Food Research International
October 2017, Volume 100, Part 1, Pages 151-160
http://dx.doi.org/10.1016/j.foodres.2017.06.041
http://archimer.ifremer.fr/doc/00389/50011/
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The effect of algae diets (*Skeletonema costatum* and *Rhodomonas baltica*) on the biochemical composition and sensory characteristics of Pacific cupped oysters (*Crassostrea gigas*) during land-based refinement

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Abstract :

Oyster refinement, a common practice in France, is aimed at increasing the weight of oyster tissue and influencing the taste properties of the refined oysters. Refinement usually takes place in land-based systems where the oysters are fed with relatively high concentrations of microalgae. In this study the impact of feeding *Skeletonema costatum* and *Rhodomonas baltica* on the biochemical composition and sensory characteristics of Pacific cupped oysters (*Crassostrea gigas*) from the Eastern Scheldt during land-based refinement was studied.

After a feeding period of four and seven weeks market-sized oysters were sampled for the analysis of fatty acids, free amino acids and volatile organic compounds and for a sensory evaluation by consumers and an expert panel.

The algae *Skeletonema costatum* showed a lower ∑ PUFA, ∑ n − 3, ∑ n − 6, C18:2n6, C18:3n3, C18:4n3, C22:6n3 content as compared with *Rhodomonas baltica*. These differences were also reflected in the fatty acid profile of the oysters fed with the corresponding algae diets. Furthermore, general linear model and principal component analysis showed marked differences in free amino acids and volatile organic compound content between *Skeletonema*, *Rhodomonas* fed oysters and reference oysters. For example, threonine, glutamine, leucine, histidine, (E)-2-hexenal, (E)-2octenal, (E)-2-octen-1-ol, (E,E)-2,4-octadien-1-ol, (E,Z)-3,6-nonadien-1-ol and (Z,E)-2,6-nonadienal contents were higher in *Skeletonema* fed oysters compared to *Rhodomonas* fed oysters. Sensory differences between the experimental oyster groups were shown. *Skeletonema* fed Pacific cupped oysters were characterized

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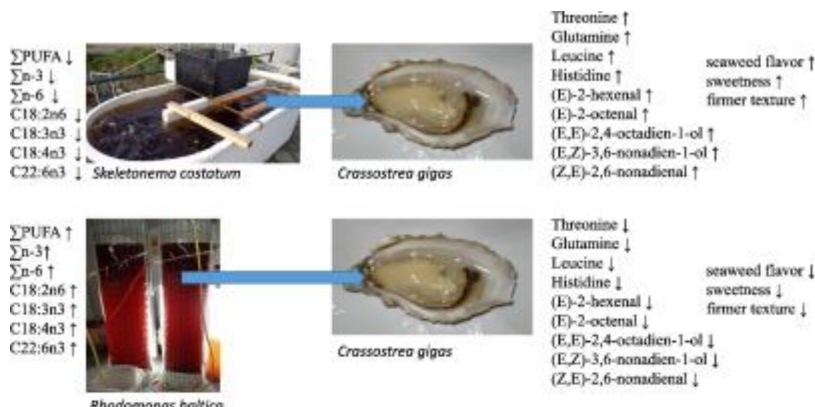
by a stronger seaweed flavor, higher perceived sweetness and a firmer texture in comparison with *Rhodomonas* fed oysters. Naïve consumers were only able to differentiate between *Rhodomonas* fed oysters and reference oysters.

Graphical abstract



Highlights

► The fatty acid profile of the algae and the oysters fed with the algae are similar. ► ∑ PUFA, ∑ n − 3 and ∑ n − 6 content is highest in *Rhodomonas* fed oysters. ► Total volatile organic compound content decreased during the experiment. ► PCA shows sensory differences between *Skeletonema* and *Rhodomonas* fed oysters. ► Consumers perceive differences between reference and *Rhodomonas* fed oysters.

Keywords : *Crassostrea gigas*, *Skeletonema costatum*, *Rhodomonas baltica*, Sensory properties, Free amino acids, Fatty acids

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1. Introduction

Shellfish refinement also known as a fattening procedure is considered a common practice in France (Soletchnik et al., 2001; Piveteau, Gandemer, Baud. & Demaimay, 1999; Robert, 1990). Market sized Pacific cupped oysters (*Crassostrea gigas*) are kept in basins and fed with naturally occurring algae in order to promote growth and to modify the taste of the oysters (Soletchnik et al., 2001; Piveteau et al., 1999). The oysters are kept in land-based systems where they are usually fed with relatively high concentrations of algae (approx. 45 mg l^{-1} DW algae per oyster) during a period varying from one to four months in order to increase the weight of oyster tissue. Shellfish refinement can lead to up to a 40 % increase in the market value for Pacific cupped oysters (Barille, Bougrier, Geairon, & Robert, 1994).

In many studies regarding the effects of refinement on oysters the diatom *Skeletonema costatum* has been used (Pennarun, Prost, Haure & Demaimay, 2003a; Pennarun, Prost, Haure. & Demaimay, 2003b; Soletchnik et al., 2001; Méléder et al., 2001; Piveteau et al., 1999). *Skeletonema costatum* naturally occurs in the fattening ponds in the West of France. This diatom is easy to cultivate using the natural saline groundwater sources in the area (Baud, Haure & Garnier, 1990; Baud & Bacher, 1990). Soletchnik et al. (2001) and Piveteau et al. (1999) showed

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an increase in the weight of oyster tissue in their experiments showing the efficiency of using *Skeletonema costatum* as a refinement diet. On the other hand, many other studies suggest that both the biochemical composition and therefore also the sensory characteristics of oysters can vary due to their respective diet. For instance Pennarun et al. (2003b) studied the effect of microalgal diets (*Skeletonema costatum* and *Isochrysis galbana*) on the VOC composition and the sensory properties of Pacific cupped oysters. Differences found in the VOC content of oysters were attributed to their algal diets and more specifically to the fatty acid content of both algae used. Also clear sensory distinctions were observed by a trained sensory expert panel between both algae fed oysters. Also Cochet, Brown, Kube, Elliott and Delahunty (2013) studied the effects of cultivation location on the sensory and biochemical characteristics of the Pacific cupped oyster. Clear distinction could be made between oysters cultivated in South Australia, New South Wales and Tasmania based upon their sensory characteristics. The authors hypothesized that some of these differences could be attributed to differences in the dietary composition between the different geographical locations.

The aim of our study was to investigate the effects of different algal diets (*Skeletonema costatum* and *Rhodomonas baltica*) with different fatty acid profiles on the **biochemical composition (in particular fatty acids, FAAs and VOCs) and sensory characteristics of the Pacific cupped oyster (*Crassostrea gigas*) originating from the most important Dutch cultivation area (Eastern Scheldt). In addition changes in the gross composition and condition index were measured. Furthermore an expert sensory panel as well as an oyster consumer panel was used to evaluate sensory aspects of the oysters during the refinement. This in-depth investigation will contribute to the development of innovative land-based cultivation of oysters in the Netherlands. In addition new refinement varieties might lead to the**

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development of new markets and improving the exploitation of the market potential of the Dutch oyster sector.

2. Materials and methods

2.1. Experimental design

Market size (80 to 120 g) Pacific cupped oysters cultivated in the Eastern Scheldt obtained from a Dutch shellfish company (Koninklijke Prins en Dingemanse, Yerseke, The Netherlands) in November 2014 were kept in small basins (1 m^3) in saline groundwater (30 g l^{-1}) for a period of seven weeks at the experimental facilities of the HZ University of Applied Sciences (Vlissingen, The Netherlands). The oysters were fed either *Skeletonema costatum* or *Rhodomans baltica* during the experimental period (November-December 2014). Water temperature in the basins was maintained at 13 ± 1 °C by means of a cooling and heating unit (TECO TC20, Italy). Salinity and oxygen content were measured daily. Salinity was measured using a conductivity meter (WTW, USA). Oxygen content was determined using a dissolved oxygen meter (WTW, USA). Aeration of the basins mixed the water column thus distributing the algae evenly in the water. Furthermore, aeration provided the oysters with the necessary oxygen. The complete water volume was replaced weekly with fresh saline groundwater. Oysters were sampled at the start of the experiment and after four and seven weeks of experimentation for analysis (gross biochemical composition, condition index, fatty acid composition, FAAs, VOCs) and sensory evaluation by an expert and a consumer panel. Oysters originating from the same cultivation location in the Eastern Scheldt, the Netherlands were used as reference samples.

2.2. Algal diets

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The diatom *Skeletonema costatum* was grown in a semi-continuous culture in outdoor raceway systems (2.5 m^3). The temperature gradually decreased during the experiment from 14.4 ± 1 °C at the start to 12.3 ± 1 °C at the end of the experiment due to decreasing air temperature during the experimental period. The flagellate *Rhodomonas baltica* was cultivated indoors in 200 l plastic bag microalgae continuous cultivation reactors (SeaCAPS, UK) with continuous aeration. Ambient room temperature (20 ± 1 °C) was maintained for this culture, whilst 24 hours illumination was provided (150 µmol m^{-2} s^{-1}). Both algae culture systems were filled with filtered (1 µm) saline groundwater (30 g l^{-1}). Walne-medium (Walne, 1970) was used in all algal cultures. The algal cultures were kept in the exponential growth phase by daily harvesting. Cellular densities were determined daily by using a Bürker-Türk hemocytometer before feeding the oysters.

