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AWI

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Temperature dependent filtration rates and ^{13}C-NMR-enrichment analysis of substrate utilization in *Pecten maximus*

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Abstract

With the seawater temperature rising more than 1.5°C (IPCC) from the pre-industrial time, marine organisms are facing more and more severe climate changes. As temperature is an important factor influencing the physiology of animals, species specific adaptations has been well observed. Subtidal species are one of the most seawater temperature influenced animals. In previous researches, NMR metabolic profiling has been proved to be a decent technique of animal physiological studies. In this work, the king scallop, *Pecten maximus* was studied to test if (1) consuming labeled phytoplankton would be a stable way of ^{13}C labeling marine filter feeders such as scallops; (2) the metabolism of *P. maximus* would also change with increasing temperature, which reflects as the different filtration rates from the outside and changing metabolic pathway inside organs. The scallop *P. maximus* were incubated under two different temperatures, 15°C and 20°C, fed with ^{13}C labeled diatom *Phaeodactylum tricornutum*. After three days' filtration rate measurement, the tissue samples of digestive gland and striated adductor muscle were dissected and extracted. Both qualitatively and quantitatively metabolic profiling was done via ^{13}C NMR analyzation.

The performance of experiment animal, *Pecten maximus* were quite different under two temperature treatments. Higher filtration rate was observed at 20°C whereas faster digestion and incorporation of algal lipids was also found inside the digestive gland from 20°C treatment. As for the muscle tissues, incorporation of ^{13}C labeling was observed in both temperature groups, proving this labeling technique is applicable for marine filter feeders.

Figures and tables

Figures

Tables

Table 9 The metabolite list for the peaks in ^{13}C spectra of digestive glands, scallop ID with 1503 from 15°C group and ID 2003 from 20°C group were shown. Relative integrals (absolute integral divided by 4X10^7, see M&M) were calculated. Mean values and standard deviations were calculated from relative integrals of the three scallops from the same group. Source from UT Southwestern Medical Center. 34

Abbreviations

α	level of significance	kid.	kidney
ANOVA	analysis of variance	L	liter
anu.	anus	lip.	lips
C	concentration	M	molar (mol/L)
°C	degree celsius	m	meter
CDCl3	chloroform-d solvent	man.	mantle
Chl a	Chlorophyll a	min	minute(s)
cho.	chondrophore	mL	milliliter
CR	clearance rate	mg	milligram
d	day	NMR	nuclear magnetic resonance
df	degrees of freedom	O_2	oxygen
D_2O	deuterium oxide	p	probability value
DHA	docosahexaenoic acid	pal.	labial palps
dig.	digestive gland	ppm	parts per million
EPA	eicosapentaenoic acid	PUFA	polyunsaturated fatty acids
fgo.	female gonad	s	second
FR	filtration rate	Si	silicon
g	gramm	T	temperature
gil.	gill	t	time
h	hour(s)	V	volume
HCO_3^-	bicarbonate ion	Wt	weight
H_2O	(Distilled) water	µm	micrometer
int.	intestine	η2	same as r^2 or R^2 in statistics, represents the effect size
hea.	heart		

1. Introduction

1.1 Climate change and its impact

Climate change, which refers to changes in climate over time, includes changes of natural variability (e.g. temperature, precipitation, solar radiation, relative humidity and wind speed) and human impacts (e.g. CO_2 emission) (IPCC; Hulme, 1999). It appears and influences on global, subcontinental, national and local levels (Gupta, 2007). The rise of temperature is quite significant and evident worldwide (Bozinovic et al., 2015), which has already lead to observable changes such as rising seawater temperature, melting sea ice and changing sea surface levels (NASA). This leads to changes on marine ecosystem such as geographical distribution, population, migration, biological seasonal timings and food availability will in turn influence the physiology of marine organisms (Pörtner and Farrell, 2008). From the special report of IPCC (2018), a temperature increase of 1.5°C above pre-industrial levels has been observed. However, the temperature changes on regions differ from each other (Fig.1), whereas the impact of global warming also works differently for various marine organisms (Kroeker et al., 2013).

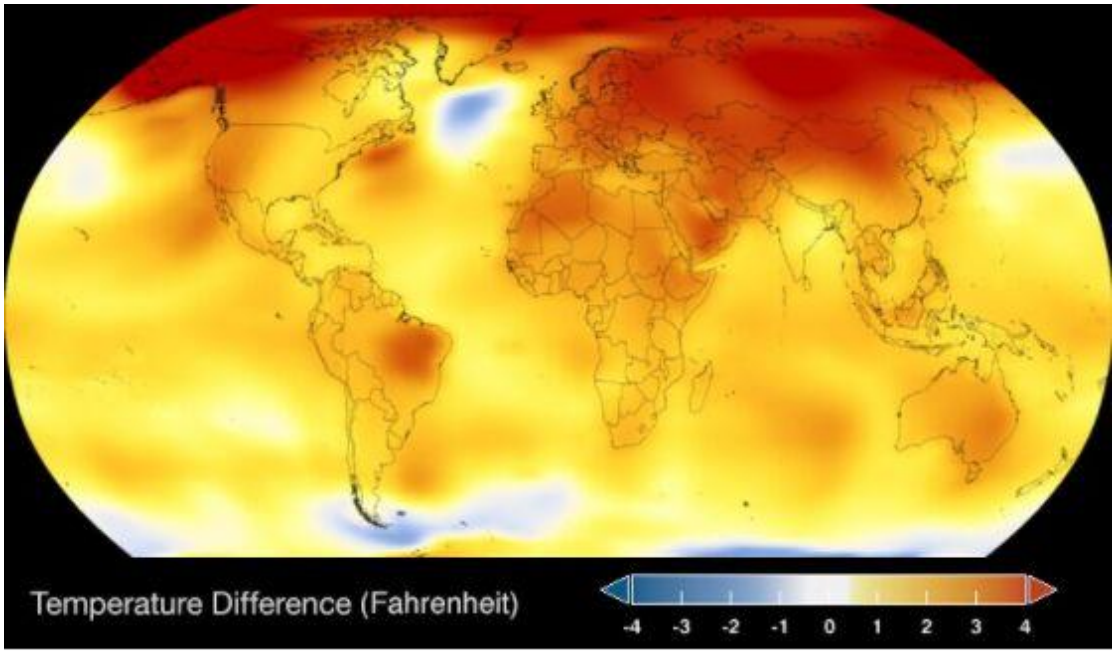


Figure 1 Temperature change in global surface temperature relative to 1951-1980 average temperatures of year 2017. Data source: NASA/GISS.

An increase of seawater in Atlantic Ocean was observed. In the research region: Vigo, Spain, the seawater temperature ranges from 15°C to 20°C (Fig. 2). The scallops,

P.maximus were mostly subtidal species (Artigaud, 2014; Saavedra, 2005), which means the seawater temperature would be the environment temperature for them.

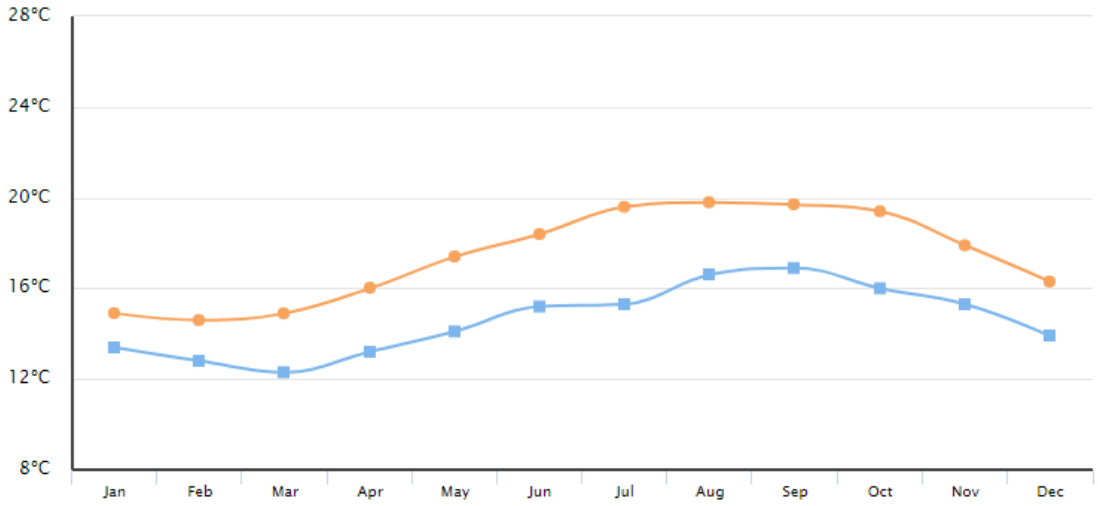


Figure 2 Monthly seawater temperature change in research region: Vigo, Spain. Orange line represents the maximum temperature while the blue line shows the minimum temperature. Source from World Sea temperature.

Temperature as an important influential factor on animal physiology as it could affect food consumption (Laing, 2014), energy uptake (MacDonald, 1986), metabolic rates (Robson, 2016) and has been investigated in many previous studies. Study by Huey (2012) suggested that the ability of animal species adapting to climate change depends on its sensitivity, the extent of exposure and disturbance as well as its potential of adaptation. Thermal windows of aquatic animals have been reported by Pörtner in 2008, describing the animal performance according to the changing temperature (Fig.3). In the graph, when temperature is outside the pejus temperature range, the animal's performance is turning worse; when outside the critical temperature range, the metabolism will switch from aerobic to anaerobic.

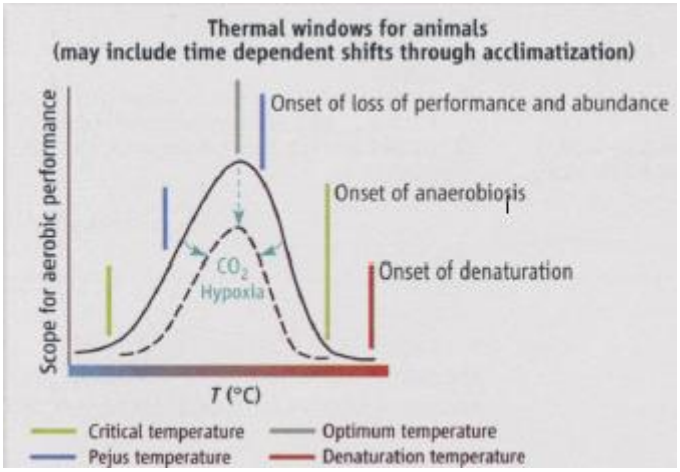


Figure 3 Thermal window showing temperature effects on aquatic animals. Source from Pörtner, 2008

High temperature, which always combined with hypoxia, could lead to stress and worse performance on marine animals, such as reduced feeding and anaerobic metabolism (Artigaud et al., 2015), change in metabolic rates (Gillooly et al., 2001), less reproduction and mortality (Doney et al., 2012).

1.2 *Pecten maximus*

1.2.1 Morphology and ecology

Pecten maximus, also known as the great scallop, or the king scallop, is a marine bivalve mollusc in the family Pectinidae (Pechenik, 2010), mainly distributed in northeast Atlantic Ocean (Fig.4). The common size for *Pecten maximus* is from 10 to 15cm (Fig.5) and shows no separate male and female size at maturity (Charlotte Marshall & Emily Wilson). *P.maximus* has two valves: brown-pink left (upper) valve and white right valve, 12 to 17 radiating rib lines can be observed on the left valves.

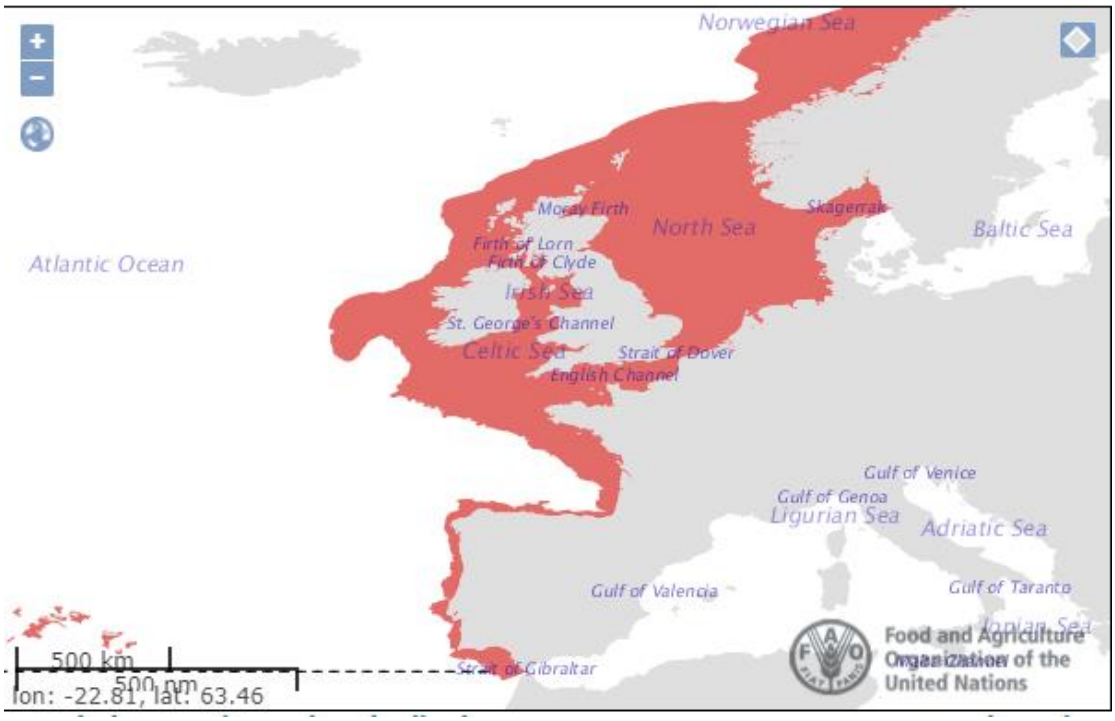


Figure 4 Distribution of *Pecten Maximus*. Source: FAO

As an intertidal benthic species, *Pecten Maximus* can be found from low tide until down to 250m. It is a free living animal, both burrower and swimmer. Its sediments include sand and mud.

Because of the wide distribution and high market value, *Pecten Maximus* is the most important European fishery scallop species (Shumway and Parsons, 2016) and well-studied in aquaculture (review by Utting, 1998).

Figure 5 *Pecten maximus* (experimental animal 1501).

Pecten maximus' reaction to predator includes closing the shell, jumping and swimming (Thomas and Gruffydd, 1971). Previous studies have looked into the swimming mechanism of *Pecten maximus* (Shumway and Parsons, 2016): *Pecten maximus* can move by clapping the valves and expelling the water on either side of the dorsal hinge.

1.2.2 Filter feeding

Adult *Pecten maximus* lives a benthic life as a filter feeder like most of the bivalves. Considering a filtration system, three elements are essential: (1) the dispersed particles, (2) the fluid medium and (3) the filter (Rubenstein, 1977).

Various food particles like pelagic phytoplankton, microphytobenthos, detrital material, nanoplankton, bacteria, zooplankton and macro-algae detritus (Lavaud, 2014) could be consumed by this filter feeder. According to the study by Lavaud (2014), *Pecten maximus* are also able to choose from the food sources to obtain the better size (less than 10 µm with an optimal range of 2 to 5 µm (Rico-Villa, 2005)), higher quality or energetic value (lipid rich) ones. Among all, the most preferred are living algae cells.

P.maximus commonly lives at the water-sediment interface with right valve buried

inside for stability and hiding from predators. *Pecten maximus* are opportunistic feeders and water flows could keep the water salinity at an acceptable level (Hardy, 2008) and help to transport the organic matter into the scallops (Shumway et al., 1987).

