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Environmental Toxicology and Chemistry
Submission date: July 2018
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Trondheim, July 2018

Ewa Cichosz

Abstract

Calanus finmarchicus is an ecologically important species in the North, Norwegian and Barents seas where marine biota is exposed to contaminants discharges due to oil and gas production and transportation. In this experiment, copepods *Calanus finmarchicus* were exposed to the water-accommodated fraction (WAF) of North Atlantic crude oil in laboratory conditions. The exposure set consisted of four different time exposures where animals where sampled after 24, 48, 72 and 96 hours. The adverse effects were investigated through metabolite profile changes using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy technique. The results indicate a potential adverse effect of oil pollution on this crucial marine type of zooplankton which may be of great importance for animals of the next trophic levels and ecosystem functioning.

Based on previous findings it was possible to perform metabolome profiling analysis which indicates changes in amino acids, their precursors and other metabolites. Changes in 12 out of 27 metabolites appear to be concurrent response to oil exposure and starvation. However, changes in concentrations of 4 of the presented metabolites seem to be induced by the oil exposure which can be observed through contradictory tendency of the metabolites concentrations after the oil exposure compared to the control samples. From these results, malonate shows the biggest changes in concentration after the oil exposure which is up to 113% increase after 96h exposure compared to the corresponding control of 96h. This finding suggests that malonate may be used as a possible biomarker of oil exposure in *Calanus finmarchicu* what can be essential for both, monitoring and decision-making processes in situations of oil spill.

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1. Introduction

There are two principal routes for crude oil to enter the marine environment. The first one is natural seepage what is estimated to consists 47% of crude oil entering the marine environment, while remaining 53% involves human activities. Main activities leading to oil pollution are results of oil extraction and transportation, refining, storage and utilization of petroleum (Kvenvolden 2003).

The growth of the human population creates an increasing need for energy and fossil fuels are still the key source of energy worldwide with the crude oil as the main source (Fortov 2014). Crude oil is mainly used in transportation and power generation (Z. H. Jiang 2010). It was estimated that the daily usage of crude oil was about 96 million barrels per day and 4.5 billion tonnes per year in 2016 (Prince 2017). Therefore, the production and transportation of oil and oil products lead to a great threat, especially for the marine environment.

The Northern Atlantic Ocean is known for an extensive oil production industry and in consequence, produced water discharges, oil transportation and accidental oil pollutions take place. Even diluted, a low concentration of oil can be a long-range threat to plankton through sub-lethal effects. Pollutants can be bioactive at low levels or accumulate to bioactive levels in organisms (B. H. Hansen 2007).

Zooplankton consists of various groups of animals that can be found in free water masses. Meso and microzooplankton can be found at concentrations higher than 10^6 per m^2 of surface area, making these animals one of the most abundant in the oceans. They are outnumbered only by viruses, bacteria and eukaryotic microscopic organisms (Sakshaug 2009).

There are many species of Copepoda found in the Atlantic Ocean, and *Calanus finmarchicus* constitutes a great part of zooplankton biomass in the northern part of the ocean. Copepods are considered an important route of energy transfer in the marine food chain. They feed on phytoplankton and are a significant prey for commercial fish. This makes them a potential route of transfer for environmental contaminants. Moreover, *C. finmarchicus* can contain up to 50% fat (based on wet/wet concentrations) what makes them vulnerable to lipophilic organic contaminants (B. H. Hansen 2007).

As the oil production is moving towards the Arctic, *C. finmarchicus* may be a suitable model organism of complex oil mixtures and oil components in the Arctic climate (Hallanger 2011).

1.1. Crude oil composition

Crude oil is a complex mixture of various molecular weights hydrocarbons and other organic compounds exhibiting different toxicity to living organisms (Olsvik 2012). Crude oil occurs naturally and is generated in geological and geochemical processes under high pressure and temperature. Various oils differ in their composition and physical properties like density, viscosity and color. Crude oil is composed of saturated hydrocarbons, aromatic hydrocarbons, resins, asphaltenes, organic compounds containing nitrogen, sulfur and oxygen but also metallic compounds (Willsch 1997). The n-alkanes are considered as the most readily degraded components of petroleum. Oil can be present in aquatic environment in both, dissolved and particulate phase. The highest concentration of dissolved petroleum hydrocarbons is found in the water accommodated fraction (WAF) which consists of BTEX (benzene, toluene, ethylbenzene and xylene), alkylation of benzene homologues, polycyclic aromatic hydrocarbons (PAHs), petroleum hydrocarbon and other complex mixtures (Faksness 2008). Thus, WAF contains portion of substances from oil that are the most toxic for aquatic organisms (Abbriano 2011).

1.1.1. The fate of crude oil in the aquatic environment

In the sea, crude oil is subjected to a series of physical, chemical and biological processes known as weathering processes. These processes result in the partial dissolution or accommodation of the crude oil. Therefore, WAF contains the most toxic substances from oil. That as well as reproducibility of the results of WAF contributed to its usage as an exposure medium in toxicity experiments when evaluating the risk of oil contamination for aquatic organisms (Z. H. Jiang 2012).

Hydrophobic pollutants associate with organic-rich phases as sediments and biological tissues or escape aqueous phase by evaporation. Evaporation is a weathering process of a great importance especially for light crude oils since it can lead to a loss of up to 50-60% (Brandvik 2009). The fate of oil depends on physicochemical and biological parameters of compounds such as vapor pressure, solubility, lipophilicity chemical stability and resistance to biodegradation. However, characteristics of particular aquatic environment such as temperature, pH, oxygen content, dissolved organic matter, content of organic carbon and water currents are also of great importance (Jaffé 1991).

In fact, ocean currents and wind/wave conditions are considered as the key mechanisms of an oil drift, however different forcing variables may change depending on condition and location (Broström 2011).

