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| Wageningen UR |
| Lipid composition measurements in Gammarids |
| Final Thesis |

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| Visschedijk, Niek  5/1/2017 |

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Yerseke, May, 2017

**Final thesis: version 1.0**

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# Summary

Daily life is greatly influenced by fossil fuels. New technological inventions and the reduction of the ice coverage due to climate change makes it possible for humans to be present and undertake activities, such as gathering oil in previously inaccessible areas like the North Pole (Sagerup, Nahrgang, Frantzen, Larsen, & Geraudie, 2016). However, oil drilling goes alongside a few dangers. When a spill is caused oil will discharge into the Arctic waters where it will present itself as toxic towards aquatic organisms. Because of low temperatures and extreme seasonality the polar regions have a low ecological recovery rate. The effect of temperature on the acute toxicity of marine fuel oil with type DMA has already been tested on the amphipod species *G. locusta & G. setosus.* This research showed that the Arctic amphipod species *G. setosus* has a lower sensitivity to marine fuel oil than the temperate *G. locusta.*It is suspected that this lower sensitivity has something to do with a difference in lipid concentration and/or composition. Another study showed the possibility of using medical test kits to determine lipid composition in marine invertebrates. These test kits are normally used for researching the blood or tissue of mammals but if possible to use on marine invertebrates, the test kits can save time as well as money. This research will therefore also investigate the use and profitability of the medical test kits. Two test kits were tested. These test kits were the phosphatidylcholine - and triglyceride colorimetric assay kits. These two kits were chosen because according to literature the *Gammarus sp.* would show the biggest differences within these lipid classes. When researched the arctic species contained more lipid in total and also a higher triglyceride content, the differences in phosphatidylcholine content are small. There can be concluded that the higher total lipid content and/or the higher triglyceride content in the Arctic *G. setosus* explains the increase in oil toxicity, however it shows a possible answer which needs more research which can be done by using medical test kits.

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# Introduction

Daily life is greatly influenced by fossil fuels. For instance almost all motorised vehicles run on some form of fossil fuels and these fuels are also used in the manufacturing of daily products like plastics and clothing.

These days fossil fuels are used for 86% of the worlds energy consumption. This is a 3% decrease from 1985 (Tverberg, 2017). Oil wells are running dry (Roberts, 2004). New technological inventions and the reduction of the ice coverage due to climate change makes it possible for humans to be present and undertake activities, such as gathering oil in previously inaccessible areas like the North Pole (Sagerup, Nahrgang, Frantzen, Larsen, & Geraudie, 2016). The Arctic is an interesting place to drill for oil because it contains an estimated 13% of all of earth’s undiscovered oil (Mclendon, 2015).However, oil drilling goes alongside a few dangers. First of all, the noise of oil drilling can be a disturbance to marine mammals. Secondly, the sea ice and remoteness of the polar region makes it hard to contain an oil spill. Lastly, because of low temperatures and extreme seasonality the polar regions have a low ecological recovery rate, this will make the impact of an oil spill even greater than when it happens at a location with a higher ecological recovery rate (Mclendon, 2015). When a spill is caused oil will discharge into the Arctic waters where it will present itself as toxic towards aquatic organisms. When present in high enough concentrations, it can harm the animals by internal and external routes of exposure (NOAA, 2017). The oil can harm animals when it enters the body through ingestion or inhalation and from external exposure through skin and eyes (NOAA, 2017). The effects of oil may be assessed by using marine invertebrate species.

The effect of temperature on the acute toxicity of marine fuel oil with type DMA has already been tested on the amphipod species Gammarus locusta and Gammarus setosus (Klaassen, 2016). Marine fuel oil with type DMA is a 100% distillate type fuel, without additives or residues. During distillation it has been boiled into a gas and then condensed into liquid. Marine fuel oil with type DMA is common fuel for boats and drilling rigs in the Arctic (legend oil trading & bunkering DMCC, 2014).

The research concluded that the temperature has a significant effect on the acute toxicity of marine fuel oil with type DMA, with increasing temperature leading to an increasing toxicity of this oil. This means that at lower temperatures the studied amphipod species where more resistant toward the toxic effect of marine fuel oil. The research also showed that the Arctic amphipod species *G. setosus* has a lower sensitivity to marine fuel oil than the temperate *G. locusta.*

However, whether the lipid concentration or the lipid composition of the studied amphipod species influences the acute toxicity of marine fuel oil at different temperatures is still unknown.

There are several methods to measure the lipid concentration and lipid composition of marine invertebrates. The lipid extraction can either be done using the Folch method (Folch, Lees, & Sloane-Stanley, 1957) or the Bligh and Dyer method (Bligh & Dyer, 1959). During this research one of the two lipid extraction methods will be used to assess the total lipid content of the Gammarid species. Methods to assess the lipid composition exist and include chromatography, thin layer chromatography and high pressure liquid chromatography (Langezaal, 2016). However, these methods for extracting and determining lipids from marine invertebrates are often costly, time consuming or both (Langenzaal 2016). Langenzaal studied the option of using medical test kits to extract and assess the lipids within amphipod species. He recommended further research for the use of test kits that are developed to analyze human blood serums for marine invertebrate research. The availability and use of these particular research kits will be investigated during this research.

This research is therefore a continuation of earlier studies on the toxicity of marine fuel oil and lipid analysis in marine invertebrates. (Klaassen, 2016; Langezaal 2016) .

The aim of the research is to asses if the total lipid concentration and/or the lipid composition of amphipod species differs between temperate and Arctic species and if this may explain the differences the acute toxicity of marine fuel oil at different temperatures. This will be assessed with a total lipid assessment and the use of test kits to extract and assess the lipid types in the *G. locusta* and *G. setosus.*

Each different lipid class requires a different test kit. Part of this research is to identify which lipid classes can be assessed by using a test kit and whether the use of the test kit can be applied to assess lipids in Gammarus species.

When the test kits prove useful during the extraction and determination of the lipids they can be used in future fieldwork expeditions. The use of test kits during field experiments will be beneficial, not only because the use of the kits is less time and money consuming but they are relatively small and can be taken along on field trips. The information gained during this research will explain the difference in sensitivity to oil between the studied *G*. *locusta* and *G. setosus.* that has been found during earlier research (Klaassen, 2016).

## Research questions.

1. Do total lipid concentration and lipid composition differ between temperate and Arctic gammarids and can this explain differences in oil toxicity as was found by Klaassen (2016)?

*Hypothesis: it is expected that an increase in lipid total concentration in Arctic gammarids will increase the resistance toward oil toxicity*

*Hypothesis: it is expected that the Arctic G. setosus will contain a higer triglyceride concentration and a lower phosphatidylcholine concentration than the temperate G. locusta*

2. Does total lipid composition and concentration in *Gammarus sp.* Differ between size classes?

*Hypothesis: it is expected that the total lipid composition and concentration will not differ between size classes*

**Sub – question**

* Which test kits and lipid extraction methods are needed to conduct relevant lipid extractions and analyses in the indicator *Gammarus sp.*?

# Background.

This part of the plan of approach will discuss earlier research, give more information on the research species and explain the use of test kits during the research.

As it has been stated in ‘1.0 Introduction’ this research is a follow up on two earlier studies, one being “*The effect of temperature on the acute toxicity of marine fuel oil type to the temperate amphipod, G. locusta -“* a research carried out by Klaassen, M.A. , the second is ´*Small scale lipid analysis in marine invertebrates.*” by Langezaal, H. These reports will be discussed during this chapter of the plan of approach and are an important source of information during the whole research.

## 

## **2.**1 Research species.

The two animal species used in this research are Gammarus locusta and Gammarus setosus. When addressing both species at the same time Gammarus sp. will refer to both species

The reason why these species are used is because they have been used during previous tests. They are sensitive species and therefore suitable to use in toxicity tests. *Gammarus sp.* are common and easy to collect. Furthermore they can be kept in storage rooms for later use and they are big enough for visual experiments.

***G. locusta.***

Kingdom: Animalia  
Phylum: Arthropoda  
Class: Crustacea  
Subclass: Malacostraca  
Superorder: Peracarida  
Order: Amphipoda  
Suborder: Gammaridea  
Family: Gammaridae  
Genus: *Gammarus*  
Species: *locusta*

**Ongeldige bron opgegeven.**

Image 1: Gammarus locusta

A temperate species of amphipod, it is

*G. locusta* is a temperate amphipod species. It is very common and present along the Dutch coast. *G*. *locusta*. used in these experiments are caught in the Oosterschelde on a monthly basis.



***G. setosus.***

Kingdom: Animalia  
Phylum: Arthropoda  
Class: Crustacea  
Subclass: Malacostraca  
Superorder: Peracarida  
Order: Amphipoda  
Suborder: Gammaridea  
Family: Gammaridae  
Genus: *Gammarus*  
Species: *setosus*

**Ongeldige bron opgegeven.**

Image 2: Gammarus setosus (picture by M. vd Heuvel-Greve)

G. setosus is an Arctic species of amphipod which has been collected in Spitsbergen in June 2016.

This species is also common along the coast of Svalbard (Greenland) and easy to collect from the beach.

## 2.2 Earlier research.

**Research by M.A. Klaassen (2016)**

Klaassen studied the effect of temperature on the acute toxicity of marine fuel oil type to the temperate amphipod, *G. locusta.*

The temperature test were conducted at three different temperatures:

At 4°C, to resemble the water temperature in the Oosterschelde during the month of March.

At 12°C, as midpoint between 4 and 20°C,

At 20°C, to resemble the water temperature in the Oosterschelde during the month of June.