When the particles entered the scallop, the gills work as sieves to mechanically filter the water and retain the nutritious particles while the unusable materials are ejected as pseudo-faeces (Lavaud, 2014).

1.3 Filtration rate

Filtration rate (FR), is a fundamental parameter in bioenergetics studies of suspension feeding bivalves (Riisgård, 1991). Filtration rates are dependent on both exogenous and endogenous factors including water temperature, salinity, particle concentration, body size (Jørgensen 1990; Bayne 1998; Riisgård 2001c; Petersen et al, 2004; Cranford & Grant 1990, Alber & Valiela 1996, Shumway et al. 1997, Velasco 2006; Aya et al, 2013). Studies (Bayne and Newell, 1983; Bayne B.L. 1993) have shown that size of scallops and temperature are two important factors determining energy balance.

Earlier studies (Petersen et al, 2004) have tested three different methods of measuring filtration rates: (1) the flow-through method, (2) the bio-deposition method and (3) the indirect or filtration method. In the flow through method, seawater runs through the chambers and the particle concentration before and after feeding were measured for filtration rates determination. In the bio-deposition method, filtration rate will be determined by the egested and rejected inorganic material. Concerning the lab conditions and the experimental design, in this study the filtration rate will be measured by the (3) indirect method, which the decrease of the feed particle concentration in a closed system over a fixed time interval will be measured as filtration rate.

According to Owen (1974), using indirect methods for filtration rates measurement always based on following assumptions:

(1) Filtration is the only reason for the particle concentration decrease; (2) the exhalant flow ('pumping rate') of the bivalve is constant; (3) A constant percentage of particles are retained (usually 100%); (4) and the particles are evenly suspended inside the medium (Coughlan, 1969).

Several filtration rates (clearance rates) expressions on scallops could be found in prior studies (Strohmeier et al., 2009; Comeau et al., 2008; Aya et al. 2013; Laing, 2004). In most of the cases, the FR is always linked with dry weight of bivalves' tissues. However, in this research, as the tissues were needed for metabolite studies and the dry weight could not be obtained, the filtration rates would be expressed as (Riisgård , 2001b):

$$FR = (\ln C_1 - \ln C_2) \times V \times T^{-1}$$

, where C_1 and C_2 are the algal cell concentrations at the beginning and end of a time interval; V is the volume (5L) of the water and T is the time interval.

1.4 Digestion and energy storage

After processed in pallial organs including gill, mantle, labial palp and lip (Shumway and Parsons, 2016), the particles will then go into the digestive system, including important organs such as digestive gland and stomach.

In *Pecten maximus*'s structure, the stomach is embedded in the digestive gland, and in this research, they are not separated from each other. The ingested material were transported to the stomach and together with digestive gland, they were digested and absorbed. The digestive gland also stores energy in terms of lipid (main) and glycogen (Pennec, 2001).

The adductor muscles of *Pecten maximus* consist of striated adductor muscle (large) and smooth adductor muscle (small), also regarded as phasic muscle and tonic muscle. The phasic muscle is the largest organ inside the scallop, responsible for fast reactions of scallops including opening and closing of the valves, swimming by expelling the water inside (Wilkens, 2006) mainly by anaerobic metabolism (Zwaan et al., 1980). The tonic muscle is much smaller comparing to the phasic muscle next by, and also smoother. The tonic muscle is able to keep the shell closed for long periods of time with little expenditure of energy, by producing energy through slow contraction (Shumway and Parsons, 2016).

The adductor muscle is the most important energy storage site in many marine bivalves (Shumway and Parsons, 2016). The muscle tissues function as reserves in two ways: (1) nutrient storage and (2) nutrient mobilization (Mathieu, 1993). In *Pecten maximus*, the phasic adductor muscle is rich in nutrients such as glycogen (5% to 22% dry weight (DW))and protein (ranging between 85% and 61% DW), which could also be provided for the reproduction of this species (Lee, 2015; Mathieu, 1993), while the lipid content is not much, only 2.5% to 4% DW (Pazos, 1997).

1.5 Temperature influences on *P.maximus*

The direct pattern of climate change is the rise of temperature, which reflects on seawater environment condition changes and would contribute to the changing living conditions of *Pecten maximus*.

1.5.1 Food availability and quality

Phytoplankton as the typical primary producers, are easily affected by sea environmental changes. Shifts of the rhyme of phytoplankton (Wiltshire, 2008; Sommer, 2008; Edwards, 2004) have been observed in several sea areas. As living algal cells are the most preferred food of scallops (Lavaud, 2014), the food availability for scallops might also get influenced, as a mismatch could take place under rising temperature.

Concerning the energy budget of scallops, lipid acts an important role in

reproduction processes (Shumway and Parsons, 2016), apart from which, the proteins and vitamins from phytoplankton are also important in nutritious activities. The high temperature impact on phytoplankton would also lead to the lower food quality of scallops.

1.5.2 Metabolic rate of *P. maximus*

The most commonly used energy budget was: C= P + R + F +U. C represents the food consumption, while the energy expenditures were: production (P), respiratory heat loss (R), faecal loss (F), executory products (U) (Shumway and Parsons, 2016).

Regarding to the biochemical, physiological and behavioral processes of animals, temperature plays an important role as it is a fundamental factor, influencing metabolic activities including cell maintenance, enzyme activity, energy uptake, reproduction (Somero, 2002; Schulte, 2015).

According to the study of Robson (2016), *Pecten maximus* showed an incline of routine metabolic rate (measured by O_2 consumption) under increasing temperature from 5°C to 20°C. The highest total metabolic rate appeared at 13°C, which in Robson's study was recognized to be the optimum temperature for king scallop *P.maximus*.

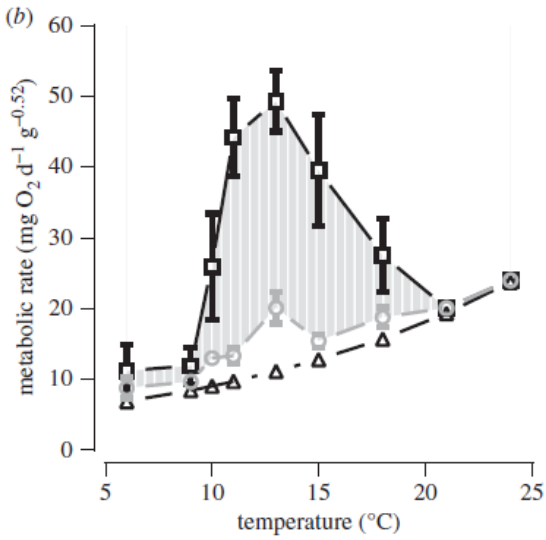


Figure 6 *P. maximus* metabolic rate against temperature: RMR (routine metabolic rate, scallops without moving): triangle-line; (2) total MR (total metabolic rate): square-line; (3) total MR excluding swimming and spinning: circle-line; (4) AMR (activity metabolic rate of swimming and spinning). Source from Robson (2016).

1.6 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear Magnetic Resonance (NMR) Spectroscopy has been proved to be a useful

and standard tool for organic structure determination (Fan, 1996). Using the magnetic properties of nuclei from isotopes such as $^{1}\mathrm{H}$ and $^{13}\mathrm{C}$, NMR can separate these isotopes in their specific chemical surrounding and is frequently used for structure elucidation and compound analysis of complex mixtures. For example, he $^{13}\mathrm{C}$ chemical shifts of 20 free amino acids were reported by Prabhu (1996) by research on *Arabidopsis*. Chauton (2003) used ^{1}H NMR spectroscopy for detecting the metabolites inside the microalgae *Phaeodactylum tricornutum*.

Metabolic profiling, as Clark (2008) has described, is "the measurement in biological systems of the complement of low-molecular-weight metabolites and their intermediates that reflects the dynamic response to genetic modification and physiological, pathophysiological, and/or developmental stimuli", usually based on the NMR spectroscopy by checking animal body fluids, tissue and/or cell extracts (Beckonert, 2007).

With the help of $^{1}\mathrm{H}$ NMR spectroscopy, the metabolites inside the American oysters were studied (Tikunov, 2010). While in the year 2014, Tikunov studied the metabolism of eastern oyster with isotope $^{13}\mathrm{C}$ and$^{15}\mathrm{N}-NMR\ spectroscopy$. By injecting 2-$^{13}C/^{15}N$-glycine and U-$^{13}C$-glucose, the metabolic profiling of four different tissues (gill, mantle, adductor muscle, digestive gland) was studied. Through metabolic profiling study, the substrates utilization, energy uptake as well as metabolic pathways were well-studied.

A previous study (Aursand, 1992) has studied the fatty acids of atlantic salmon (*Salmo salar*). By $^{13}\mathrm{C}$ labeling and lipid extraction, the compounds were identified quantitatively and fatty acids classified.

1.7 *Phaeoductylum tricornutum*

Phaeodactylum tricornutum is a common unicellular diatom which can be found in both brackish water and seawater (Prestegard, 2016). The diatom contains 7–35 wt% of lipids in the form of fatty acids (Shoji, 2004) and has high lipid productivity up to 26.75 $\mathrm{mgL^{-1}d^{-1}}$ (Longworth, 2016). Due to the simple structure, fast growth rate as well as cell composition, it is widely used as biofuels (Kim, 2015), also could serve as feed for marine filter feeders such as bivalves.

As has been discussed above, scallops prefer living microalgae as feed rather than other organic particles (Lavaud, 2014). Due to the reason that they could not produce polyunsaturated fatty acids (PUFAs) themselves (Shumway and Parsons, 2016), the only way of obtain these PUFAs is by consuming phytoplankton. *Phaeoductylum tricornutum* as food source could provide these essential fatty acids (PUFAs) to scallops, however, the cell walls of *P.tricornutum* also makes it difficult for scallops to digest (Spencer, 2008).

1.8 Aim of the study and working hypotheses

As discussed above, temperature is an important influential factor to the life and metabolism of scallop, *Pecten maximus*. On facing the more and more serious climate change causing seawater temperature rise, this study will test the following hypotheses:

(1) An increase of seawater temperature has an impact on the energy uptake on the scallop *Pecten maximus*. Filtration rates as a direct outer performance would also change accordingly. By rising the temperature from 15°C to 20°C, the filtration rate will also increase.

(2) By consuming isotope labeled material, in this study, ^{13}C labeled algae *Pheodactylum tricornutum*, the filter feeder, *P. maximus* will be properly labeled for metabolic profiling via NMR spectroscopy.

(3) With changing temperature, substrates utilization and metabolic pathways also change accordingly, which also differ between tissues.

2. Material and methods

2.1 Label material

^{13}C-labeled diatoms of *Phaeodactylum tricornutum* was used as labeled substrate source for bivalves. Before the experiment started, the diatom has been incubated in a 5L glass bottle with f/2+Si medium (Guillard, 1975, see Appendix), 3mM sodium ^{13}C-bicarbonate was added inside as label material and carbon source.

The labeling of *P.tricornutum* was not checked via NMR as we already have successful experience with it and the diatom incubation conditions were not changed. The diatom *P.tricornutum* was assumed to be well labeled after 10 days of incubation without disturbance.

2.2. Acquisition and incubation of *Pecten maximus*

The *Pecten maximus* used in this experiment were all collected from Vigo, Spain, transported to AWI Bremerhaven, Germany and incubated in the aquarium at 15°C for 1 month for acclimation before the experiment.

Table 1 Number of scallops used in each group

	unlabeled	Labeled
Test group	1	1
15°C	0	3
20°C	0	3

A pre-experiment was set up to test the labeling method. Two scallops were incubated at 15°C, with one scallop feed with labeled *Phaeoductylum tricornutum* and the other scallop with unlabeled. The incubation took place in the 15°C TK (temperature control) room. Each scallop was put inside a 10L black bucket, filled with 10L 0.2µm filtered seawater (by GF/C filter). Every day $2X10^9$ ^{13}C *Phaeodactylum tricornutum* cells were provided to each scallop.

Two temperature groups of 15°C and 20°C were set up. For each group, 3 *Pecten maximus* were used. Each scallop was put inside a 10L black bucket, filled with 5L 0.2µm filtered seawater (by GF/C filter). Every day $4X10^9$ ^{13}C labeled *Phaeodactylum tricornutum* cells were provided to each scallop.

The scallops of 15°C group were incubated in a TK room with a constant temperature of 15±0.1°C. Temperature control of the 20°C group was managed placing the set-up in a cooling box in a room at around 22°C by cooling thermostats (Launda eco re630 gold), with water temperature around the bucket set to 19.8°C

(Fig. 7). Air stones were used for bubbling and mixing up the water to keep water flow for the filter feeders. Also, iron “donuts” were used for the right valve of the *Pecten maximus* to maintain the stability and keep them at the same position.

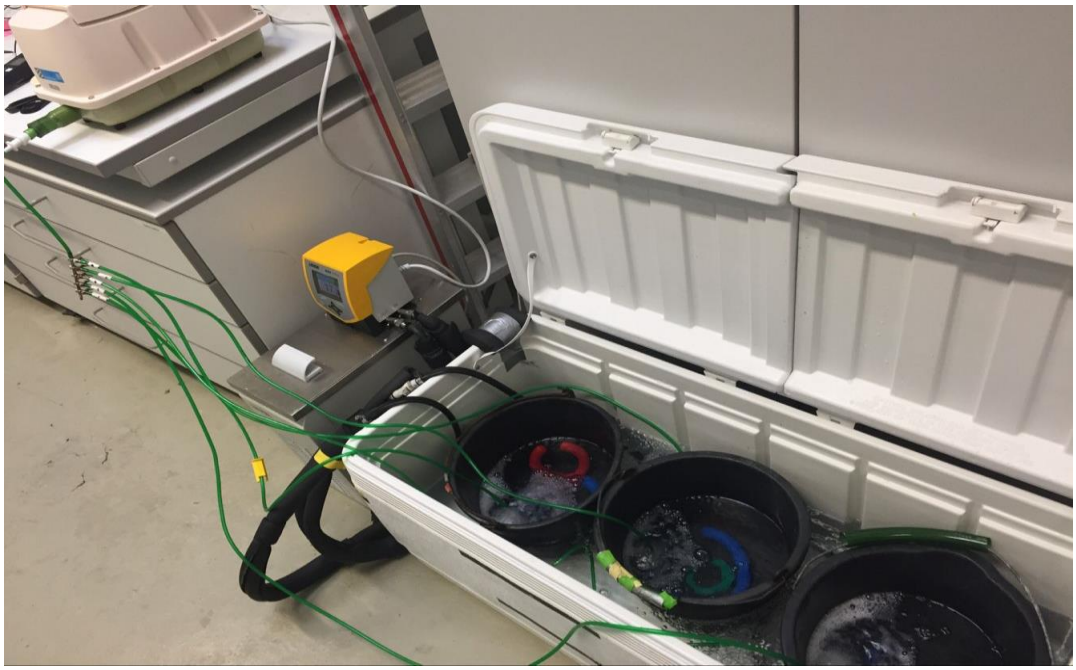


Figure 7 Temperature control set up for the 20°C group

The incubation lasted for four days, including a first day of starving and temperature acclimation and then three days of feeding. Water samples from each bucket were taken at the starting time (0h), 1h, 2h and 4h to measure the cell concentration of *Phaoductylum tricornutum*. The water temperature of every bucket was also taken at the same time interval to ensure the right temperature (Table 3). Water was changed every 24h to remove residual algae and for water quality. Also, during the experiment time, the least human disturbance was ensured to keep the feeding going continuously.

2.3 Scallop dissection and tissue extraction

2.3.1 Dissection

After three days’ feeding, the scallops were dissected and tissues separated. Five tissue blocks including: (1) digestive gland, (2) striated adductor muscle, (3) smooth adductor muscle, (4) gill and (5) mantle, were wrapped in aluminum folie and immediately frozen in liquid nitrogen. Tissues were then kept at -80°C.