1.1.2. Toxicity of oil compounds

BTEX are low molecular weight monoaromatic hydrocarbons and are moderately soluble and highly volatile. BTEX affinity for partitioning into tissue lipids of aquatic organisms and sorption to sediment organic matter is described as moderate (K_{ow} 2.13-3.2). Physical and chemical properties of BTEX make them non-persistent in seawater, but they weakly bind to marine sediments and are bioaccumulated to low concentrations by aquatic organisms (J. M. Neff 2002). However, in the North Sea, produced water from the gas wells usually contains higher concentrations of BTEX than produced water from oil wells (J. M. Neff 1996).

Polycyclic aromatic hydrocarbons (PAH) is the group of compounds found in crude oil that is of a great concern especially for the marine biota because of their toxicity and persistence in marine biota (J. M. Neff 1987). Studies show that the toxicity of oil increases after dissolving in solution, since it becomes more bioavailable for the organisms (Carls 2008). PAHs are known for their genotoxic, carcinogenic and reproductive effects. In addition, PAHs can bioaccumulate and thus transport further through the food chain. Studies also show that PAHs cause toxicity through narcotic mode of action (H.J.M. Verhaar 1992) and membrane destabilization in cells (B. A. Hansen 2017). Studies on dispersed oil toxicity proved reduced food uptake in cod larvae (B. H. Hansen 2016). Adverse effects of high concentrations of oil (4.1 – 5.6 mg oil/L) in *C. finmarchicus* can be observed as carapace discoloration and reduced swimming activity whereas low concentrations (0,08 mg oil/L) reduce feeding activity (B. A. Hansen 2017). Oils differ in composition and thus in toxicity, but in general oil toxicity increases with the length of the carbon chain and amount of benzene rings (Z. H. Jiang 2010).

Alkylphenols are considered as natural components of crude oil but are also applied as surfactants and emulsifying agents in many industrial products. They have hormone-disrupting effects affecting reproduction of fish and were found to be released in the North Sea with produced water (Meier 2007). Study by Meier et al. (2007) shows that alkylphenols are able to induce estrogen-resembling effects in male fish, affect steroid levels and disturb gonadal development in both, male and female fish at very low doses.

1.2. Calanus finmarchicus

Copepods are a major group of crustacean-plankton found worldwide. Among copepods important in the Barents Sea are C*alanus hyporboreus, Calanus glacialis* and *Calanus finmarchicus. C. finmarchicus* is predominantly herbivorous and dominates in the southern and western parts of the Barents Sea. The female is 2.6-4.0 mm long on average and reproduces around the time of phytoplankton spring bloom (Sakshaug 2009). This particular copepod is the main food source for many fish in the Northern Atlantic, making it an

economically important species since it plays a key role in the energy transfer between primary producers and many planktivorous fish and larvae (Runge 1988).

Development of *C. finmarchicus* starts from eggs and leads to six nauplii stages (N1-N6) where consecutive molting stages lead to larger and morphologically more complex larvae. Subsequent molting in copepodite stages (C1-C5) results in sexually mature, adult animals (C6) (Skaret 2014). Copepods grow and develop until summer or early autumn, then start preparing for migration into deeper water for over-wintering. *C. finmarchicus* is able to complete two generations in one year, whereas it takes one year to complete one life cycle north of approximately 68˚N (Sakshaug 2009).

C. finmarchicus begins to store lipids in an oil sac from the copepodite stage C3 and by stage C5 this oil sac reaches its maximum size which can comprise as much as 50% of the body volume (Miller 2000). In order to prepare for the diapause, *C. finmarchicus* delay processes like development and molt progression to sequester lipids in the form of wax esters which serve as an energy source for starvation during the diapause (Rey-Rassat 2002). The lipid reserve is used by the copepod during the food deprivation periods but it also contributes in the reproductive process (Mayor 2009).

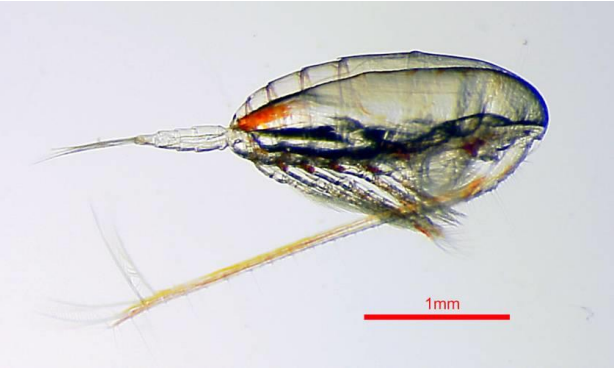


Figure 1. *Calanus finmarchicus* in CV stage with large lipid storage (Photo: Dag Altin, BioTrix)

1.3. Metabolomics

Metabolites are small molecules, either intermediate or products of metabolism, that can provide a functional metabolic profile of cellular biochemistry. Thanks to technologies such as NMR and mass spectrometry, even thousands of metabolites can be quantitatively or semi-quantitatively measured from a minimal amount of biological sample, making systems-level analysis possible (Reo 2002). There are two different types of metabolomics analysis: targeted analysis based on a hypothesis where a set of metabolites related to a one or more pathways are defined or untargeted analysis where many metabolites are measured and compared between the samples (Vinaixa 2012). Untargeted metabolomics is an important technique

based on global metabolite profiling used in analyses linking cellular pathways with biological mechanisms. By using such a technique, it is possible to understand complicated mechanisms by categorizing organ-specific toxicity as well as monitoring onset and progression of toxicological effects and to ultimately identify biomarkers of toxicity (Patti 2012).

Metabolomics serves as a very powerful tool in analysis of metabolites referring to the whole metabolic profile of the cell. Information obtained from NMR analysis when combined with genomics and proteomics can be used to construct computer network models to describe cellular functions (Reo 2002).

Metabolomics is also a useful technique for assessing interactions of organisms with the environment which also helps to evaluate organism function and health at the molecular level. This technique allows the analysis of interactions from the individual to the population level. Such results can be used to interpret from instantaneous effect to those occurring over evolutionary time scales also enabling studies of genetic adaptation. Ecophysiology studies of metabolomics in combination with factors such as changes in temperature, water, food availability, light, and atmospheric gases are increasingly popular in attempts to understand complicated processes. Similarly, ecotoxicogenomics discusses changes induced in both, aquatic and terrestrial ecotoxicology to establish organismal responses to xenobiotics in both, field and laboratory (Bundy 2009). Results of both these fields of studies allow for critical evaluation of the contribution of metabolomics to the environmental sciences and discuss recommendations for future uses.