Feeding rates were set at 30 mg dry weight algae day^{-1} $oyster^{-1}$. Dry weight (DW) for the *Skeletonema costatum* was 45 pg algae $cell^{-1}$ while the DW of *Rhodomonas baltica* was 116 pg algae $cell^{-1}$. The oysters were fed once during the day at a fixed time in the morning.

2.3. Production

In order to assess oyster production the total wet weight and oyster tissue wet weight were recorded for 50 individual oysters in all experimental treatments. The condition index (AFNOR, 1985) was measured in order to evaluate the amount of tissue in relation to the total weight of the oyster.

2.4 Biochemical composition

The biochemical composition (DW, ash, protein, carbohydrate, lipid, fatty acid profile and FAA content) was analyzed for both the algae diets (*Skeletonema costatum* and *Rhodomonas baltica*) and the reference and *Skeletonema* and *Rhodomonas* fed oysters at the start of the experiment

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and after four and seven weeks of feeding, respectively. The VOCs were only analyzed in the oyster samples.

2.4.1. Gross biochemical composition

In order to determine the gross biochemical composition (protein, lipid and carbohydrate content) of the algal diets three 50 ml samples of the algae culture were filtered over a Whatman GF/C filter paper. The biochemical composition (DW, ash, protein, lipid and carbohydrate content) of the oysters was determined for three pooled samples of 50 oysters The oysters were homogenized by using a Ultra Turrax T25 homogenizer (IKA Werke GmbH, Germany) prior to the analysis.

Dry matter content of the oysters was determined by drying 5 g of homogenate at 105 °C for 48 hours. The ash content of the oysters was determined by incinerating 1 g of homogenate in a muffle oven at 550 °C for 16 hours. Protein content for both algal diets and oysters was analyzed using the method described by Lowry et al. (1951). A commercial test kit (BIORAD, 500–0112) was used while Bovine serum albumin was used as a standard. Lipid fraction was extracted by the method of Bligh and Dyer (1959) using chloroform-methanol (1:2, v/v). The lipid content was measured spectrophotometrically according to Marsh and Weinstein (1966) after carbonization (200 °C for 15 min) with tripalmitin as a standard. Carbohydrate content was analyzed spectrophotometrically using the method of DuBois, Gilles, Hamilton, Rebers and Smith (1956). Glucose solutions were used as a standards in order to measure the carbohydrate concentration.*2.4.2. Fatty acids profile*

The fatty acid profiles of both algal species were determined of three 6 ml samples taken from the algae cultivation systems as described by Caramujo, Boschker and Admiraal (2008). While the fatty acid profile of the oysters was determined for three pooled samples of 50 oysters.

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Briefly, the modified Bligh and Dyer method of Findlay, King and Watling (1989) was used for lipid extraction. Fatty acid methyl esters (FAME) were obtained from total lipid extracts using derivatization with 2.5 % H_2SO_4 inmethanol at 80 ˚C for 1.5 hours according to Guckert, Antworth, Nichols and White, 1985. FAMEs were analyzed by capillary gas chromatography with flame ionization detection using a Varian 3400 gas chromatograph equipped with a Varian SPI injector, which was coupled via a Type II combustion interface to a Finnigan Delta S isotope ratio mass spectrometer. An a-polar analytical column (Hewlett-Packard Ultra-2 (50 m in length, 0.32 mm ID and 0.17 μm film thickness) was used. Helium was used as the carrier gas. The oven temperature increased from 80°C (1 min) to 130 °C at 40 °C min^{-1} and subsequently from 130 to 260 °C at 3 °C min^{-1}.

Identification of FAME was based on retention time data of known standards, later confirmed by mass spectrometry (Hewlett-Packard Mass Selective detector, U.S.A.).

2.4.3. Free amino acids

For the algal diets three samples (approx. 50 g filtrated fresh algae paste) were freeze-dried. For the oysters, 10 individual oysters (80 - 120 g) were freeze-dried (at -70 ˚C) for each experimental group.

Free amino acids were extracted according to Mierke-Klemeyer et al. (2008) with modifications described by Maehre, Malde, Eilertsen and Elvevoll (2014), by dissolving approximately 0.2 g freeze-dried material in a mixture of 9 ml distilled H_2O and 1 ml 20 mmol l^{-1} norleucine (internal standard), followed by homogenization for 15 seconds with an Ultra Turrax T25 homogenizer (IKA Werke GmbH, Germany). One ml of 35 % sulfosalicylic acid was added for removal of proteins and large peptides, followed by homogenization for another 15 seconds and

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centrifugation at 4000 g for 10 minutes. Aliquots of 200 µl of the supernatants were diluted to a suitable concentration in lithium citrate buffer at pH 2.2 and submitted to analysis.

Analysis of FAAs was performed on a Biochrom 30 amino acid analyzer (Biochrom Co., UK). The amino acids were chromatographically separated on an ion exchange column, followed by post-column derivatization with ninhydrin and detection of UV signal at 440 nm and 570 nm (Spackman, Stein & Moore, 1958). UV-signals were analyzed by Chromeleon software (Dionex, USA) and compared with A9906 physiological amino acids standard (Sigma Chemicals Co., USA).

2.4.4. Volatile organic compounds

The VOCs were analyzed for five individual oyster (80-120 g) per experimental group. For every individual oyster 10 ml of NaCl saturated ultrapure water was added to 5 g oyster tissue prior to homogenization. Homogenization was performed for one minute using a T25 Ultra Turrax homogenizer (IKA Werke GmbH, Germany) ensuring that the sample remained in ice preventing excess heat. VOCs were extracted by HeadSpace Solid Phase Micro Extraction (HS-SPME) and analyzed by Gas Chromatography–Mass Spectrometry (GC-MS) according to Fratini, Lois, Pazos, Parisi and Medina (2012). GC–MS analysis was performed in a Thermo Finnigan ThermoQuest (USA) gas chromatograph equipped with a split/splitless injector and coupled to a trace quadrupole mass detector (Thermo Finnigan ThermoQuest, USA). Compounds were separated in a capillary column (30 m × 0.250 mm × 1 µm film thickness, fused silica DB-1701, Agilent Technologies, USA). All analyses were performed setting ionization energy at 70 eV, filament emission current at 150 µA and the electron multiplier voltage at 500 V (Fratini et al., 2012) and the spectra were acquired in full scan mode.

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Identification of the components was based on computer matching with the reference mass spectra of the Wiley 6, Mainlib and Replib libraries and by comparison of mass spectra and retention times with commercial standards. Quantification of volatiles was performed by the method of internal standards using 3-methyl-3-buten-1-ol as described by Fratini et al. (2012). The standards: 2-Ethylfuran, 2,3-pentanedione, (E)-2-pentenal, hexanal, (E)-2-hexenal, heptanal, (Z)-4-heptenal, (E,E)-2,4-heptadienal, (E)-2-octen-1-ol and 3-methyl-3-buten-1-ol (used as internal standard) were purchased from Sigma-Aldrich (Germany). 1-Penten-3-ol was obtained from Fluka (Switzerland) and 2,4-octadien-1-ol was purchased from Alfa Aesar (USA).

2.5. Sensory evaluation

Sensory evaluation of the reference oysters, the *Skeletonema* and the *Rhodomonas* fed oysters was conducted by both naïve consumers and trained panelists. In the consumer evaluation a three Alternative Forced Choice (3-AFC) method was used in order to investigate whether naïve consumers were able to discriminate between the experimental groups of oysters. The trained panelist's evaluation consisted of a Quantitative Descriptive Analysis (QDA).

2.5.1. Consumer evaluation

Up to 56 consumers were selected from a pool of consumers recruited in a previous study (van Houcke, Altintzoglou, Stieger, Linssen & Luten, 2016a). Consumers in this pool were selected on the criteria that they were oyster consumers and had no allergies to shellfish or specifically, oysters. The majority of the consumers was male (approx. 75 %) and above 55 years of age (approx. 55 %). Consumers did not receive a financial reimbursement for participating in the study. No information on the actual aim or the experimental design was disclosed to the consumers.

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Before sample preparation the oysters had a depuration period of 24 hours. Oysters were opened by hand-shucking, the adductor muscles were cut on both sides and the oyster tissue was replaced on the lower shell (half-shell product). All samples were coded with a randomized three-digit code.

In order to evaluate consumer discrimination between *Skeletonema* fed oysters, *Rhodomonas* fed oysters or reference oysters a full factorial design was implemented in this study, rendering into three possible combinations: two reference oyster vs. one *Skeletonema* fed oyster, two reference oysters vs. one *Skeletonema* fed oyster and two *Skeletonema* fed oysters vs. one *Rhodomonas* fed oyster. The sample consisting of three half-shell oysters was presented on a plastic plate. Consumers were asked to indicate which of the oysters they considered different from the other oysters based upon outer appearance, smell and taste. Each consumer participated in two 3-AFC tests. The presentation order of the samples and 3-AFC combinations was completely randomized.

The evaluation sessions were of approximately 40 minutes duration. Each 3-AFC test took 15 minutes with a break of 10 minutes in between in which the consumers could take a sip of water and a cracker.

