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Abstract

Mytilus edulis culture depends on natural plankton with seasonal variations in dietary nutritional value. The microbial communities assimilated by M. edulis in Magdalen Islands lagoons (St. Lawrence Gulf, Canada), are mainly dominated by small heterotrophic eukaryotes, which vary taxonomically and in terms of lipid composition over the growing season. Particular lipids classes and fatty acids found in the mussels' food source may have an effect on their physiological condition. From May to October, four physiological measurement series (oxygen consumption and clearance rates), were performed on 1- and 2- years-old mussels under two nutritional conditions. The first group of mussels was fed with their natural diet and the second group was fed microalgae rich in essential fatty acids. Both diets were characterized according to their composition in lipid classes and fatty acids. The relationship between metabolic rates and diets quality, in terms of lipids class and fatty acids components was compared. No significant differences in mussels' metabolic rates were found between two different diets. Indeed, only significant changes over time and between mussel's ages were noted for clearance rate. These diets were different in their fatty composition and associated with significant differences in the mussel's gills fatty acids composition, particularly in polyunsaturated fatty acids.

Keywords: Metabolic rates; Lipids; Fatty acids; *Mytilus edulis*; Diets

Introduction

Since 30 years blue mussel *Mytilus edulis* L. is cultured in the lagoons of the Magdalen Islands in the southern Gulf of St. Lawrence, Canada. One of many particularities of these lagoons is that nutrients (nitrogen, phosphates and silicates) are present in very low concentration [1-3], characteristics of an oligotrophic environment [4]. In spite of these weak nutrients inputs, bacterial remineralisation, rapid access to nutrients due to the continuous mixing of the water column by winds [5] and deep light penetration still do provide suitable conditions for the production of autotrophs cells in the lagoons. Together these favourable conditions can help for primary productivity in this particular environment [1]. However, except for few sporadic diatoms blooms, autotrophs are not the dominating species in these lagoons characterized by a strong dominance of small heterotrophic species [1, 2, 6, 7]. Bivalves like *M. edulis* feed by filtrating surrounding water column and capture various microscopic particles using complex organs [8]. Highly selective food acquisition is made through a muco-ciliated mechanism located on the gills and labial palps [9]. The bivalves gill is the site of interaction with the environment. Gills create water circulation in the pallial cavity which is tightly related with respiration, particle processing, bioaccumulation and evacuation of wastes [10]. Many authors have reported that *M. edulis* optimum ingestion is for particles between 1 and 50 µm [11, 12] with a nominal efficiency for 4 µm particles [13]. However, some authors have also reported that mussels can select particles as small as 1µm [14] with a retention efficiency of 14-64% [15]. Mussels can therefore consume a variety of suspended particles like bacteria, phytoplankton, detritus and sometimes microzooplankton [16-18]. Among particles potentially available for mussels in the lagoons some still have an unknown nutritional value. As for many animal species, mussels need polyunsaturated fatty acids (PUFA) or more precisely essential fatty acids (EFA) to support normal metabolic functions and even to survive in cold temperatures [19, 20]. EFA are typically biosynthesized by primary producers like autotroph phytoplankton and bivalves have a very limited ability to synthesize de novo EFA due the limited activity of specific elongase and desaturases [21-23]. Consequently, phytoplankton must provide mussels with EFA needed for survival, growth and reproduction [21]. Dietary lipids affect the lipid composition of membranes and tissues which in turn can affect the whole animal physiology and behaviour [24]. Hulbert [25] showed that cells membranes and their lipids can play a pacemaker role in metabolism. Membranes lipids specific composition would exert their effect in changing molecular activity of membranes proteins [26]. Thus, how an oligotrophic environment dominated by small heterotrophic species in the lagoons of Magdalen Islandscan maintain mussel's productivity? Objective of this project was to compare the lipid profiles of natural diet (ND) from the House Harbour lagoon (HH) in Magdalen Islands to a microalgal cocktail diet (MD) rich in EFA and to examine their influence on metabolism and clearance rate of one- and two-year-old cultured mussels. Three hypotheses were tested. The first hypothesis was that natural diet from the lagoon is deficient in EFA due to the low abundance of autotroph phytoplankton. If natural food from the HH lagoon is really poor in EFA, then mussels would probably have developed a compensatory mechanism to ensure a healthy physiological condition. The second hypothesis was that one- and two-year-old mussels fed MD have a better physiological condition than those fed with ND because of their higher EFA content. The physiological condition of mussels has been characterized according to their oxygen consumption and clearance rates. Finally, the third hypothesis was that EFA content in cells membranes of the mussel's gills are related to oxygen consumption and clearance rates as explained in Hulbert's pacemaker theory.

Material and Methods

Experimental design

The study area was located in the House Harbour (HH) lagoon, Magdalen Islands (Gulf of St-Lawrence, Québec, Canada; Figure 1) (47º 25, 730 N; 61º 48, 832 W). One- and two-year-old mussels from suspension culture were provided by a local mussel grower. Mussels were cleaned from epibionts and transferred in 24 pearl-nets with 1 cm^2 mesh size, (50 mussels by pearl net) in mid-May 2009. Pearl nets were suspended on a long-line kept at 2 m from surface besides the aquaculture site. Measurements were realized between May and October to characterize mussels physiological conditions before, during and after the main spawning event [27]. Four series of measurements (commonly name as sampling periods) were then performed: (1) June 3-5 and June 8-10 (early June), (2) July 8-10 and July 13-14 (early July), (3) August 17 to 21 (mid-August) and (4) October 13-16 and 21 (mid-October). Prior to each sampling periods, fifty one- and two-year-old mussels were randomly taken in the pearl-nets [24] previously installed at experimental site. After that, mussels were brought at Merinov installations, and placed in a pearl net in the experimental tank. Theses mussels were fed with microalgae diet (MD), in the experimental tank, for a minimum of five days. Shin et al. [28] showed that a period of five days is long enough to generate a modification of fatty acids composition in the mussel tissues after a diet change. To obtain MD, seawater from HH lagoon was filtered at 1 µm through a sieve and supplemented with a mixture of *Nannochloropsis occulata, Isochrysis galbana* and *Pavlova lutheri*, which is a standard quality hatchery food rich in EFA (29). Water trajectory, from HH lagoon to experimental tank, is shown as letters in order from a to j (Figure 2). Microalgae mixture was stocked in a 60 L Nalgene reserve tank. Microalgae were provided continuously to the experimental mussels at flow of 20 ml min^{-1} with a peristaltic pump (Masterflex, Cole Parmer Instruments Co). At the beginning of and during each measurement period, dry weight of both ND and MD diets (in experimental tank) was determined in order to calculate equivalent rations of MD similar to that of the ND found in the lagoon at this time. ND and MD dry weight analysis were based on a method

described in Aminot & Chaussepied [30]. A chiller was also used to cool water in the microalgae tank in order to adjust it with the temperature of lagoon water. When sampling periods began (after the five acclimatising days), three one- and three two-year-old mussels were sampled from both HH lagoon (ND) and experimental tank (MD) to determine their oxygen consumption and clearance rate, and this for each day during the four given period. So, physiological measurements were performed on 12 mussels per day (Figure 3). Over sampling periods observations were based on an N=15 for each treatment except for the first sampling period in June N=20.

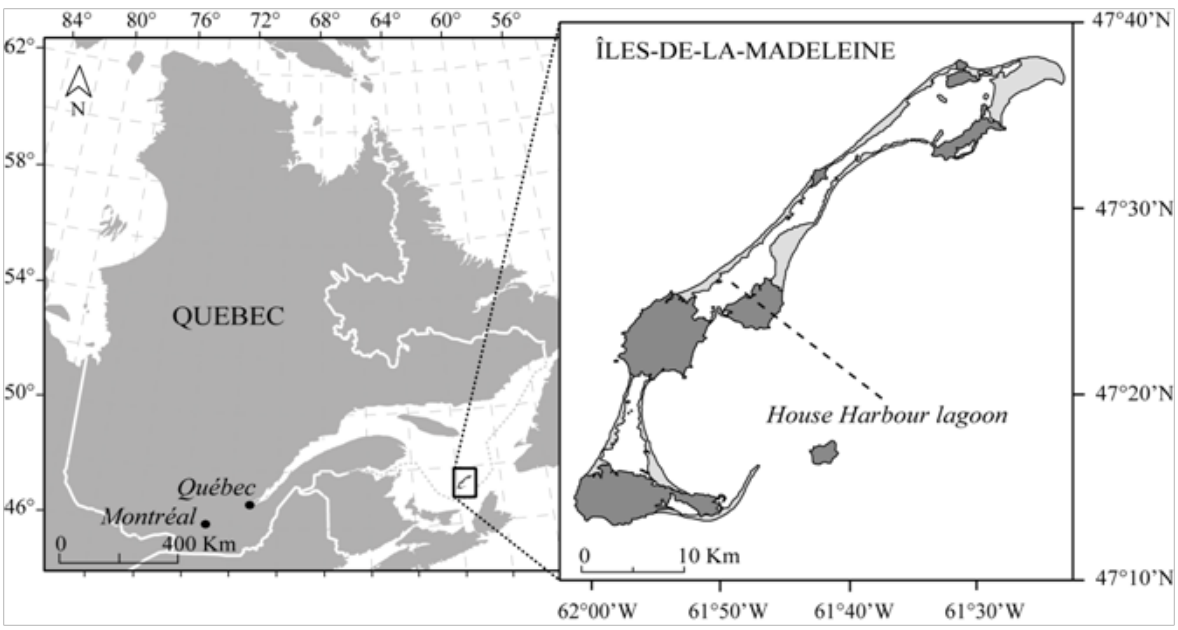

Figure 1: The experimental site in House Harbour lagoon (HH).