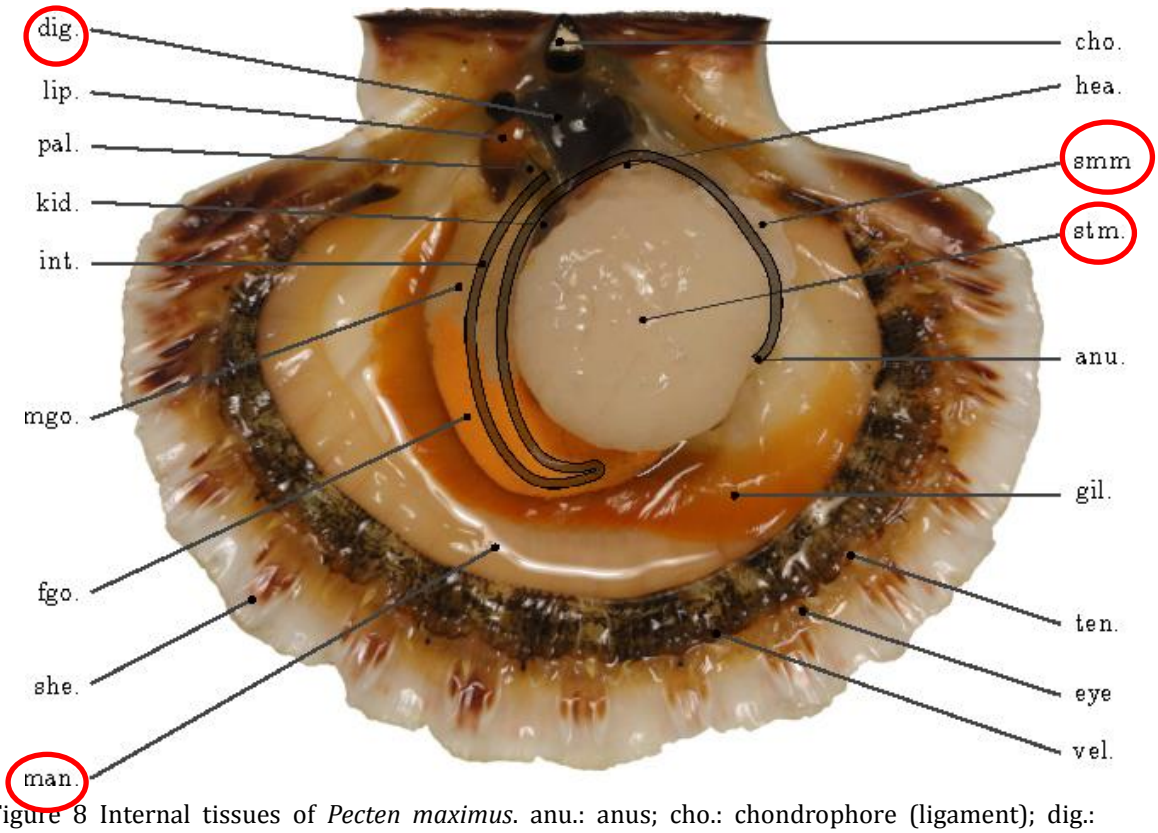


Figure 8 Internal tissues of *Pecten maximus*. anu.: anus; cho.: chondrophore (ligament); dig.: digestive gland; fgo.: female gonad; gil.: gill (right fold); hea.: heart; int.: intestine (not seen, drawn); kid.: kidney; lip.: lips; pal.: labial palps; man.: mantle; stm. :striated adductor muscle; smm.: smooth adductor muscle. Source from Lavaud (2014).

2.3.2 Tissue extraction

Methanol-chloroform extraction (adapted from Bligh, 1959) was done for 2 tissue blocks: (1) digestive gland and (2) striated adductor muscle. Around 120mg frozen tissue (Table 2) were taken from each tissues sample. After grounding, around 60mg tissue pieces were weighed and transferred to ice chilled precyllys tubes (Precellys® 2 mL Hard Tissue Homogenizing Ceramic Beads Kit (CK28)) filled with 400µL methanol and 125mL milli-Q water. Then the samples were all taken to the tissue homogenizer (Precellys 24, Bertin) with 2 cycles of 20s at 6000rpm. 400µL chloroform and 400µL milli-Q water were added to the tubes and vortexed for 15s, then the tubes were settled on ice for 10min. After that, the samples were centrifuged 10min for 3000rpm at 4°C. The upper layer containing metabolites were transferred to 1.5mL Eppendorf tubes and were dried by the vacuum concentrator (Christ RVC 2-18 CD plus) for 12h. The lower part of lipid extractions were stored in open brown glass under a fume hood overnight.

Table 2 The fresh weight of tissue samples used for methanol-chloroform extractions.

	Test	15°C	20°C

	unlabeled	labeled	1501	1502	1503	2001	2002	2003
Digestive gland (mg)	117.6	122.8	118.2	119	112.2	112.7	111.7	103.2
Striated adductor muscle (mg)	109.8	112.4	114.6	108.9	114.8	109.9	116.9	110.4

2.4 ^{1}H and ^{13}C NMR spectroscopy

After extraction, (1) digestive gland and (2) striated adductor muscle samples were dissolved for NMR measurements. 40µL D_2O was used as solvent for each metabolite sample, whereas 70µL chloroform was used for every lipid sample. All samples were measured by a vertical 400 MHz NMR spectrometer (AVANCE III HD 400 WB; Bruker BioSpin GmbH) at 20°C NMR probe temperature.

In this research, ^{13}C NMR was used to check the incorporation of isotope labeling to identify the specific substrates. Proton (^{1}H) NMR spectrum was also obtained for unlabeled metabolic profiling, however, as it is not the main study aim of this work, relevant spectra were not shown.

2.5 Data analysis and statistics

2.5.1 Analysis of ^{13}C NMR spectra

In this study, the NMR signal peaks were not only qualified but also quantified: (1) qualitative analysis: signals and related shifts were first identified on the ^{13}C spectra, then, based on literatures and relevant databases, the signals were assigned to specific substrates/ compounds/ metabolites; (2) quantitative analysis: Integrals were done for each signal via Bruker TopSpin Software for comparisons. Absolute integrals were obtained, and for better comparisons, relative integrals were calculated. For the lipid measurement, as for each sample, same amount (70µL) of chloroform-d ($CDCl_3$) was used as solvent, therefore the absolute integral of chloroform signals (approx.4X10^8) was used as standard. As for the metabolite spectra, there was no standard signal, therefore all absolute integrals were divided by 4X10^7 as relative integral. This is because the metabolites' signals were much smaller than lipid signals and if divided by 4X10^8, it would be too low for comparison.

2.5.2 Statistics

A Chi-squared test was performed for checking the size class of experiment animal

Pecten maximus. Kruskal-Wallis test was used to determine the temperature control for the 20°C group as well as the sample weight. The filtration rate was checked by ANOVA, before ANOVA, Bartlett's test was first performed, with p-value>0.05. All statistics were done by R software and with α=0.05.

3. Results

3.1 Incubation parameters

All experimental animals, *Pecten maximus* were from the similar sizes (Table 3). No mortality occurred in all groups during the experiment.

Table 3 Size information of the *Pecten maximus* used

	Scallop ID	Length (cm)	Width (cm)	Height (cm)
15°C	1501	10.8	9.4	3.2
	1502	10.2	9.1	2.8
	1503	9.9	8.9	2.6
20°C	2001	10.6	9.3	3.0
	2002	11.5	9.7	3.1
	2003	9.9	9.0	2.6

Chi-squared test was performed on checking if the shell length, width and height of *Pecten maximus* in each group were from the same size group. With p-values of 0.99, 1 and 0.99, we could reach the conclusion that size is not an influential parameter for this experiment.

As the scallops of 15°C group were incubated in 15°C TK rooms and water were also pre-cooled inside, we didn't check the temperature during the experiment. However, 20°C group temperature control was managed by the cooling thermostats, however, easily influenced by the room temperature. The water inside was measured everytime water sample was taken to ensure the experimental conditions. All temperatures are shown below in Table 4.

Table 4 Temperature changes in three buckets for 20°C group. Surrounding temperature represents the water inside the white box surrounding the 3 buckets. The last column represents the average temperature ± standard deviation.

Day 1	Bucket 1 (°C)	Bucket 2 (°C)	Bucket 3 (°C)	Surrounding temperature (°C)
0h	19.6	19.6	19.4	19.8
1h	19.7	19.7	19.6	19.7
2h	19.6	19.5	19.5	19.4
4h	19.6	19.6	19.4	19.8
mean±stdev	19.63±0.05	19.60±0.08	19.48±0.10	19.68±0.19

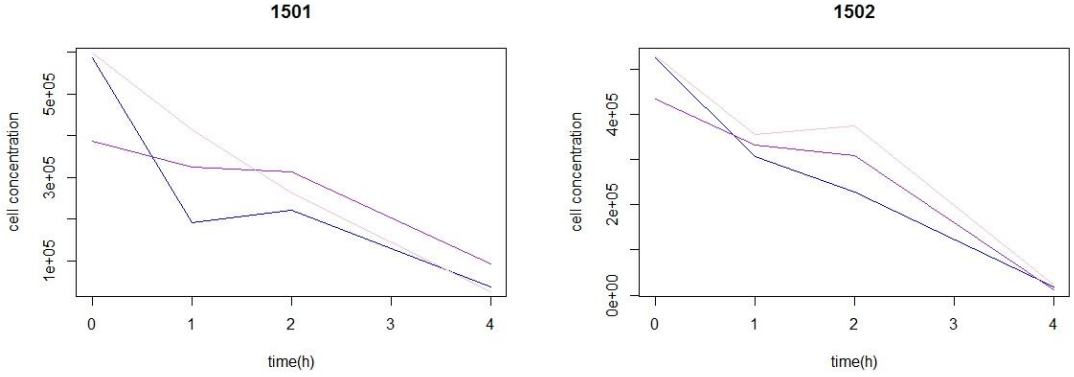
Day 2	Bucket 1 (°C)	Bucket 2 (°C)	Bucket 3 (°C)	Surrounding temperature (°C)
0h	19.2	19.2	19.3	19.5
1h	19.6	19.6	19.6	19.7
2h	19.8	19.8	19.8	19.9
4h	20.0	20.0	20.0	20.1
mean±stdev	19.65±0.34	19.65±0.34	19.68±0.30	19.80±0.26

Day 3	Bucket 1 (°C)	Bucket 2 (°C)	Bucket 3 (°C)	Surrounding temperature (°C)
0h	19.2	19.1	19.2	20.0
1h	19.5	19.5	19.5	19.9
2h	19.9	19.9	19.8	19.9
4h	20.0	20.0	19.9	20.1
mean±stdev	19.65±0.37	19.63±0.41	19.60±0.32	19.98±0.10

A Kruskal-Wallis test was performed to test if the water temperature from the three days were within the same range and no significant differences were observed (p-value=0.5375), we could say the three days' water temperature were quite stable.

3.2 Filtration rates

The filtration rates' experiment for each temperature group was performed in 3 days as triplicates. The cell concentration curves were plotted for each individual in Fig.9.



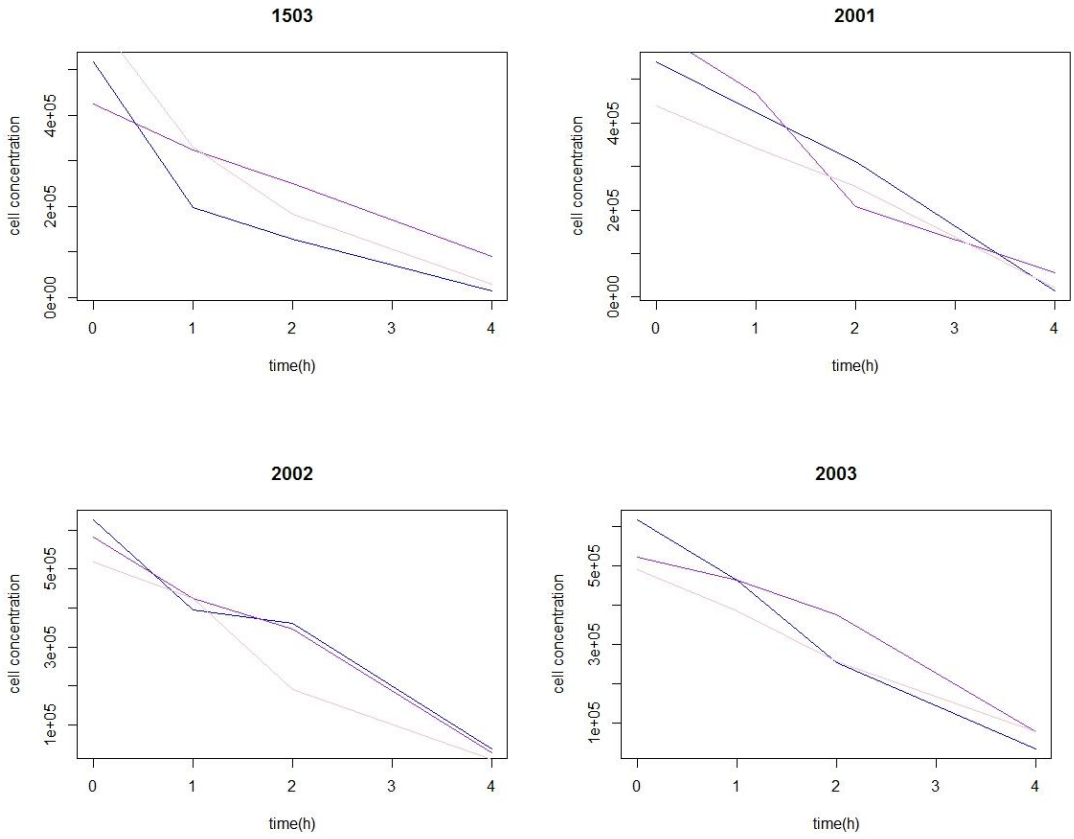


Figure 9 Line graph of cell concentration changes according to time during feeding of each scallop. In each graph, the blue line represents day1, purple line for day 2 and day 3 was plotted with pink line.

From the figures above, a decrease of cell concentration overtime was observed in every individual. The filtration rates were calculated and the mean filtration rates within each group are summarized in Table 5 with standard deviation.

Table 5 Filtration rates (FR) of *Pecten maximus* from the 2 temperature groups under different time intervals. Mean filtration rates were calculated from the 3 scallops in each group (also shown as 'sample size') and their filtration rates in 3 days' experiment

Temperature (°C)	Time (h)	Sample size	Filtration rates ($L \cdot h^{-1} \cdot ind^{-1}$)	
			mean	Standard deviation
15	1	3	2.63	0.56
	2	3	1.09	0.93
	4	3	3.36	1.72
	All day	3	2.61	1.32
20	1	3	1.32	0.27

2	3	2.08	0.27
4	3	4.72	0.91
All day	3	3.21	0.86

Statistics were performed (check appendix) to check the differences of filtration rates within same temperature groups and between two different temperature treatments. And from the statistics we could find out that: (1) No significant difference was observed in the three days for each individual with in the two groups; (2) No significant difference was observed between individuals within the same temperature group; (3) Scallops under different temperature treatments varied in feeding rates: filtration rates in 20°C was higher than 15°C.

Pecten maximus from both groups showed the same highest filtration rates during the time interval from 2h to 4h, with 3.36 ± 1.72 for 15°C and 4.72 ± 0.91 for 20°C ($\mathrm{L\cdot h^{-1}\cdot ind^{-1}}$). There is also an increase of filtration rates according to time in the 20°C group. Due to the high standard deviation, no significant trend could be concluded from the 15°C group.