Tests were carried out at room temperature in different classrooms at the HZ University of Applied Sciences in Vlissingen, The Netherlands. Consumers were seated approximately 1 m apart in the classrooms. Consumers were seated at random, received a participant number for anonymity and were instructed not to speak to each other during and between the tests.

2.5.2. Trained panelists evaluation

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The Quantitative and Descriptive Analysis (ISO, 2003) tests were performed using 14 trained panelists (internal panel Ifremer, Nantes, France) experienced in seafood sensory evaluation and already involved in oyster assessment.

Prior to the QDA tests two sessions were organized for attribute generation, selection and consensus on the chosen attributes. The samples used in these sessions were reference oysters from the Eastern Scheldt and five weeks algae fed oysters (both with *Skeletonema costatum* and *Rhodomonas baltica*). The agreed attributes were: overall odor, marine odor, fruit odor, mud odor, darkness of the visceral mass, darkness of the gills, darkness of the mantle edge, fullness of the shell, crunchy texture, melting texture, chewiness, marine taste, algal taste, saltiness, sweetness, bitterness, metallic taste and astringency. In total three QDA tests were carried out with oysters derived from the start of the experiment and at the four and seven weeks' time feeding periods. All tests (including the two sessions on attribute generation and consensus on the attributes) took place in the sensory room at Ifremer in Nantes (France) at ambient temperature under daylight in isolated booths (ISO, 1988). Data were collected with a computerised system (Fizz, Biosystèmes, Dijon, France).

Oysters were presented as half-shell products with the shell of the top to preserve the odor. The samples assessed consisted of two reference oysters, two *Skeletonema* fed oysters and two *Rhodomonas* fed oysters. Samples were assigned with three digit numbers and randomized for the order presentation within panelists (Latin square design). In the tests panelists were asked to score the perceived intensity of the 18 different attributes on a 16 cm unstructured line for each oyster presented. Anchors corresponded with no intensity and very strong intensity on the left hand anchor and the right-hand anchor, respectively. Water and crackers were available during the tests and panelists were free to use these at will.

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2.6. Statistical data analysis

The data on biochemical composition, fatty acids, FAAs and VOCs of the oysters were processed by ANOVA using a two-way (diet, time) GLM model. Both the factors diet and time consisted of three levels (reference, Skeletonema and Rhodomonas and T0, T4 and T7, respectively). Furthermore standardized Principal Component Analysis (PCA) was performed on the fatty acids, FAAs and VOCs data.

Consumer evaluation data were tested for significant differences with the Chi-square test. While, trained panelist evaluation data were analyzed using both a two-way (diet, time) GLM model and PCA.

The data in the tables are expressed as mean ± standard deviation.

Three levels of significance are presented in this study: $P < 0.001$ (***), $P < 0.01$ (**) and $P < 0.05$ (*).

3 Results and Discussion

3.1. Production

AFNOR condition indexes showed a slight increase over time for both refinement diets although this was not significant. While reference oysters had a condition index of 16.0 *Rhodomonas* fed oysters showed a condition index of 16.4 and 16.9 after four and seven weeks of feeding respectively. *Skeletonema* fed oysters increased to a condition index of 16.7 after seven weeks even though there was a decrease in condition index after four weeks of feeding (15.9). Pacific cupped oyster fed with *Skeletonema costatum* in France showed increases in (AFNOR) condition indexes from 6 - 9 to 10 - 14 during six week periods (Pennarun et al., 2003a; Haure et al., 2003; Soletchnik et al., 2001, Baud, Brisset & Cardinal, 1995). A lack of significant increases in the

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condition indexes in our results could probably be explained by the relatively high condition indexes of the reference oysters at the start of the experiment.

3.2. Biochemical composition

3.2.1. Gross biochemical composition

Table 1 shows the gross biochemical composition of the algal diets, reference oysters and the oysters after four and seven weeks of feeding with *Skeletonema costatum* or *Rhodomonas baltica*. Significant diet and time effects are shown for the dry weight, protein and carbohydrate content of the oysters. Relatively low DW content (13.3 % WW) was found in the reference oysters at the seven weeks sampling interval compared with the reference oysters at the beginning of the experiment and at the four weeks sampling interval (16.2 and 15.5 % WW, respectively).

Highest protein content was measured in the reference oysters at the four and seven week sampling intervals (60.9 and 59.4 % DW, respectively) and lowest protein content in the *Rhodomonas* fed oysters after four weeks (52.7 % DW). The carbohydrate content was highest in both the *Skeletonema* (25.5 % DW) and *Rhodomonas* fed oysters (25.3 % DW) after the four week sampling interval. The lowest content was found in the reference oysters at the four and seven week sampling intervals (17.3 and 19.2 % DW, respectively). The lipid levels in the oysters remained relatively stable ranging from 7.8 to 9.1 % DW.

The changes found in the biochemical composition in our study are in line with studies of Soletchnik et al. (2001) and Pennarun et al. (2003a). Soletchnik et al. (2001) reported significant increases in the carbohydrate content of Pacific cupped oysters supplemented with *Skeletonema costatum* in comparison with non-supplemented oysters kept in semi-closed land-based pond systems. The increase found in the carbohydrate content was mainly due to an increase in the

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glycogen content. No effect was found on the lipid content while the protein content showed a trend to lower levels for the *Skeletonema* fed oysters in the study of Soletchnik et al. (2001). Pennarun et al. (2003a) reported increasing carbohydrate (and glycogen) and decreasing protein content for *Skeletonema costatum* and *Isochrysis galbana* fed oysters. Pennarun et al. (2003a) showed a significant increase in the lipid content which is in contrast with our results. Furthermore Pennarun et al. (2003a) also showed a varying lipid content between oysters fed with the different algal diets. The carbohydrate, lipid and ash contents in our study are comparable with data from Pacific cupped oysters from Ireland (Linehan, O'Conner & Burnell, 1999), Germany (Pogoda, Buck, Saborowski & Hagen, 2013) and France (Pennarun et al., 2003a; Solechnik et al., 2001). The protein content, however, seemed to be quite high in our study and is only comparable with protein content measured in Pacific cupped oysters from Ireland sampled in August (Linehan, et al., 1999).

3.2.2. Fatty acids

The fatty acid profile of the algal diets, the reference oysters and the oysters after four and seven weeks of feeding with *Skeletonema costatum* or *Rhodomonas baltica* are shown in table 2. The algal diets show differences between total saturated fatty acid (∑SFA), total mono-unsaturated fatty acid (∑MUFA) and total poly-unsaturated fatty acid (∑PUFA) content. The diatom *Skeletonema costatum* shows a higher ∑SFA and ∑MUFA content (17.3 and 21.1 %, respectively) in comparison with the flagellate *Rhodomonas baltica* (13.3 and 7.6 %, respectively). The largest difference between both algae species is however found in the ∑PUFA content (79.0 % in *Rhodomonas baltica* and 61.7 % in *Skeletonema costatum*). This difference in ∑PUFA content could mainly be attributed to differences in total n3 fatty acid (∑n-3) content of both algae species (22.1 % for *Skeletonema costatum* and 71.8 % for *Rhodomonas baltica*).

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Rhodomonas baltica shows the highest C18:3n3, C18:4n3 and C22:6n3 content (28.0, 27.2 and 6.2 %, respectively) in comparison with *Skeletonema costatum* (0.8, 4.4 and 2.9 %, respectively). These findings are in line with literature on the fatty acid profile of both *Rhodomonas baltica* (Kreibich, Saborowski, Hagen & Niehoff, 2008) and *Skeletonema costatum* (Pennarun et al., 2003a).

As table 2 shows differences in the fatty acid profiles of the Pacific cupped oysters are due to both the diet and the time interval. For instance, the ∑SFA content is slightly higher for the reference oysters in comparison with *Skeletonema* and *Rhodomonas* fed oysters. The ∑MUFA content seems to be lower for *Rhodomonas* fed oysters and higher for *Skeletonema* fed oysters in comparison with the reference oysters. While the ∑PUFA content is found to be higher for *Rhodomonas* fed oysters in comparison with either *Skeletonema* fed oysters or reference oysters. Furthermore the ∑SFA content shows a decreasing trend over the experimental period while the ∑PUFA content shows lower values at the four week interval. In addition, PCA based on the total fatty acid profile characteristics of both the reference oysters and the oysters fed with either *Skeletonema costatum* or *Rhodomomans baltica* (figure 1) shows a clear separation between experimental groups of oysters. The separation of the reference oysters from the algae fed oysters indicates that the fatty acid profiles of the reference oysters are most divergent and varied during the experiment. The separation of the seven weeks *Rhodomonas* fed oysters from the other algae fed oysters is mainly caused by the dietary and time effect found in the C14:0, C16:1n7, C18:2n6, C18:3n3, C18:4n3, C20:5n3 and C22:6n3 content.