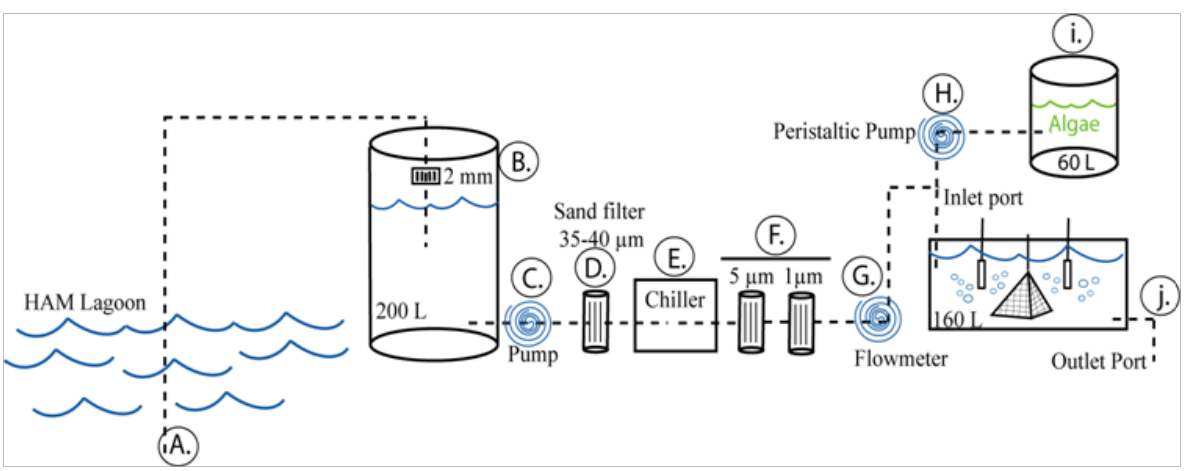

Figure 2: Microalgae diet setting.

Diets characterization

Each day during sampling periods, ND samples (triplicate) were pumped (Pump Rule 3700, ITT Co., Gloucester, MA, USA) at 2.5 m below the surface, at the experimental site, into opaque 2-L Nalgene bottles. Each 2 litres sample was sorted through a 177-µm mesh size Nitex and then collected on a Whatman GF/C filter pre-combusted at 450°C. For determination of lipid and fatty acids composition, filters were stored at -80°C in lipid-free amber glass vial with Teflon-lined caps under nitrogen with 1 ml dichloromethane and BHT (0.1% w/v) to prevent sample degradation. To determine dry weight, samples were washed with ammonium formate (3%), dried at 70°C for 24 h and weighed to obtain the total particulate matter (TPM). Then they were burned for 4 h at 450°C and reweighed

to obtain the particulate inorganic matter (PIM). Particulate organic matter (POM) was estimated by subtracting the PIM from the TPM. Triplicates of 2 litres MD were sampled in the experimental tank used to feed mussels in opaque 2-L Nalgene bottles to determine dry weight, lipid and fatty acids composition as already described for ND. Theses water characterizations were also done on each sampling days of four sampling periods. Water temperature and salinity were monitored in the reserve tank each day with a multiparameter sonde YSI30 (YSI instrument, Yellow Springs, OH, US). Water samples for nutrients analysis and for flux cytometry were pumped out at 2.5 meters below the surface and temperature and salinity measured (for ND). Theses analyses were made in triplicates. For nutrient analysis, water (1 ml) was filtered through a LCR membrane 0.45µm filter (Millipore, Billerica, MA, USA) fixed on a syringe into pre-indentified centrifugation tube to preserve nutrient samples. These samples were frozen at -80ºC until analysis with a Bran Luebbe Auto analyzer 3 (SEAL Analytical, ltd. Mequon, WI, US) using standard methods of Amstrong et al. [31] for nitrates, Murphy & Riley [32] for phosphates and Grasshoff et al. [33] for silicates. ND samples for flux cytometry (4.5 ml) were filtered on a 20-µm Nitex filter and preserved in a cryovial. For MD, flux cytometry was done with cells from two microalgae batches, used during the four experimental periods. These samples were also preserved in a cryovial (without filtration). Glutaraldéhyde 25 % (final concentration 0.1%) was added to the samples which were kept in the dark for 15 minutes for cells fixation. Then the samples were frozen at -80ºC until analysis with an Epic Altra flux cytometer (Beckman coulter inc., Brea, CA, USA). Six groups of planktonic cells were quantified: heterotrophic bacteria separated according to their nucleic acid content (LNA and HNA for low and high nucleic acid, respectively), in addition to eukaryotes and cyanobacteria according to the pico (0.2-2 µm) and nano (2-20 µm) size classes using the method described in Belzile et al. [34].

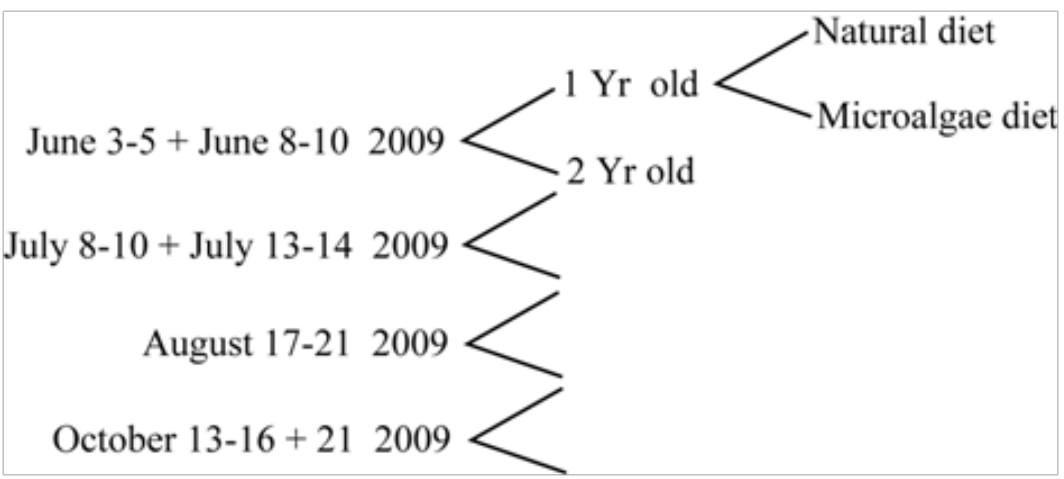

Figure 3: Experimental design.

Physiological measurements

Epibionts were gently removed from the experimental mussels and these mussels were identified with a white maker depending on their diet and age. Before measurements, individual mussels were placed in six independent 250 ml metabolic chambers with fresh aerated filtered (1 µm) seawater kept well-mixed with a magnetic stirrer for 1-2 hours. Physiological measurements were done in a temperature-controlled room set at the same water temperature as the one measured in the lagoon. Physiological measurements began with oxygen consumption (VO_2) recording. The metabolic chambers were sealed and the decrease in dissolved oxygen was measured with a YSI (5331) polarographic electrode coupled to YSI (5300) micro-oxymeters (YSI instrument, Yellow Springs, OH, USA) and flatbed recorders (Servogor 102, mini-insturments Inc, Skelmersdale, UK). The output signal was monitored continuously until a minimum decrease of 20% in dissolved O_2 was obtained. Mussel respiration was expressed in $mlO_2 \cdot h^{-1} \cdot g^{-1}$ dry weight with an allometric coefficient of 0.65 [35]. A control chamber with no mussels has been used for each measure set. Two sets were done each measurement day. When mussels spawned during the physiological determinations, the data were eliminated. Clearance rate measurements (CR) were performed after VO_2 measurements with the same experimental mussels. Each mussel was transferred into a 500ml chamber filled with the experimental diet, i.e. water from the lagoon (ND) or the microalgae diet (MD). For each measurement set (total of two per day) six chambers plus one control chamber were used. The experimental diet was kept in suspension through a gentle aeration all around the chamber. Water samples (20 ml) were taken at every 15 minutes for 45 minutes (T0, T1, T2, T3) and stored in borosilicate tubes with 80µl Lugol acid (0.4 % v/v) at 4°C until estimation of particles concentrations with a Beckman Coulter-counter Z2 fitted with a 70-µm orifice tube (Beckman Coulter Canada,