3.4 ^{13}C labelling of *Pecten maximus*

The test group was used to check the labeling technique. The tissue samples of (1) digestive gland and (2) striated adductor muscle were measured to check for $^{13}\mathrm{C}$ NMR signals in scallops fed with unlabeled and $^{13}\mathrm{C}$ labeled algae.

3.4.1 Metabolites' labeling

Figure 10 shows the $^{13}\mathrm{C}$-NMR spectrum from digestive gland extract (of cytosol/ methanol fraction) of a scallop fed with unlabeled algae in comparison of a labeled tissue sample. The chemical shifts represent the environment of $^{13}\mathrm{C}$ atoms. For metabolites' signals, generally speaking, most of the amino acids' signals appears at range 30 ppm (parts per million) to 70 ppm; carbohydrates' signals are expected be observed in 70 ppm to 80 ppm regions, formate ($\mathrm{CHOO^-}$) and relative signals normally could be found at around 170 ppm. Also, in a NMR spectrum, the insignificant and disordered signals are noise and cannot be assigned to specific metabolites. Clear, significant signals are the signals used in analysis of chemical substrates.

In this pre-experiment, the unlabeled scallop was used to represent the natural abundance of $^{13}\mathrm{C}$ while the signal difference between labeled and unlabeled scallop represents the labeling results.

The digestive gland from the test group was first checked for $^{13}\mathrm{C}$ signals. By comparing the unlabeled (red) and labeled (blue) $^{13}\mathrm{C}$ signals (Fig.10), we could see (1) the peaks in unlabeled (14 peaks) was more than the labeled (3 peaks); (2) height of the peaks of unlabeled was higher than the labeled. And if we compare the $^{13}\mathrm{C}$

spectra from the digestive gland with the spectrum from the labeled algae (Fig.11), we could see the chemical shifts of the signals were quite similar.

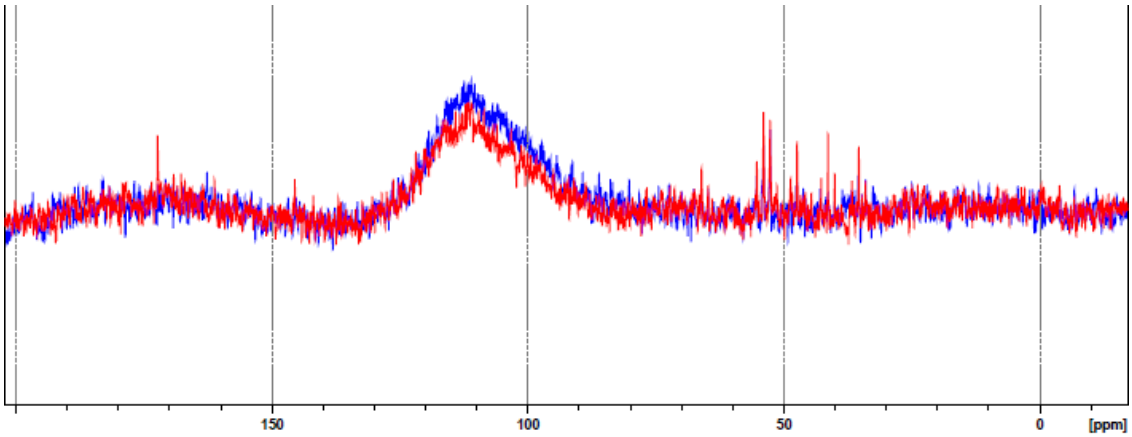


Figure 10 ^{13}C signals of unlabeled (red) and labeled digestive gland (blue) of *Pecten maximus* metabolites from test group.

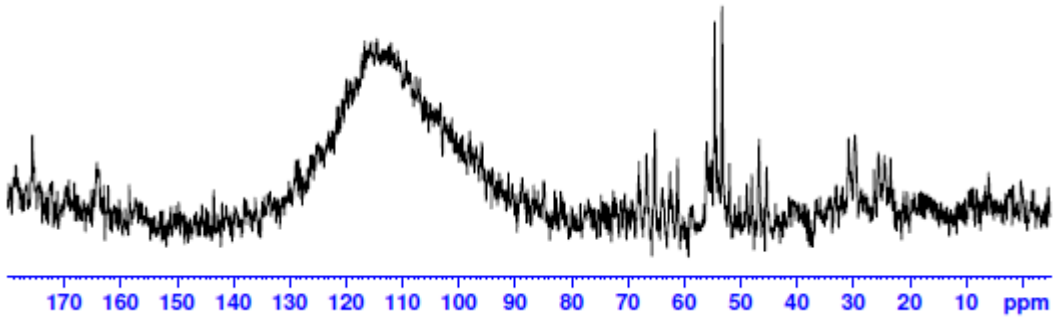


Figure 11 ^{13}C spectrum of the labeled algae *P. tricornutum*.

However, significant difference in spectra of the striated adductor muscle samples (Fig. 12) could be observed. From the spectra of labeled (blue) and unlabeled (red) muscle, significant differences could be observed: (1) 33 ^{13}C signals (peaks) were found in the labeled spectra, while only 14 ^{13}C signals (peaks) were observed in the unlabeled spectra; (2) the height of labeled signals were much higher than the unlabeled.

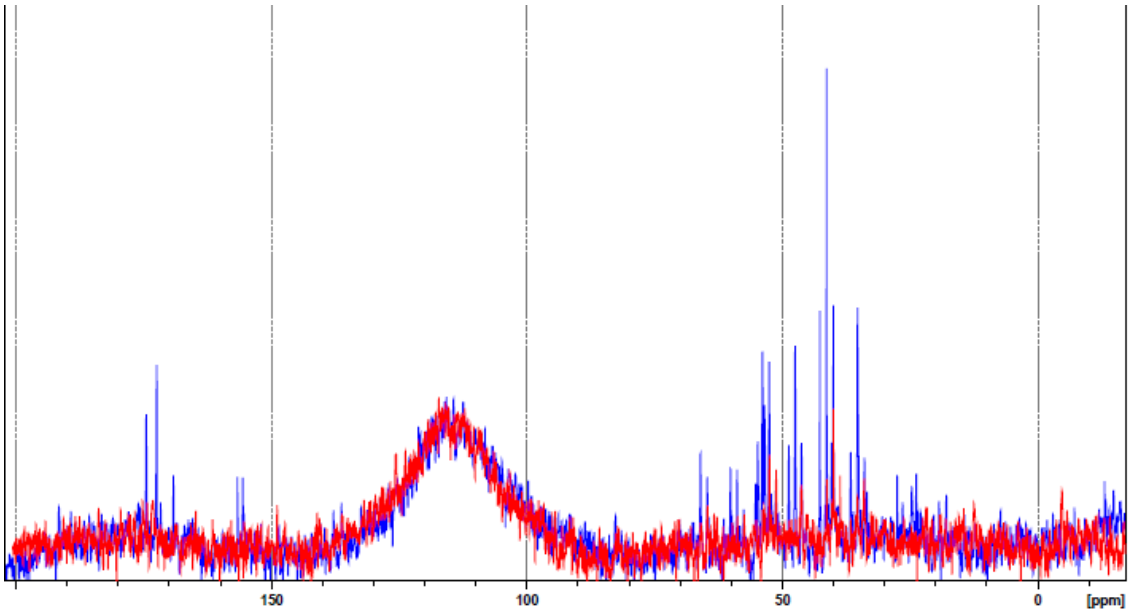


Figure 12 ^{13}C signals of unlabeled (red) and labeled adductor muscle (blue) of *Pecten maximus* metabolites from test group.

From the difference between unlabeled and labeled muscle spectra, we could found that the ^{13}C atoms were well incorporated and this labeling method is working well.

3.4.2 Lipid's labeling

Like the metabolites samples, lipid extractions from (1) digestive gland and (2) striated adductor muscle were also checked. Unlabeled samples represent the natural abundance of ^{13}C.

The ^{13}C from lipids of the digestive gland was first checked (Fig.13). By comparing the two spectra we could observe that peaks around 130 ppm and 175 ppm only appeared in the labeled digestive gland sample, this could also prove that some fatty acids were already incorporated with ^{13}C atoms.

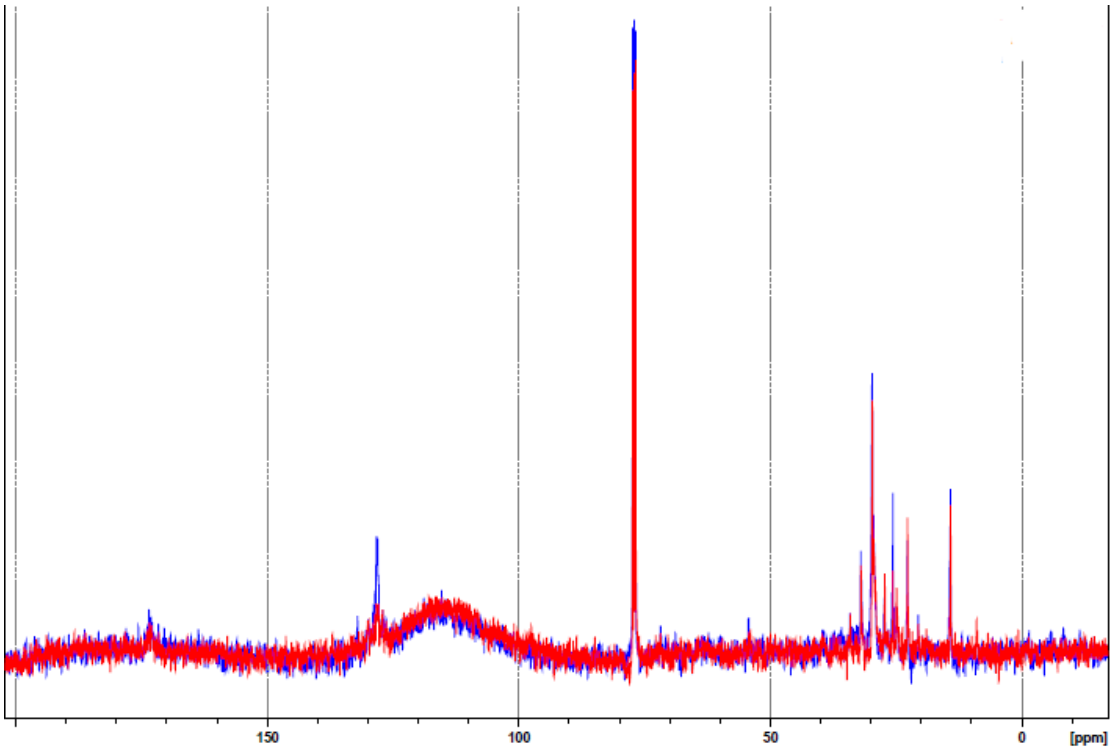


Figure 13 ^{13}C signals of unlabeled (red) and labeled adductor digestive gland (blue) of *Pecten maximus* lipids from test group.

However, when the muscle lipid samples were checked, the spectra of unlabeled and labeled samples look quite similar: both two spectra have 6 peaks; the integral of relevant peaks were in the same level (Fig.14).

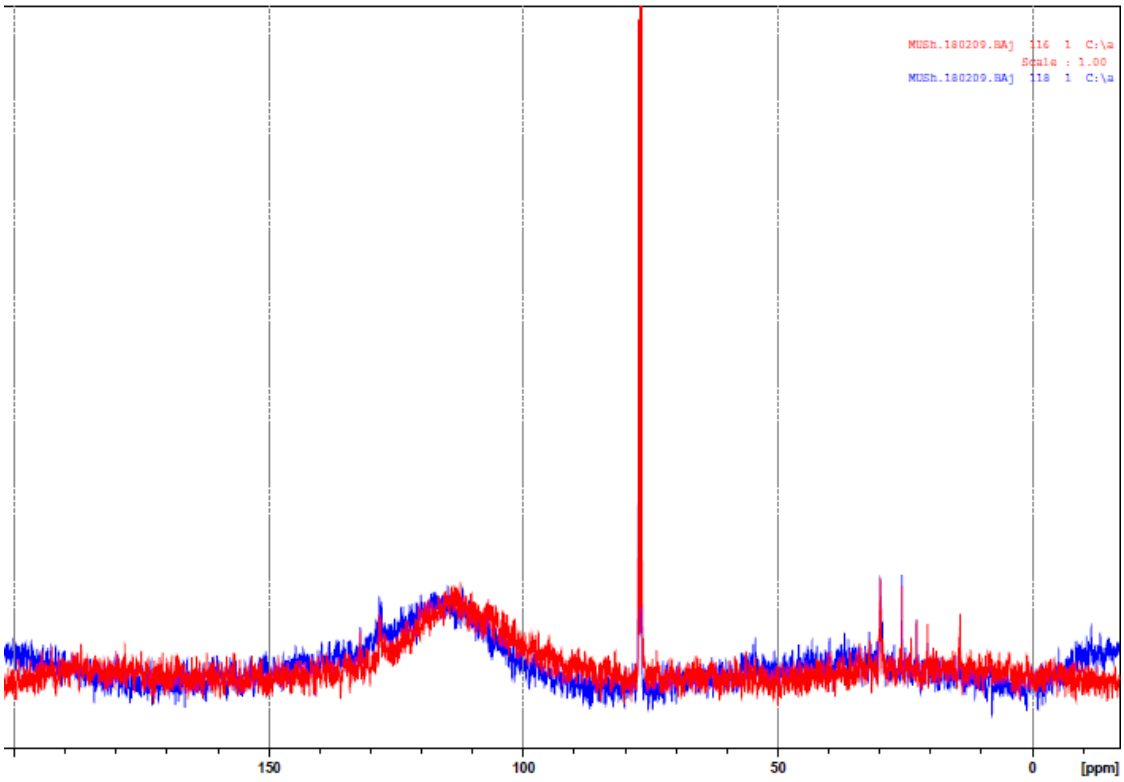


Figure 14 ^{13}C signals of unlabeled (red) and labeled adductor muscle (blue) of *Pecten maximus* lipids from test group.

3.5 Metabolic profiling in digestive gland

3.5.1 Metabolites in digestive gland

Unlike the test group, quite a lot of ^{13}C labeled metabolites inside the digestive gland under both temperature treatments were observed in the spectra (Fig.15). Substrates were identified and lists of important metabolites were shown (Table. 6).