The test species *G. locusta* were exposed to water accommodated fractions (WAF) of oil.

Different concentrations were used in order to determine the LC50 values. Survival was written down every 24 hours for five days (120 hours). At the end of every experiment all gammarids were weighted and frozen and the mortality data was used to create dose-response curves.

The dose response curves were used to determine LC50 values by using GraphPAD® Prism (version 7.0).

The obtained LC50 values showed that with an increase in temperature the sensitivity increased as well. This means that there was a higher mortality rate at higher temperatures.

Secondly the Critical Body Residue (CBR) of *G. locusta* was determined. To do this three concentrations were chosen according to the results of the LC50 experiment. A control concentration of C0 and a high concentration of C6. The medium concentration differed per temperature. Every 15 minutes the gammarids were observed for a full 48 hour-period. Dead gammarids were removed directly. Time of death, weight and length were noted and after the measurements the gammarids were stored at -20°C. The chemical analysis to assess the CBR on different temperatures was planned but the analyses did not take place before the report was written, and therefore not reported. However, a significant difference in time of death in *G. locusta* exposed to 0.3 ml/L was found between temperatures. With increasing temperatures the time to death decreased. Length and weight did not have influence on the time until death. Both experiments as mentioned above where also carried out in the Arctic region of Spitsbergen in 2016, usingthe Arctic species *G. setosus.*

Compared to *G. locusta, G. setosus* showed a higher LC50 value of 0,04489ml/L and the time until death was higher.

In conclusion, temperature significantly influenced sensitivity to oil, expressed as LC50-values and time of death. Also, toxicity data gained from the temperate *G. locusta* cannot be used directly for risk assessment to oil of the Arctic *G. setosus* as arctic gammarids responded less sensitively, unless a worst-case scenario is found acceptable or a correction factor is applied.

**Research by H. Langezaal (2016)**

Langezaal studied a possible method to analyze the full spectrum of lipid classes that are present in marine invertebrates on a small scale, with a the focus on the analysis of samples from the amphipod *Corophium sp..* by use of medical test kits used for human blood serum analysis for the analysis of lipid classes in marine invertebrates. What medical kits for lipid analysis are available.

The lipids found in marine invertebrates are: wax esters, phospholipids, fatty acids, sterols and triacylglycerol’s with a big difference in wax ester and phospholipid content between Arctic and tropical marine invertebrates (Kanazawa, 2001). The use of lipids range from energy provider up until hormone creation, however this is not of great importance for this follow up research.

Methods for lipid extraction are either the Folch or the Bligh & Dyer method and current methods for consist of: chromatography, thin layer chromatography, high pressure liquid chromatography and gas chromatography.

Research towards the use of medical kits is done because traditional methods for determining lipid classes cost a lot of money as well as time.

The problem with the medical kits is that there is not a kit for every lipid class. However, the research concludes that the use of medical kits could mean an advantage over currently used methods for small scale lipid analysis in marine invertebrates.

Langenzaal (2016) found that there are medical test kits available for the following lipid classes: triglyceride, phospholipids, cholesterol and free fatty acids. After the sample preparation the lipid mixture can be determined with a spectrophotometer at 570 nanometer or a fluorometer at around 540 nanometer (Biovision Inc. n.d.). If done correctly the use of medical kits will roughly take up an hour. Tissue from the invertebrates can be used as material even though the test kits are designed to determine lipids in body tissue, urine, bloodplasma – or serum, or other biological fluids of mammals.

Use of the test kit for small scale lipid analysis has not yet been done. Whether the kits will work when invertebrate material is used will be determined during this follow up research.

In conclusion there is no recommended method for small scale lipid analysis of *Corophium sp.* because every study uses a different method. The use of medical test kits can however have an advantage over currently used lipid determination methods. Because of the speed and low costs compared to traditional methods.

**Literature on lipids in marine invertebrates**

During this research the differences in lipid content and lipid composition in *G. setosus* and *G. locusta* will be studied. By use of literature reviews a theoretical difference between *G. setosus* and *G. locusta* can be developed.

Literature shows that zooplankton originating from cold waters show a wax ester content of 20 % of their total lipid content, making it their primary lipid class. Their tropical counterparts only show a 10% wax ester content of their total lipid content (Kanazawa, 2001). This is because the Arctic marine invertebrates depend strongly on short and intense algae blooms and therefore must migrate to deeper colder waters when food becomes less available in the shallow parts. In more tropical waters, food in the form of algae blooms is constantly available throughout the year and therefore the tropical marine invertebrates have a higher phospholipid content than Arctic marine invertebrates, making phospholipids the primary lipid class in tropical marine invertebrates (Connelly, Deibel, Parrish, & Trela, 2015).

|  |  |  |  |
| --- | --- | --- | --- |
| **Lipids in amphipoda** | **Present in Arctic species (%)** | **Present in temperate species (%)** | **references** |
| Wax esters | >20  42 | 1.4  <10 | (Copeman & Parrish, 2003); (Langezaal, 2016); (Auel, 2002) |
| Triacylglycerols | 31 | 4.6 | (Copeman & Parrish, 2003); (Auel, 2002) |
| phospholipids | 12 | 54.6 | (Copeman & Parrish, 2003); (Auel, 2002) |
| free fatty acids | 9 | 0.0 | (Copeman & Parrish, 2003) |
| sterols | 5 | 22.1 | (Copeman & Parrish, 2003); (Auel, 2002) |
| Triglycerides | 23 | x | (Auel, 2002) |

Table 1: lipid percentage in Arctic and temperate amphipods according to literature.

Wax esters form a big part of the lipid composition in Arctic amphipods (see table 1). This potentially complicates the goal in the use of medical kits for assessing lipid composition in marine invertebrates as there is currently no medical test kit for wax esters available. Either a new medical test kit must be developed or another method must be used in order to measure the wax ester content in the amphipods.

# Method

This part of the report will contain the materials used during the research and the method used to gain information in order to give answers to the research questions.

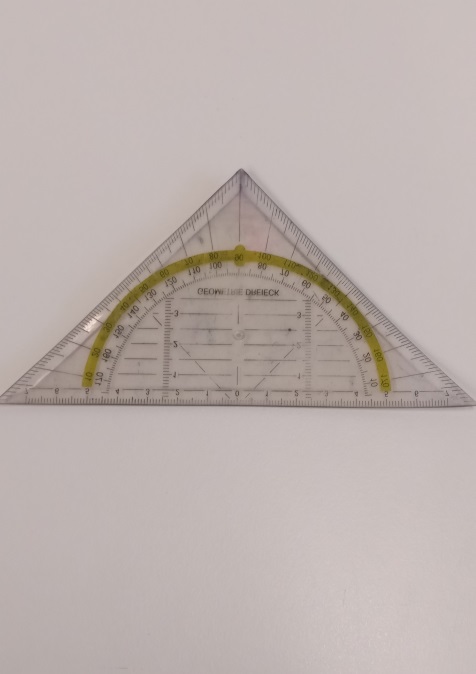
## Collection samples.