Mississauga, ON, Canada). The greatest difference between two particles measurements (between T0 and T1, or T1 and T2, or T2 and T3) was used for the CR calculation (36, 37). The CR ($l \cdot h^{-1} \cdot g^{-1}$ dry weight) was calculated as $[\ln(C_i/C_f)/(t_f-t_i) \times V]/m^{\alpha}$, where C_i = initial particle concentration (T0 or T1 or T2), C_f = final particle concentration (T1 or T2 or T3), (t_f-t_i) = duration between C_f and C_i measurement (always 0.25 hours), V = chamber volume (l), m = mussel dry weight tissue (g) and α = allometric coefficient of 0.72 as in Riisgard & Molhenberg [38]. A chamber control with no mussel was used in case there were cells sedimentation and/or cells proliferation. Correction factor calculated as $[\ln(C_i/C_f)/0.75\ h]$ and subtracted from calculated CR values [39]. The mean correction factors for cells sedimentation (positive value) or proliferation (negative value) were between 0.05 and 0.29 $l h^{-1}$. At the end of the physiological measurements, we separated the flesh mass of mussels into two halves, each containing half of the organs present. One half was dried for 72 h at 65°C to assess mussel dry mass and the other frozen at -80°C for later lipids analysis. Shells were also measured and weighted. Shell lengths of mussels used for measurements increased with a mean (± SE) of 36 ± 3.9 mm in early June to 52.4 ± 10 mm in mid-October for one-year-old mussel and 55.1 ± 5.6 mm to 65.2 ± 2.4 mm for two-years-old mussels.

Lipids analysis

Lipids were extracted in dichloromethane-methanol like Parrish technique [40] using a modified Folch procedure [41]. Extracts were spotted on silica gel-coated chromarods (SIII, Shell USA). Lipid classes were determined according to Parrish [42] to determine wax esters (WE), ketones (KET), triacylglycerols (TAG), free fatty acids (FFA), free aliphatic alcohol (ALC), sterols (ST), acetone-mobile polar lipids (AMPL), phospholipids (PL) contents by thin-layer chromatography using flame ionization detection (TLC/FID) with an Iatroscan MK-6 (Shell USA, Fredericksburg, VA, USA). Lipid classes were evaluated in $mg \cdot g^{-1}$ of wet tissue, summed and expressed as a percentage of total lipids. Afterward, lipid extracts were separated into neutral (including triglycerides, free fatty acids and sterols) and polar lipids (including mainly phospholipids) fractioned by silica gel (30 X 5 mm i.d, packed with Kieselgel 60, 70-230 mesh, (Merck, Darmstadt, Germany) hydrated with 6% water and eluted by dichloromethane: methanol and methanol, respectively [43]. Neutral fraction was further eluted on an activated silica gel with hexane and diethyl ether to eliminate the free sterols. Fatty acids methyl esters (FAMEs) from each neutral and polar fraction were prepared by addition of sulphuric acid and heating at 100ºC for ten minutes and analyzed in MSMS scan mode (ionic range: 60 - 650 m/z) on a Polaris Q ion trap coupled to a Trace GC (Thermo Finnigan, Mississauga, ON, Canada) equipped with a VB-5® mass spec grade fused silica capillary column (30 m x 0.25 mm i.d; ValcoBond®, VICI Valco Instruments Co. Inc., Broakville, ON, Canada) with high purity helium as the carrier gas. External calibration was used with appropriate dilution in hexane: dichloromethane of Supelco 37 component FAME Mix standard solution (Supleco Inc., Belfonte, PA, USA) to obtain five-point calibration plots, from 6.25 to 100µg ml^{-1}. Mid-August samples have been lost before lipids and fatty acids analysis, so this period in not included in analyses.

Statistical analyses

Three-way ANOVAs (analyse of variance) were used to study the effects of sampling period, mussels age and diet on the physiological parameters, and on lipids classes. Two-way ANOVA were used to determine differences in the content of organic (POM), inorganic (PIM), and total particulate matter (TPM) in both natural and microalgal diets for the four sampling periods. Finally two-way ANOVA were used to determine differences among dry weight for one- and two-year-old mussels in both diets for the four sampling periods. Normality was tested on residuals with a Shapiro-Wilk test. Homogeneity of variance was graphically verified and data were independent for each other. When application conditions were not satisfied, data were transformed. In physiological measurements, VO2 and CR data were log-transformed and the percentage of lipid classes was square root-transformed. For POM and PIM, after all possible transformations have been tried unsuccessfully to satisfied application conditions, two diet means were randomly eliminated in the June dataset to obtain equal sample sizes [44]. Untransformed data are presented in tables and figures. When differences were detected, a posteriori comparisons were performed using the Tukey test. All statistics tests have been performed on JMP 7 statistical software (SAS institute inc., Cary, NC, USA). To visualize differences in fatty acids between ND and MD, a multidimensional scaling (MDS) using PRIMER-E 5.2.2 statistical software was used (PML, Plymouth, Devon, UK). MDS was realized from a similarity matrix (Bray-Curtis) on unprocessed data. ANOSIM (analysis of similarity) was also performed on samples to look at dissimilarities within and between each group (MD and ND). Negative response indicates that dissimilarities are greater within groups compared to differences between groups while positive response indicates greater dissimilarities between each group compare to dissimilarities within group [44]. T-tests were also performed to look at differences in percentage of total FA among both diets for each fatty acid. When application conditions were not satisfied log transformations were done. Sequential Bonferroni correction [45] was applied on the t-tests results. Finally, as for diets, similar analyses were realised to determine differences between fatty acids content in mussel gills fed with the two diets.

Results

Temperature and salinity

Temperature during each sampling period was 11.5 ± 2.2 °C in June, 14.5 ± 1.1°C in July, 18.3 ± 2.2°C in August, and 8.1 ± 1.0°C at the end of October (mean ± SE). The salinity at study site was stable over the experimental period and varied only between 30.0 and 31.1 ‰ from the beginning of June to the end of October.

Natural diet (ND) and microalgae diet (MD) characteristics

No significant interactions between factors (date x diet) for TPM, PIM, and POM were observed. There were also no significant differences among TPM content in both diets in contrast to POM and PIM contents (Table 1). MD contained a higher POM content ($mg \cdot l^{-1}$) compare to ND, while ND contained higher PIM compare to MD. Not surprisingly, there were significant differences for all seston components among the four sampling periods from June to October. There was an increase in TPM, PIM and POM from mid-August to October comparatively to early June (Figure 4).

Table 1: Summary of two-way ANOVA for organic particulate matter (POM), inorganic particulate matter (PIM) and total particulate matter (TPM) in both diets and for all sampling periods. Bold values indicate significant effect.

Variation		**POM**		**PIM**		**TPM**	
Sources	***df***	***f***	***p***	***f***	***p***	***f***	***p***
Date	3	3.11	**0.04**	12.93	**<0.0001**	8.51	**0.0002**
Diet	1	6.63	**0.015**	12.4	**0.001**	0.14	0.711
Date x Diet	3	2.29	0.097	1.11	0.359	0.41	0.744
Error	32						