1.4. Aims of the study

The aim of the presented study was to assess the adverse effects of oil pollution reflected in changes in metabolome profile in the copepod *C. finmarchicus* which was chosen as a suitable representation of zooplankton characteristic for the cold climate.

The WAF exposure was selected as a surrogate for exposure to oil spill pollution.

The experimental hypothesis was that the exposure to WAF of crude oil would result in temporal changes in concentrations of selected metabolites in copepod *C. finmarchicus*.

2. Materials and methods

The experiment was conducted in April 2017 at NTNUs Centre of Fisheries and Aquaculture (SeaLab). The test organisms, *C. finmarchicus* were adult, nonovulating females. The water accommodated fraction of oil was prepared from a naphthenic North Sea crude oil obtained from a Norwegian oil platform situated in the North Sea. The experiment consisted of an exposure time series where samples of *C. finmarchicus* exposed to oil were obtained after 24, 48, 72 and 96 hours of exposure, with four replicates at each time point. Additionally, 4 replicates were taken at time 0 (hours) from the same population at the start of the experiment, these samples served as a control.

2.1. Test organisms

Calanus finmarchicus was supplied from the company BioTrix, Trondheim. This inhouse culture was established in 2004 and consists of a multigeneration copepod culture from the Trondheim Fjord. Copepods were maintained in 280 L containers supplied with filtered sea water at 8-10˚C (Salinity: 30%). In the culture, feeding was adjusted to maintain the level of algal carbon in the cultures in excess of 150 µg C/L in order to keep normal growth and development of the copepods (B. A. Hansen 2017).

2.2. Preparation of WAF

Preparation of the WAF of oil was done at SeaLab, NTNU according to the standardized method recommended by CROSERF (Chemical Response to Oil Spills – Ecological effects Research Forum) guidelines, which allowed obtaining comparable results. The WAF of the oil was prepared with North Atlantic crude oil using a loading ratio of 1:40 of oil to filtered sea water. The oil was carefully added on the surface to create a slick and the mixture was submitted to gentle low energy magnetic stirring at ~13˚C to avoid the formation of oil droplets. After 72h of stirring, the water phase was siphoned off and cooled down before the start of the exposure experiment. A sample of WAF was taken for chemical analysis. Gas chromatography combined with mass spectrometry was used for analysis of total hydrocarbon content and individual oil components.

The WAF is a standardized medium used in oil exposure experiments because it is very difficult to generate environmentally realistic exposure scenarios with oil. In natural marine oil spills, the oil can be present in many different forms. Components of the oil can be volatile and/or water soluble and may evaporate and/or dissolve into the water column, causing the formation of WAF. Oil droplets may also form in the water column to a varying extent, resulting in physical rather than chemical effects. This makes it difficult to have a standard, replicable laboratory procedure that will be suitable for examining all possible effects of oil spills on marine organisms, such as both toxic effects of the chemical fraction of the oil in the water and

the physical effects of the oil droplets in the water. Thus, a standardized WAF exposure is often applied for ecotoxicological studies. However, in a real spill scenario, concentrations of WAF vary depending on factors like distance, weather, temperature and many others (Singer 2000).

2.3. Exposure medium

The concentration of oil (i.e., the WAF) used in the final experiment was established by determining the median LC_{50} after 96h of exposure in an additional acute exposure experiment. The LC_{50} corresponds to the concentration where 50% of the population dies. Thus, choosing an exposure concentration well below the LC_{50} amount allows for the study of sublethal toxic effects in copepods without causing pronounced lethality. The exposure media concentration consisted of a 9.85% dilution of the WAF concentration. Prepared WAF was transferred into 5L bottles and diluted with filtered sea water to the desired concentration. Bottles were closed with a Teflon-lined caps to prevent evaporation, then placed in 10˚C.

2.4. Exposure system

The exposure system consisted of 32 bottles (5L, Schott, Germany) containing the desired WAF concentration (exposure series) diluted in filtered seawater. 155 copepods were placed in each bottle. Control samples consisted of copepods kept in the filtered sea water without WAF. During the experiment animals were kept in in low light conditions, at 10˚C. Copepods were not fed during the exposure time.

2.5. Sample preparation

After the exposure, *Calanus finmarchicus* were gently poured down from the glass bottle into the glass bowl. Using sieves, copepods were caught and divided for different analyses. For metabolomic analysis 25 copepods from each bottle were transferred to marked glass vials, frozen in liquid nitrogen, then kept in freezer at -80°C for later analysis.

2.6. Sample extraction

Samples (25 copepods per sample) were homogenized in 2 mL standard tubes with 0.5g of 1.4 mm ceramic (zirconium oxide) beads in Precellys®24 tissue homogenizer with 390 µl of MeOH:H_2O 2:1. The program used for the homogenization consisted of two 15-second sessions with 5 second pause between the grinding sessions of multi-directional movement with a speed of 5500 rpm. Homogenized samples were transferred quantitatively to 2 mL glass vials using 210 µl of MeOH:H_2O 2:1 and 160 µl of distilled H_2O for flushing Precellys tubes. Next 400 µl of high purity (99.8%) chloroform (LiChrosolv®) was added, samples were vortexed two times for 30 seconds, then left on ice for 15 minutes. Samples were centrifuged for 5 minutes at 3000 rpm in 4°C for phase separation. One sample of 400 µl was taken from methanol phase for NMR

analysis and a second sample of 150 µl was taken for other analysis. From the chloroform phase, a sample of 250 µl was taken into a glass vial. Samples from the chloroform phase were dried under a stream of nitrogen for 30 minutes using TurboVap® LV concentration station. Samples from the methanol phase were dried using a Hetovac VR-1 centrifuge for 3h. Samples were kept on ice during the whole procedure. After drying, all samples were frozen at -80°C for further analyses.