The main fatty acids in all experimental oysters are C16:0, C20:5n3 and C22:6n3. The highest C16:0 content was found in the reference oysters at the four week sampling interval (21.0 %) while the lowest content was found in *Rhodomonas* fed oysters after seven weeks of feeding

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(17.9 %). Both C20:5n3 and C22:6n3 showed lowest content in the reference oysters at the seven week interval (25.0 and 16.5 %, respectively). Highest content of C20:5n3 was found in the reference oysters at the start of the experiment and the *Skeletonema* fed oysters after seven weeks of feeding (28.6 %). Highest content of C22:6n3 was found in *Skeletonema* fed oysters after seven weeks of feeding (16.5 %). The levels of these essential fatty acids are comparable with the ranges found for Pacific cupped oysters in the literature (van Houcke, Medina, Linssen & Luten, 2016b; Pogoda et al., 2013; Fratini et al., 2013; Linehan et al., 1999; Pazos, Ruez, Garcia-Martin, Abad & Sanchez, 1996).

From the literature it is known that the fatty acid profile of the algae diet can affect the fatty acid profile of the oysters. Pennarun et al. (2003a) and Piveteau et al. (1999) showed an increase in C18:1n9, C18:2n6, C18:3n3 content when Pacific cupped oysters were fed with *Isochrysis galbana*. When oysters were fed with *Skeletonema costatum* their C20:5n3 and C22:6n3 content increased. In our study higher contents of C18:1n9 and C20:5n3 were measured in the algae *Skeletonema costatum* and also a higher content of C22:6n3 in the algae *Rhodomonas baltica* which could explain the differences in the fatty acid profile of the oysters. The marked differences in the C18:3n3 and C18:4n3 content of both algae is also reflected in the oysters fed with the microalgae. Highest content for both C18:3n3 and C18:4n3 was measured in *Rhodomonas* fed oysters. Also the C16:1n7 and C18:2n6 content in the oysters show a clear dietary effect. It is known that diatom algae species such as *Skeletonema costatum* have a high content of C16:1n7 as compared to other algae (Sargent, Parkes, Mueller-Harvey & Henderson, 1987).

3.2.3. Free Amino Acids

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Table 3 shows the FAAs of the algal diets, the reference oysters and the oysters after four and seven weeks of feeding with *Skeletonema costatum* or *Rhodomonas baltica*.

In both algal diets the largest differences are found in the content of aspartic acid, asparagine , glutamine, proline and alanine. In *Skeletonema costatum* the aspartic acid (1.8 mg g^{-1} DW), asparagine (3.7 mg g^{-1} DW) and glutamine (3.4 mg g^{-1} DW) content is higher than in *Rhodomonas baltica* (0.6, 2.7 and 1.7 mg g^{-1} DW, respectively). Proline was only detected in *Rhodomonas baltica* (1.3 mg g^{-1} DW) while alanine content was higher in *Rhodomonas baltica* (1.5 mg g^{-1} DW) as compared to *Skeletonema costatum* (0.6 mg g^{-1} DW).

Threonine content was higher in *Skeletonema costatum* (0.4 mg l^{-1} DW) in comparison with *Rhodomonas baltica* (0.1 mg g^{-1} DW). Leucine and histidine were not detected within the algae *Rhodomonas baltica* while concentrations of 0.3 and 0.2 mg g^{-1} DW were found in the algae *Skeletonema costatum*, respectively.

The FAA composition of the oysters is dominated by taurine. The concentrations range from 40.9 to 44.8 mg g^{-1} DW. Other major FAAs are alanine, proline, glycine, and glutamate. These FAAs have also been reported as the main FAAs in Pacific cupped oysters from Australia (Cochet et al., 2013), from Japan (Sakaguchi & Murata, 1989; Murata & Sakaguchi, 1986) and China (Hong et al., 2002). Also taurine was the dominating FAA and the concentrations in these studies are comparable with our results.

Diet effects were shown for aspartic acid, threonine, serine, glutamine, glycine, leucine, ornithine and histidine. For aspartate and ornithine lowest content (ranging from 3.6 - 3.8 and 0.1 - 0.2 mg g^{-1} DW) was measured in reference oysters in comparison to *Skeletonema* fed oysters (ranging from 4.5 - 4.9 and 0.2 - 0.3 mg g^{-1} DW) and *Rhodomonas* fed oysters (ranging from 5.0 - 5.2 and 0.2 - 0.2 mg g^{-1} DW). However for serine the highest content was measured in reference

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oysters (ranging from 0.6 - 0.9 mg g^{-1} DW) when compared with *Skeletonema* (0.4 mg g^{-1} DW) and *Rhodomonas* fed oysters (0.6 mg g^{-1} DW).

The FAA content of the Pacific cupped oysters seems to be related to the FAA content of the algal diets. The glutamine, threonine, leucine and histidine contents were significantly higher for *Skeletonema* fed oysters (3.3, 0.8, 0.4 and 0.4 mg g^{-1} DW) in comparison with *Rhodomonas* fed oysters (2.0, 0.5, 0.3 and 0.2 mg g^{-1} DW).

For threonine, serine, glutamine, alanine, histidine and arginine content in the oysters significant time effects are also shown in table 3. In addition the PCA plot of the FAA profiles of the oysters (figure 2) shows that separation between the algae fed oysters are due to both diet and time effects. Cochet et al. (2013) hypothesized that the differences found in the FAA profile from Pacific cupped oysters from different cultivation locations in Australia could be attributed either to differences in the microalgae populations and concentrations at the different locations or timing differences in the reproductive cycles of the oysters.

3.2.4. Volatile Organic Compounds

During the experimental period the total VOC content decreased from 9.2 mg kg^{-1} at the start of the experiment to 5.2 - 6.0 mg kg^{-1} at the seven week sampling interval (table 4). The VOCs found are alcohols (6), aldehydes (4), ketones (1) and furans (1). The main VOCs found were the alcohols 1,5-octadien-3-ol, (E,E)-2,4-octadien-1-ol and 1-penten-3-ol. All VOCs, except (E,E)-2,4-Octadien-1-ol, were also identified in earlier studies with Pacific cupped oysters by Van Houcke et al. (2016b), Fratini et al. (2013) and Pennarun et al. (2003b) and Pennarun, Prost and Demaimay (2002). (E,E)-2,4-Octadien-1-ol was however measured by Fratini et al. (2012) in the pullet carpet shells (*Venerupis pullastra*).

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Almost all VOCs showed significant diet x time interactions as shown in table 4. The highest 1,5-octadien-3-ol content was measured in reference oysters at the start of the experiment and at the four week sampling interval (4.5 and 4.6 mg kg^{-1}, respectively). The lowest content was measured after four weeks of feeding with *Skeletonema costatum* (2.3 mg kg^{-1}). The (E,E)-2,4-octadien-1-ol content showed a decline in the reference oyster (from 2.0 mg kg^{-1} at the start of the experiment to 0.7 mg kg^{-1} at the seven week sampling interval). Levels in both *Skeletonema* and *Rhodomonas* fed oysters have already declined after four weeks of feeding (to 0.5 and 1.0 mg kg^{-1}, respectively). The 1-penten-3-ol content increases over time in the reference (from 0.5 at the start of the experiment to 0.7 mg kg^{-1} at the seven week sampling interval) and the *Rhodomonas* fed oysters (up to 0.9 mg kg^{-1} at the seven week sampling interval) while the (E)-2-pentenal content increases in the reference (from 0.3 at the start of the experiment to 0.4 mg kg^{-1} at the seven week sampling interval) and the *Skeletonema* fed oysters (up to 0.5 mg kg^{-1} at the seven week sampling interval). The higher 1-penten-3-ol content in *Rhodomonas* fed oysters is most likely related to the higher n-3 PUFA content in the algal diet. The higher (E)-2-pentenal, also known to originate from n-3 PUFA degradation, content in *Skeletonema* fed oysters might be related to the fatty acid C20:5n3, This n-3 PUFA is the only one measured with a higher content in *Skeletonema costatum* diet in comparison to the *Rhodomonas baltica* diet. C20:5n3 is, as mentioned earlier, found in relatively high concentrations in diatoms such as *Skeletonema costatum*. The other VOCs show similar trends with decreasing content over time for all treatments. The PCA plot of the VOCs (figure 3) also shows clear separation of the experimental groups due to diet and time effects.

3.3. Sensory evaluation

3.3.1. Consumer evaluation

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In the 3-AFC test the chance level was 33.3 % for consumers to choose the divergent oyster from the three oysters presented. Results show that consumers were only able to discriminate between reference and *Rhodomonas* fed oysters. After the four week sampling interval 52.5 % of the consumers chose *Rhodomonas* fed oysters to be divergent from reference oysters (k = 21, n = 40). At the seven week sampling interval 50.0 % of the consumers chose *Rhodomonas* fed oysters to be divergent from the reference oysters (k = 20, n = 40).

The 3-AFC test with reference and *Skeletonema* fed oysters showed no significant difference. In total only 37.5 % (k = 15, n = 40) and 39.5 % (k = 15, n = 38) of the consumers were able to discriminate between both oysters at the four and seven week sampling intervals, respectively. In the 3-AFC composed with oysters fed with the different algal diets consumers were also not able to discriminate between oysters (40 %, k = 16, n = 40 and 28.9 %, k = 11, n = 38 after four and seven weeks of feeding).