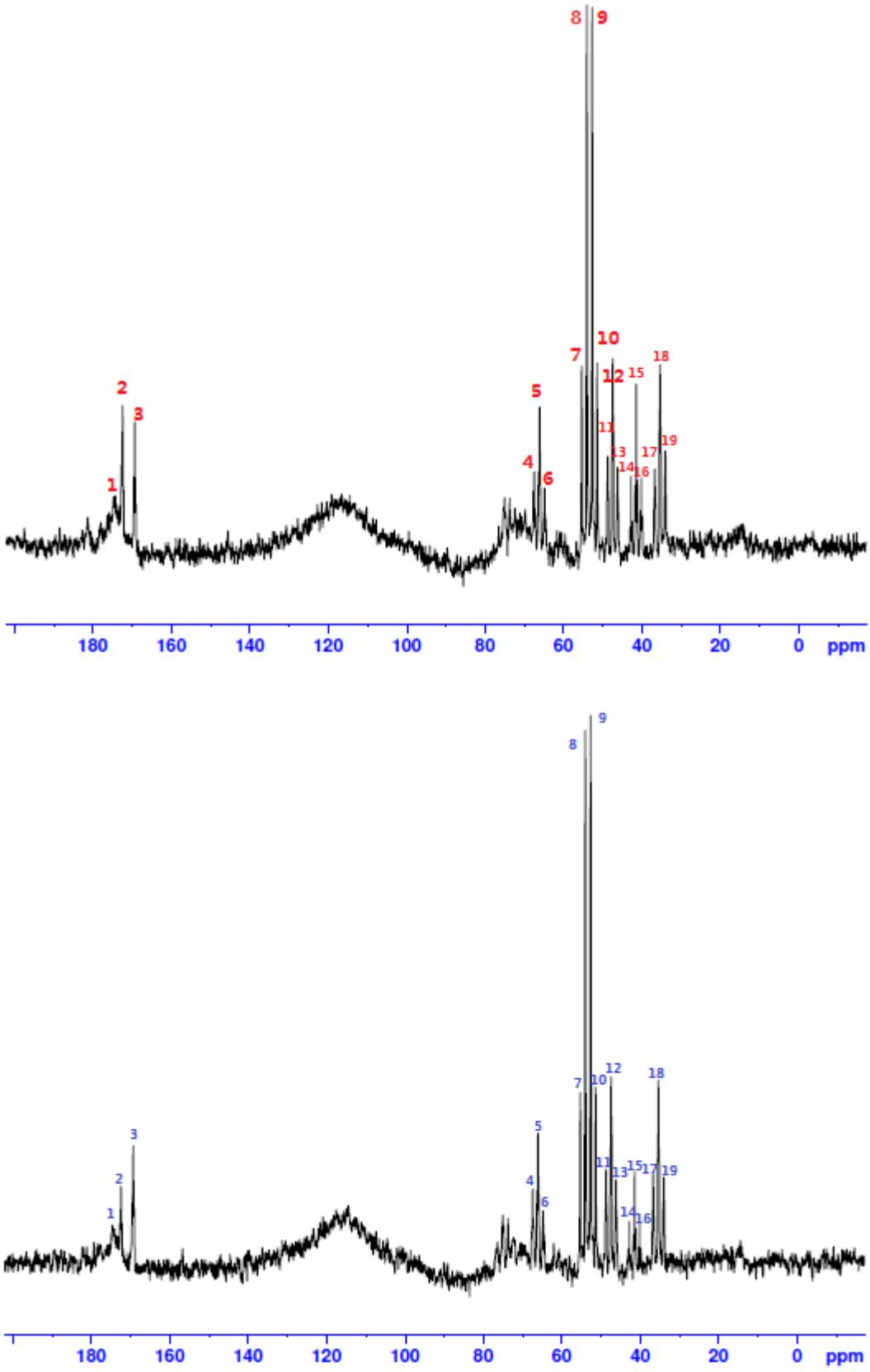


Figure 15 Digestive gland metabolites' spectra of 15°C (upper) and 20°C (lower). Important metabolite peaks were labeled and listed in the table.

Table 6 The metabolite list for the peaks in ^{13}C spectra of digestive glands, scallop ID with 1503 from 15°C group and ID 2003 from 20°C group were shown. Relative integrals (absolute integral divided by 4X10^7, see M&M) were calculated. Mean values and standard deviations were calculated from relative integrals of the three scallops from the same group. Source from UT Southwestern Medical Center.

Object	Metabolite	Shifts (ppm)	15° C		20° C	
			mean	stdev	mean	stdev
Integral 1	Citrate C1,C5	174.46	2.885	1.271	2.284	0.446
Integral 2	Formate	172.54	3.398	0.476	3.631	2.293
Integral 3	Undefined	169.28	2.570	0.282	3.358	1.398
Integral 4	Threonine C3	67.41	1.780	0.237	2.619	1.506
Integral 5	Betaine C2	66.12	3.121	0.402	4.236	2.419
Integral 6	Undefined	64.77	1.138	0.282	2.036	1.570
Integral 7	Glutamine C2 or Glutamate C2	55.35	2.323	0.198	3.305	2.099
Integral 8	Betaine C3	53.95	7.810	0.672	9.160	4.103
Integral 9	Aspartate C2	52.65	7.365	0.631	9.376	4.429
Integral 10	L-Alanine C2	51.31	2.405	0.109	3.441	2.304
Integral 11	Taurine (N)	48.70	1.744	0.177	2.367	1.639
Integral 12	Proline C5	47.36	3.689	0.370	5.013	3.066
Integral 13	Undefined	46.10	1.644	0.078	2.614	2.022
Integral 14	Glycine C2	42.75	0.671	0.330	0.715	1.198
Integral 15	Leucine C3	41.40	2.199	0.345	2.056	1.776
Integral 16	Undefined	40.16	1.070	0.264	0.931	1.231
Integral 17	Taurine (S)	36.65	1.587	0.249	2.334	1.226
Integral 18	Succinate (C2)	35.37	3.409	0.559	4.443	2.003
Integral 19	Glutamate C4	34.03	1.835	0.399	2.094	1.085

While checking the metabolite list, some related metabolisms were quite obvious. In digestive gland, Krebs cycle related metabolites including citrate, succinate and glutamine were identified; Small serine signals could be observed at 61.5 ppm, while the glycine peaks are also not very high (less than 1) comparing to other metabolites and serine signals were not observed, suggesting the glycine-serine metabolism was not found in the digestive gland. Osmolytes such as taurine (1.587±0.249), betaine (7.810±0.672 C3, 3.121±0.402 C2), L-alanine (2.405±0.109) were found to be the most abundant ones.

The identified ^{13}C signals in the spectra from the two temperature treatments were identical except for 2 peaks at 70-80 ppm (75.44 ppm and 73.97 ppm) that were only observed in the 20°C group. These peaks were identified as carbon from carbonhydrates and could be related to the glycolysis.

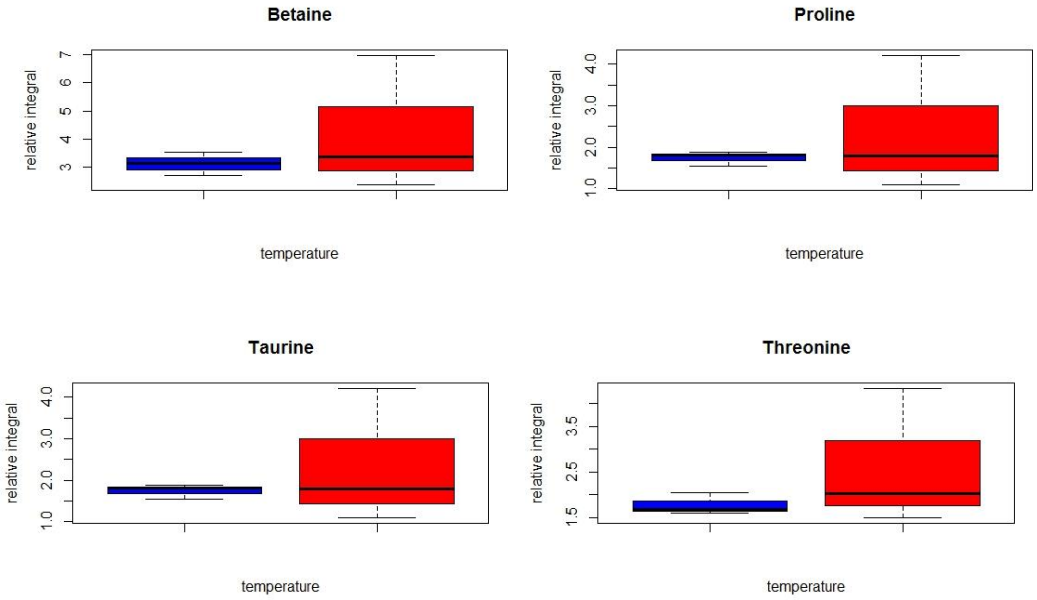


Figure 16 Substrates' content under different temperature treatments: blue box represents 15°C while red box represents 20°C.

If we compare the integrals of metabolites of the two temperature groups, it is quite obvious that except for citrate signals at 174.46 ppm, other metabolites were all higher in the 20°C than the 15°C group (Table 6). In figure 16, four metabolites were plotted for more clear comparison.

3.5.2 Lipids in digestive gland

As has been discussed above, the scallops from the test group had an "empty stomach" with no metabolites being found in the digestive glands. We also compared the lipid phase from the digestive gland extraction of the labeled and unlabeled scallops (Fig.17) and the results were shown:

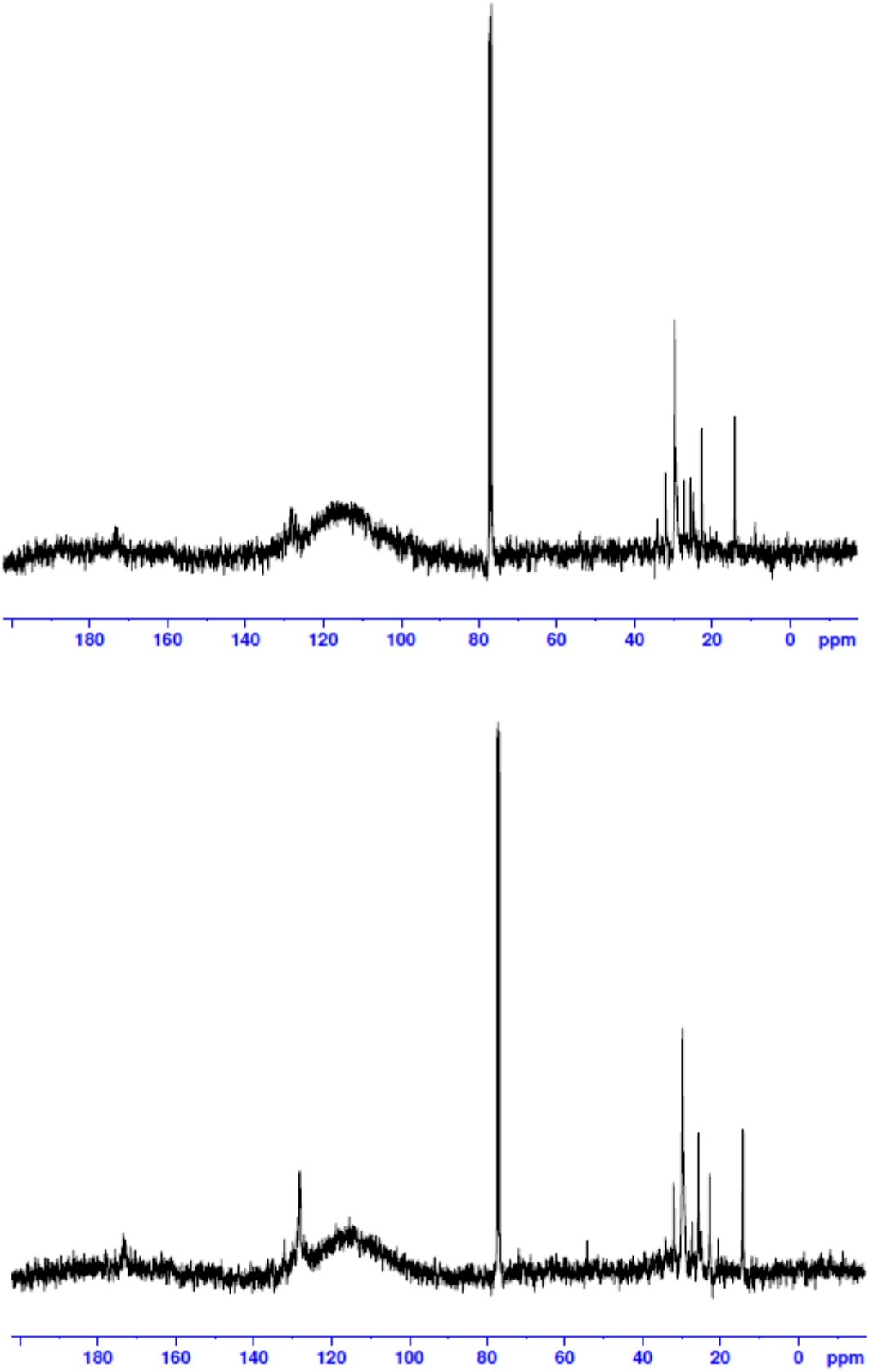


Figure 17 Lipid spectra of the digestive gland from the test group: unlabeled (above) and labeled (under).

The spectrum of the unlabeled muscle represents the natural abundance of ^{13}C inside the digestive gland. By comparing the two spectra, only small peaks were found to be different. Special structures were used for identification of chemical substrates. In this case, the double bound of the unsaturated fatty acid was examined. The double bound (Aursand, 1992) inside DHA (20:5(n-3)) is at C4 (127.58 ppm) and C5 (129.64 ppm), while the one inside EPA (22:6(n-3)) is at C5 (128.92 ppm) and C6 (128.71ppm).

The digestive gland from the 15°C was extracted and ^{13}C spectra were obtained (Fig.18). The substrates were identified and specific shifts listed (Table 7).

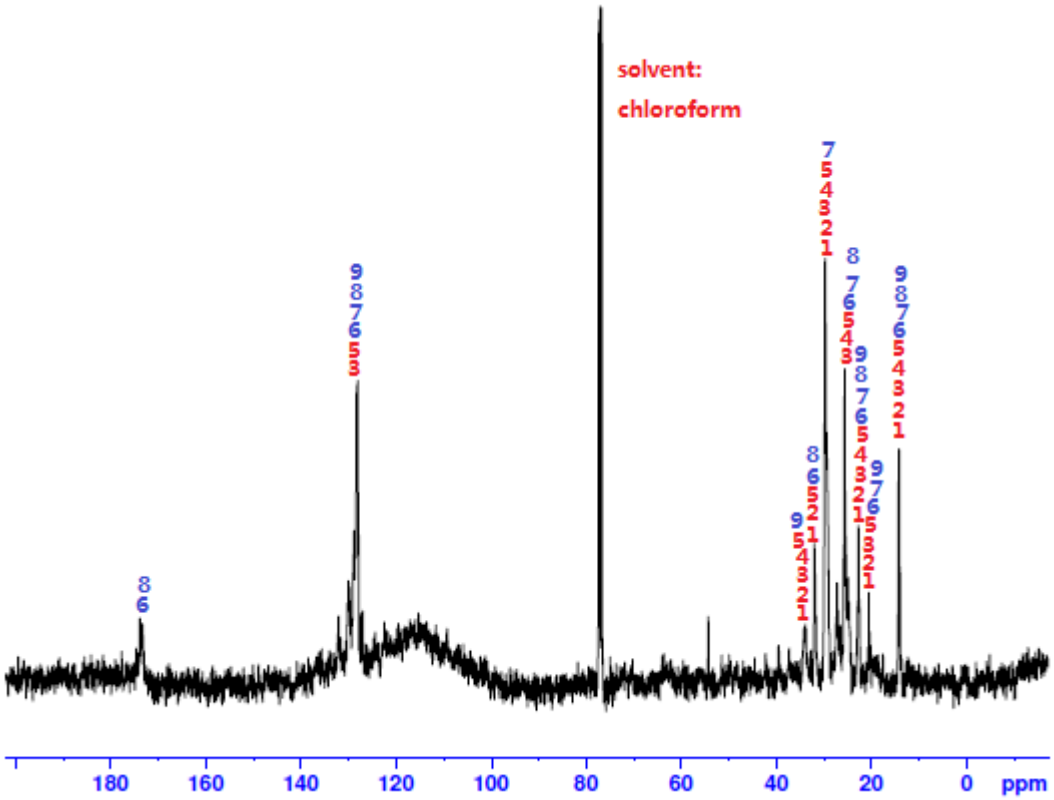


Figure 18 The digestive glands' ^{13}C spectra of three experimental animals from 15°C group: 1501 (upper), 1502 (middle), 1503 (under). The chemical shifts were labeled in the first graph and the labeling represent: (1) C14:0 (2) C16:0 (3) C16:1n-7 (4) C18:0 (5) C18:1n-9 (6) C18:1n-7 (7) C18:4n-3 (8) EPA (9) DHA

Table 7 Chemical shifts of major fatty acids, carbon related to the double bounds inside substrates were in bold. Source from Aursand (1992).