**Materials**

Wading suit ->  Fishing nets -> 

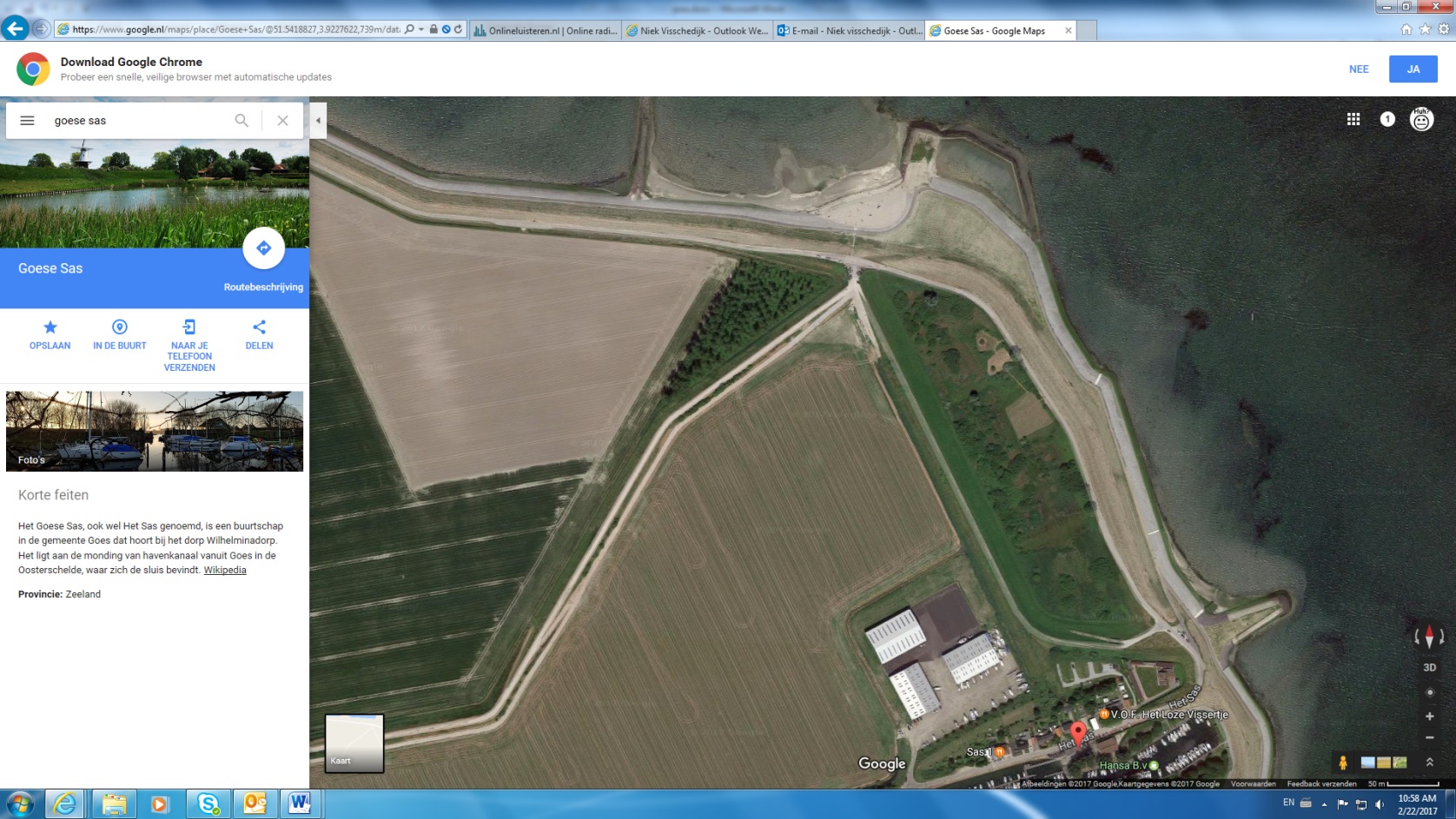
Bucket ->  Small barrel with lid ->  

Microbalance -> Linen cloth -> 

Tweezers -> Protractor ->

**Method**

*G. locusta* was collected at Goese Sas, a location at the Oosterschelde (image 3).



**Sampling sites**

**Car parking**

Image 3: a picture showing the sampling sites at Goese Sas (google, 2017)

This location was chosen because Japanese wireweed *(Sargassum muticum)* was apparent in large quantities. This weed was very useful during the collection of *G. locusta* as *G.locusta* finds shelter within these strings of Japanese wireweed.

By running a small fishnet along the strings of the wireweed *G. locusta* was caught. This process was easier when the fishing net had a square or rectangular form because that way there is more contact area with the wireweed, allowing more Gammarus to get caught.

In order to prevent the smaller individuals to escape through the holes of the fishing net, it was necessary to hold the fishing net directly above the bucket after running it along the wireweed.

Small *G*. *locusta* would fall into the bucket but the bigger ones had to be taken out of the fishing net and put into the bucket by hand. When the tide came back up, the captured gammarids were moved from the bucket into the barrel with the lid in order to provide safe transport back to the lab.

Back in the lab the first batches of Gammarus were all individually measured and weighted in order to gain more insight into the weight and length distribution that could be expected. Later batches were directly divided in four length classes: <6cm, 6-10cm, 11-15cm and >15cm.

Image 4: measuring Gammarus locusta



Image 4: measuring Gammarus locusta

Measuring of the gammarids was done by using a protractor and a small spoon or a pair of tweezers in order to keep the animal from curling into a ball (image 4). The length of the animal was measured without it’s antennae, so from the tail to the start of the head.

After drying the gammarids using a linen cloth it was weighted by using a microbalance.

Lastly the gammarids were divided into the different size classes and placed in a -80 degree Celsius freezer per size class.

## Lipid extraction

In order to measure the total lipid content of the *Gammarus sp.* the lipids had to be extracted.

Two effective methods are the Folch method (Folch, Lees, & Sloane-Stanley, 1957) and the Bligh and Dyer method (Bligh & Dyer, 1959). In the study done by Langenzaal (2015) it is stated that the Folch method is recommended when samples have an estimated lipid content greater than 25%. The Bligh and Dyer method is the most recommended method for determining the total lipid content in biological tissue (Smedes & Askland, 1999). The Bligh and Dyer method is often used to determine lipid content in various fish species (Payne, Johnson, & Otto, 1999). 95% of the lipid can be recovered using the Bligh & Dyer method and the method can be applied to any tissue containing at least 80% water (Bligh & Dyer, 1959).

Because of these arguments and the available materials, the Bligh and Dyer method was chosen to extract the lipids from the *Gammarus sp.* The applied Bligh and Dyer method in this research is a protocol set up by E. van Barneveld and it is property of Wageningen Marine Research.

### Materials for lipid extraction.

* Chloroform
* Methanol, technical quality
* Demineralized water
* 80ml centrifuge tubes with screw on cap
* 25 or 50 ml flat bottom flasks.
* 10ml finn-pipet
* Pipettes and/or dosage flask of maximum 30ml
* Centrifuge EC-4 (IEC)
* Ultra turrax T25
* Hotplate IKA C-MAS HS7 or similar
* Sartorius ED3202S digital college balance or similar (accuracy 0,01 g)
* Analytical balance, Mettler AT201 or similar (accuracy 0,1 mg)
* beakers 50ml, 100ml

### 3.2.2 Bligh & Dyer method for lipid extraction

This method was done with five samples. The first two consisted of 1.7 grams of herring/mackerel samples with a known lipid percentage of 11,54%. These two samples functioned as the control samples. Sample three consisted of 8 grams of the *Gammarus locusta,* which could not be conducted in duplo because there was a lack of test species. The last two samples with the numbers four and five consisted of the Arctic *G. setosus* species in which number four had specimens with a length of around 17 cm and number five had specimens with a length of around 22 cm.

After weighing in the samples demineralized water was added using a finn-pipette. The amount of added water was calculated using the formula

A = sample weight in grams. P= liquid percentage of the sample.

After adding the water, 20 ml of methanol was added directly from the dosage flask as well as 10 ml of chloroform also directly from the dosage flask. The weight of the added chloroform was noted.

For 30 seconds the solution was homogenized using the Ultra Turrax at 8000 rpm. 10 ml of chloroform was added again and the weight of the 10 ml of chloroform was noted. The solution was then homogenized agian for 30 second with the Ultra Turrax again at 8000 rpm. Lastly, 10 ml of demineralized water was added using a finn pipette. The whole solution was again homogenized using the Ultra Turrax at 8000rpm for 30 second.

Pieces of the *Gammarus* tended to get stuck in the Ultra Turrax and therefore it was cleaned with a pair of tweezers and by running the Turrax with only demineralized water, in order to keep it from getting stuck.

Next, the samples were put in the centrifuge for 10 minutes at 3000 rpm. When the samples came out of the centrifuge, three layers were formed. The upper layer being a water – methanol layer, the second layer was residue excluding fat and the third layer was a chloroform layer including the dissolved fat.

The upper layer was removed using a pump that sucked the upper layer up. With a pasteur pipette a small hole was created in the thick second layer so 10 ml the third layer could be retrieved by using a finn pipette. The 10 ml of chloroform/fat samples were put into small beakers. The 10 ml of the third layer was weighted and the weight was noted.

The chloroform/fat solutions in the beakers were paced on a hot plate which had a temperature of 175⁰C. The beakers were left on the hot plate until all the chloroform was evaporated and only the fat remained. After weighing the beakers with the fat and subtracting the initial weight of the beakers, the fat percentage can be calculated using the following formula. Fat%=

Va= total weight of chloroform (g)

Vb= pipetted weight from chloroform/fat layer (g)

a= weight of the sample (g)

b= amout of weighted fat (g)

While carrying out this method the control sample (herring/mackerel) in duplo was used to verify the quality of the experiment. The results of the control samples will not be shown in the results because the values are irrelevant for this research and are only used to verify the quality of the experiment.

## Lipid assessment

The lipid assessment was done using medical test kits. Part of this research was to find out which test kits were relevant and useable for future research. In order to do this, the study done by Langezaal (2015) was used as an important source of information. After having set up a table with the different lipid classes and their respectable percentage of total lipid content in marine invertebrates as found in literature(table 1), contact with potential suppliers was made.

|  |  |  |  |
| --- | --- | --- | --- |
| **Lipid types in amphipods** | **Percentage in Arctic species (%)** | **Percentage in temperate species (%)** | **References** |
| Wax esters | >20  42 | 1.4  <10 | (Copeman & Parrish, 2003); (Langezaal, 2016); (Auel, 2002) |
| Triacylglycerols | 31 | 4.6 | (Copeman & Parrish, 2003); (Auel, 2002) |
| Phospholipids | 12 | 54.6 | (Copeman & Parrish, 2003); (Auel, 2002) |
| Free fatty acids | 9 | 0.0 | (Copeman & Parrish, 2003) |
| Sterols | 5 | 22.1 | (Copeman & Parrish, 2003); (Auel, 2002) |
| Triglycerides | 23 | x | (Auel, 2002) |

Table 1: lipid class concentrations in amphipod species as found in literature.

As no medical test kits were available to determine the wax esters lipid class, test kits to determine phosphatidylcholine (which corresponds with phospholipids) and triglyceride (which corresponds with triacylglycerols) were selected and ordered at the company Sanbio. These two test kits are from the brand Cayman chemical and are called:

* Triglyceride colorimetric assay kit
* Phosphatidylcholine colorimetric assay kit

### 3.3.1 Materials for lipid extraction

**triglyceride colorimetric assay kit**

* triglyceride standard solution
* standard diluent assay reagent
* NP40 substitute assay reagent
* Sodium phosphate assay buffer
* Triglyceride enzyme mixture

**Needed materials, not supplied with the kit**

* plate reader (measure absorbance between 530 – 550 nm.)
* adjustable pipettes and a multichannel or repeating pipette
* pure water
* test tubes
* 15 ml centrifuge tubes
* Aluminum foil

**Phosphatidylcholine colorimetric assay kit**

* PC buffer
* PC color detector
* PC enzyme mixture
* PC-specific PLD
* Phosphatidylcholine standard solution
* PC detergent solution

**Needed materials, not supplied with the kit**

* plate reader (measure absorbance between 585 – 600 nm.)
* adjustable pipettes and a multichannel pipette
* pure water

### 3.3.2 Method for lipid assessment (test kit use)

**Triglyceride colorimetric assay kit and phosphatidylcholine colorimetric assay kit**

**sample preparation**

Firstly the gammarids were divided into size classes, weighted and minced into small pieces.

