this is not consistent with the results from this trial as the SCD diet contained significantly higher amounts of carbohydrates than other diets although it wasn't the best performing diet. This suggests that requirements may be fulfilled at lower levels, and that other nutritional factors must be met to facilitate all potential growth. It is also possible that the detrital component of the MA-SCD diet was not as palatable or digestible as the MA-UF diet and was therefore not ingested or digested at the same rate. Furthermore, the increased carbohydrates content combined with a richer n-3 fatty acid profile of the MA-UF diet could be at the root of the better growth performances of the oyster fed this diet.

As expected, the three treatments that included the live microalgae mix performed the best overall. The MA and AP diets were intended to establish an industry consistent benchmark and it was not anticipated that any diet would perform better than the live microalgae diet. Surprisingly, individual wet weight of oysters fed the MA-UF was instead significantly higher than that of animals fed live microalgae alone. This diet also outperformed both the SCD and the algae paste diets that were previously shown to possess potential as live microalgae replacement in the hatchery production of oyster juveniles (Schiener et al., 2015). These findings suggest that the MA-UF diet was either the most nutritionally complete (i.e. more suitable carbohydrate content and fatty acid profile) and/or most bioavailable. The AP diet showed similar levels of nutrients to the MA diet; however, growth in the AP treatments was significantly slower. Likewise, the MA-SCD and MA-UF diets had very similar nutritional profiles despite the MA-UF diet performing significantly better overall. This suggests that beside nutrient density there is a much more complex range of parameters, such as settling rate, ingestion rate and assimilation rate, that contribute to the success of a diet and highlights the need for successive studies to ascertain the key factors that allowed the UF feed to be so successful when used in conjunction with a multi-specific algal diet.

New research is currently underway to explore the potential of macroalgae for the production of biofuels (Suutari et al., 2015) as hexose sugars can be extracted from macroalgae and processed into biofuels by anaerobic digestion and fermentation (Goh and Lee, 2010; Chen et al., 2015). This process generates significant waste biomass, which can, in theory, be utilised and further processed into an SCD product. Sea urchin digestion process is still under-researched and the findings from this study suggest that digestive enzymes and/or the microbiota associated with echinoderms digestive processes could provide valuable information for the advancement on marine biomass exploitation and, at the same time, produce residuals that may prove to be advantageous for the aquaculture industry. Nonetheless, the actual economic implications of this hypothetical partnership are, difficult to speculate due to both industries infancy and collaborative interdisciplinary research should be conducted to evaluate the technical and economic scope of such initiative.

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