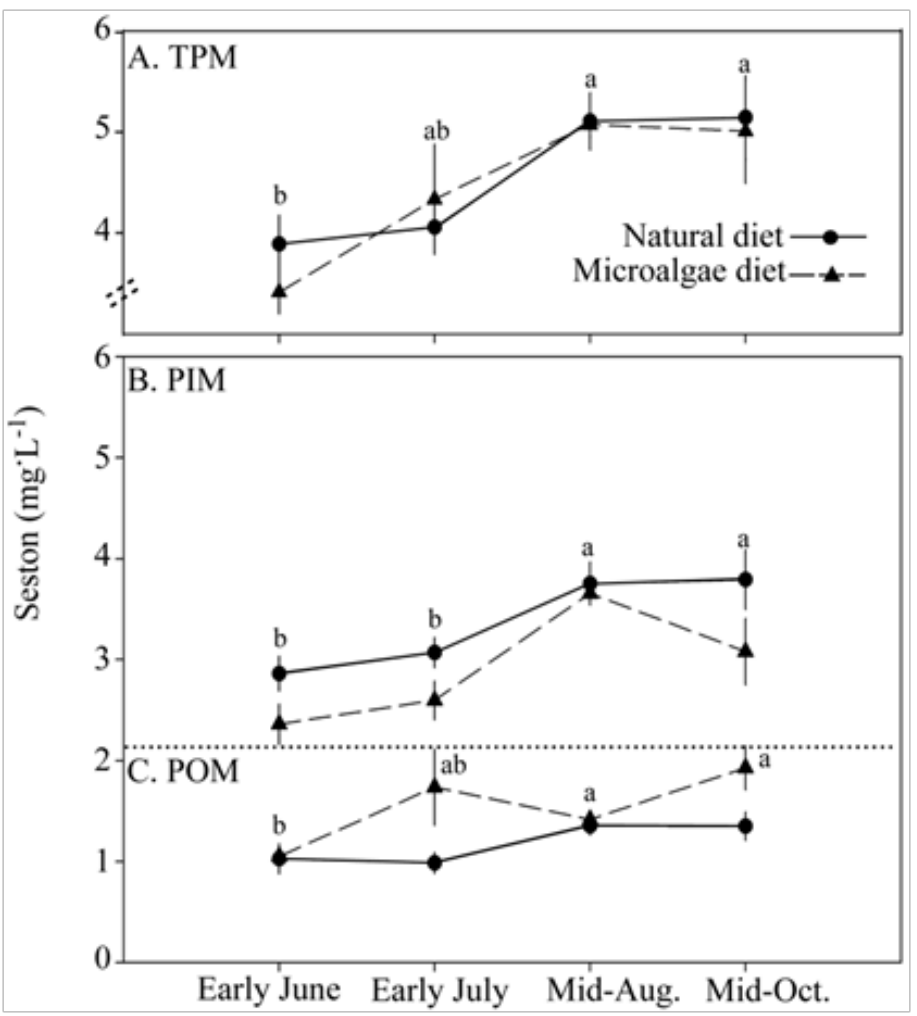

Figure 4: Characteristics of both the natural (ND) and microalgae (MD) diets in terms of seston components during the four experiment periods (mean ± SE).Different letters represent significant difference at $p<0.05$.

Nutrients

Nutrients in HH lagoon (ND diets) were really low over the four sampling periods with overall means of 0.15 ± 0.06 µmol $PO_4^{3-} \cdot l^{-1}$ (phosphates), 0.91 ± 0.02 µmol $NO_2^-/NO_3^- \cdot l^{-1}$ (inorganic nitrogen), and 0.96 ± 0.14 µmol $Si(OH)_4^- \cdot l^{-1}$ (silicates) (mean ± SE).

Bacteria and microalgae concentrations

MD contained higher microalgae cells abundance compared to ND (from 2 to 10 times higher levels depending of time). Microalgae cells in the natural diet were present in a higher proportion in early July (Figure 5). ND diet was characterized by a high proportion of picocyanobacteria and picoeukaryotes with 68 to 93 % of total contribution. The counts of bacteria cells in the diets (Figure 6) gave a contrasting picture relative to the algal cells. Bacteria were much more abundant in the natural diet (over 25 time higher) than in both batches of MD. LNA bacteria were present in higher proportions than HNA (percentage relative to total counts) in the natural diet during the first three sampling periods: 60.2 % in June, 53.9 % in July and 50.5 % in August (Figure 6). In contrast, LNA represented 45.4% of the total bacteria count in October. In the MD, LNA bacteria were present in a smaller proportion than in the ND with: 10.2 % in batch #1 and 23.6 % in batch #2.

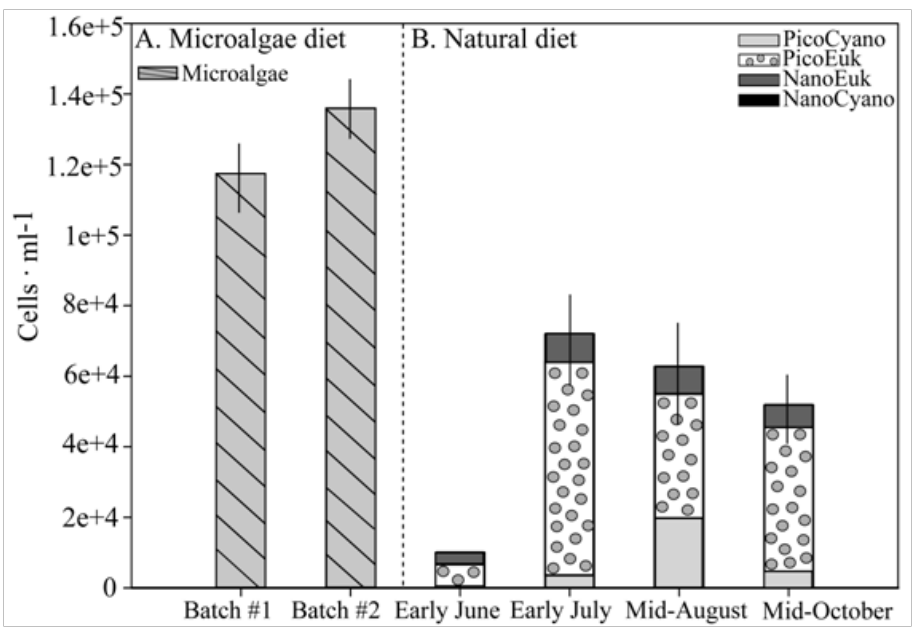

Figure 5: Autotroph cells (pico-cyanobacteria, pico-eukaryotes, nano-eukaryotes, and nano-cyanobacteria) count sin both natural (ND) and microalgae (MD) diets (mean ± SE).

Mussels dry weight

In mussel's dry weight measurements, no significant interactions between factors were noted (date x diet). Mussels dry weight increase from early July for one-year-old mussels ($F_{(3, 95)}$ = 130, p<0.0001) and from mid-August for two year-old mussel ($F_{(3, 96)}$ = 39.4, p<0.0001) in relation to their post-spawning period (Figure 7). Dry weights were similar among diets.

Physiological measurements: oxygen consumption and clearance rates

No significant double or triple interaction (date, age and diet) were detectable for both VO_2 and CR (Table 2). No diet and mussels age effects were observed on VO_2. No diet effect has been observed on clearance rates. The only significant differences in VO_2 were among sampling periods with higher oxygen consumption in July and August compared to early June and mid-October (Figure 8). CR was significantly different among mussels ages and sampling periods (Figure 9). CR decrease from early June to mid-October.

Lipids classes in diets and mussels gills

Significant variations of lipids classes were observed between diets or sampling periods (Table 3). In the TAG content (mean ± SE), significant variations were related only to sampling periods with a higher level in early June (24 ± 14%) comparatively to 9.6 ± 5.5% and 12.7 ± 8.1% for early July and mid-October respectively. ST, AMPL and PL showed variations only to diet factor with higher level of ST and AMPL in the ND (ST: 4.2 ± 2.1% and AMPL: 53.9 ± 9.6%) comparatively to MD (ST: 0.9 ± 2.4% and AMPL: 38.2 ± 12.5%). PL showed higher level in microalgae diet with values of 48.9 ± 20.8% comparatively to 22.2 ± 12.9% for natural diet. WE, FFA, KET and ALC level were negligible with less than 2% of contribution and were not included in

analyses. Overall, mussels gills were mainly constituted by PL (84 ± 6.8%), followed by AMPL (9.2 ± 5.6%), ST (5.1 ± 2.8%) and TAG (1.8 ± 2.1%). TAG, WE, FFA, KET and ALC were excluded from analyses due to negligible level (less that 2% contribution). Significant date and diet interaction have been observed for ST content in the mussel gills (Table 4), with higher levels in mid-October (Table 5). AMPL showed significant date and age interaction (Table 4) and where characterized generally by higher level in gill in one-year-old mussels except in mid-October (Table 5). For PL, similar date and age interaction have been observed (Table 4) and two years-old mussels exhibited higher level in their gills except in mid-October (Table 5).