The size classes for *G. locusta*  were: <6cm, 6-10cm, 11-15cm, and >15 cm.

The size classes for *G. setosus* were: Medium(15 -20cm), Medium/large (20-25cm),

Large (25-30 cm) and Extra-large (>30 cm).

A 12 ml vial of NP40 was diluted with pure water till 48 ml.

2 ml of the diluted NP40 was homogenized with 350-400 mg of the minced *Gammarus* tissue.

The homogenized solution was centrifuged at 10,000 x g for 10 minutes at 4 °C,

After the being centrifuged the solution was stored in a box with dry ice at a temperature of -79°C, to be able to be transported to another location where it was stored in a freezer at – 80 °C for five days before the assessments were done.

**Lipid assessment calculations**

The results of the medical test kits will show the amount of triglyceride and the amount of phosphatidylcholine that was present in the test species. All the results are gained by reading the prepared samples with a spectrophotometer. The results that the spectrophotometer gives are the absorbance values. To calculate the concentration of the researched lipids the formula below is used.

**Triglyceride/Phosphatidylcholine (mg/dl)** =

The corrected absorbance value (CAV) is the absorbance value minus the 0 mg/dl absorbance value of the standard preparation values.

The preparation of the standard curve is part of the test kit use and a complete explanation on how the test kits were used can be found in appendix II.

the y – intercept and the slope are obtained with the formula that belongs to the plotted standard curve. Table 2 & 3 below show the concentrations that the standard curve contains according to the test kit manual.

|  |  |  |  |
| --- | --- | --- | --- |
| **Tube** | **200 mg/dl PC stock (µl)** | **PC detergent solution (µl)** | **PC concentration (mg/dl)** |
| A | 0 | 500 | 0 |
| B | 50 | 450 | 20 |
| C | 100 | 400 | 40 |
| D | 150 | 350 | 60 |
| E | 200 | 300 | 80 |
| F | 250 | 250 | 100 |
| G | 375 | 125 | 150 |

Table 2: the concentration of phosphatidylcholine in the standard curve.

|  |  |
| --- | --- |
| **tube** | **Triglyceride concentration (mg/dl)** |
| A | 200 |
| B | 100 |
| C | 50 |
| D | 25 |
| E | 12.5 |
| F | 6.25 |
| G | 3.125 |
| H | 0 |

Table 3: the concentration of triglyceride in the standard curve.

the corrected absorbance value in the formula is sample value gained from the spectrophotometer minus the 0 mg/dl absorbance value of the standard curve.

### 3.3.3 hypothesis testing

The P values have not been tested, this is due to the low sample size in this pilot research.

In order to conduct statistical test more replicates are needed per sample to be able to see statistical differences between experiments. This means however that this report does not include any P value testing.

# 4.0 Results

Because this particular research is a pilot research, part of the results will be an explanation as to why the two chosen test kits were used instead of other viable test kits, why only two test kits were used instead of more and why certain methods where used when there were multiple options.

The other part of the results will consist of data that was gained by carrying out this research. The data will be shown in the form of tables and graphs.

## 4.1 Method set up

**Selection of the total lipid method**

There are two popular methods to extract lipids from animal tissue, the Folch method and the Bligh & Dyer method.

The Folch method was recommended by Langenzaal (2015) when the used tissue has an estimated lipid percentage greater than 25%. However, *Gammarus* species do not have a lipid content greater than 5 percent.

The Bligh & Dyer method is often used to determine lipids in fish species and can only be used with tissue containing at least 80% water.

*Gammarus* species do not have a high enough lipid content to apply the Folch method. The requirements to conduct the Bligh & Dyer method were met with the used tissue, the materials to apply the Bligh & Dyer were available and therefore the Bligh and Dyer method was the chosen method that was applied during this research.

**Lipid assessment test kits**

Table 1 (page 14) shows the results of the literature research on which lipids are present in *Gammarus* species.

Test kits were chosen focused on the types of lipid that showed the biggest differences between the Arctic and temperate species.

The top three lipid classes with the biggest differences were:

1- phospholipids 2- wax esters 3- triacylglycerols

The test kits were quite expensive and because only the use of the medical test kits was supposed to be tested during this research, two test kits were chosen.

The used test kit were a ‘phosphatidylcholine colorimetric assay kit’ which measures a phospholipid class and a ‘triglyceride colorimetric assay kit’ which measures a form of triacylglycerols.

These test kits were chosen for a few reasons.

Firstly, it was certain that at least one of these kits would work with tissue instead of with blood serum or plasma.

Secondly, there was no available test kit for wax esters so instead triacylglycerol was chosen as third lipid group showing differences between regions.

Furthermore, the instruments needed for the test kits were available and useable.

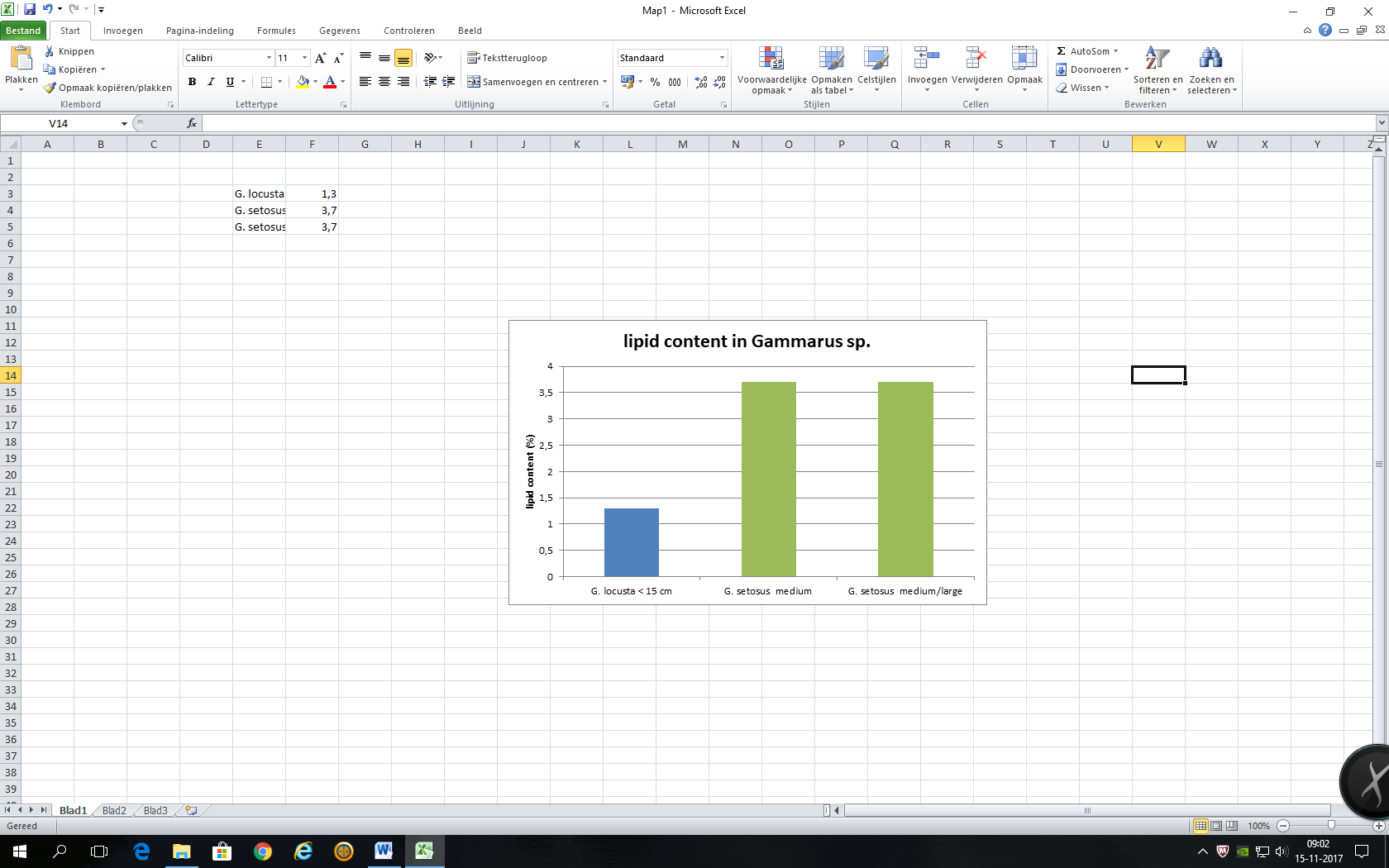
4.2 Lipid extraction

Table 2 and graph 1 show the total lipid content in the Arctic and temperate *Gammarus* species, based on the Bligh and Dyer method. These show that the Arctic *G*. *setosus* contains a higher concentration of total lipids. The total lipid content of the temperate *G. Locusta* is 1.3%,and the total lipid content of the Arctic *G.* *setosus* is 3.7 % a factor 2.8 higher. There is no difference in total lipid concentration between the medium and medium/large sized individuals of the *G. setosus.*

**Total lipid content in the *Gammarus sp.***

|  |  |  |
| --- | --- | --- |
| ***Gammarus sp.*** | **Region** | **Total lipid content (%)** |
| *Gammarus locusta (< 15cm)* | Temperate | 1.3 |
| *Gammarus setosus (medium size)* | Arctic | 3.7 |
| *Gammarus setosus (medium/large size)* | Arctic | 3.7 |

Table 4: The total lipid content in the Gammarus sp.