3.3.2. Trained panelists evaluation

GLM analysis of the panelists evaluation data shows both dietary and time effects for the attributes overall odor and the fullness of shell (table 5). Overall odor intensity was highest in reference oysters at the start of the experiment (score of 5.4) and was lowest in *Rhodomonas* fed oysters after seven weeks of feeding (score of 2.9). A decline of overall odor intensity was found in all dietary treatments. Similar declines were observed for the marine odor and fruit odor intensity. Reference oysters at the start of the experiment scored highest (score of 5.0 and 1.4 respectively) while either *Rhodomonas* fed oysters after seven weeks of feeding and *Skeletonema* fed oysters after four weeks of feeding sored lowest (score of 2.2 and 0.4 respectively). Fullness of the shell decreased over time in the reference oysters from a score of 5.4 at the start of the experiment to 3.5 at the seven week sampling interval. In the algae fed oysters the score for the

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fullness of the shell increased to 5.8 and 6.1 for *Skeletonema* and *Rhodomonas* fed oysters respectively. Standardized PCA of the QDA data based on average panel scores (figure 4) shows separation of the experimental groups. Separation was mainly due to the characterization of reference oysters as having a high overall odor intensity, high saltiness and a strong marine flavor in comparison with the algae fed oysters. Reference oysters sampled at the different time intervals also show separation in the PCA plot. Darkness of the visceral mass, darkness of the gills and the blackness on the mantle edge seem to play an important role in the separation of the reference oysters.

Differences in the QDA scores between *Skeletonema* fed oysters and *Rhodomonas* fed oysters are small. However, a separation in the PCA between *Skeletonema* fed oysters and *Rhodomonas* fed oysters is also shown in figure 4. *Skeletonema* fed oysters are characterized by a stronger seaweed flavor, and are more sweet in comparison with *Rhodomonas* fed oysters. Pennarun et al. (2003b) also found that algae fed oysters, with the algae *Skeletonema costatum* and *Isochrysis galbana*, were described by a sweeter taste in comparison to the reference oysters in their study. Pennarun et al. (2003b) attributed this sweeter taste to an increased carbohydrate content.

A firmer texture for *Rhodomonas* fed oysters and a more melting texture for *Skeletonema* fed oysters were observed by the trained sensory panel. In the study of Pennarun et al. (2003b) *Skeletonema costatum* fed oysters were also described by a more melting texture as opposed to a firmer texture for *Isochrysis galbana* fed oysters. This difference was attributed to a higher glycogen and lipid content in *Skeletonema* fed oysters.

In our study both the *Skeletonema* and *Rhodomonas* fed oysters, from the four and seven week sample intervals, are mainly separated due to increasing fullness of the shell over time.

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The role of carbohydrates in the texture and the fullness of the shell seem eminent in our results. Feeding oysters with both algal diets increases the carbohydrate level and therefore the oysters are considered to be have a higher chewiness and appear larger (higher fullness of the shell). The main reported reason for textural changes in oysters is the glycogen level (Pennarun et al., 2003b). Glycogen is the natural energy reserve of oysters and shows a seasonal variation with low levels just after spawning (Dridi, Romdhane & Elcafsi, 2007). Furthermore Dridi et al. (2007) also showed the relationship between the glycogen level and the condition index (a measurement of fullness of the shell), with higher condition indexes found for oysters with higher glycogen levels.

The observed differences in flavor properties could not be explained by the FAAs content in the different experimental oyster groups. Several FAAs are known to contribute to flavor development in seafood. For instance glutamate is known to decrease perceived sweetness in shellfish such as scallop (Wantanabe, Lan, Yamaguchi & Konosu, 1990) and Manilla clam (Fuke & Konosu, 1991). Glycine has been mentioned (Hong et al., 2002) as contributing to a fresh sweetness in Pacific cupped oysters. However, the glycine content in *Skeletonema* fed oysters in our study is lower in comparison with that in *Rhodomonas* fed oysters, while the highest perceived sweetness, in the QDA from the trained panelists, was scored for *Skeletonema* fed oysters. Conversely, alanine is also known to affect the perceived sweetness (Wantanabe et al., 1990). Even though no significant dietary effect was found for the alanine content in our study, higher alanine content was found for *Skeletonema* fed oysters (5.3 mg g^{-1} DW) in comparison with *Rhodomonas* fed oysters (4.1 mg g^{-1} DW).

The total VOC content decreases over time in all experimental oyster groups concomitantly with the perceived overall odor intensity as determined by the expert panel. However, the overall odor

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intensity for the reference oysters at the seven week sampling interval was considered stronger in comparison with both algae fed oysters while the total VOC content showed similar levels at the seven week sampling interval. Direct comparison between the odor scores in the QDA and the odor concentrations of the VOCs is difficult. The decrease in the marine odor (and possibly the marine taste) might be related to the decrease of (E,Z)-3,6-nonadien-1-ol which has been reported to have a marine, cucumber odor (Piveteau et al. 2000; Pennarun et al. 2003b). Nowadays *Skeletonema costatum* is often used as the refinement diet (Soletchnik et al., 2001; Piveteau, Gandemer, Baud. & Demaimay, 1999; Robert, 1990) while refinement using *Rhodomonas baltica* is not yet commercialized. However differences in both the biochemical composition and sensory characteristics of the oysters within this research are shown to occur between all experimental groups. Furthermore, naïve consumers are able to perceive differences between *Rhodomonas* refined oysters and reference oysters whilst this is not the case for *Skeletonema* refined oysters and reference oysters. *Rhodomonas* refined oysters could therefore be marketed as a 'new' oyster refinement variety. The high PUFA content of the *Rhodomonas* refined oysters might be used as a marketing tool for further commercialization.

4. Conclusion

In our study dietary effects were found for the gross biochemical, fatty acid, FAA and VOC composition of Pacific cupped oysters. *Skeletonema* fed oysters have a significant lower ∑PUFA, ∑n-3, ∑n-6, C18:2n6, C18:3n3, C18:4n3, C22:6n3, glycine, (E)-2-pentenal and (Z)-2-penten-1-ol content in comparison with *Rhodomonas* fed oysters. The content of ∑MUFA, C16:1n7, C20:5n3, threonine, glutamine, leucine, histidine, (E)-2-hexenal, (E)-2octenal, (E)-2-octen-1-ol,

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(E,E)-2,4-octadien-1-ol, (E,Z)-3,6-nonadien-1-ol and (Z,E)-2,6-nonadienal was found to be significantly higher in *Skeletonema* fed oysters in comparison with *Rhodomonas* fed oysters. Refinement with different algal diets has an effect on the sensory characteristics of Pacific cupped oysters. PCA analysis of the QDA data showed separation between all experimental groups. Reference oysters are mainly characterized by a high overall odor intensity, high saltiness and a strong marine flavor. All algae fed Pacific cupped oysters are characterized by a high fullness of the shell. Furthermore *Skeletonema* fed Pacific cupped oysters are separated from *Rhodomonas* fed Pacific cupped oysters mainly by a stronger seaweed flavor, higher perceived sweetness and a firmer texture in *Skeletonema* fed oysters. Naïve consumers only perceive differences between *Rhodomonas* fed oysters and reference oysters. Therefore refinement with Rhodomonas baltica seems more apparent. Differences in both biochemical composition and sensory characteristics of the oysters increased over time suggesting a minimum refinement time of seven weeks.

5. Acknowledgements

The authors gratefully acknowledge Pim van Dalen ,Wessel Bakhuizen and María Jesús González with their help in the experimental work. The Ifremer panelists and consumer panelists are acknowledged for their contribution in the sensory evaluation of the oysters. This research was partially funded by the project Zilte Parels (Nationaal Regieorgaan Praktijkgericht Onderzoek SIA) and by the Interreg IV Project AquaVlan.

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Caption figure 1

Figure 1. Principal component analysis for the fatty acid profiles (A) and the n-3 fatty acid profiles (B) of reference oysters (Ref) and oysters after four and seven weeks of feeding with *Skeletonema costatum* (Ske) or *Rhodomonas baltica* (Rho).

Caption figure 2

Figure 2. Principal component analysis of the free amino acid profiles of reference oysters (Ref) and oysters after four and seven weeks of feeding with *Skeletonema costatum* (Ske) or *Rhodomonas baltica* (Rho).

Caption figure 3

Figure 3. Principal component analysis of the volatile organic compound profiles of reference oysters (Ref) and oysters after four and seven weeks of feeding with *Skeletonema costatum* (Ske) or *Rhodomonas baltica* (Rho).

Caption figure 4

Figure 4. Principal component analysis of quantitative descriptive analysis data from reference oysters (Ref) and oysters after four and seven weeks of feeding with *Skeletonema costatum* (Ske) or *Rhodomonas baltica* (Rho).