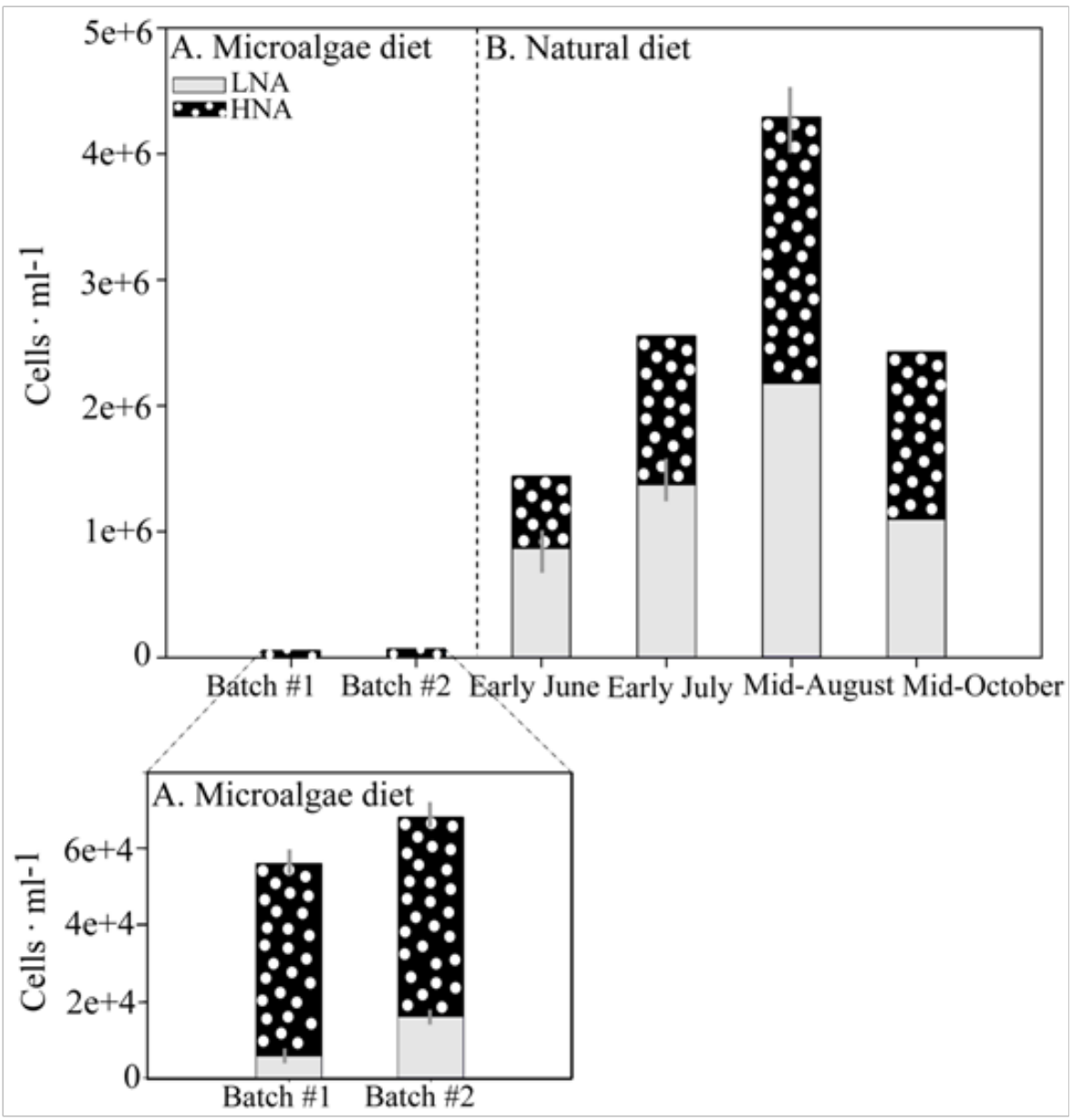

Figure 6: Counts of high (HNA) and low nucleic acid bacteria (LNA) cells in both natural (ND) and microalgae (MD) diets (mean ± SE).

Fatty acids in diets

A multidimensional scaling (MDS) was performed on fatty acids found in ND and MD. Only FA contributing to > 2 % of the total, were included in the analysis. MDS shows similarity between sampling dates for a given diet. The FA found in the MD from different sampling period seemed to be less variable than those in the natural diet, as symbols are closer on the MDS (Figure 10). Both diets seemed different for all experimental samplings. Overall representativeness of the test was reliable (stress=0.07) (44). Global R provided by ANOSIM was 0.765 indicating greater differences between

each diet than within sampling dates (p=0.001). A series of t-tests were performed to look at the FA contributing the observed dissimilarity among ND and MD (Table 6). Only 18:4n3, 18:3n3, 20:4n6 (AA), 22:6n3 (DHA), sum of PUFA and EFA showed no significant differences between both diets. Total contribution of FA with at least one unsaturation (MUFA) was 7% higher in MD than in ND, particularly because of higher levels of 16:1 and 18:1n9. ND was characterized by 7% more saturated fatty acid (SFA), than the MD. There were no significant differences between total EFA found in both diets, but the level of EPA was about 3% higher in MD. The mass of total FA (TFA) was about twice higher in MD comparatively to ND.

Table 2: Summary of three-way ANOVA showing the effects of age, diet and sampling period on VO2 and CR for one- and two-year-old mussels fed with microalgae or natural diet. Bold represent significant effect.

	Oxygen consumption(VO2)			**Clearance Rates (CR)**		
Variation sources	df	f	p	df	f	p
Date	3	87.93	<0.0001	2	6.11	0.003
Age	1	2.9	0.09	1	11.92	0.001
Date x Age	3	1.37	0.255	2	0.06	0.943
Diet	1	0.004	0.951	1	1.42	0.235
Date x Diet	3	1.05	0.371	2	1.25	0.29
Age x Diet	1	0.96	0.33	1	2.04	0.155
Date x Age x Diet	3	1.03	0.38	2	0.54	0.585
Error	212			149		

Table 3: Diets characterisation: Two-way ANOVA showing the effects of date and diet on lipids classes in% for triacylglycerols (TAG), sterols (ST), acetone-mobile polar lipids(AMPL) and phospholipids (PL) in natural (ND) or microalgae (MD) diet. Bold represent significant effect.

Variation	***TAG***			***ST***		***AMPL***		***PL***	
sources	***df***	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
Date	2	5.58	0.01	0.24	0.789	0.3	0.747	2.14	0.138
Diet	1	2.47	0.128	44.12	<0.0001	5.03	0.034	22.23	<0.0001
Date x Diet	2	0.19	0.831	1.31	0.289	2.22	0.129	0.11	0.897
Error	26								

Table 4: Mussels gills characterisation: Three-way ANOVAs showing the effect of date, age and diet on lipid classes in% for sterols (ST), acetone-mobile polar lipids(AMPL) and phospholipids (PL)for one- and two-year-old mussels fed with natural (ND) or microalgae (MD) diet. Bold represent significant effect.

Variation	***ST***			***Ampl***		***PL***	
sources	***df***	***f***	***p***	***f***	***p***	***f***	***p***
Date	2	17.2	<0.001	0.84	0.433	2.83	0.062
Age	1	60.61	<0.001	8.88	0.003	0.06	0.798
Date x Age	2	0.49	0.613	8.35	0.004	6.46	0.002
Diet	1	1	0.319	0.69	0.407	2.18	0.142
Date x Diet	2	5.11	0.007	0.2	0.817	0.38	0.68
Age x Diet	1	1.36	0.245	2.42	0.121	0.98	0.324
Date X Age X Diet	2	0.35	0.707	0.49	0.611	0.55	0.58
Error	154						

Table 5: Variations of lipid classes in gills of mussels in relation to significant interactions between factors as highlight in Table 4(mean ± SE). Different letters indicate significant differences inside these lipids classes.

	Variation sources	Early June	Early July	Mid-October
ST	Natural diet	4.6 ± 0.5bc	3.9 ± 0,6c	6.3 ± 0.4ab
	Microalgae diet	3.6 ± 0.7c	5.2 ± 0.6abc	6.9 ± 0.5a
AMPL	One-yr-old mussels	11.1 ± 2.1ab	12.5 ± 1.9a	7.7 ± 0.8bc
	Two-yr-old mussels	7.7 ± 1.2c	7.1 ± 1.8bc	9.7 ± 0.8abc
PL	One-yr-old mussels	82.9 ± 2.4ab	83.3 ± 2.3ab	86.6 ± 1.0a
	Two-yr-old mussels	84.6 ± 1.6ab	86.1 ± 1.5a	81.1 ± 1.1b

Table 6: FA composition for natural and microalgae diets for pooled values of early June, early July and mid-October* indicate fatty acids with significant differences after sequential Bonferroni correction. All values represent mean ± SE with N=5.