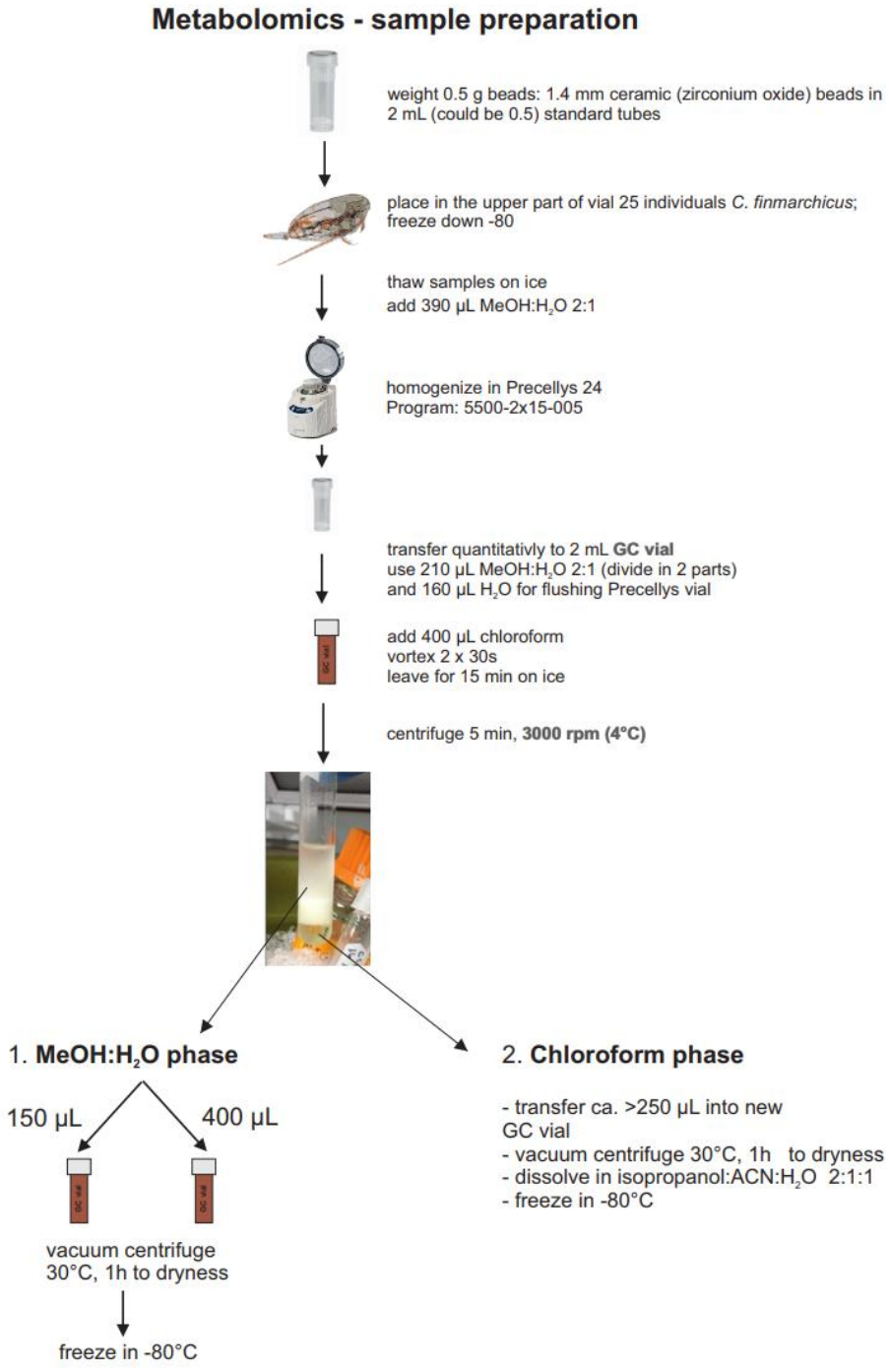


Fig.2 Scheme of the sample preparation procedure.

2.7. Chemical analysis

Chemical analyses of the semi-volatile compounds (SVOC) and volatile organic compounds (VOC) of the crude oil and prepared WAF were performed at SINTEF in Trondheim. Gas chromatography – mass spectrometry (GC-MS) techniques were used to analyze SVOC and Purge and Trap GC-MS for the analysis of VOC. The analyses of SVOC were performed on a single crude oil sample, a generated WAF (1:40 oil: water ratio), and the WAF exposure medium (which corresponds to 9,85% of generated WAF), and the WAF medium after 96h. For VOC analysis triplicates of crude oil were analyzed, while WAF analyses were run in duplicates. Results of the chemical analyses can be found in Appendix A and B.

2.8. NMR analysis

Nuclear magnetic resonance analysis was performed on methanol phase samples taken from homogenized and freeze-dried samples of *C. finmarchicus*. This was performed according to Hansen et al. (B. H. Hansen 2016). Samples were resuspended in 200 µl deuterated water (D_2O) solution containing 1 mM trimethylsilylpropionate (TSP) as an internal reference and transferred to 3-mm NMR tubes for analysis. ^{1}H-NMR spectra were recorded with a Bruker DRU 600 US+ spectrometer fitted with a cryogenic probe at 600MHz. Samples were stored at 4°C in the Bruker Samplejet autosampler during the analysis. Acquisition was conducted at 300 K, and the next sample was heated and dried during the acquisition of the preceding sample. 64 no of transients were recorded into data points using an acquisition time of 2.73 s. The used pulse program was Bruker noesygppr1d with a NOESY mixing time of 10 ms.

2.9. Statistical analysis

The Chenomx (Chenomx Inc., Edmonton) program was used to process and quantify metabolites. MestreNova (Mestrelab Research, S.L., Santiago de Compostela) was used to process and extract fingerprints for the untargeted analysis with PCA. The PCA was made with SIMCA (Umetrics, Sweden).

Data were transferred to Excel and statistical analysis of NMR results were performed using IBM SPSS statistical software. Analysis of variance (ANOVA) was used to compare between the treatment effects. Dunnett's test was used to compare the exposed groups against the control. The significance level was set to $p < 0.05$ for all testes.