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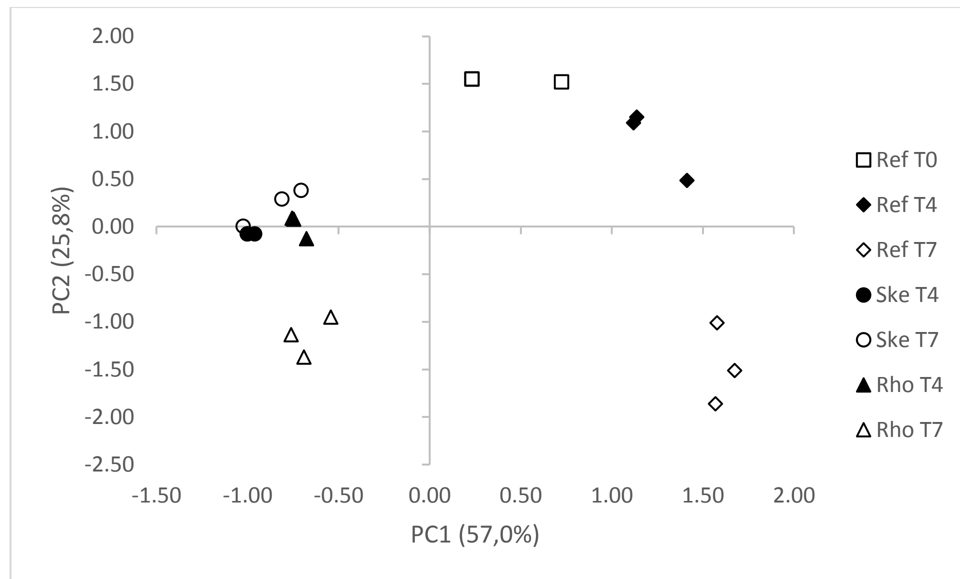

Fig. 1A

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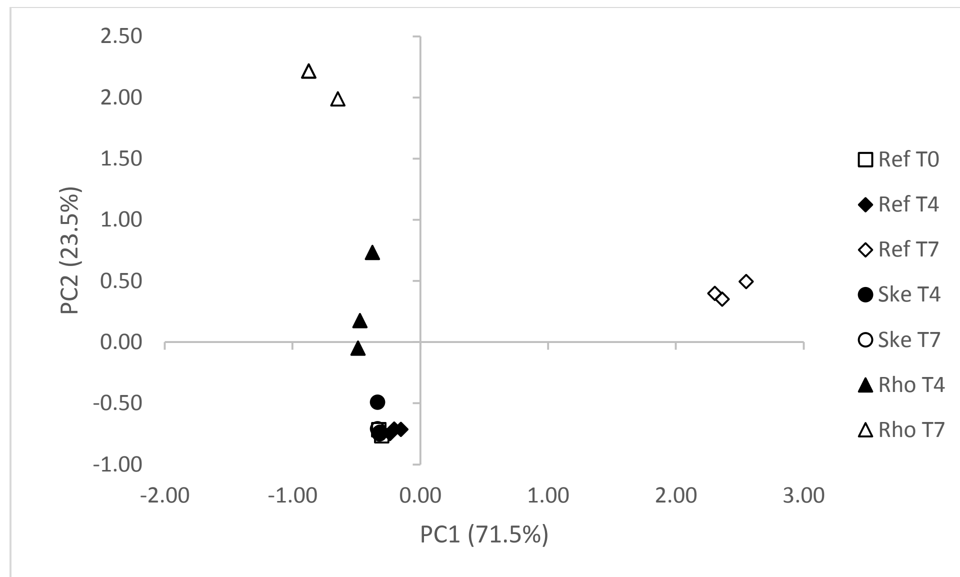

Fig. 1B

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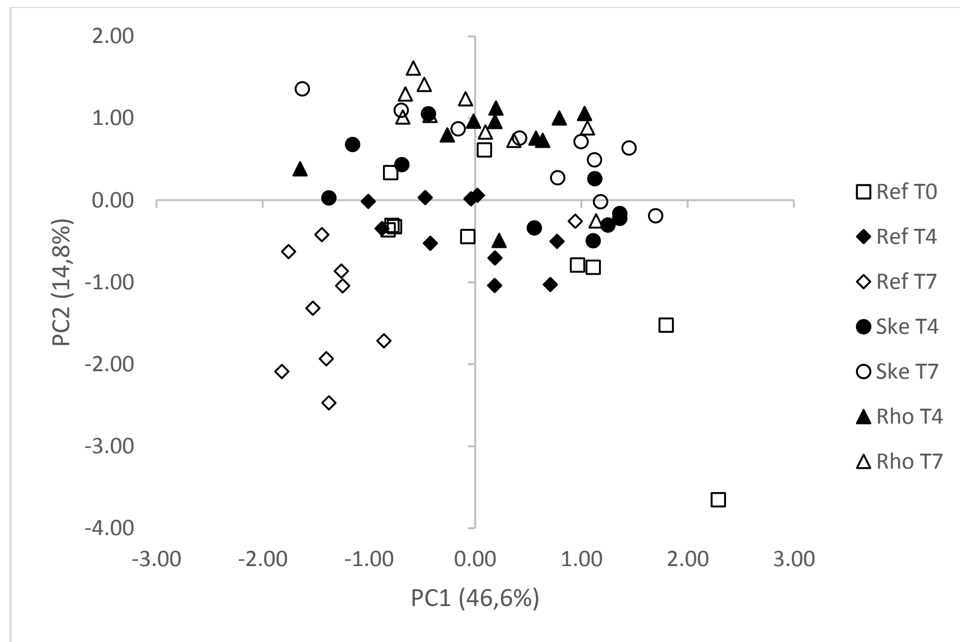

Fig. 2

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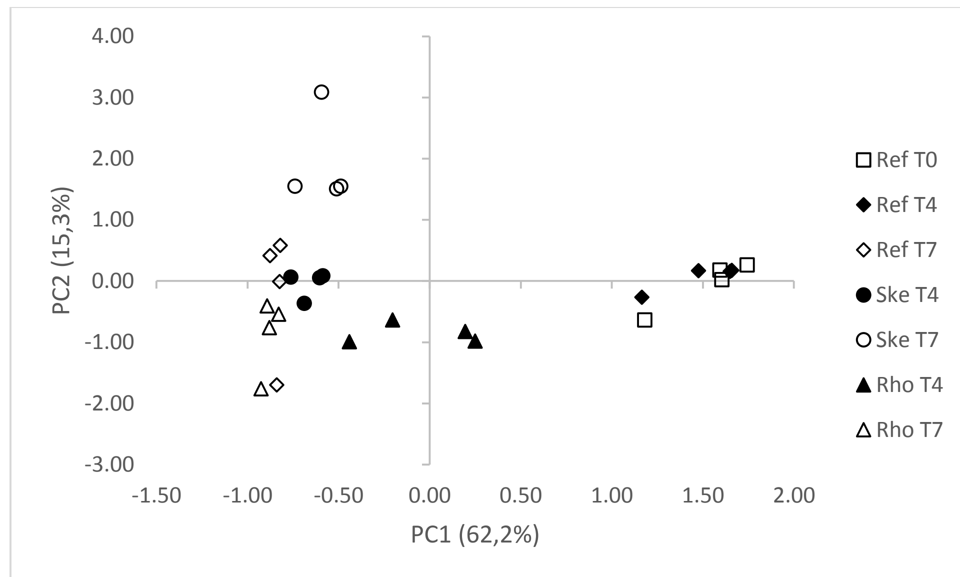

Fig. 3

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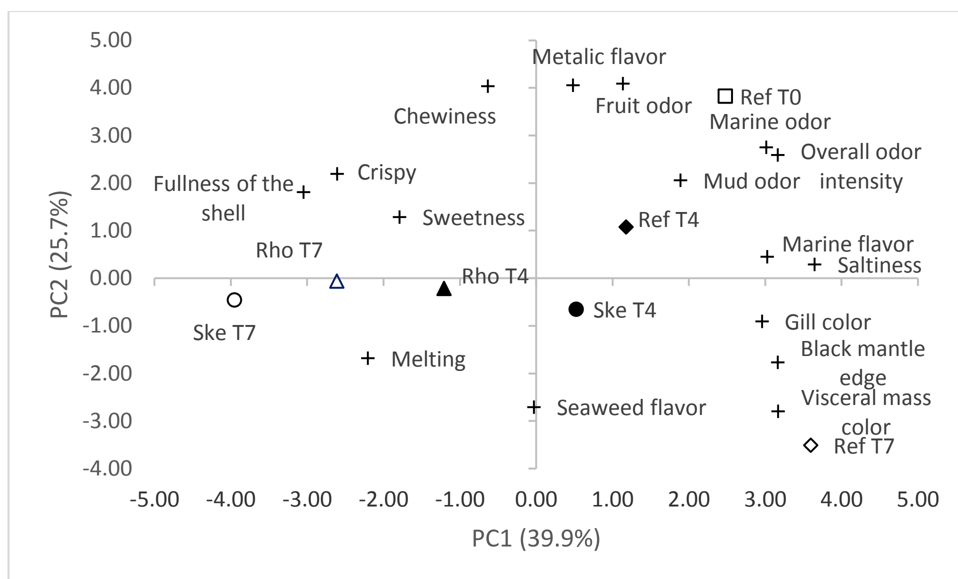

Fig. 4

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Caption table 1

Table 1. Gross biochemical composition of algal diets, reference oysters (Ref) and oysters after four and seven weeks of feeding with *Skeletonema costatum* (Ske) or *Rhodomonas baltica* (Rho). Values for dry matter are expressed as % of wet weight (WW), while other values are expressed as % of dry weight (DW). *** stand for $P<0.001$, ** stands for $P<0.01$, * stands for $P<0.05$

Caption table 2

Table 2. Fatty acid profile of algal diets, reference oysters (Ref) and oysters after four and seven weeks of feeding with *Skeletonema costatum* (Ske) or *Rhodomonas baltica* (Rho). Values are expressed as % of total fatty acid content. *** stand for $P<0.001$, ** stands for $P<0.01$, * stands for $P<0.05$