	C14:0	C16:0	C16:1n-7	C18:0	C18:1n-9
C1	180.62	180.58	180.65	180.62	180.58

C2	34.20	34.23	34.19	34.22	34.16
C3	24.74	24.80	24.73	24.77	24.17
C4	29.72	29.21	29.06	29.18	29.09
C5	29.72	29.37	29.11	29.34	29.12
C6	29.72	29.57	29.21	29.54	29.38
C7	29.72	29.81	29.74	29.78	29.38
C8	29.49	29.81	27.21	29.78	27.20
C9	29.42	29.81	**129.79**	29.78	**129.75**
C10	29.31	29.81	**130.05**	29.78	**130.04**
C11	29.13	29.81	27.29	29.78	27.27
C12	31.99	29.81	29.81	29.78	29.83
C13	22.74	29.49	29.13	29.78	29.73
C14	14.12	32.05	31.83	29.78	29.51
C15		22.79	22.59	29.48	29.20
C16		14.14	14.13	32.04	31.97
C17				22.77	22.74
C18				14.12	14.13

	C18:1n-7	C18:4n-3	C20:5n-3 (EPA)	C22:6n-3 (DHA)
C1	173.32	180.07	179.87	179.38
C2	34.01	34.01	33.42	34.02
C3	24.86	24.35	24.54	22.54
C4	-	29.07	26.51	**127.58**
C5	-	26.91	129.10	**129.64**
C6	-	129.58	128.81	25.63
C7	-	128.60	25.68	128.01
C8	-	25.60	128.22	128.35
C9	-	128.32	128.23	25.68
C10	27.21	128.18	25.68	128.28
C11	129.78	25.71	128.14	128.31
C12	129.88	**127.98**	**128.32**	25.68
C13	27.23	**128.35**	**25.68**	128.13
C14	-	25.69	127.94	128.13
C15	-	127.12	128.63	25.57
C16	31.81	132.05	25.59	127.92
C17	22.67	20.61	127.08	128.60
C18	14.07	14.29	132.10	25.57
C19			20.61	127.08
C20			14.31	132.03
C21				20.59
C22				14.27

As for the test group, under same temperature and time conditions, very few signals were observed and lipids were supposed to be unincorporated (except for DHA and EPA). Combining the chemical shifts of 15°C with the test group spectra, it is understandable to suppose that the signals we observe were mostly from the *Phaeodactylum tricornutum* remained inside the digestive gland. Therefore, we checked the labeled *P. tricornutum* ^{13}C spectra for comparison (Fig.19).

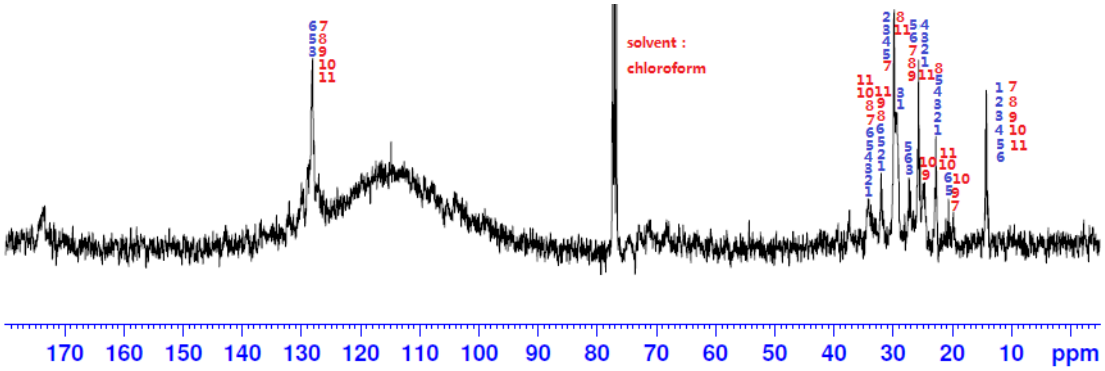


Figure 19 ^{13}C chemical shifts of lipid extractions in *Phaeodactylum tricornutum.* Whereas (1) C14:0 (2) C16:0 (3) C16:n-7 (4) C18:0 (5) C18:1n-9 (6) C18:2n-6 (7) 18:n-3 (8) C20:4n-6 (9) C20:5n-3 (10) C22:6n-3 (11) C24:0.

The ^{13}C peaks inside the *P.tricornutum* spectrum was quite identical to the spectra from the 15°C group except for small peak at chemical shift of 62.06 ppm, which could be identified as TAG (triglyceride) (Hatzakis, 2011).

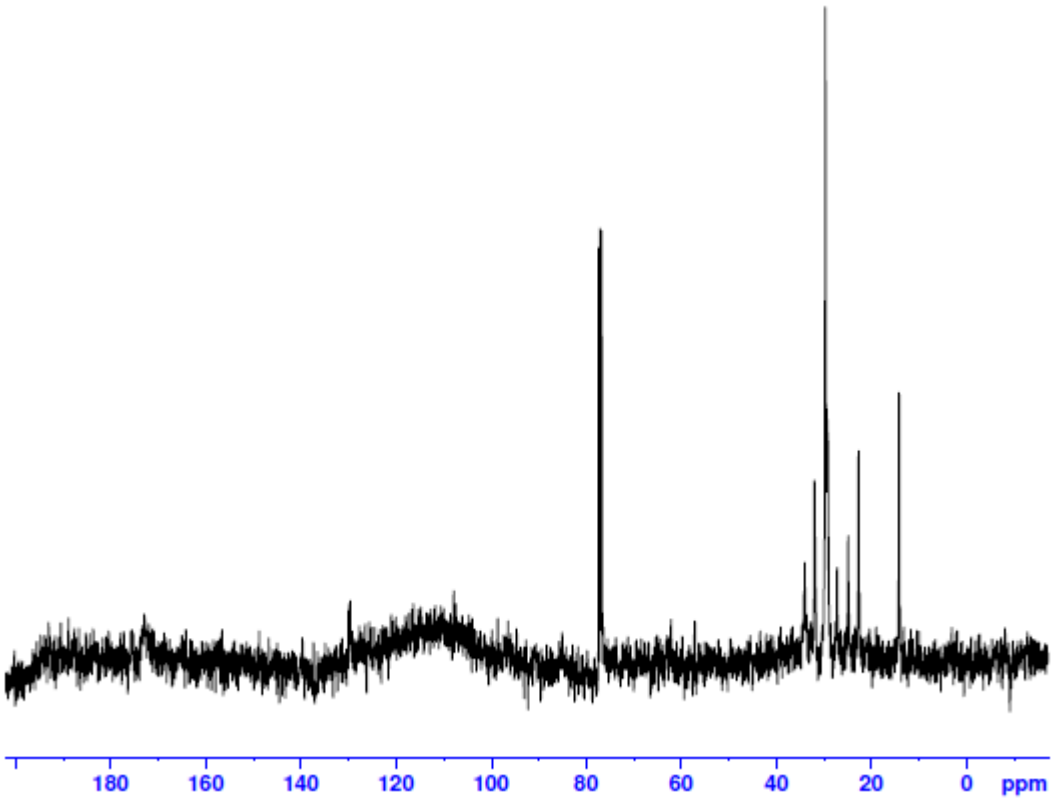


Figure 20 Digestive gland ^{13}C spectrum of *Pecten maximus* at 20°C treatment. Scallop ID 2003 was taken as example.

Then we take a look at the ^{13}C spectra from the 20°C group (Fig.20). No obvious signals could be found around 130 ppm in the 20°C spectrum in contrast to 15°C, where distinct and pronounced signals could be identified. Great differences were observed at chemical shifts around 130 ppm: 0.251±0.053 at 128.89 ppm; 0.212±0.185 at 128.34 ppm; 0.321±0.280 at 128.16 ppm while signals were hardly found for the 20°C group (less than 0.1). Signals around 130 ppm can be contributed to unsaturated fatty acids, as the double bounds were at around 130ppm (Table 7). And if we check the ^{13}C shifts list and compare it with the integral (Table 8), we could also see the chemical shifts related to unsaturated fatty acids between 20 ppm to 50 ppm were also smaller comparing to the 15°C group. Therefore, for the 20°C group, unsaturated fatty acids were consumed and disappeared from the digestive gland.

Table 8 The list of integrals for the peaks in ^{13}C spectra of lipid inside digestive gland. Chloroform was taken as the standard (77.05 ppm) and relative integrals were calculated accordingly. Mean integral was calculated from relative integral of the three scallops from the same group, "stdev" represents the standard deviation. Unfound shifts were left in blank.

Shift	15°C		20°C	
(ppm)	mean	stdev	mean	Stdev

173.38	0.137	0.114	0.065	0.071
173.10	0.061	0.020	0.082	0.081
129.89	0.251	0.053	0.088	0.084
129.01	0.171	0.095	0.034	0.032
128.80	0.106	0.095	0.052	0.045
128.57	0.094	0.082	0.059	0.057
128.34	0.212	0.185	0.056	0.097
128.16	0.321	0.280	0.050	0.087
128.02	0.155	0.136	0.068	0.118
127.82	0.132	0.021	0.043	0.075
127.02	0.042	0.037	0.017	0.029
77.05	1.000		1.000	
62.10			0.090	0.075
54.48	0.047	0.016	0.013	0.012
32.14	0.108	0.134	0.131	0.066
31.97	0.111	0.020	0.165	0.065
31.80	0.103	0.028	0.117	0.057
29.73	1.119	0.300	1.131	0.362
29.45	0.063	0.109	0.378	0.182
29.27	0.280	0.289	0.185	0.162
29.13	0.374	0.264	0.286	0.107
28.96			0.124	0.014
27.20	0.117	0.049	0.127	0.045
25.83	0.116	0.040	0.066	0.048
25.62	0.287	0.048		
25.39	0.136	0.035		
24.90	0.113	0.098	0.232	0.074
22.81	0.299	0.064	0.264	0.086
20.55	0.046	0.006		
14.34	0.111	0.031		
14.17	0.186	0.062	0.247	0.105

3.6 Metabolic profiling in striated adductor muscle

3.6.1 Metabolites in adductor muscle

From the spectra (Fig.21) and list (Table.9) below, we could find that: (1) Muscle tissues have quite different metabolite types comparing to digestive gland; (2) The concentration of substrates inside the muscle tissue is much less than in the digestive gland.

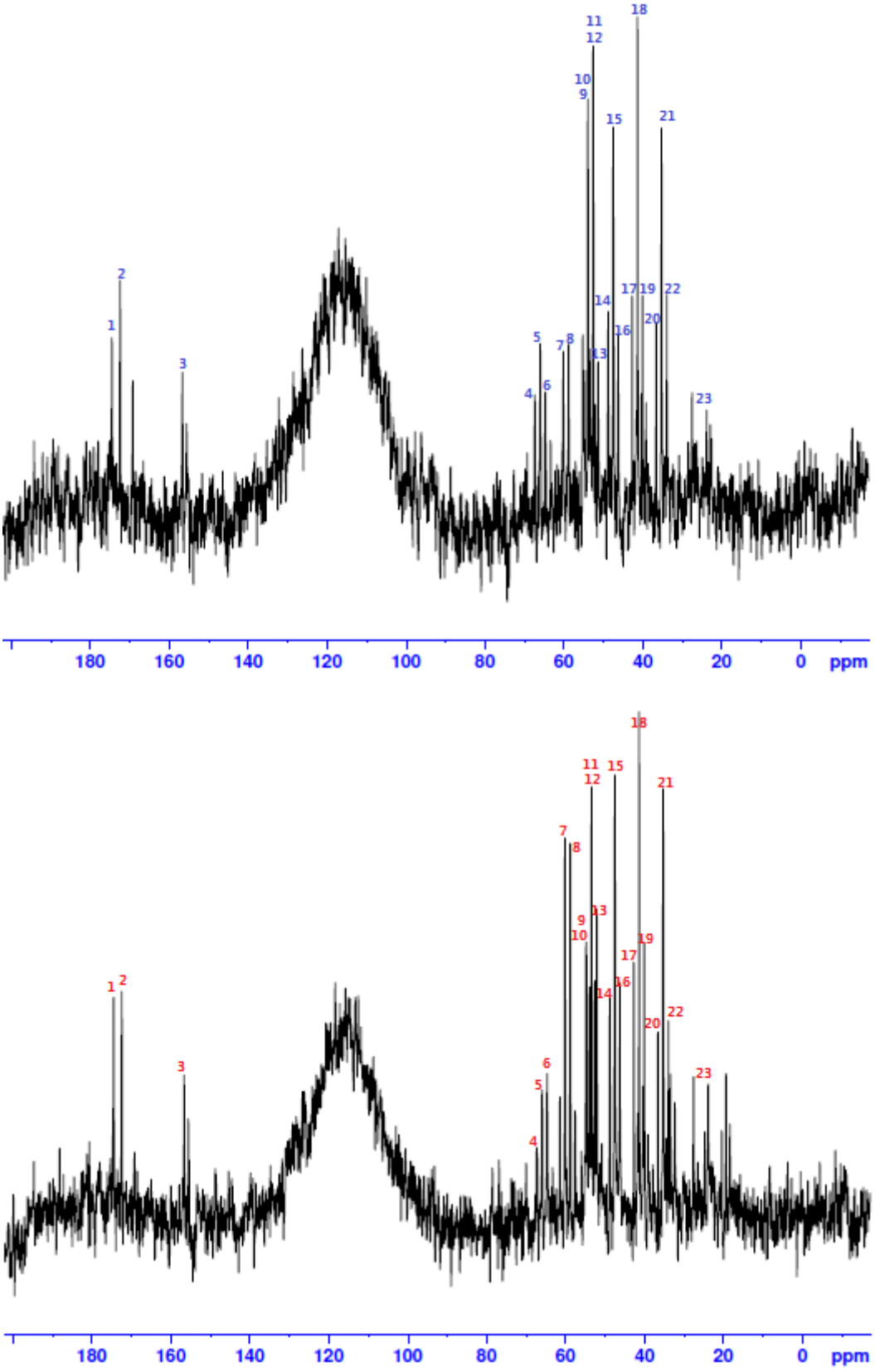


Figure 21 Adductor muscle metabolites' spectra of 15°C (upper) and 20°C (lower). Important metabolite peaks were labeled and listed in the table.

Table 9 The metabolite list for the peaks in ^{13}C spectra of digestive glands, scallop ID with 1503 from 15°C group and ID 2003 from 20°C group were shown. Relative integrals (absolute integral divided by 4X10^7, see M&M) were calculated. Mean values and standard deviations were calculated from relative integrals of the three scallops from the same group. Source from UT Southwestern Medical Center.