3. Results

3.1. Chemical composition of the North Atlantic naphthenic crude oil

Semi-volatile compounds including phenols, naphthalenes and three- to five-ring polycyclic aromatic hydrocarbons were analyzed by gas chromatography-mass spectrometry (GC-MS) operated in selected ion monitoring (SIM) mode according to the method used in Hansen (2017).

3.1.1. Volatile organic compounds

Dominating substances detected in the crude oil VOC profile were methylcyclohexane (20.31 g/kg), cyclohexane (9.38 g/kg) and methylcyclopentane (6.36 g/kg). On the other hand, in the WAF samples toluene (1.61 g/kg), m-xylene (1.36 g/kg) and benzene (0.92 g/kg) dominated. Table 1 presents the concentrations of BTEX (benzene, toluene, ethylbenzene and three xylene isomers), which are well-known toxicants for both terrestrial and aquatic organisms (An 2004). This group comprises the majority of volatile compounds detected in the WAF medium (see Appendix A for more information).

Table 1. Concentrations of BTEX compounds found in oil (g/kg) and in WAF (µg/kg).

BTEX	Oil		WAF	
	Average [g/kg]	SD	Average [µg/kg]	SD
Benzene	0.5	0	918.02	96.55
Toluene	2.9	0.03	1611.76	268.06
Ethylbenzene	1.57	0.01	400.61	9.88
m-Xylene	5.38	0.06	1363.21	92.12
p-Xylene	1.73	0.02	128.50	16.49
o-Xylene	1.52	0.01	481.10	31.97
Sum BTEX	13.61	0.14	4903.02	534.12

3.1.2. Semi-volatile organic compounds

In crude oil, C1-C4-naphthalenes (7.55 g/kg) and C1-C4-decalins (5.51 g/kg) were the dominating compounds while in WAF samples naphthalene and C1-C4-alkylated homologues were prevailing (for more detailed information see Appendix B). Phenols were not detected in any of the samples but chemical analysis shows that majority of all detectable WAF compounds constitute naphthalene and homologues.

Table 2. Main classes of SVOC detected in WAF, WAF exposure medium and WAF after 96h. Concentrations are given in (µg/L). TEM stands for total extractable material.

Compound group	Mass [µg/L]		
	WAF	WAF exp. med.	WAF after 96h
TEM	3088.963	2786.592	198.625
∑ All identifiable compounds	269.946	218.197	21.675
∑ Decaline and C1-C4-alkylated homologues	0.806	0.706	0.023
∑ Naphthalene and C1-C4-alkylated homologues	253.502	207.417	19.896
∑ Phenantrene /anthracene and C1-C4-alkylated homologues	3.094	2.675	0.188
∑ Dibenzothiphene and C1-C4-alkylated homologues	1.312	1.136	0.108
∑ PAH 2 + rings*	12.369	10.780	0.895
∑ Phenols and C1-C5-alkylated homologues	0.000	0.000	0.000

*∑ PAH 2+ rings: benzothiophenes (C1–C4), acenaphthylene, acenaphthene, dibenzofuran, fluorenes (C1–C3), phenanthrenes (C1–C4), anthracenes (C1–C4), dibenzothiophenes (C1–C4), fluoranthenes (C1–C3), pyrenes (C1–C3), benz(a)anthracene, chrysenes (C1–C4), benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene, indeno(1,2,3-c,d)pyrene, dibenz(a,h)anthracene and benzo(g,h,i)perylene.

3.2. NMR profiling

There are 27 metabolites identified through ^{1}H-NMR spectroscopy technique: alanine, arginine, aspartate, betaine, choline, dimethylamine, glutamine, glycine, glycylproline, inosine, isoleucine, leucine, lysine, malonate, methionine, o-acetylcholine, o-phosphocholine, phenylalanine, proline, pyroglutamate, sarcosine, taurine, threonine, trimethyl-amine N-oxide, tyrosine, valine and sn-glycero-3-phosphocholine.

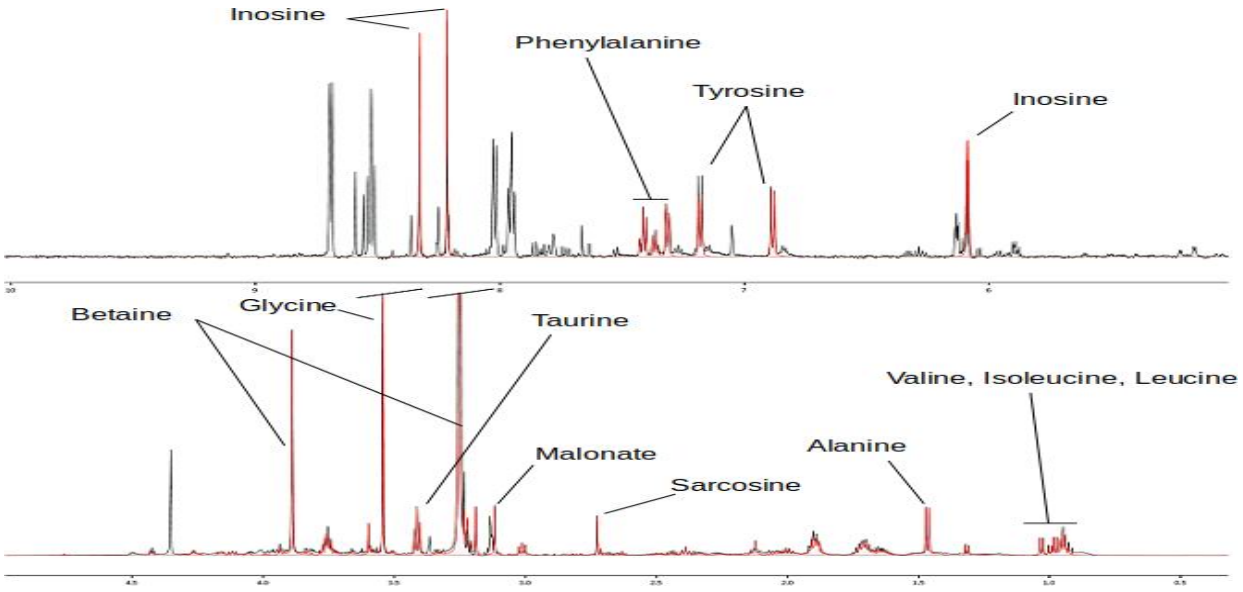


Figure 3. ^{1}H-NMR spectrum with annotations of the dominant peaks. The two regions of the NMR spectrum shown here are scaled to best represent the region and the intensities are not comparable between regions. Black is the full spectrum, whereas the red spectrum represents the signal from quantified components quantified using the Chenomx software.