Caption table 3

Table 3. Free amino acid profile of algal diets, reference oysters (Ref) and oysters after four and seven weeks of feeding with *Skeletonema costatum* (Ske) or *Rhodomonas baltica* (Rho). The values are expressed as mg g^{-1} DW. *** stand for $P<0.001$, ** stands for $P<0.01$, * stands for $P<0.05$

Caption table 4

Table 4. Volatile organic compound profile of reference oysters and oysters (Ref) after four and seven weeks of feeding with *Skeletonema costatum* (Ske) or *Rhodomonas baltica* (Rho). The values are expressed as mg g^{-1} DW. *** stand for $P<0.001$, ** stands for $P<0.01$, * stands

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for P<0.05

Caption table 5

Table 5. Quantitative descriptive analysis scores of reference oysters (Ref) and oysters after four and seven weeks of feeding with *Skeletonema costatum* (Ske) or *Rhodomonas baltica* (Rho). *** stand for P<0.001, ** stands for P<0.01, * stands for P<0.05

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Table 1

	Ske		Rho		T0 Ref		Ref T4		Ref T7		Ske T4		Ske T7		Rho T4		Rho T7		GLM analysis		
	Ave[1]	Stdev[2]	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Diet (D)	Time (T)	D x T
Dry Matter (% WW)					16,2	2,5	15,5	2,3	13,3	2,1	17,7	2,7	16,6	2,3	17,7	2,6	17,7	2,7	***	***	**
Ash (% DW)					12,8	3,0	14,5	4,0	14,0	3,1	14,1	3,7	13,6	2,9	14,5	4,1	14,0	3,0	ns	ns	ns
Protein (% DW)	23,3	2,5	29,1	0,1	54,2	1,9	60,9	1,9	59,4	2,4	54,1	2,4	54,2	1,8	52,7	2,3	55,5	1,9	***	**	ns
Lipids (% DW)	16,2	0,6	12,1	0,5	8,8	0,6	7,8	0,7	9,1	1,0	8,5	0,9	8,2	0,9	9,0	0,6	8,7	0,9	ns	ns	ns
Carbohydrates (% DW)	26,4	0,7	11,7	0,8	23,8	1,4	17,3	2,0	19,2	2,3	25,5	2,3	23,8	1,1	25,3	1,4	23,5	2,3	***	**	ns

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[1] Ave: Average
[2] Stdev: Standard deviation

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Table 2

	Ske		Rho		T0 Ref		Ref T4		Ref T7		Ske T4		Ske T7		Rho T4		Rho T7		GLM analysis		
	Ave[3]	Stdev[4]	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Diet (D)	Time (T)	D x T
∑SFA	17,3	1,1	13,3	0,4	29,9	0,3	31,2	0,5	31,5	0,3	29,4	0,2	29,3	0,1	29,3	0,5	28,4	0,4	***	**	ns
∑MUFA	21,1	1,1	7,6	0,2	16,9	0,4	18,0	0,3	18,0	0,4	21,0	0,2	19,4	0,3	17,8	0,2	18,7	0,1	***	ns	ns
∑PUFA	61,7	3,3	79,0	0,5	53,2	0,7	50,7	0,4	50,5	0,4	49,6	0,0	51,3	0,3	52,9	0,6	52,9	0,3	***	**	ns
∑n-3	22,1	1,2	71,8	0,8	46,8	0,6	46,3	0,4	46,0	0,6	44,4	0,1	45,6	0,6	46,8	0,9	46,7	0,3	***	*	*
∑n-6	4,6	0,7	5,7	0,3	6,5	0,7	4,4	0,1	4,5	0,1	5,2	0,1	5,6	0,5	6,1	0,1	6,2	0,0	***	***	ns
C14:0	9,6	0,5	6,7	0,2	3,9	0,1	4,4	0,1	3,5	0,8	4,2	0,0	4,2	0,2	4,0	0,1	3,5	0,6	ns	ns	ns
C16:0	6,4	0,3	6,3	0,0	20,8	0,4	21,0	0,6	20,5	0,6	18,9	0,2	19,1	0,4	19,2	0,4	18,7	0,1	***	ns	ns
C16:1n7	18,9	0,9	2,6	0,2	3,9	0,1	3,9	0,8	3,0	0,1	5,2	0,1	4,2	0,2	4,0	0,1	3,1	0,0	***	***	ns
C16:2n4	5,1	0,3	0,4	0,0	nd		nd		nd		nd		nd		nd		nd		-	-	-
C16:2n7	2,3	0,1	0,1	0,0	nd		nd		nd		nd		nd		nd		nd		-	-	-
C16:3n4	16,3	0,8	0,3	0,1	nd		nd		nd		nd		nd		nd		nd		-	-	-
C16:4n1	11,2	0,6	0,8	0,1	nd		nd		nd		nd		nd		nd		nd		-	-	-
C17:0	0,1	0,0	0,1	0,0	nd		nd		1,5	0,0	1,0	0,0	1,1	0,0	1,0	0,0	1,0	0,0	***	ns	ns
C18:0	1,1	0,1	0,3	0,0	5,2	0,1	5,9	0,1	6,0	0,1	5,2	0,1	4,9	0,5	5,1	0,1	5,2	0,0	***	***	ns
C18:1n9	1,0	0,0	0,6	0,0	2,6	0,0	2,9	0,0	4,5	0,1	3,1	0,0	3,2	0,1	3,0	0,1	3,1	0,0	***	***	***
C18:1n other	1,1	0,1	4,5	0,0	7,8	0,1	8,3	0,8	7,5	0,2	9,4	0,1	9,2	0,4	7,7	0,4	9,3	1,8	*	ns	*
C18:2n6	1,4	0,1	4,3	0,0	1,3	0,0	nd		nd		1,0	0,0	1,1	0,0	2,0	0,0	2,1	0,0	***	**	*
C18:3n3	0,8	0,1	28,0	0,5	1,3	0,0	nd		nd		1,4	0,6	1,1	0,0	2,7	0,6	3,1	0,0	***	ns	ns
C18:4n3	4,4	0,3	27,2	0,2	2,6	0,0	2,9	0,0	3,0	0,1	3,1	0,0	3,2	0,1	4,0	0,1	4,2	0,0	***	***	ns
C20:1n11	nd		nd		2,6	0,0	2,9	0,0	3,0	0,1	3,1	0,0	2,8	0,5	3,0	0,1	3,1	0,0	ns	ns	ns
C20:4n6	0,2	0,0	0,5	0,0	3,9	0,1	4,4	0,1	4,5	0,1	3,1	0,0	3,2	0,1	3,0	0,1	3,1	0,0	***	***	ns
C20:5n	1	0,7	1	0,	2	0,	2	0,	2	0,	2	0,	2	0,	2	0,	26,	0,	***	***	**

[3] Ave: Average

[4] Stdev: Standard deviation

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3	4,0		0,2	1	8,6	5	7,3	6	5,0	6	7,3	3	8,6	1	6,9	0	0	1			*
C22:2n6	2,8	0,7	0,4	0,2	nd		nd		nd		nd		nd		nd		nd		-	-	-
C22:4n6	0,2	0,0	0,5	0,0	1,3	0,0	nd		nd		1,0	0,0	1,4	0,6	1,0	0,0	1,0	0,0	ns	ns	ns
C22:5n3	0,1	0,0	0,2	0,1	2,6	0,0	2,9	0,0	1,5	0,0	2,1	0,0	2,1	0,1	2,0	0,0	2,1	0,0	***	***	***
C22:6n3	2,9	0,1	6,2	0,1	11,7	0,2	13,2	0,2	16,5	0,3	10,5	0,1	10,6	0,4	11,1	0,3	11,4	0,1	***	***	***