Variable	Natural Diet	Microalgal Diet
14:0*	6.44 ± 0.35	12.29 ± 0.38
15:0*	1.70 ± 0.07	0.50 ± 0.04
16:0*	16.96 ± 0.62	19.41 ± 0.47
17:0*	1.77 ± 0.12	0.42 ± 0.03
18:0*	6.19 ± 0.52	1.42 ± 0.16
20:0*	3.13 ± 0.26	0.70 ± 0.06
22:0*	3.31 ± 0.28	0.87 ± 0.06
24:0*	2.95 ± 0.22	0.48 ± 0.11
Σ SFA*	42.83 ± 1.21	36.26 ± 0.84
16:1*	8.73 ± 0.64	13.28 ± 0.27
18:1n9*	7.63 ± 0.63	11.36 ± 0.75
20:1*	1.24 ± 0.37	0.26 ± 0.08
22:1n9*	1.13 ± 0.24	0.40 ± 0.05
Σ MUFA*	19.42 ± 1.21	26.30 ± 0.81
18:3n6*	1.55 ± 0.50	0.92 ± 0.04
18:4n3	2.97 ± 0.24	3.00 ± 0.24
18:2n6*	4.38 ± 0.33	5.40 ± 0.19
18:3n3	3.47 ± 0.17	2.93 ± 0.05
20:4n6 (AA)	1.87 ± 0.12	2.15 ± 0.07
20:5n3 (EPA)*	8.50 ± 0.64	11.20 ± 0.49
20:3*	1.68 ± 0.24	0.58 ± 0.08
20:2*	0.81 ± 0.37	0.48 ± 0.03
22:6n3 (DHA)	10.93 ± 1.18	10.40 ± 0.33
22:5n3 (DPA)*	1.23 ± 0.42	0.10 ± 0.10
Σ PUFA	37.76 ± 1.52	37.43 ± 0.97
Σ EFA	21.31 ± 1.47	23.75 ± 0.82
TFA (μg Mg^{-1})*	12.58 ± 1.67	24.80 ± 5.43

Fatty acids composition is given as % of total fatty acids.

Only fatty acids contributing > 2% in at least one sample were reported.

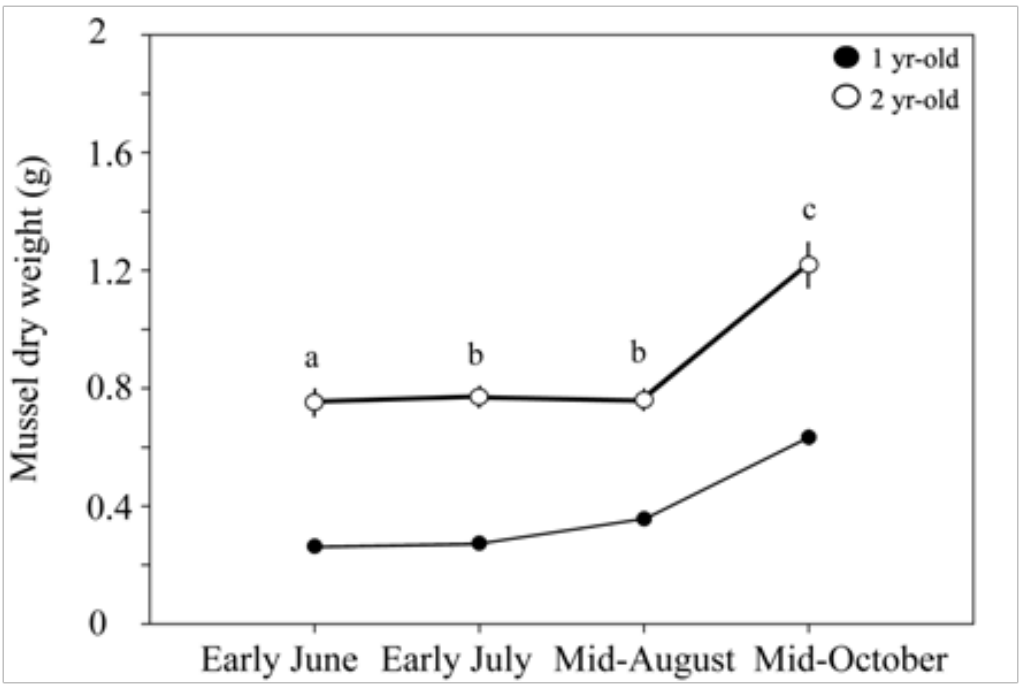

Figure 7: Mussels dry weight (g) used during the four sampling periods for one- and two-year-old mussels (mean ± SE). Different letters represent significant difference at $p<0.05$. Same letters for 1 year-old mussels as no temporal difference were observed.

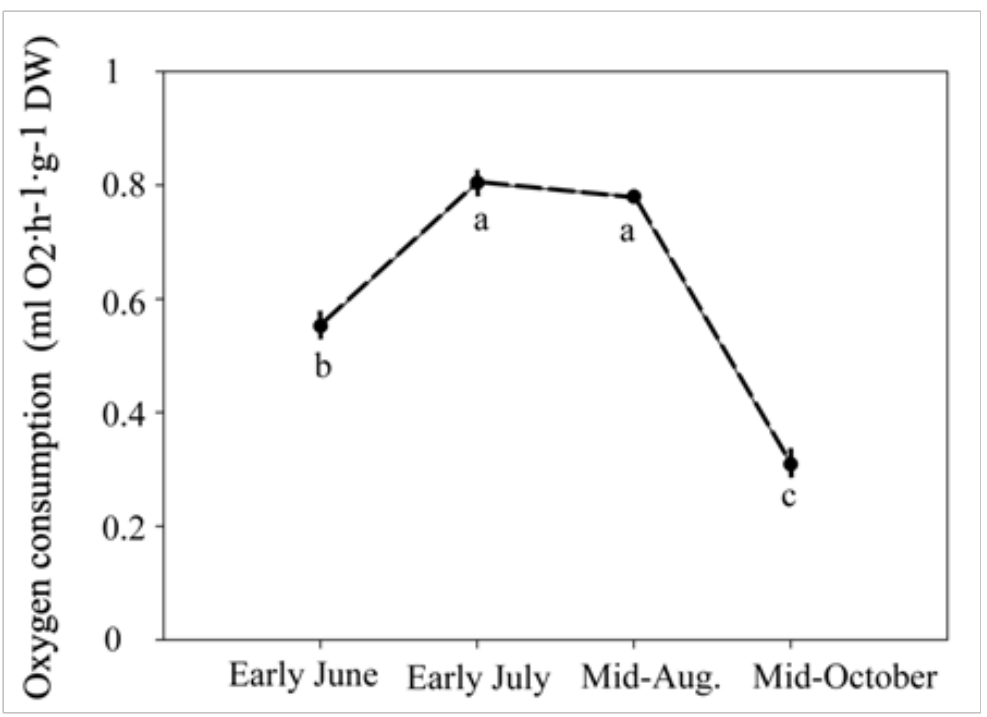

Figure 8: Oxygen consumption (VO2) as a function of sampling period (mean ± SE). Data from different diets and age classes were pooled together as there were no significant interactions. Different letters indicate significant difference sat $p<0.05$.

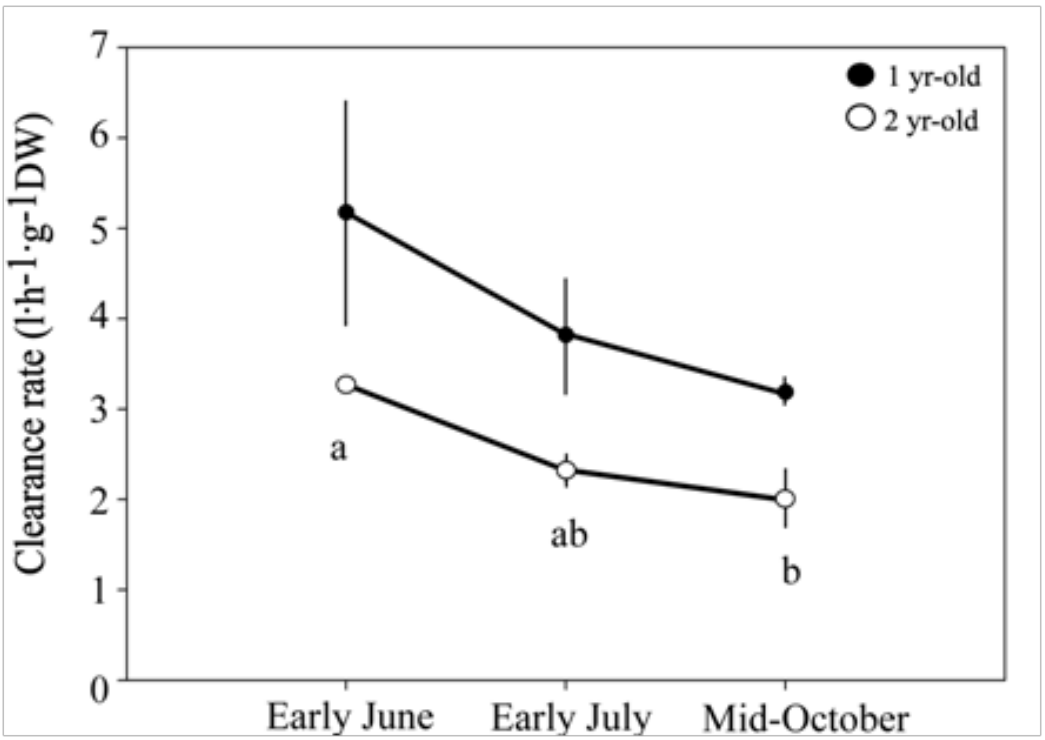

Figure 9: CR as a function of time and age (mean ± SE). Data from different diets were pooled together as these effects were not significant. Different letters indicate significant differences between sampling dates at $p<0.05$. Same letters for 2 year-old mussels as no temporal difference were observed.