The results of the selected set of metabolites (whenever statistical changes were detected) in the experiment are presented as graphs below. Complete results of the statistical analysis can be found in the Appendix C. In total, 16 out of the total 27 detected metabolites were detected with statistically significant changes in the concentration after the exposure.

The betaine concentrations show statistical significant difference between the treatments (p=0.0031) and the concentration in exposed groups is lower compared to the control (Fig. 4). The largest difference in concentration is after 96h where it decreased 22% in the exposed sample compared to the control sample. Dunnett's test results show that significant differences from the control (C00) are E48 (p=0.0393), E72 (p=0.0361) and E96 (p=0.0035).

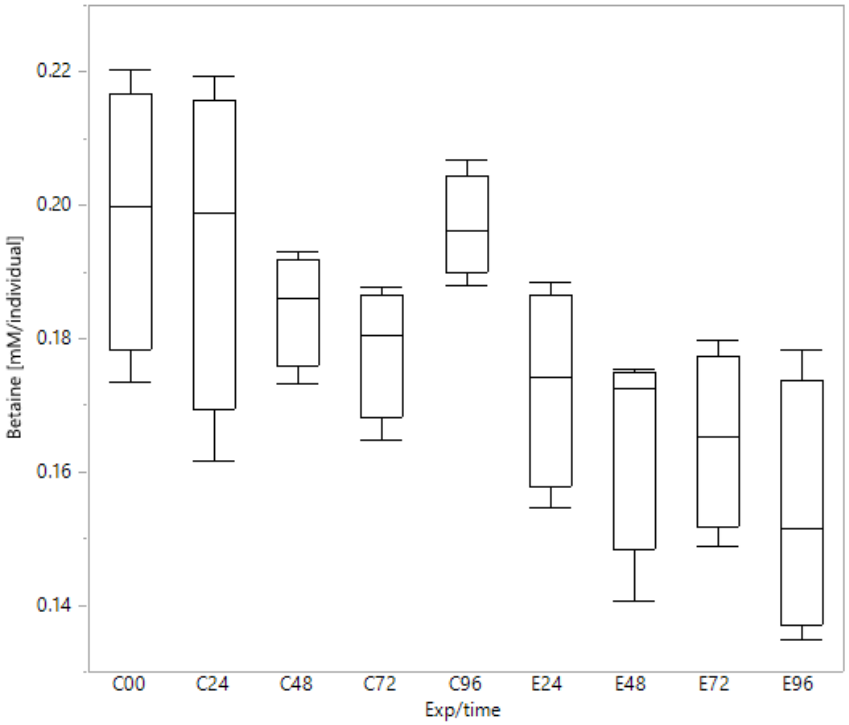


Figure 4. Change in concentration of betaine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is 0.0031. Results of Dunnett's test show that significantly different pairs are E48, E72 and E96. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

The glutamine concentrations show statistical significant difference between the treatments ($p<0.0001$) and concentrations were lower in the exposed groups compared to the control (Fig. 5). As compared to the controls, the glutamine concentrations were lower in the exposed groups: 59% after 24h, 50% after 48h, 48% after 72h, and the largest difference was observed after 96h when it was 63% lower in the exposed animals as compared to the corresponding control (C96). Dunnett's test results show that pairs of means that are significantly different from the control (C00) are C96 ($p<0.0001$), C72 ($p=0.0028$), E96 ($p=0.0153$), E48 ($p=0.0147$) and E24 ($p=0.0007$).

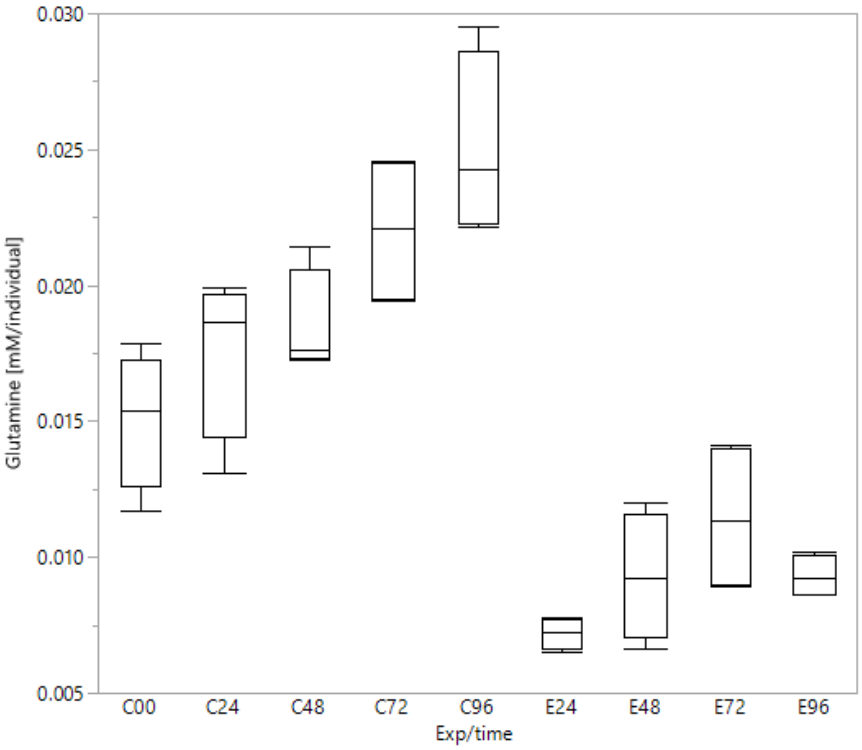


Figure 5. Change in concentration of glutamine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is <0.0001. Results of Dunnett's test show that significantly different pairs are C96, C72, E96, E48 and E24. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

The glycylproline concentration show statistical significant difference between the treatments (p=0.0067, Fig. 6). There is a slight decrease in concentration of glycylproline in exposed samples when compared to the controls. The biggest difference in the concentration can be observed after 72h of WAF medium exposure with a decrease of 40% compared to the corresponding control (C72). Dunnett's test results show that pairs of means that are significantly different from the control (C00) are E48 (p=0.0343), E24 (p=0.0184), E72 (p=0.0021), E96 (p=0.0018).