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Table 3

	Ske		Rho		T0 ref		T4 ref		T7 ref		T4 Skel		T7 Skel		T4 Rho		T7 Rho		GLM analysis		
	Ave[5]	Stdev[6]	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Diet (D)	Time (T)	DxT
Tau	n.d.		0,1	0,0	44,8	7,3	42,9	3,4	44,8	3,1	43,8	3,0	42,0	5,1	40,9	2,5	40,8	2,7	ns	ns	ns
Asp	1,8	0,0	0,6	0,1	3,8	0,7	3,6	0,9	3,8	0,6	4,5	0,6	4,9	0,4	5,0	0,6	5,2	0,6	***	ns	ns
Thr	0,4	0,0	0,1	0,1	1,0	1,0	0,5	0,1	0,3	0,2	0,7	0,4	0,9	0,6	0,5	0,2	0,5	0,2	*	**	ns
Ser	0,1	0,0	0,1	0,1	0,6	0,3	0,9	0,3	0,6	0,2	0,4	0,2	0,4	0,2	0,6	0,1	0,6	0,2	**	*	*
Asn	3,7	0,2	2,7	0,1	1,5	1,2	0,8	0,6	0,4	0,2	1,2	0,6	1,2	0,8	1,0	0,5	1,0	0,9	ns	ns	ns
Glu	3,0	0,2	3,6	0,5	6,0	1,4	5,8	0,7	5,4	0,7	5,8	1,0	5,4	1,2	5,2	0,8	5,0	0,7	ns	ns	ns
Gln	3,4	0,2	1,7	0,7	3,7	3,8	2,1	1,1	1,0	0,8	3,3	1,8	3,3	2,0	2,0	0,9	1,9	1,3	*	*	ns
Pro	nd		1,3	0,6	6,6	2,8	7,1	2,5	3,7	3,4	6,8	2,8	6,5	2,7	6,3	2,3	7,1	1,9	ns	ns	*
Gly	0,1	0,0	0,1	0,0	6,1	3,9	6,6	2,5	3,9	1,5	4,3	1,5	4,7	1,8	6,7	2,5	6,8	1,7	*	ns	ns
Ala	0,6	0,0	1,5	0,2	9,0	3,6	6,3	1,7	4,3	1,1	6,0	1,6	4,7	1,7	4,2	0,9	4,0	1,3	ns	***	ns
Val	0,4	0,0	0,1	0,0	nd		nd		nd		nd		nd		nd		nd		-	-	-
Ile	0,3	0,0	nd		nd		nd		nd		nd		nd		nd		nd		-	-	-
Leu	0,3	0,0	nd		0,2	0,1	0,2	0,1	0,2	0,0	0,4	0,2	0,4	0,3	0,3	0,1	0,3	0,1	***	ns	ns
Tyr	0,3	0,0	nd		nd		nd		nd		nd		nd		nd		nd		-	-	-
b-Ala	nd		nd		2,9	1,4	3,5	1,2	1,9	0,8	3,2	0,9	3,2	0,7	3,1	1,3	3,5	1,5	ns	ns	*
Phe	0,1	0,0	nd		nd		nd		nd		nd		nd		nd		nd		-	-	-
Orn	0,3	0,0	0,1	0,0	0,2	0,1	0,1	0,0	0,1	0,1	0,2	0,1	0,3	0,2	0,2	0,1	0,2	0,1	***	ns	ns
Lys	0,3	0,0	0,2	0,0	0,6	0,3	0,5	0,1	0,4	0,3	0,5	0,2	0,6	0,3	0,5	0,2	0,4	0,2	ns	ns	ns
His	0,2	0,0	nd		0,4	0,3	0,2	0,1	0,1	0,1	0,3	0,1	0,4	0,2	0,2	0,1	0,2	0,1	**	*	ns
Arg	0,8	0,1	1,0	0,3	2,0	1,0	2,0	0,4	1,1	0,5	1,6	0,2	1,7	0,4	1,7	0,3	1,9	0,4	ns	*	**

[5] Ave: Average

[6] Stdev: Standard deviation

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Table 4

	T0 ref		T4 ref		T7 ref		T4 Skel		T7 Skel		T4 Rho		T7 Rho		GLM analysis		
	Ave[7]	Stdev[8]	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Diet (D)	Time (T)	D x T
<u>Aldehydes</u>	*1,0*	*0,1*	*1,0*	*0,2*	*0,5*	*0,1*	*0,6*	*0,1*	*0,9*	*0,1*	*1,2*	*0,2*	*0,5*	*0,0*	*ns*	***	***
(E)-2-pentenal	0,3	0,0	0,3	0,0	0,4	0,0	0,3	0,0	0,5	0,0	0,3	0,0	0,3	0,0	***	***	***
(E)-2-hexenal	0,2	0,0	0,1	0,0	0,1	0,0	0,1	0,0	0,1	0,0	0,2	0,0	0,1	0,0	***	***	***
(E)-2-octenal	0,1	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,1	0,0	0,1	0,0	0,0	0,0	***	***	***
(Z,E)-2,6-nonadienal	0,4	0,1	0,5	0,1	0,1	0,0	0,2	0,0	0,2	0,0	0,5	0,1	0,1	0,0	ns	***	***
Alcohols	*8,1*	*0,9*	*7,8*	*0,5*	*4,5*	*0,7*	*3,7*	*0,2*	*4,4*	*0,4*	*5,4*	*0,7*	*5,4*	*0,9*	***	***	***
1-penten-3-ol	0,5	0,0	0,5	0,0	0,7	0,0	0,7	0,1	0,6	0,0	0,7	0,0	0,9	0,1	***	*	*
(Z)-2-penten-1-ol	0,1	0,0	0,2	0,0	0,2	0,0	0,2	0,0	0,2	0,0	0,2	0,0	0,3	0,0	***	***	ns
(5Z)-octa-1,5-dien-3-ol	4,5	0,6	4,6	0,3	2,8	0,5	2,3	0,1	2,8	0,3	3,0	0,3	3,3	0,3	***	**	***
(E)-2-octen-1-ol	0,2	0,0	0,2	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,1	0,0	0,1	0,0	***	***	***
(E,E)-2,4-octadien-1-ol	2,0	0,2	1,6	0,2	0,7	0,2	0,5	0,1	0,7	0,1	1,0	0,2	0,8	0,0	***	***	***
(E,Z)-3,6-nonadien-1-ol	0,7	0,1	0,7	0,1	0,0	0,0	0,1	0,0	0,0	0,0	0,3	0,1	0,1	0,0	***	***	***
Ketones	*0,1*	*0,0*	*0,1*	*0,0*	*0,1*	*0,0*	*0,1*	*0,0*	*0,1*	*0,0*	*0,1*	*0,0*	*0,1*	*0,0*	**	*ns*	*ns*
2,3-pentanodione	0,1	0,0	0,1	0,0	0,1	0,0	0,1	0,0	0,1	0,0	0,1	0,0	0,1	0,0	**	ns	ns
Furans	*0,1*	*0,0*	*0,1*	*0,0*	*0,0*	*0,0*	*0,1*	*0,0*	*0,1*	*0,0*	*0,1*	*0,0*	*0,0*	*0,0*	***	*	***
2-ethylfuran	0,1	0,0	0,1	0,0	0,0	0,0	0,1	0,0	0,1	0,0	0,1	0,0	0,0	0,0	***	*	***

[7] Ave: Average
[8] Stdev: Standard deviation

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Table 5

	T0 Ref		Ref T4		Ref T7		Ske T4		Ske T7		Rho T4		Rho T7		GLM analysis		
	Ave[9]	Stdev[10]	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Diet (D)	Time (T)	D x T
Overall odor	5,4	1,4	5,0	2,0	4,1	1,4	4,2	2,1	3,0	1,9	3,3	1,6	2,9	1,6	**	*	ns
Marine odor	5,0	1,8	4,0	2,2	3,5	1,8	3,7	2,2	2,6	2,4	3,4	1,9	2,2	1,5	ns	*	ns
Fruit odor	1,4	1,0	0,6	0,9	0,5	0,6	0,4	0,6	0,5	0,7	0,7	1,0	0,6	0,8	ns	**	ns
Mud odor	1,1	1,0	1,6	2,2	0,7	1,8	0,8	1,3	0,4	0,6	0,2	0,2	0,8	1,4	ns	ns	ns
Darkness of visceral mass	3,4	1,9	3,3	2,4	4,7	1,9	3,6	1,1	2,7	1,5	3,3	1,2	3,2	1,6	ns	ns	*
Darkness of gills	4,8	1,7	3,8	1,7	5,2	1,4	4,2	1,3	4,0	1,4	3,7	1,7	3,9	2,0	ns	ns	ns
Darkness of mantle edge	5,9	1,2	5,1	1,1	6,9	1,2	5,6	1,1	4,7	1,9	5,1	1,7	5,4	1,7	ns	ns	**
Fullness of shell	5,4	1,5	4,0	1,0	3,5	1,9	5,4	1,2	5,8	1,3	5,6	1,3	6,1	1,8	***	**	ns
Crunchy texture	5,2	1,9	4,8	1,8	4,5	2,0	5,1	1,4	5,4	2,1	4,7	1,3	5,4	2,0	ns	ns	ns
Melting texture	3,9	2,1	3,3	2,3	4,3	2,2	3,7	1,8	5,2	2,0	4,0	2,2	4,1	1,6	ns	ns	ns
Chewiness	4,3	1,4	3,9	2,2	2,9	1,5	3,3	1,6	3,8	1,7	3,0	1,6	3,9	1,8	ns	ns	ns
Marine taste	5,8	1,7	5,0	2,0	5,7	1,8	5,5	1,8	4,8	1,8	5,3	2,2	4,0	1,6	ns	ns	ns
Algal taste	1,7	1,8	1,7	1,4	2,4	1,2	2,3	1,6	2,1	2,2	1,3	1,4	2,3	1,6	ns	ns	ns
Saltiness	4,6	1,5	3,9	1,9	4,6	2,0	3,9	1,5	3,2	1,6	3,4	1,8	3,7	2,2	ns	ns	ns
Sweetness	2,5	1,6	1,9	1,1	2,0	1,4	1,7	1,1	3,0	2,2	2,3	1,6	2,0	1,9	ns	ns	ns
Bitterness	1,8	1,6	1,8	1,7	1,5	1,4	2,1	2,1	1,3	1,7	1,8	2,1	2,0	1,6	ns	ns	ns
Metallic taste	2,4	1,8	1,8	1,8	1,6	1,4	1,9	2,2	1,7	1,8	2,0	2,2	2,0	1,8	ns	ns	ns
Astringency	1,9	1,5	2,4	2,3	2,1	1,8	2,8	2,4	1,9	2,5	1,8	1,7	2,5	1,9	ns	ns	ns

[9] Ave: Average
[10] Stdev: Standard deviation

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