Object	Metabolites	Shifts (ppm)	15° C		20° C	
			average	stdev	average	Stdev
Integral 1	Citrate C1,C5	174.46	0.743	0.164	0.877	0.287
Integral 2	Formate	172.54	0.761	0.353	0.902	0.319
Integral 3	Arginine C6	156.65	0.308	0.026	0.389	0.172
Integral 4	Threonine C3	67.41	0.215	0.205	0.136	0.049
Integral 5	Betaine C2	66.12	0.608	0.226	0.505	0.049
Integral 6	Undefined	64.77	0.414	0.011	0.413	0.151
Integral 7	Isoleucine C2	60.07	0.691	0.572	0.768	0.791
Integral 8	Serine C2	58.78	0.795	0.440	0.752	0.407
Integral 9	Cysteine C2	54.82	1.209	0.615	1.231	0.519
Integral 10	Betaine C3	53.95	1.107	0.477	1.231	0.405
Integral 11	Aspartate C2	53.47	1.892	1.413	1.218	0.791
Integral 12	Aspartate C2	52.65	1.566	0.625	1.161	0.250
Integral 13	Alanine C2	51.31	0.591	0.386	0.469	0.087
Integral 14	Taurine (N)	48.7	0.576	0.055	0.565	0.183
Integral 15	Proline C5	47.36	1.237	0.114	1.290	0.554
Integral 16	Citrate C2,C4	46.1	0.578	0.073	0.696	0.430
Integral 17	Glycine C2	42.75	0.797	0.579	0.841	0.280
Integral 18	Leucine C3	41.4	1.550	0.348	1.823	0.268
Integral 19	Cysteine C3	40.16	0.965	0.505	1.415	0.312
Integral 20	Taurine (S)	36.65	0.560	0.132	0.560	0.124
Integral 21	Succinate C2	35.37	1.169	0.128	1.366	0.298
Integral 22	Glutamate C4	34.03	0.693	0.131	0.683	0.105
Integral 23	Lysine C5	27.44	0.345	0.140	0.403	0.119

Apart from the metabolic pathways and metabolites that have already been described in the digestive gland, we also observed in the muscle tissues from both temperature treatments, the glycine and serine were found, which relates to the glycine-serine biosynthesis.

The substrates' types, as well as the ratios between each other in both temperature groups are quite alike. Metabolites with the highest concentration in both temperature groups were: Cysteine, Betaine, Aspartate and Leucine.

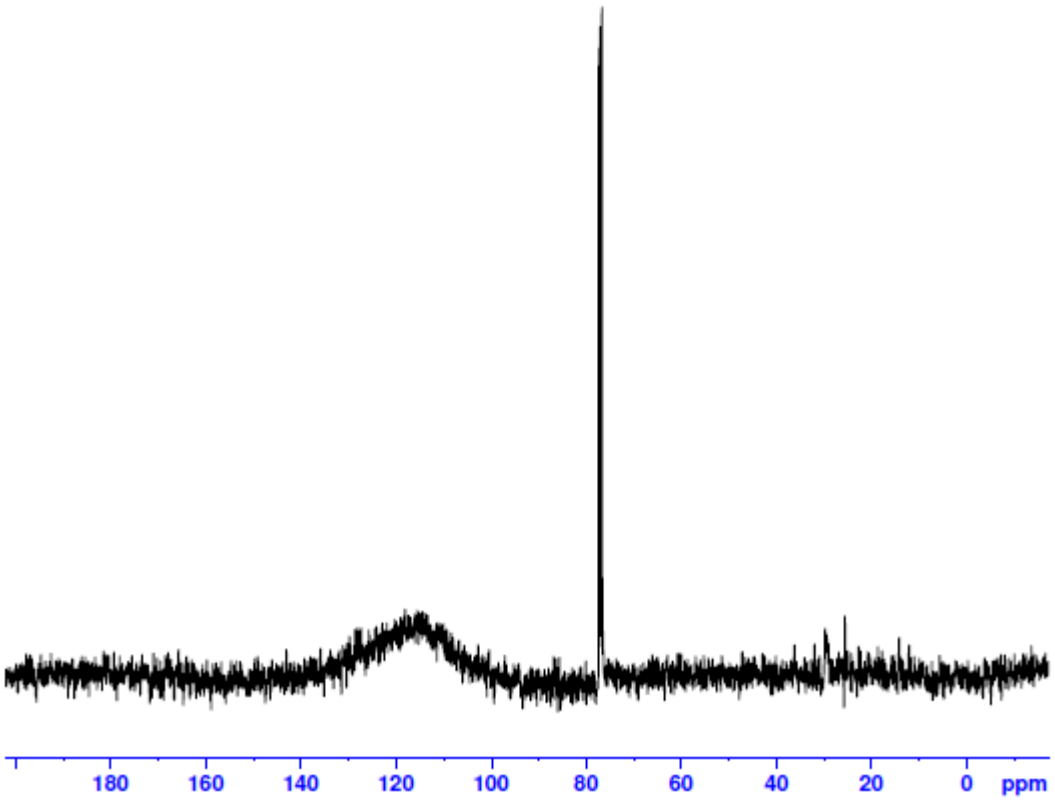
When comparing the substrates concentration under the two different temperature treatments, no significant difference could be identified.

It is quite interesting that no signal was found at 70-80 ppm was found, which

means no carbohydrate was observed in the muscle tissue.

3.6.2 Lipids in adductor muscle

Through this experiment, almost no significant signals from fatty acids were observed in adductor muscles in both temperature treatment groups (Fig.22). No ^{13}C labeled lipids were found inside the muscle.



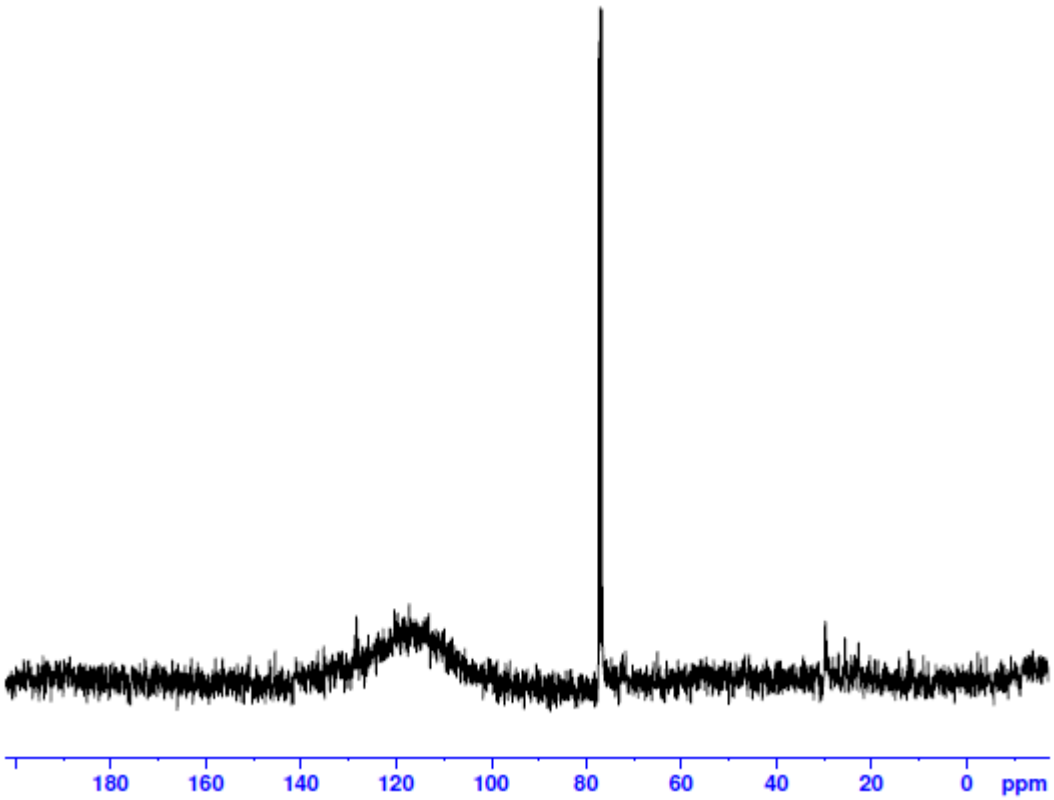


Figure 22 Muscle lipid ^{13}C spectra of both temperature groups: 15°C (individual ID1503 was used as example) and 20°C (individual ID2003 was used as example), the only signal belongs to the solvent chloroform (CDCl3).

4. Discussion

4.1 Filtration rates determination of *Pecten maximus*

All experimental animals fed on *P. tricornutum* has shown decline in cell concentration (Fig.9). However, no specific decline trend could be found. This could be because only 4 algal concentrations were obtained from each scallop. A clearer trend could be observed if more time intervals and measurements were set.

According to the present data, the filtration rate of 20°C is higher than 15°C (check table 5). This result is similar to the previous study by Laing (2014), they reported the temperature influence on juvenile king scallop *P. maximus* under different temperature treatments. A significant increase with temperature could be observed (Fig.23).

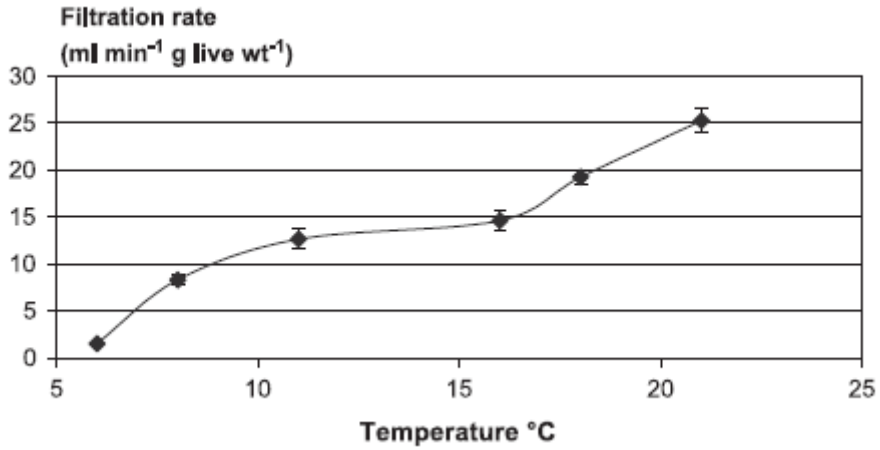


Figure 23 Relationship between king scallop filtration rate and temperature by Laing (2014)

Apart from the temperature influence, it is also observed in present study that the FR of scallops increased with time and the highest FR appeared at time period of 2-4h.

In Laing's research, the experiment animal, *P. maximus* has shown a reduced filtration rate when cell concentration increased form $2X10^5$ cells/mL to $3X10^5$ cells/mL, which is comparable with the present experiment: when the cell concentration decreased from $3X10^5$ cells/mL (1h to 2h) to $2X10^5$ cells/mL (2h to 4h), the FR increased.

The king scallop *Pecten maximus* and blue mussel *Mytilus edulis* are both marine filter feeding species and has similar feeding diet. A study by Riisgard (1981) has shown that beyond $3X10^4$ *P. tricornutum* cells/mL, the filtration rate will decrease with increasing cell concentrations for *M. edulis* and *vice versa*. This result could be comparable for the filtration rate of *P. maximus* regarding to the cell concentration: with the *P. maximus* ingesting diatoms, the cell concentration is decreasing overtime, and with the declined algal concentration inside the water, the FR will in turn to

increase.

The experimental animal, *Pecten maximus* is quite sensitive to environmental changes, including light, salinity as well as other surrounding water parameters (Chauvaud, 2005). Physical manipulation (Richardson, 1990) also contributes to the disturbance of this sensitive animal. When they sense the change of environment, the valves were closed and feeding immediately stopped.

In the present study, all scallops were well placed on iron “donuts” for stability. They were all incubated inside black buckets to avoid light influences. For the 15°C groups, the TK room is well dark controlled with limited light condition, whereas for the 20°C group, the incubation box was closed for light inhibition.

It is also observed in the present experiment that sound might also has an impact on the great scallop, *P. maximus*. No relevant research was found regarding to this species before. However, during the present experiment, especially for the 15°C group, the TK room was not completely isolated, the sound inside the room may have caused closure of the shells. Also, with the sound of slamming doors, the closing shells were also observed from personal observation.

As has been discussed above, *P. maximus* is very sensitive to surrounding environmental changes. Water samples were taken from the buckets for cell concentration determination. Although this was a fast procedure, the open of lids, stirring water and position check of *Pecten maximus* also caused the closure of cells. Therefore, the scallops were not in continuous feeding state but rather in small time periods as it took time for them to get to the filter feeding state. However, this systematical error occurs in every scallop inside each group and does not influence the comparison of temperature influence on filtration rates.

The cell concentration was determined by manually counting under the Axio Observer rather than automatically counting with coulter counter as the diatom used as fed, *Phaeodactylum tricornutum* forms cluster and could not be precisely determined by the devices. For more precise cell counting, *Thalassiosira pseudonana* could also be a nice choice of feeding material provided to the *Pecten maximus.*

4.2 Labeling method test

The ^{13}C labeling procedure in this experiment was: (1) ^{13}C -bicarbonate ($\mathrm{NaH}^{13}\mathrm{CO}_3$) was provided to the marine diatom *P. tricornutum*; (2) After 10 days’ incubation, the diatom *P.tricornutum* was well incorporated with ^{13}C atoms; (3) ^{13}C labeled algae was provided to the scallop *P. maximus* as feed; (4) By consuming the ^{13}C labeled diatom, ^{13}C atoms would be well-incorporated into the scallop and used for metabolic profiling.

As has been described in the M&M part (2.1) in this study, the labeling efficiency of diatoms was not checked, therefore all the diatoms were assumed to similar labelled as presented in the student research project (Fig.11&19) labeled and fed to the scallops. The scallop labeling was successful by the comparison of ^{13}C signals of two scallops’ muscle tissues from the test group: the increased signals numbers and heights of ^{13}C labelled metabolites in the muscle tissue could not be explained by

natural abundance only, the ^{13}C labeling were from external sources. This proves that the ^{13}C compounds from the algae were incorporated and metabolized in the scallops.

However, no significant signals could be identified as external $^{13}\mathrm{C}$ in the digestive gland. There could be two possibilities behind it: (1) the labeling method did not work out and almost no $^{13}\mathrm{C}$ atoms were incorporated; (2) The algae has already been completely digested by the scallop when the organs were dissected. As we could prove the success of labeling from the muscle spectra, the actual reason for no significant $^{13}\mathrm{C}$ peak is not a failure in labeling but rather "an empty stomach".

The same labeling technique was repeated in the following test of two temperature groups (15°C and 20°C) with 3 scallops in each group. Muscle spectra obtained all show significant difference in comparison to the unlabeled one (natural abundance) and were similar to the labeled tissue sample from the test group. With successful labeling for three replications, we could say this is a repeatable, stable labeling method.

Also, in both temperature experiments, the initial cell concentration was increased compared to the test group (see M&M). This change is because only small signals were observed in the test group and an "empty stomach" was found in the digestive glands. With increasing algal cell number, significant $^{13}\mathrm{C}$ signals were observed from the digestive gland samples. However, an assignment of the origin $^{13}\mathrm{C}$ atoms could be given to confirm the signals were from the remaining *P.tricornutum* inside the digestive gland or the metabolites from the organ, or maybe both.

4.2 Effects of increasing temperature on digestive gland

By comparing the integrals of two different temperatures (Table 6), we could find that apart from citrate, other metabolites' contents from 20°C group are higher than 15°C. Two possible explanations for this phenomenon could be: (1) When the water temperature increased, the ventilation rate (respiration rate) of scallop also increased (Artigaud, 2014). While breathing, scallops open their shells and the use the gill to obtain oxygen from the water (Shumway and Parsons, 2016), the filter feeding, as discussed in the introduction part (1.2) was also done by the gills. Therefore, higher ventilation would also lead to a higher filtration rate as the filter feeding was also done by the gills. Due to the higher filtration rate at 20°C, more diatoms were remained in the digestive gland; (2) When temperature increased from 15°C to 20°C, higher metabolic rate (Robson, 2016) (higher metabolic energy losses (Newell, 1980)) was observed. More energy consumption as well as faster metabolism could be inferred, which could be the possible explanation for the higher metabolites concentration inside at higher temperature.

From the lipid samples of two temperature treatments, we could found that only in the 20°C group and not in the 15°C group: (1) Triglycerides (TAGs) were identified in the spectra; (2) unsaturated fatty acids were not found.