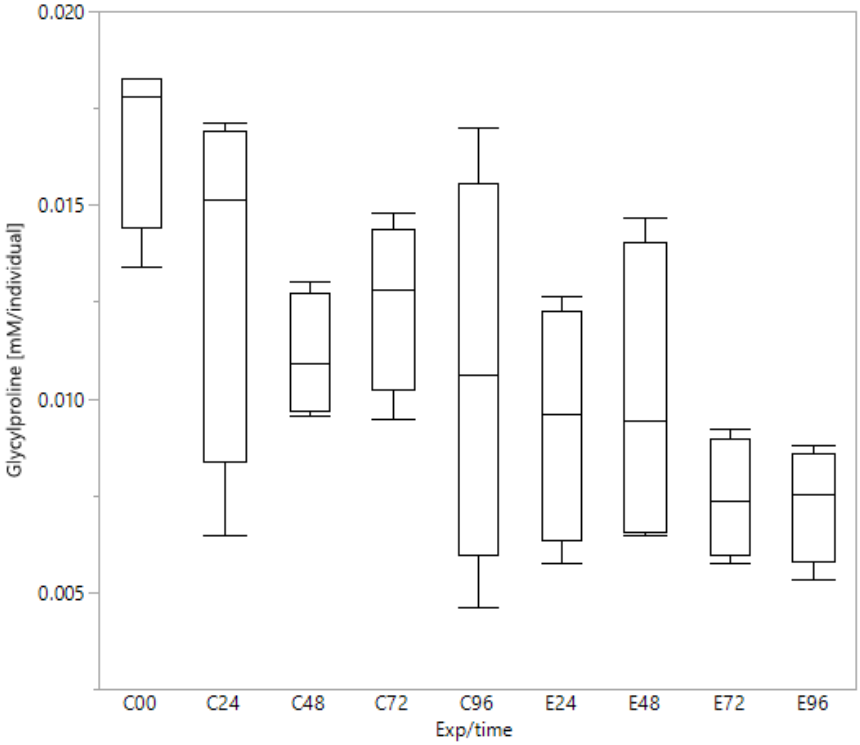


Figure 6. Change in concentration of glycylproline in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is 0.0067. Results of Dunnett's test show that significantly different pairs are E48, E24, E72, and E96. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

The isoleucine concentrations show statistical significant difference between the treatments (Fig. 7, p=0.0049). The concentration of isoleucine was lower in the exposed samples as compared to C00, and seems to stay relatively constant over time. In the controls there was a decline after 24h and after that the concentrations started to slowly increase. The difference in the concentrations among the control and exposed samples is rather small with the highest difference of 28% occurring after 96h. Dunnett's test results show that the concentrations in the exposed animals were significantly different from the control (C00) for E96 (p=0.0260), E72 (p=0.0221) and E24 (p=0.0040).

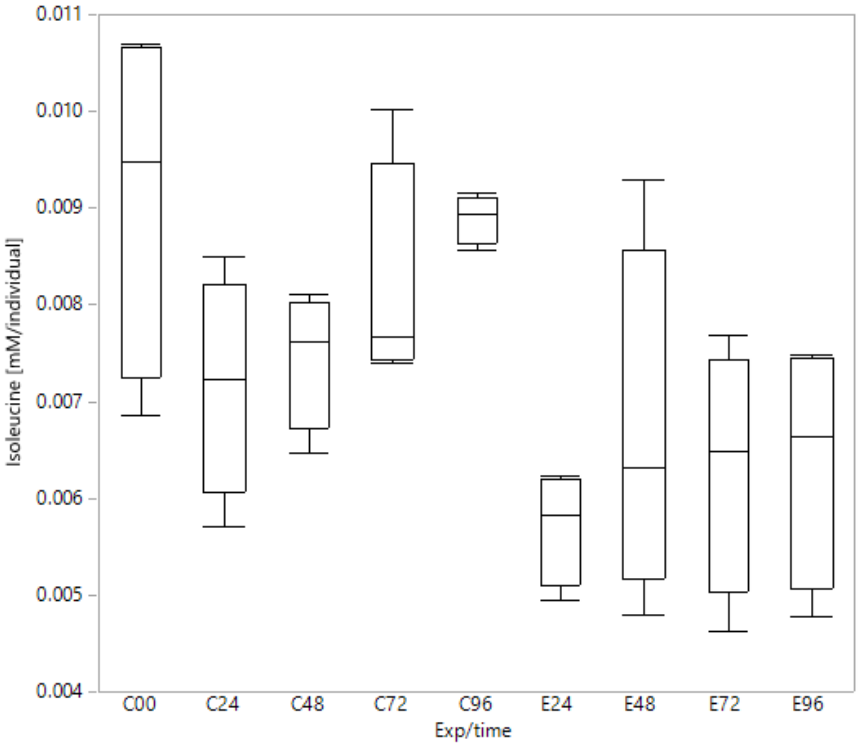


Figure 7. Change in concentration of isoleucine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is 0.0049. Results of Dunnett's test show that significantly different pairs are E96, E72 and E24. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

The leucine concentrations show statistical significant difference between the treatments (Fig. 8, p=0.0010). As compared to C00, the concentrations of leucine were lower in all experimental samples, controls and exposed. There is no big difference in concentration within the groups (control and exposed) over time. The largest difference between the groups (control and exposed) is 19% after 24h exposure to WAF medium. Dunnett's test results show that the experimental groups that are significantly different from the control (C00) are: C96 (p=0.0325), C24 (p=0.0118), C72 (p=0.0067), C48 (p=0.0048), E96 (p=0.0011), E48 (p=0.0007), E72 (p=0.0006) and E24 (p<0.0001).