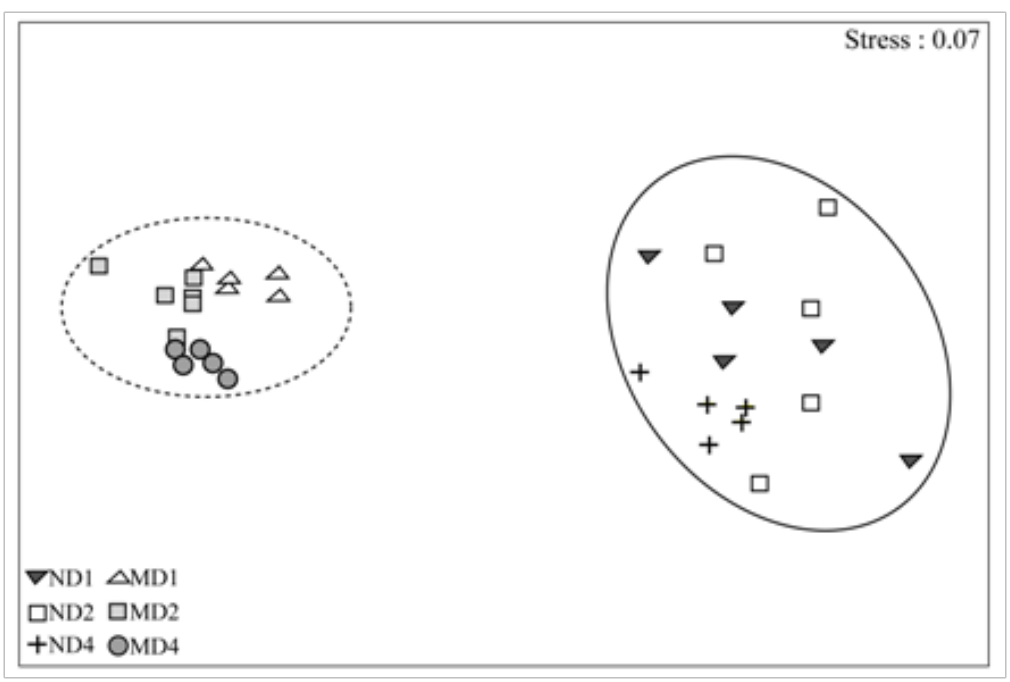

Figure 10: MDS on natural (ND, plane line) and microalgae (MD, dash line) diets for sampling period of early June (1), early July (2) and mid-October (4). Each symbols corresponds to a replicate (N=5).

Fatty acids in mussels gills

Multidimensional scalings (MDS) was also performed on fatty acids found in mussels gills fed with either the ND or MD (Figure 11). Only FA contributing to >2 % of the total, were included in the analysis. MDS ere performed for one- and two-year-old mussels separately. The stress value provided with a MDS indicates inter-objects distances to dissimilarities. Stress value for one-year-old mussel (0.14) is acceptable but not the one for two-year-old mussels (0.21) [44]. Thus, further analyses were only done for one-year old mussel group. The global R from ANOSIM was 0.119 (p=0.001) for one-year-old mussels and 0.089 (p=0.001) for two-year-old ones. A series of t-tests was done to look which FA's made inter-group differences as shown in MDS with 1-yr-old mussels (Table 7). In SFA or MUFA groups, slight significant differences (<1%) were found for 16:0, 16:1, 18:1n9, and 18:1n7 between mussel gills fed with both diets. PUFA showed 3% higher level, particularly in essential fatty acids in mussels gills fed with ND. Total FA's mass was similar in the gills of mussels fed with both diets with about 2.3 $\mu g \cdot mg^{-1}$.Note that no NMID were found in mussels gills. An indicator of the nutritional quality of the experimental diets, could be defined as the ratio of the polar fraction of fatty acids (phospholipids constituting cell membranes) in mussels gills relatively to the fatty acid in each diet. This ratio indicates selective incorporation or elimination in the cell membrane of gills for a given dietary fatty acid. A level of fatty acid similar between mussel's gills and diet (equal relative proportion) confirm a presumably satisfied nutritional requirement for this fatty acid. Only results for some essential PUFAs are presented (Figure 12). PUFA ratio around 1.5 indicating selective incorporation of these FA by mussels. AA showed the most important selectivity (ratio over 3.7) followed by DHA (over 1.8) for both diets. However, EPA selectivity were observed only for mussel's gills fed with ND.

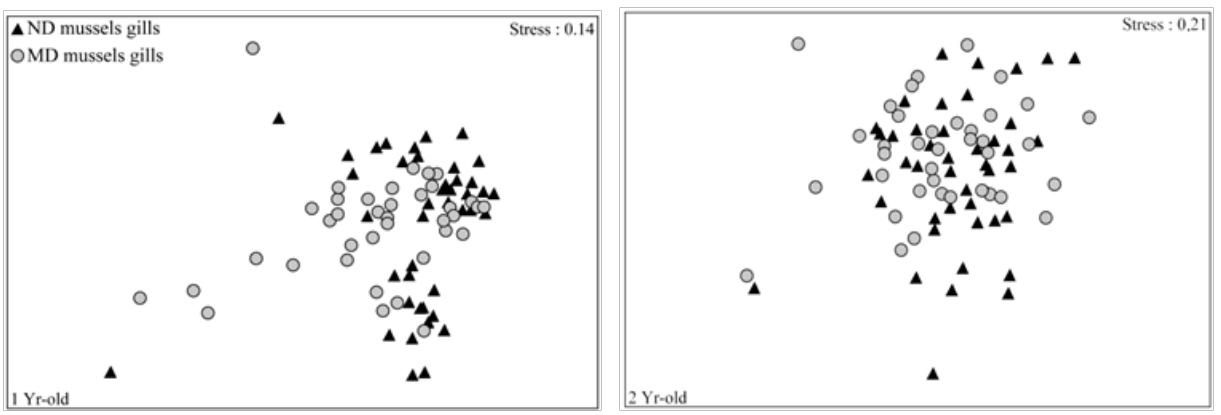

Figure 11: MDS representation for fatty acids contents in gills from one- and two-year-old mussels fed with both natural (ND) and microalgae (MD) diets. Symbols are the number of replicate for all sampling period.

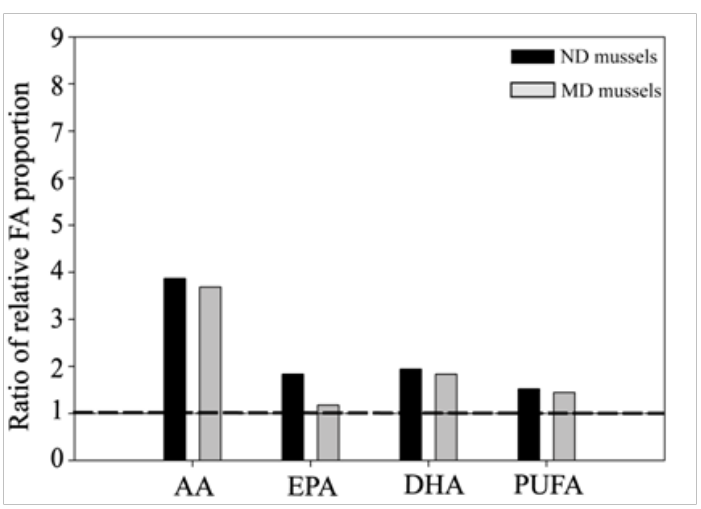

Figure 12: Relative proportion of some essential fatty acids (AA, EPA, and DHA) and polyunsaturated fatty acids in lipid polar fraction of mussel's gills feed on natural (ND)diet from oligotrophic lagoon and from cultivated microalgae diet (MD). The dashed line indicates the proportion of fatty acids in the gills equal to those in the diets.

Table 7: Fatty acids composition for one-year-old mussels (gills) fed with either natural (ND) ormicroalgae (MD) diets. * indicate fatty acids showing significant differences after sequential Bonferroni correction. All values represent mean ± SE (N=15).