Graph 1: The total lipid content in Gammarus sp. in Arctic and temperate regions.

## 4.3 Lipid composition assesment

### 4.3.1 Triglyceride

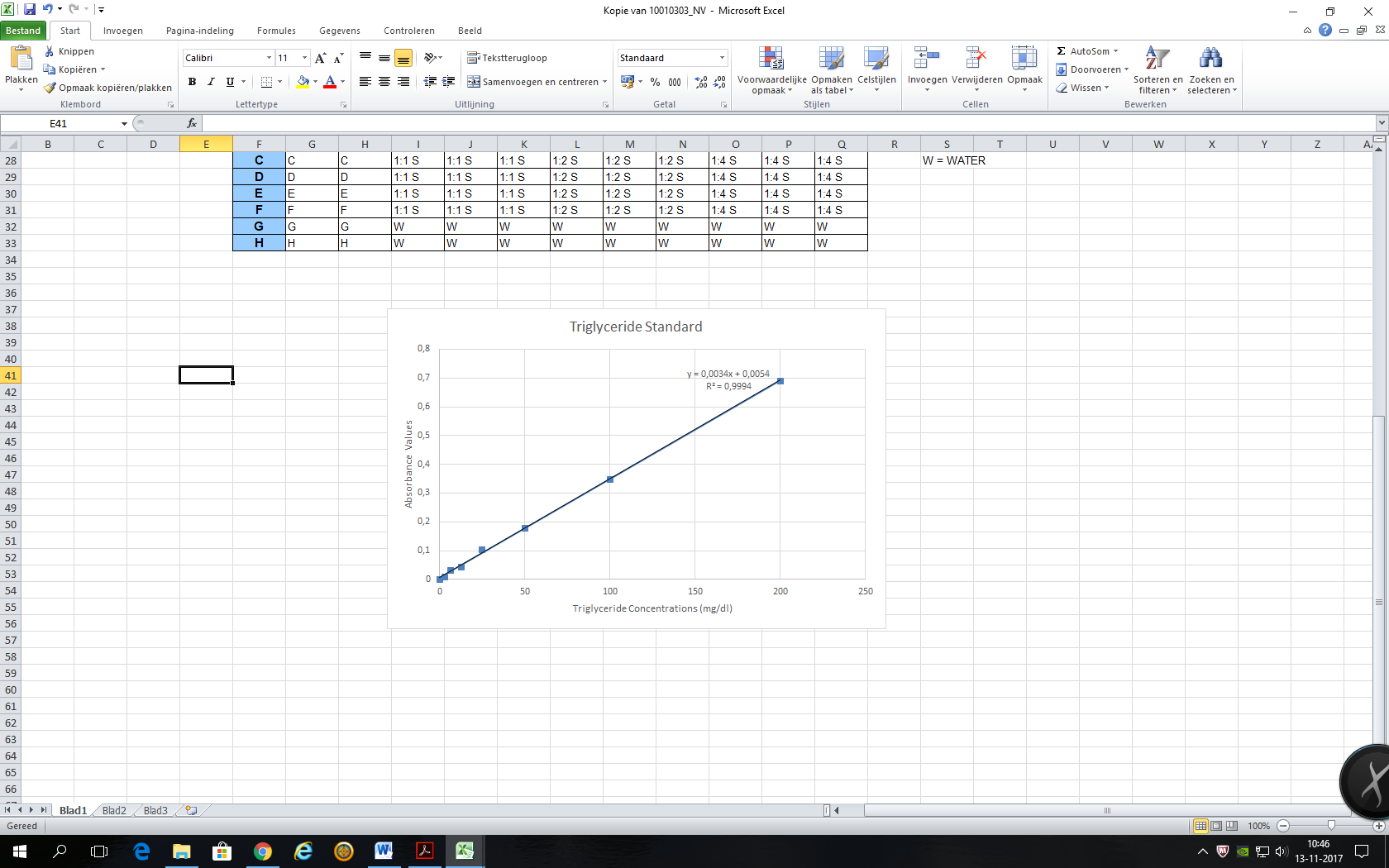
Table 5 shows the values of the standard curve gained from the spectrophotometer. Column H is a sample used to make the standard curve and contains a 0.0 mg/dl triglyceride concentration. H has in this case a value of 0.116 and is used to calculate the corrected absorbance values(CAV).

CAV = sample – H

**Absorbance values of the triglyceride standard curve on 540 nm**

|  |  |  |
| --- | --- | --- |
| **Row**  **Column** | **Standard curve** | **CAV standard curve** |
| A. | 0.808 | 0.692 |
| B. | 0.465 | 0.349 |
| C. | 0.295 | 0.179 |
| D. | 0.219 | 0.103 |
| E. | 0.161 | 0.045 |
| F. | 0.148 | 0.032 |
| G. | 0.124 | 0.008 |
| H. | 0.116 | 0.0 |

Table 5: absorbance values of the triglyceride samples on 540 nm in the triglyceride medical test kit.



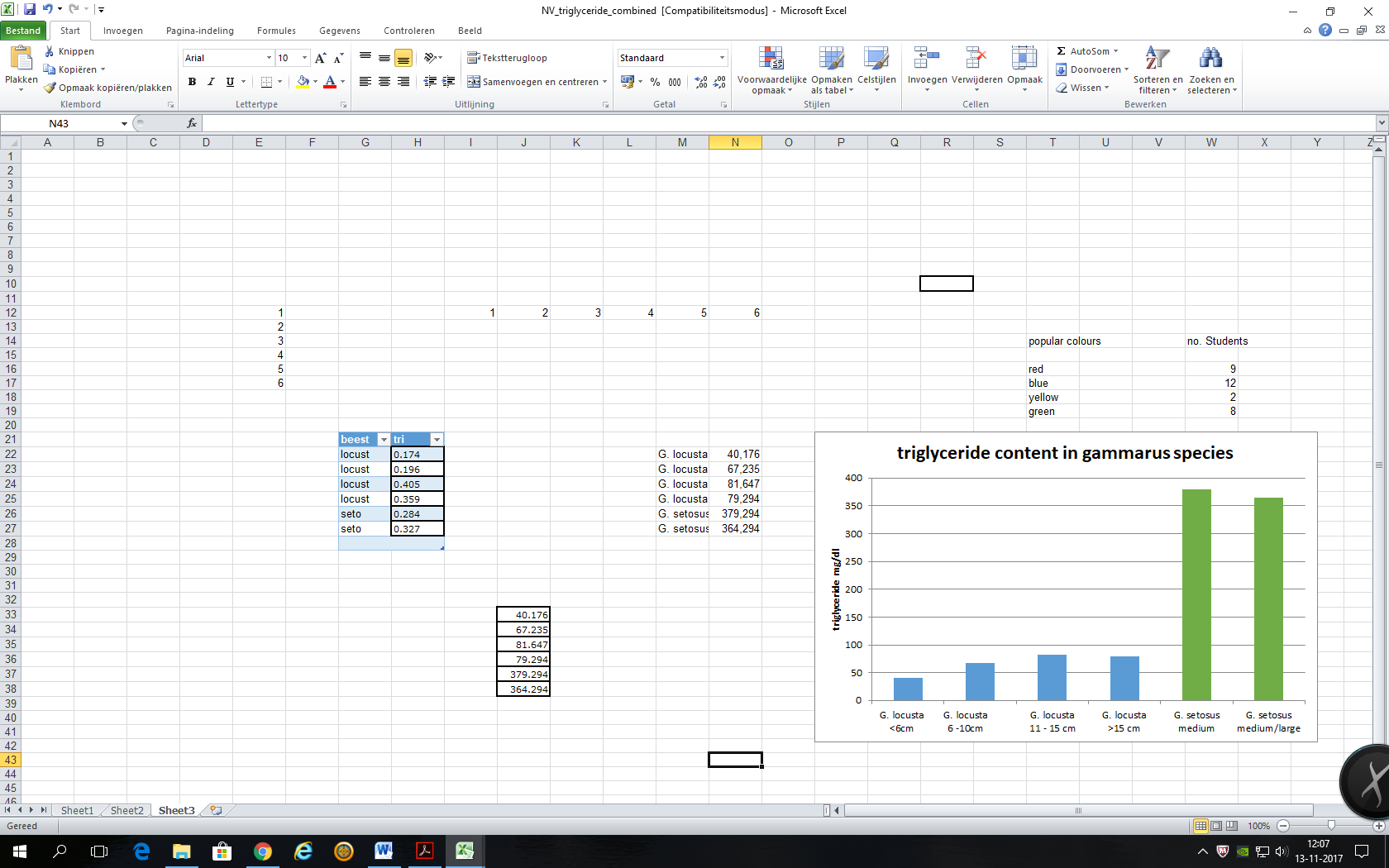
Graph 2: Triglyceride standard curve gained by subtracting standard H absorbance value from all values. ( y – intercept = 0.0054 & slope = 0.0034)