The digestive gland served as an organ of lipid storage in bivalves (Shumway and

Parsons, 2016), inside which, fatty acids were mostly stored in the form of triglycerides (Berg, Tymoczko and Stryer, 2002). The existence of this signal indicates that the (1) synthesis of fatty acids had started; (2) after digestion, some ^{13}C labeled lipids were incorporated as storage in the digestive gland.

As has been described at the introduction part (1.7), *P. maximus* is not able to produce PUFAs (such as EPA and DHA) themselves and the only way of obtaining these essential lipids is by consuming phytoplankton (Shumway and Parsons, 2016). Comparing the spectra of two temperature groups, unsaturated fatty acids were only disappeared from digestive gland in the 20°C group (check 3.5.2), suggesting a faster fatty acid incorporation under higher temperature.

A faster metabolic rate was observed in the 20°C group: unsaturated fatty acids were consumed and incorporated faster than the 15°C group. Therefore, from the metabolite concentration as well as the lipid incorporation, it is not difficult to find that with increasing temperature, the metabolism inside the digestive gland is higher.

Considering the unfound unsaturated fatty acids inside the digestive gland at 20°C, another question would be: Where were the unsaturated fatty acids transported to? A possible explanation would be the lipids were used for gonad development.

Two major factors could influence the gonad development of P.maximus: temperature (Paulet, 1991) and nutrient (Pazos, 1997; Devauchelle, 1991;).

According to Paulet (1991), increase of temperature can induce gonad development of *P. maximus*. Paulet (1991) also found out that the spawning season for *P. maximus* was July, when the seawater temperature was around 16-18°C, which was quite close to the experimental temperature of the present study.

For scallops' reproduction, carbohydrates and proteins from adductor muscle, as well as lipids from digestive gland would be involved in the gonads' development (Shumway and Parsons, 2016). Study by Pazos (1997) showed that PUFAs were the predominant (36.19%–60.85%) of total fatty acids in triacylglycerol fraction of *P. maximus* gonads, among which, the EPA (22:6(n-3)) and DHA (20:5(n-3)) have the highest content. Pazos (1997) also discovered that the PUFAs content follows the change of scallop's gonad (Fig.24), which is in accordance with Devauchelle (1991), in whose research, food availability and quality influence on the reproduction of *P. maximus* and a relationship between fatty acids and eggs was found. *P. tricornutum* is a phytoplankton quite rich in EPA (2.6–3.1% of the diatom's dry biomass) (Atalah, 2007).

Therefore, due to the influence of nice food and higher temperature, a faster gonad development might have appeared in the 20°C group and more lipids (especially PUFAs) were needed for the reproduction. However, unfortunately no gonad samples were taken in the present experiment, otherwise it would have been interesting to check the ^{13}C signals of these organs.

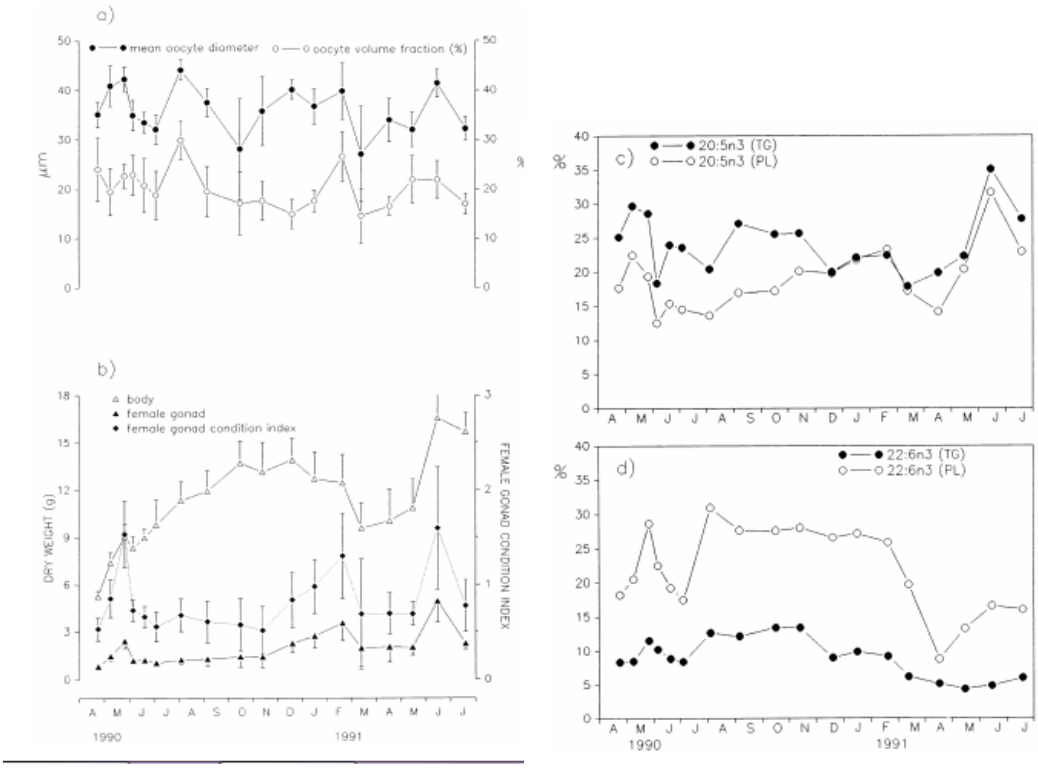


Figure 24 Seasonal changes in the mean oocyte diameter (a), oocyte volume fraction (a), dry weight of the total body (b) and female gonad of *Pecten maximus* (b) and female gonad condition index (b). Seasonal variations of DHA (22:5n-3) (c) and EPA (22:6n-3) (d) (Results were represented in the percent of triacylglycerol or phospholipid fatty acids. Source from Pazos, 1997

4.3 Effects of increasing temperature on adductor muscle

The main substrates inside scallop organs differ from each other: for the digestive gland, carbohydrates such as glycogen and free glucose content were low, less than <4.5% of the dry weight (Pazos, 1997) but a high content of lipids could be observed. As for the adductor muscle, the case was quite different, protein was the major component inside the striated adductor muscle (Pazos, 1997), whereas a large amount of glycogen worked as energy storage in the muscle (Shumway and Parsons, 2016).

In this study, under both temperatures, no carbohydrates were discovered in the adductor muscle. One reason might be the short incubation time (3 days). We compared the results with Tikunov (2014), who observed incorporated ^{13}C glucose signals in eastern oysters in three days' labeling period. In Tikunov's research, labeled glucose was injected directly to the oyster while in the present study, the scallops were provided with *P. tricornutum* (carbohydrate only 31% of dry weight, (Bai, 2016)), which requires time for the scallops to ingest and digest.

No significant differences in metabolite's contents could be identified in muscle under the two temperature treatments, which was the same case for the lipid extractions. Possible explanations could be: (1) as has been discussed above (4.2), after digestion, the lipid wasn't transported to the adductor muscle but to the gonad

(which is the most likely explanation); (2) the digestion and incorporation of fatty acids requires time and was not started in the muscle during the incubation time (second likely). However, another possible explanation for no-significant signals in the muscle spectra could be because of (3) the low lipid content (less than 4% (Pazos, 1997) inside the muscle tissue.

4.4 Methodological improvements

As has been described in the material and methods part, five tissues from the scallops were obtained. However, due to time reasons, we only managed to measure two of them, which were the striated adductor muscle and digestive gland. It would be very interesting if other tissues (gill, mantle and smooth adductor muscle) could also be studied for substrate incorporation. With all these results, a schematic picture of metabolism inside the *Pecten maximus* could possibly be drawn, which would help to better understand the animal's physiology (also under changing temperature).

In this experiment, the weight of the tissues were not obtained, therefore a weight related analyzation was not performed. This was because the tissue sample needs to be frozen immediately to keep the metabolism as precise as possible, the dry weight, as well as the wet weight could not be measured.

The incubation for each group lasts for 3 days. As we did not find much lipid signals inside the muscle tissues as we expected, the reason behind this could be the short incubation time, lipids were not well incorporated. Further experiments could have longer feeding time to check the lipid metabolism.

4.5 Perspectives

In further studies, it would be interesting if the faeces of *P. maximus* could be collected and $^{1}\mathrm{H}$ and $^{13}\mathrm{C}$ spectroscopy applied to faeces study of the scallop: analyzing (1) amount of faeces production; (2) $^{13}\mathrm{C}$ labeling in the faeces. Faeces production and filtration rate together would help to understand the organic matter uptake as well as the energy flow of the *P.maximus*, and the $^{13}\mathrm{C}$ labeling in faeces could also work as an evidence of $^{13}\mathrm{C}$ incorporation inside the animal.

For further study on how global warming impacts on the physiological processes on *P. maximus*, more temperature steps would be needed. It remains to be seen, how the scallop *P. maximus* reacts to temperatures out of the optimal temperature regime (e.g. 25°C) (Artigaud, 2014). Apart from temperature influence, the salinity shift was also observed under climate change conditions, it would be really interesting if the salinity impact could be investigated as well as the combined effects of temperature and salinity.

5. Conclusion

The incorporation of ^{13}C labelled phytoplankton was observed in the scallop *P. maximus*, proving this technique is applicable for ^{13}C labelled NMR metabolic profiling on marine filter feeders. In future, more relevant research could be conducted via this technique.

By increasing temperature from 15°C to 20°C, no mortality of *P. maximus* was observed and acclimation was performed in the way of increasing feeding and potentially faster lipid incorporation. However, further research will be needed to test the adaptation of king scallop *P. maximus* under the influence of strengthening global warming.

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Appendix

Table A1. Specific filtration rates of *P.maximus* at 15°C.

Time	day1			day2			day3		
	1501	1502	1503	1501	1502	1503	1501	1502	1503
0h-1h	5.599	2.703	4.811	0.872	1.344	1.348	1.850	1.984	3.127
1h-2h	-0.713	1.455	2.227	0.194	0.338	1.298	2.276	-0.254	2.976
2h-4h	4.462	0.545	5.524	3.077	2.354	2.568	5.756	1.212	4.701
All day	3.453	1.312	4.521	1.805	1.598	1.945	3.910	1.039	3.876

Table A2. Specific filtration rates of *P.maximus* at 20°C.

Time	day1			day2			day3		
	2001	2002	2003	2001	2002	2003	2001	2002	2003
0h-1h	1.224	2.306	1.438	1.355	1.546	0.596	1.231	0.984	1.203
1h-2h	1.524	0.449	3.020	4.063	1.044	1.033	1.514	4.001	2.047
2h-4h	7.778	5.591	5.022	3.286	6.303	3.913	6.152	1.394	3.000
All day	4.576	3.484	3.626	2.997	3.799	2.364	3.762	1.943	2.313

Table A3 f/2 medium contents, adapted from Guillard, 1975

Chemical	Volume (L)	amount(g)
NaNO3	0.025	1.875
NaH2PO4·2H2O	0.025	0.163
Na2EDTA·2H2O	0.025	0.121
FeCl3·4H2O	0.025	0.068
CuSO4·5H2O	0.025	0.25
ZnSO4·7H2O	0.025	0.55
CoCl2·6H2O	0.025	0.25
MnCl2·2H2O	0.025	0.368
NaMoO4·2H2O	0.025	1.575
Biotin	0.01	0.00001 (0.1mL of 0.1mg/L)
Vitamin B12	0.01	0.00001(0.01mL of 1.0mg/L)

Thiamine HCl	0.01	0.002

f/2 medium (5L)

Filtered sea water (FSW)	4750mL
NaNO3 Stock Solution	5mL
NaH2PO4 Stock Solution	5mL
Trace Metals Stock Solution	5mL
Vitamin Stock Solution	2.5mL

Table A4. Specific NMR parameters

Parameters	^{1}H metabolites	^{1}H lipids
TD (Time domain size)	70656	70656
SWH [Hz ppm] (Sweep width)	8802.82 21.9998	8802.82 21.9998
AQ [sec] (acquisition time)	4.0132608	4.0132608
RG (Receiver gain)	228	114
DW [µsec] (Dwell time)	56.800	56.800
DE [µsec] (Pre-scan delay)	6.50	6.50
D1 [sec] (relaxation delay)	4.000000000	4.000000000
d12 [sec] delay for power switching	0.00002000	0.00002000
D20 [sec] Fixed echo time	0.000222000	0.000222000
DELTA1 [sec]	0.00021882	0.00021766
DELTA2 [sec]	0.00021550	0.00021550
DS	2	2
L4 (Loop For 12 filter)	126	126
NS (total number of scans)	32	32
TD0 (Number of averages in 1D)	1	1
Channel 1		
SFO1 [MHz] (Frequency of channel 1)	400.1318812	400.1318812

O1 [Hz ppm] (Frequency of channel 1)	1881.19 4.701	1881.19 4.701
NUC1 (Nucleus for channel 1)	1H	1H
P1 [µsec] (90 degree high power pulse)	5.000	5.000
p2 [µsec] (180 degree high power pulse)	10.00	10.00
PLW1 [W, dB] (Power level for pulse default)	5 -6.99	5 -6.99
PLW9 [W, dB] (Power level for presaturation)	1.667e-005 47.78	5e-6.99 53.01

Parameters	^{13}C metabolites	^{13}C lipids
TD (Time domain size)	65536	65536
SWH [Hz ppm] (Sweep width)	22058.82 219.225	22058.82 219.225
AQ [sec] (acquisition time)	1.4854827	1.4854827
RG (Receiver gain)	2050	2050
DW [µsec] (Dwell time)	22.667	22.667
DE [µsec] (Pre-scan delay)	6.50	6.50
D1 [sec] (relaxation delay)	4.000000000	4.000000000
d11 [sec] delay for disk	0.03000000	0.03000000
DS	2	2
NS (total number of scans)	8192	8192
TD0 (Number of averages in 1D)	1	1
Channel f1		
SFO1 [MHz] (Frequency of channel 1)	100.6220690	100.6220690
O1 [Hz ppm] (Frequency of channel 1)	9300.00 92.434	9300.00 92.434

H

NUC1 (Nucleus for channel 1)	13C	13C
P1 [µsec] (90 degree high power pulse)	10.000	10.000
PLW1 [W, dB] (Power level for pulse default)	22 -13.42	22 -13.42
Channel f2		
SFO2 [MHz] (Frequency of channel 2)	400.1318810	400.1318810
O2 [Hz ppm] (Frequency of channel 2)	1880.96 4.701	1880.96 4.701
NUC2 (Nucleus for channel 2)	1H	1H
PCPD2 [µsec] (90 degree pulse for decoupling sequence)	5.00	5.00
PLW2 [W, dB] (Power PLW2)	5 -6.99	5 -6.99
PLW12 [W, dB] (Power level for CPD/BB decoupling)	0.087111 10.60	0.015432 18.12

Table A5. Summarize of statistics on filtration rates with variables including: 3 days' treatment, 3 individuals and temperature."*" represents rejected null hypothesis.

		Mean FR	
Variables and groups (ANOVA)	df	F	P
Within 15°C groups			
Time X individual (1h)	2	0.289	0.759
Time X individual (2h)	2	2.065	0.208
Time X individual (4h)	2	5.376	0.045*
Within 20°C groups			
Time X individual (1h)	2	1.035	0.411
Time X individual (2h)	2	0.097	0.909
Time X individual (4h)	2	0.569	0.594
Between groups (cor.test)	df	t	p
Time X group (1h)	7	0.376	0.718*
Time X group (2h)	7	-0.619	0.555*
Time X group (4h)	7	0.877	0.410*

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Declaration

I hereby assert that this thesis at hand is in all parts my own work. Only the quoted sources and references have been used. Any direct or indirect adaptation of the work of others has been marked and referred to as such. This thesis has not been submitted in the same or similar version, not even in part, to any other authority for grading and has not been published elsewhere.

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Tianyi Zhao

Bremen, 12/09/2018