Variable	Mussels fed with natural diet	Mussels fed with microalgal diet
16:0*	1.70 ± 0.07	2.14 ± 0.06
18:0	3.43 ± 0.10	3.31 ± 0.10
24:0	0.74 ± 0.08	0.47 ± 0.08
Σ SFA	20.06 ± 0.21	20.95 ± 0.30
16:1*	1.70 ± 0.05	2.14 ± 0.05
18:1n9*	1.65 ± 0.05	2.40 ± 0.06
18 :1n7*	1.73 ± 0.05	1.96 ± 0.04
20:01	8.09 ± 0.32	7.80 ± 0.30
Σ MUFA	15.51 ± 0.30	16.53 ± 0.30
18:2n6*	1.55 ± 0.06	1.85 ± 0.05
20:4n6 (AA)	7.23 ± 0.20	7.92 ± 0.21
20:5n3 (EPA)*	15.57 ± 0.38	13.19 ± 0.36
20:3	5.95 ± 0.12	6.30 ± 0.15
22:6n3 (DHA)*	21.20 ± 0.30	19.09 ± 0.28
22:5n3 (DPA)*	2.55 ± 0.03	2.30 ± 0.03
Σ PUFA*	57.39 ± 0.40	54.10 ± 0.56
Σ EFA*	14.67 ± 0.16	13.40 ± 0.21
TFA (µg mg^{-1} DW)	2.31 ± 0.10	2.25 ± 0.16

Fatty acids composition is given as % of total fatty acids.

Only fatty acids contributing >2% in at least one sample were reported.

Discussion

The present study confirmed that the Magdalens Islands (MI) lagoons are oligotrophic environments as previously shown by Trottet [1, 7] & Roy [2]. Nutrients found in House Harbour (HH) lagoon were present in very low concentrations (4) with nitrogen/phosphates/silicates levels always present in concentrations < 1 µmol·l^{-1} from June to end-October. The absence of rivers, and thus terrestrial runoff, may limit nutrient supply. However, rain and continuous water column mixing by wind can contribute somewhat to provide nutrients. Another potential reason to explain the low nutrient concentrations can be the quick recycled nutrient utilization. As soon as nutrients are available they may be quickly used by autotrophs or other microorganism. HH lagoon was characterized by dominance (over 70%) of pico-species, mainly picoeukaryotes, and by important concentration of bacteria. The highest density of autotrophic cells in the natural diet (ND) from the lagoon was measured in early July with 7.2 x 10^4 cells ml^{-1} while bacteria concentration was until 4.3 x 10^6 cells·ml^{-1} in mid-August. In contrast, the artificial microalgal diet (MD) adjusted to obtain similar TPM content than ND diet, contained a much higher abundance of microalgae cells (1.2 to 1.4 x 10^5 cells·ml^{-1}) but a much lower bacteria concentration (5.6 to 6.8 x 10^4 cells·ml^{-1}). Thus, there were at least 18 times more phytoplanktonic cells in MD than in ND during the present study, explaining the higher mass of total fatty acids observed in the MD, as microalgae are known to have high levels of lipids [46].

Our first hypothesis was that ND from the oligotrophic HH environment should be deficient in EFA. This diet was compared to MD diet constituted from a mixture of *Nannochloropsis occulata*, *Pavlova lutheri* and *Isochrysis galbana* known to be rich in EFA [29, 47]. A food source mostly made of heterotrophs should have low levels of PUFA and EFA [48]. In the present study, the fatty acids profiles of each diet showed similar proportions of PUFA, EFA, EPA and DHA. Thus, the oligotrophic environment of HH lagoon seems to produce PUFA and EFA during all summer since fatty acids signature was similar to autotrophic environments. Results of flow cytometry showed important picoeukaryotes concentrations at each sampling date, suggesting that the PUFA and EFA source to consumers like mussels in HH lagoon (ND) could be these picoeukaryotes cells. However, as the mass of total fatty acids was two times lower in ND than in MD, the absolute quantity of PUFA and EFA was then less important in ND. This could be problematic for mussels particularly with the lower efficiency retention of these small particles by mussels [15]. However, your physiological data seem not indicate impact on mussels feed on this natural diet from oligotrophic environment.

Mussels fed ND exhibited similar VO_2 and CR as those fed with MD at all sampling dates covering spawning cycle, rejecting the second hypothesis. These differences in diets composition mainly related to size of plankton and bacteria concentration, particularly with their similar level of PUFA, seem no enough to induce physiological changes. These results could also suggest that mussels from HH lagoon are able to filter efficiently picoplankton, probably rich in PUFA, and bacteria found in high concentration in seawater to support energy and essential fatty acids needs. Some previous studies have already reported that mussels can eat particles as small as picoplanckton [11, 12, 15, 49]. The temporal variation of VO_2 and CR of mussels from early June to mid-October could be related mainly by the sea water temperature and reproduction process [27, 35, 50]. Another explanation for the seasonal variation in CR is a possible seasonal variation in food utilization by the mussel [51]. However, this could be dismissed since the composition of the MD diet was similar at each date.

Mussel gills was mainly composed of structural lipids, like phospholipids and sterols [19] with less than 10% of energetic lipids reserves. Either gills' mussels feed with ND or MD seemed to be deficient in EFA. With these results, the third hypothesis cannot by tested. The fatty acids in mussel's gills had similar profiles between mussels of two-year-old fed with the two diets while there were slight differences in one-year-old mussels in relation to diet. One-year-old mussels fed ND had approximately 1.2 % more EFA in their gills than mussels fed MD with values around 14.7 and 13.4 %. These results seem low relatively to values reported in literature. Pernet et al. [19] found about 33% EFA in mussel's gills of *Mytilus edulis* sampled in winter and fed with a mix of microalgae culture. However, PUFA results in mussels gills where in a range of 57.4 % and 54.1% for ND and MD mussels respectively. These results are more similar to the ones obtain by Pernet [19] which showed a total of 60% PUFA in mussels gills. Mantle of *M. galloprovincialis* also showed PUFA levels around 50% and over 30% EFA [52, 53] while Shin et al. [28] observed over 50% PUFA in the mantle *Perna veridi*. Thus, it is possible that lower EFA content observed in this study comparatively to literature could be related to a sub-optimum choice of microalgae species. The MD diet was composed of *Nannochloropsis occulata, Isochrysis galbana* and *Pavlova lutheri.* Some of these microalgae species could be hard to digest for the mussels, as suggested for *Nannochloropsis atomus* [54] & *Pavlova lutheri*[55] for oyster larvae, *C. gigas*. General selective retention for all EFA in gills of mussels fed with both diets was observed as the levels of these fatty acids were near one or over two times higher in the gills that in the ones in the food. Lower ratios like EPA in MD could suggest some EFA deficit for mussels. However, without samples with higher level of EFA in the gills it is difficult to confirm a nutritional deficit. When EFA are in short supply, molluscs like mussels can extend some precursors to obtain EFA in substitution, commonly called NMID [56-58]. In the present study no NMID were found in mussels gills and that could suggest no physiological deficit in EFA. The higher level of EFA in gills could reflect only the normal physiological retention process of these tissues. Furthermore, even if mussels spawned during the first sampling periods it seemed that the FA content in their gills was not disturbed. This is another element reinforcing the idea that actually HH lagoon is providing good food quality in lipids terms for mussels.

No difference were observed between PUFA composition of natural and microalgae diets, but for the other fatty acids a clear distinction was observed. ND showing higher SFA and lower MUFA levels than MD. In ND, small quantities of 15:0 (*pentadecanoate*) and 17:0 (*heptadecanoate*) were found (1.70 and 1.77 % respectively) compared to almost nothing in MD (0.50 and 0.42 %). These specific FA indicates the presence of bacteria in environment (59-61). As previously shown in the flux cytometry result bacteria are present in a relatively high proportion in ND compared to MD. Low percentage values of FA biomarkers for bacteria in ND can be explained by their microscopic size (<1µm). Thus, even if there are present in a high abundance, the biomass of FA bacteria was small. The two diets were characterized with important level of 14:0 (*myristate*) and 16:0 (*palmitate*), but these FA are mostly ubiquitous markers identifying many things simultaneously. It is difficult to highlight FA relationship with some specific organisms in ND. Two markers of terrestrial inputs and macrophyte residues are18:2n6 and 18:3n3(62), but similarity level between the two diets suggest weak impact of this food source for mussels in HH lagoons. For the two diatoms markers (16:4n1 and 20:5n3)(20), only 20:5n3 is present but at higher level in MD diets, suggesting weak abundance of diatoms during the 4 sampling periods in HH lagoons. Low levels of zooplankton markers (20:1n11, 20:1n9, 22:1n11, 22:1n9) (20) were observed in both diets, but this results was not surprising as seawater samples were sorted through a 177-µm mesh size before lipids analyses.

Conclusion

In conclusion, HH lagoon is oligotrophic because of a limited external supply of nutrients. This lagoon is host to a specific microfauna resulting from a microbial loop and the presence of abundant bacteria and picoeukaryote cells. It seem that HH lagoon is a diversified marine environment (in microorganisms) which could provide enough PUFA and EFA quantities for mussel's well-being. These FA are important for growth and survival of mussels. No NMID were found in mussel's gills probably for that reason. As a result, no significant differences were found on physiological parameters (VO_2 and CR) of mussels according to their diet.

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