**Triglyceride concentration (mg/dl)**

Table 6 and graph 3 show the triglyceride concentrations in the tested organisms. This shows that the Arctic *G.* *setosus* contains roughly between 4.5 times more triglycerides when compared to the smallest *G. locusta* size classes and 9 times more than the larger *G. locusta* size classes*.* Triglyceride concentrations within size classes of *G. locusta* are 1.2 – 2 times lower in the smallest size classes when compared to the larger size classes. There is no significant difference between the two largest size classes of both *Gammarus* species.

|  |  |  |  |
| --- | --- | --- | --- |
| **Row**  **Column** | **Absorbance values of samples** | **CAV of samples** | **Triglyceride**  **(mg/dl)** |
| A. *(G. locusta*  <6cm) | 0.258 | 0.142 | 40.176 |
| B. (*G. locusta*  6 – 10cm) | 0.350 | 0.234 | 67.235 |
| C. (*G. locusta*  *11 – 15cm)* | 0.399 | 0.283 | 81.647 |
| D. (*G. locusta*  >15cm) | 0.391 | 0.275 | 79.294 |
| E. (*G. setosus Medium)* | 1.411 | 1.295 | 379.294 |
| F. (*G. setosus medium/large)* | 1.36 | 1.244 | 364.294 |

Table 6 : values from the spectrophotometer and the formula(formula page 15)



Graph 3 : a column graph showing the difference in triglyceride content in the gammarus species

### 4.3.2 Phosphatidylcholine

Table 7 shows the values of the standard curve gained from the spectrophotometer which was reading at 590nm. The corrected absorbance values are the ‘standard curve’ and ‘samples’ values minus the value A has in the ‘standard curve’ row. In this case the standard curve value of A is 0.12.

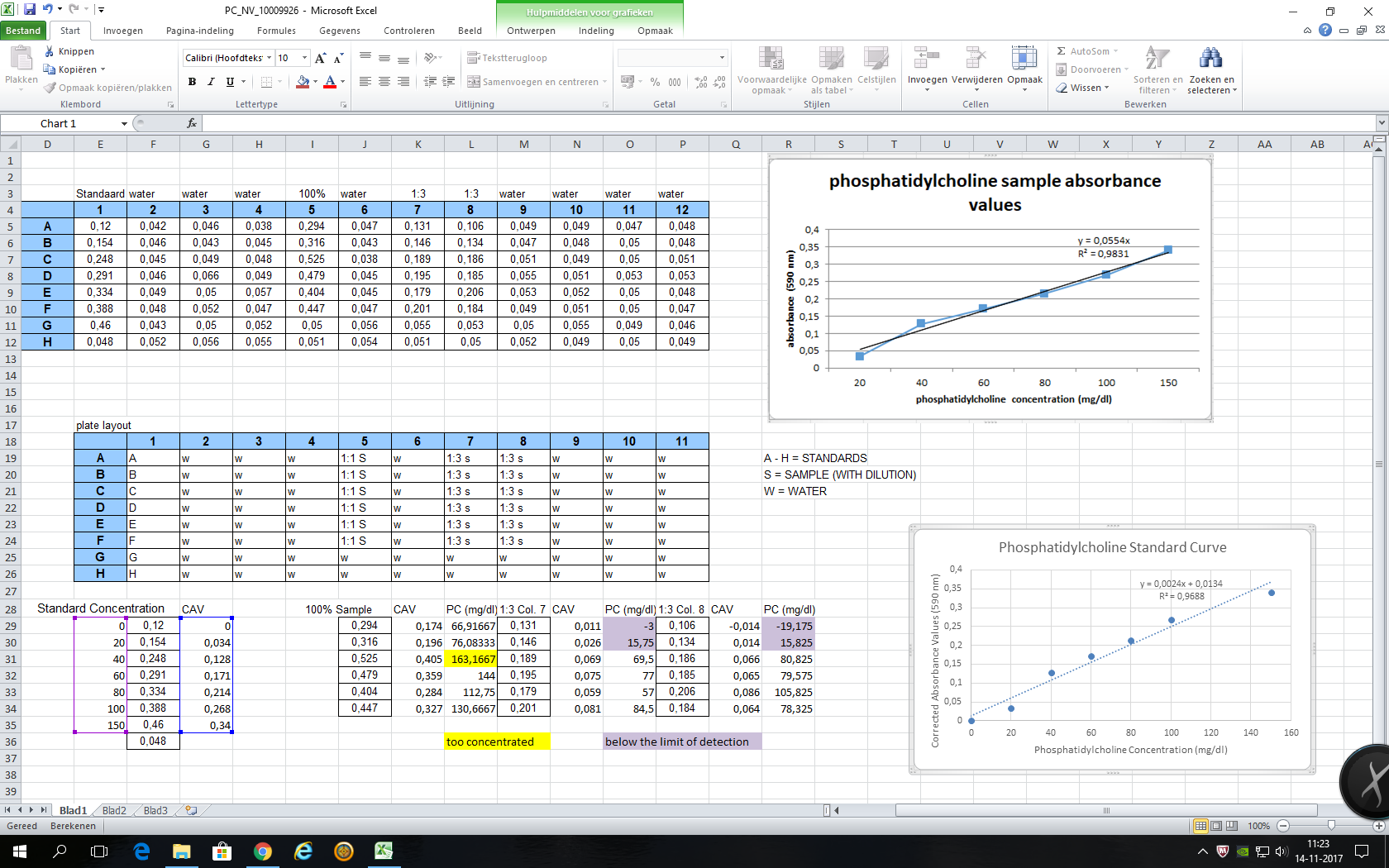
A is used because it is the 0 mg/dl concentration value for the phosphatidylcholine samples.

CAV = sample - A

**phosphatidylcholine values of the standard curve (590 nm)**

|  |  |  |
| --- | --- | --- |
| **Row**  **Column** | **Standard curve** | **CAV Standard curve** |
| A. | 0.12 | 0.00 |
| B. | 0.154 | 0.034 |
| C. | 0.248 | 0.128 |
| D. | 0.291 | 0.171 |
| E. | 0.334 | 0.214 |
| F. | 0.388 | 0.268 |
| G. | 0.46 | 0.34 |

Table 7: the results gained from the spectrophotometer after using the phosphatidylcholine medical test kit.



Graph 4: phosphatidylcholine standard curve gained by subtracting standard A absorbance value from all values. ( y – intercept = 0.0134 & slope = 0.0024)

**Phosphatidylcholine concentration (mg/dl)**

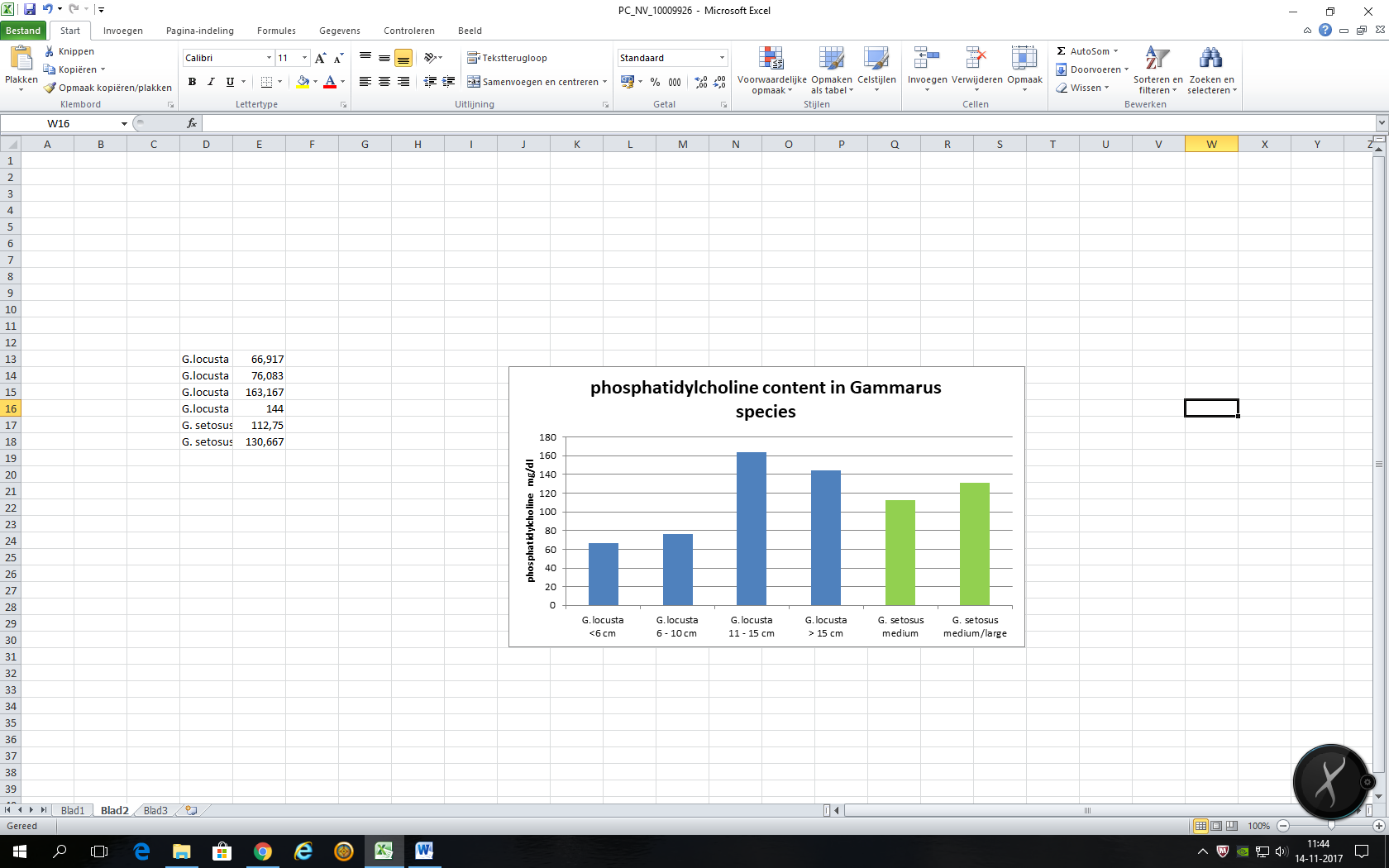
Table 6 and graph 5 were created by using the formula below, the standard curve and the values gained from the spectrophotometer. The results show that the temperate *G. locusta* has a higher concentration of phosphatidylcholine than the Arctic *G. setosus* in the size classes 11 – 15 cm and

> 15 cm. However the smaller size classes have a lower concentration of phosphatidylcholine.

The average concentration of the temperate *G. locusta* is 112.54 mg/dl and the average concentration of the Arctic *G. setosus* 158.83 mg/dl showing that the average concentrations are not that different.

|  |  |  |  |
| --- | --- | --- | --- |
| **Row(dilution)**  **Column** | **Absorbance values** | **CAV** | **Phosphatidylcholine**  **(mg/dl)** |
| A. *(G. locusta*  <6cm) | 0.294 | 0.174 | 66.917 |
| B. (*G. locusta*  6 – 10cm) | 0.316 | 0.196 | 76.083 |
| C. (*G. locusta 11 – 15cm)* | 0.525 | 0.405 | 163.167 |
| D. (*G. locusta*  >15cm) | 0.479 | 0.359 | 144.0 |
| E. (*G. setosus Medium)* | 0.404 | 0.284 | 112.75 |
| F. (*G. setosus medium/large)* | 0.447 | 0.327 | 130.667 |

Table 8 : phosphatidylcholine values from the spectrophotometer and the formula



Graph 5 :a column graph showing the phosphatidylcholine content in the gammarus species

# 5.0 Discussion

**Collection of test species**

It became apparent that the collection of sufficient individuals of the test species to conduct the experiments took up a lot of time. The *Gammarus locusta* were caught in the Oosterschelde and one person could capture a maximum of 1 gram per hour. However, this maximum was only reached once and depends on the time length of low tide.

8 grams is needed to conduct the Bligh and Dyer total lipid assessment once and 350 – 400 mg of tissue is needed to conduct the experiment with the medical test kits. This means that one person would have to spend around 20 hours capturing gammarids to be able to provide enough testing material for the experiments.

**Total lipids**

The results of the Bligh and Dyer experiment came out as expected. The Arctic *G. setosus* has a factor 2.8 higher total lipid content than the temperate *G. locusta.* This result confirms the hypothesis, it was expected that the Arctic species would have a higher total lipid content than the temperate species. this is logical because this fat content plays a big part in the heat regulation of the body, plant material near the polar regions tend to be softer and contain more nutrients allowing the animals to grow bigger and fattier (Konkel, 2012).

**Lipid composition**

Triglyceride

The results of the lipid composition experiment that were carried out by using the medical test kits.

The triglyceride test results showed that the Arctic *G. setosus* has significantly more triglyceride lipid content. When comparing the two *Gammarus sp.*  the Arctic species has a factor 4,5 to 9 times more triglyceride content. When comparing these results to earlier results found in literature (table 1), there is a confirmation that these results are correct. Literature shows that 31% of the Arctic species total lipid content contains triacycglycerols. That compared to the temperate species of which only 4.6% of the total lipid content consists of triacylglycerols.

Triacylglycerols are a way that energy is stored in the body, it stores six times the amount of energy as normal sugar. Triacylglycerols are stored just under the skin and can be used as a fat layer to keep warm in colder climates (Paul, unknown). It seems therefore logical that the arctic species has a factor 4.5 to 9 time more triacylglycerols than the temperate species.

Phosphatidylcholine

According to literature the total lipid content of the temperate species contains 54,6% phospholipids and that of the Arctic species only 12%, a difference of 42%. The test with the medical test kits shows the highest concentration of phosphatidylcholine to be present in the *Gammarus locusta* 11 – 15 cm size class(163.167 mg/dl). However, when the average is taken the Arctic species contains 158.83 mg/dl which is only a factor 1.4 higher than the 112.54 mg/dl of the temperate species. the results of the test and the found results in literature do not completely confirm each other.

When comparing the lowest phosphatidylcholine content in the temperate species with the highest phosphatidylcholine content, a difference of almost 50% shows. However, the highest phosphatidylcholine in the temperate species is 20% higher than the highest phosphatidylcholine content in the arctic species.

Phosphatidylcholine has shown to play an important role in a few areas including: maintaining cell structure, fat metabolism, memory, nerve signaling, as a precursor to important neurotransmitters, and liver health (phosphatidylcholine.org, 2011). Some of these roles the phosphatidylcholine plays in the cells might give a reason why small sized temperate gammarids have less phosphatidylcholine content in their bodies compared to the larger sized temperate and arctic species.

**Medical test kit use**

The medical test kits were supplied with a limited amount of supplies. The plate contains enough wells for the standard curve in triplicate plus three samples in triplicate to make 12 columns. However, problems are expected when working with devices and methods that are new to a person. A problem with the phosphatidylcholine test kit occurred during the incubation, which made the samples unusable.

Because of that problem there was not enough material to make the samples again in the same quantity and therefore it was decided to analyze one 100% sample and a 1:4 sample in duplicate to still gain a good view on the phosphatidylcholine content in the samples. It could be said that the conclusion is therefore questionable but, the core concept of the research which was to find out whether the test kits would be beneficial to lipid content research in marine invertebrates is still viable.

The results that were gained from the medical test kits are to be checked. The test kits have only been used once on marine invertebrates and to see whether the test kits were a success, another series of samples need to be tested to validate the results gained during this research.

A wider overview of the different lipid classes is needed to put everything in context. Further research not only test the lipid classes that are not yet tested but also phosphatidylcholine and Triglyceride again. As a duplo to this research, it will not take up a lot of time but it will check the viability of this study.

**Further use of test kits**

The use of test kits was studied in this project because their use would reduce the time and cost of lipid assessments in marine invertebrates as well as the fact that medical test kits can be brought along during field expeditions. However, These test kits will be of no use during field expeditions because the preparation of the collected samples requires heavy equipment, such as a cooling centrifuge, which is stationed at a laboratory.

Even though the test kits will be of no use when brought along during field expeditions their use still saves cost as well as time of a full lipid assessment and can be applied as proxy for lipid composition.

**Sample preparation**

Before the samples were prepared to go into the plate reader, they had to be prepared. For this preparation a centrifuge was needed that could contain a constant temperature of 4°C. That particular centrifuge was stored in IJmuiden and had not been used in more than a decade and the use of the centrifuge was stated in an old manual. Eventually the centrifuge worked. However, the only way to tell whether or not the internal temperature was actually 4°C was to trust the thermometer that was included with the centrifuge. Because it was the first time the centrifuge was used in more than a decade and even though the temperature inside the centrifuge felt like 4°C when opened, there is no way to tell for sure that this temperature was secured during centrifuging.

# 6.0 conclusion

Based on the obtained results the following conclusions can be drawn for the research – and sub questions that were identified for this project:

* Do total lipid concentration and lipid composition differ between temperate and Arctic gammarids and can this explain differences in oil toxicity as was found by Klaassen (2016)?

*Hypothesis: it is expected that an increase in lipid concentration will increase the resistance.*

The Arctic species *G. setosus* showed a higher total lipid concentration (a factor 2.8 higher). This supports the possibility that the difference in total lipid concentration influences the in oil toxicity as was found by Klaassen (2016).A higher lipid concentration can act as a buffer for oil toxicity. The hypothesis can therefore be accepted based on the results.

*Hypothesis: it is expected that the Arctic G. setosus will contain a higher triglyceride concentration and a lower phosphatidylcholine concentration than the temperate G. locusta.*

The triglyceride content of the Arctic *G. setosus* is higher than the triglyceride content of the temperate *G. locusta,* the phosphatidylcholine content is highest in the temperate *G. locusta*. The hypothesis can therefore be accepted based on the results.

* Does total lipid composition and concentration in *Gammarus sp.* Differ within size classes?

*Hypothesis: it is expected that the total lipid composition and concentration will not differ between size classes*

The lipid concentration does not seem to differ within different size classes however the lipid composition does change within different size classes.

Therefore, the hypothesis is not accepted.

**Sub – question**

* Which test kits and lipid extraction methods are needed to conduct relevant lipid extractions and analyses in the indicator *Gammarus sp.*?

The most relevant lipid extraction method to extract lipid from marine invertebrates is the Bligh and Dyer method. The most relevant test kits are the ones that detect any form of:

**-** wax esters

**-** triacylglycerols

**-** free fatty acids

**-** sterols

**-** phospholipids

However, until this day there is no medical test kits to detect wax esters.

# Appendix I

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# Appendix II – medical test kit use

The medical test kits were used as was instructed in the manual. This Appendix will show specifics on how the instructions within the manual were carried out.

The test kits were ordered at a company called Sanbio and were shipped from the United States to the Netherlands. The producer of the test kits is Cayman chemical.

*Sample preparation.*

The sample preparation for both the phosphatidylcholine and triglyceride consisted of the same steps.

A vial containing 12ml of a 5x surfactant solution was mixed with 48 ml of demineralized water. This diluted NP40 substitute assay reagent was used to prepare the tissue samples.

The collected Gammarids were divided in their respective size classes. From all these size classes 350 – 400 mg of tissue was minced and homogenized with 2 ml of the diluted NP40 Substitute Assay Reagent.

The vials with the minced tissue and NP40 solution was centrifuged at 10.000x g for ten minutes at 4°C. the centrifuged samples were placed in a box with dry ice in order to be transported at a temperature of – 80 °C.

from here the two different assay kits have different instructions and will therefore be divided.

**triglyceride colorimetric assay kit**

*Reagent preparation*

* Triglyceride standard

The 400 µl of 1.000 mg/dl solution of triglyceride standard was ready for use. The triglyceride standard was used to prepare the standard curves (graph 2 & 4).

* Standard diluent assay reagent

A 12ml vial containing a 5x salt solution. Before using, the contents were mixed with 48ml of demineralized water. This solution was used to prepare the triglyceride standards.

* Sodium phosphate assay buffer.

A 4 ml vial containing 250mM sodium phosphate buffer with a pH of 7.2. before using, the contents were mixed with 16 ml of demineralized water. The solution was used to prepare the triglyceride enzyme solution.

* Triglyceride enzyme mixture

A vial containing a lyophilized enzyme mixture. 1 ml of demineralized water was added to the vial and the contents of the vial were transferred to a 15 ml centrifuge tube wrapped in aluminum foil. 14 ml of the diluted sodium phosphate assay buffer were added.

*Standard preparation*

Eight clean test tubes were labeled A-H. 200 µl of the NP40 solution were added to tubes B – H.

400 µl of diluted standard diluent and 100 µl of triglyceride standard was added to tube A and mixed thoroughly.

To obtain the dilution series to make the standard curve 200 µl of the mixed solution was removed from tube A, added to tube B and mixed thoroughly. Next, 200 µl was removed from tube B, added to tube C and mixed thoroughly. This process was repeated for tubes D-G. tube H only contained the diluted NP40 substitute assay reagent and was used as the blank (See the table below).

|  |  |
| --- | --- |
| **Tube** | **Triglyceride concentration (mg/dl)** |
| A | 200 |
| B | 100 |
| C | 50 |
| D | 25 |
| E | 12.5 |
| F | 6.25 |
| G | 3.125 |
| H | 0 |

Table 9: triglyceride standard curve concentrations.

*Performing the assay*

10 µl of the standards (tubes A-H) were added to the designated wells (an example of how a wells plate is set up, is shown in table 12 from a top down view). 10 µl of the samples were added to two or three wells. The reaction was initiated by adding 150 µl of enzyme mixture solution to each well.

The plate was covered with a plate cover and carefully shaken for a few seconds to ensure that the solutions were mixed.

The plate was incubated at room temperature for 15 minutes and measured with the plate reader at 540 nm.

**Phosphatidylcholine colorimetric assay kit**

*Reagent preparation (needed substances)*

* PC buffer (10X)

3 ml of the PC buffer was mixed with 27 ml of demineralized water, the mixture was used for the assay and for diluting reagents.

* PC color detector

The vial contained a lyophilized powder of DAOS ( N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline) and 4-aminoantipyrine.

3 ml of the 1X PC buffer was added to the PC color detector.

* PC enzyme mixture

The vial contains a lyophilized powder of choline oxidase and horseradish peroxidase. 1 ml of the 1X PC buffer was added to the vial.

* PC specific PLD

A vial containing a solution of phosphatidylcholine specific phospholipase D. ready to use as supplied

* Phosphatidylcholine standard

A vial containing a standard phosphatidylcholine. Ready to use as supplied.

* PC detergent solution

A vial contiaining Triton X – 100 solution. It was ready to use as supplied

*Standard preparation*

1 ml of PC detergent solution was added to the phosphatidylcholine standard and briefly stirred.

200 µl of the standard was transferred to another glass vial and 1.3 ml of PC detergent solution was added to obtain a 200mg/dl PC stock solution. 7 clean test tubes were labeled A-G and the amounts of PC stock solution and PC detergent solution were added to each tube as is described in table 10.

|  |  |  |  |
| --- | --- | --- | --- |
| **Tube** | **200 mg/dl PC stock (µl)** | **PC detergent solution (µl)** | **PC concentration (mg/dl)** |
| A | 0 | 500 | 0 |
| B | 50 | 450 | 20 |
| C | 100 | 400 | 40 |
| D | 150 | 350 | 60 |
| E | 200 | 300 | 80 |
| F | 250 | 250 | 100 |
| G  Table 10: preparation of the phosphatidylcholine standards. | 375 | 125 | 150 |

*Performing the assay*

Before performing the assay the reaction mixture was made according to table 11.

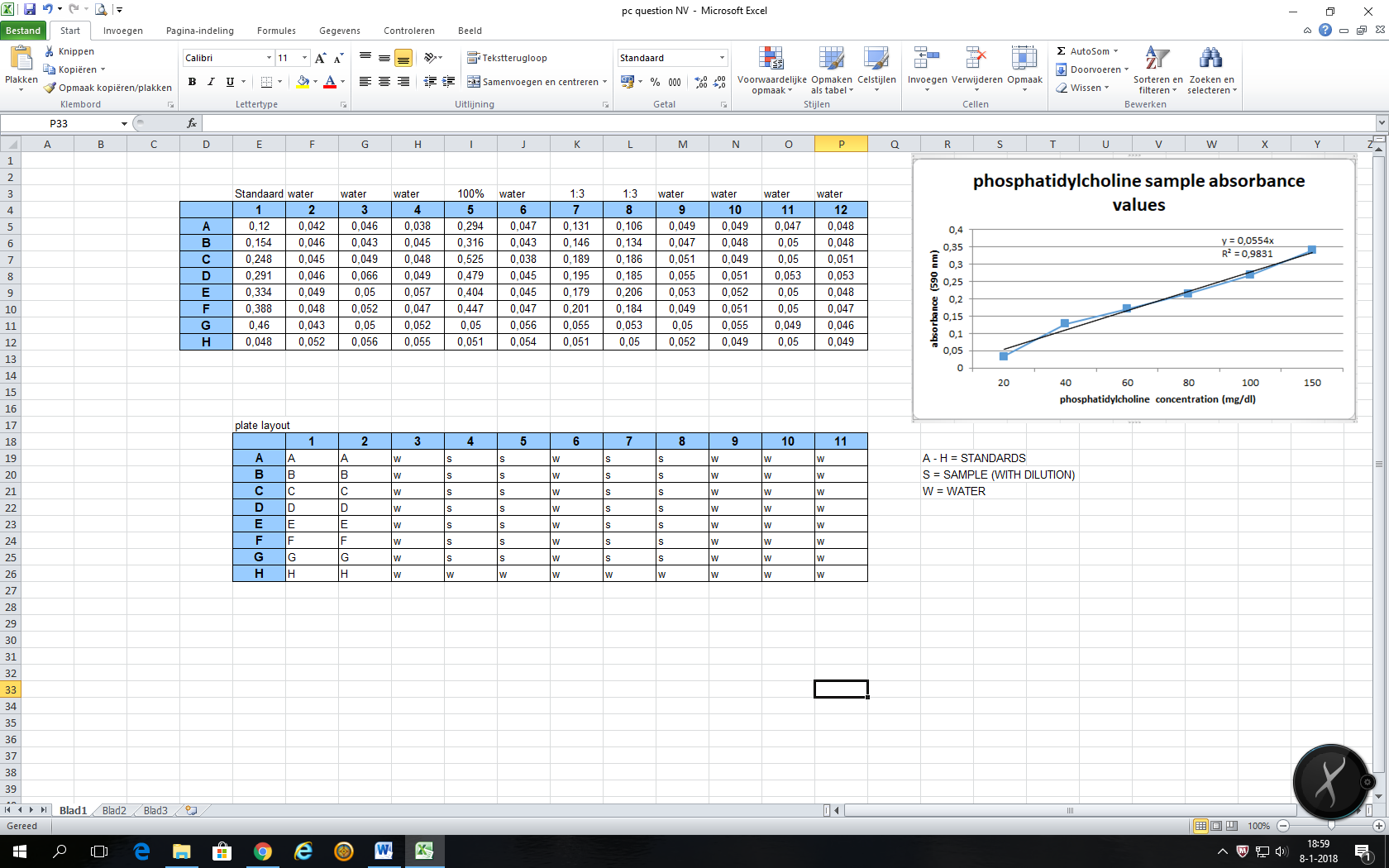
|  |  |  |
| --- | --- | --- |
| **Reagent** | **50 wells** | **100 wells** |
| PC color detector | 3 ml | 6 ml |
| PC enzyme mixture | 1 ml | 2 ml |
| PC-specific PLD | 30 µl | 60 µl |
| 1X PC buffer | 970 µl | 1.94 ml |

table 11: reaction mixture preperation.

10 µl of standard (tubes A – G) was added into the designated wells on the plate (see table 12 for an example). 10 µl of sample was added to their designated wells and the reactions were initiated by adding 100 µl of reaction mixture to each well. The plate was carefully stirred to mix the solutions in the wells. The plate was covered with a plastic plate cover and incubated at 37°C for 60 minutes.

The plate was put into the plate reader which was reading at 590 nm.

*Plate layout example*

The letters A – H are wells designated for the standard curve.

W = water

S = sample

Table 12: an example of a plate layout.