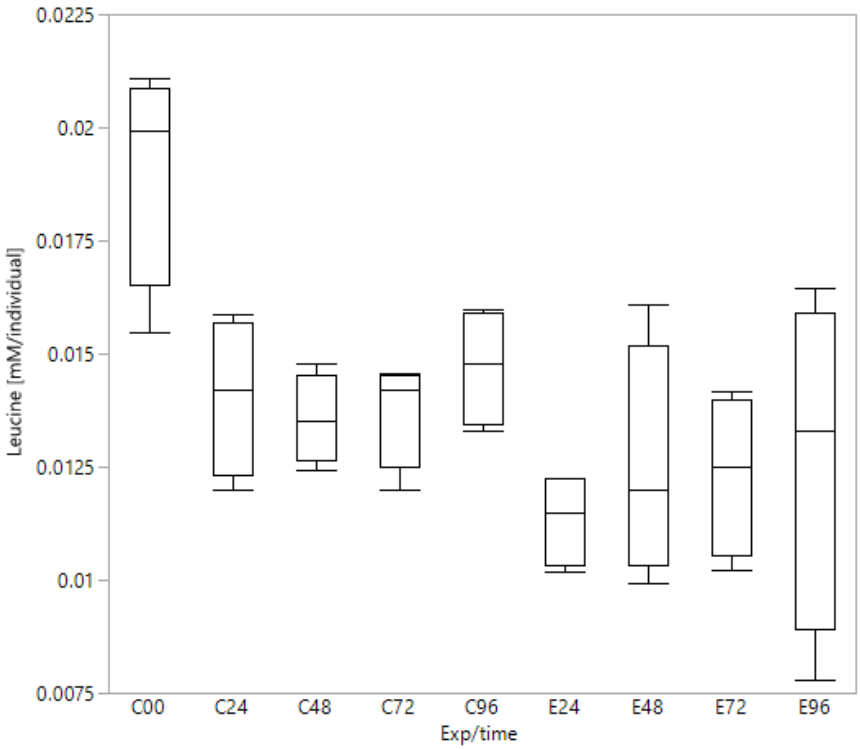


Figure 8. Change in concentration of leucine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is 0.0010. Results of Dunnett's test show that significantly different pairs are C96, C24, C72, C48, E96, E48, E72 and E24. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

The lysine concentrations show statistical significant difference between the treatments (Fig. 9, p=0.0065). As compared to C00, the concentration of lysine was lower in all samples of both control and exposed treatments. There is a delicate shift in concentration of exposed samples, where concentrations are lower than in controls but again time of the exposure does not seem to influence much these concentrations. The largest difference in concentrations when comparing control and exposed treatments is 30% after 72h. Dunnett's test results show that the experimental groups that are significantly different from the control (C00) are C48 (p=0.0190), E96 (p=0.0120), E48 (p=0.0036), E72 (p=0.0034) and E24 (p=0.0016).

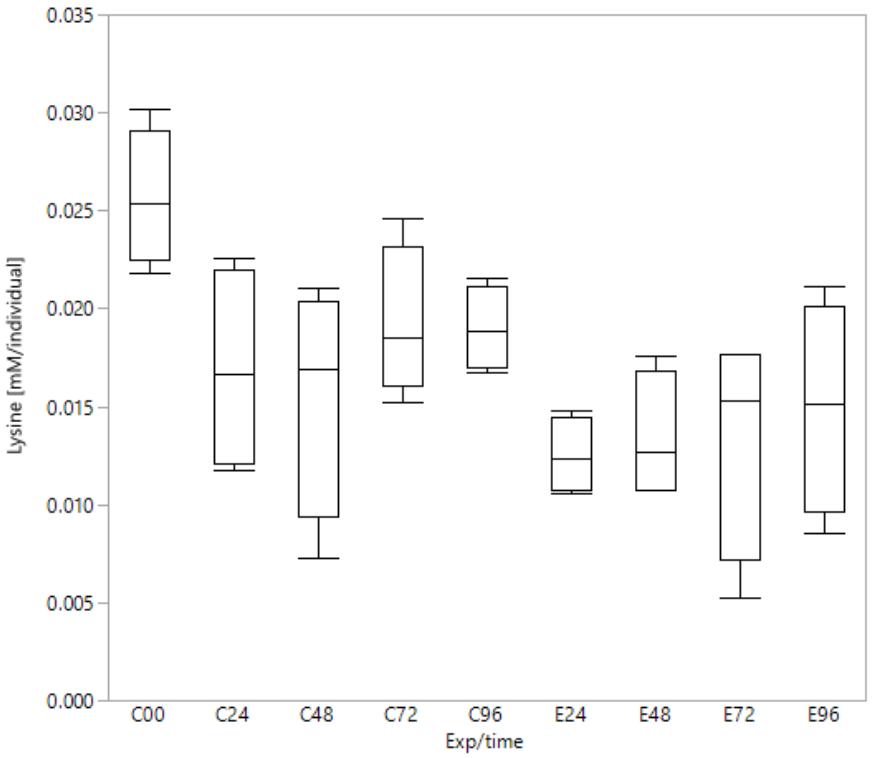


Figure 9. Change in concentration of lysine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is <0.0065. Results of Dunnett's test show that significantly different pairs are C48, E96, E48 , E72, and E24. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

The malonate concentration show statistical significant difference between the treatments (Fig. 10, $p<0.0001$). Changes in concentration of malonate present the largest change of all analyzed metabolites (18% increase after 24h, 57% after 48h, 99% after 72h and finally 113% after 96h of exposure as compared to control groups). Concentrations decreased over time in the control samples, whereas in exposed samples the concentrations increased. Dunnett's test results show that the experimental groups that are significantly different from the control (C00) are E96 ($p=0.0001$), E72 ($p=0.0053$), C96 ($p=0.0299$) and C72 ($p=0.0191$).

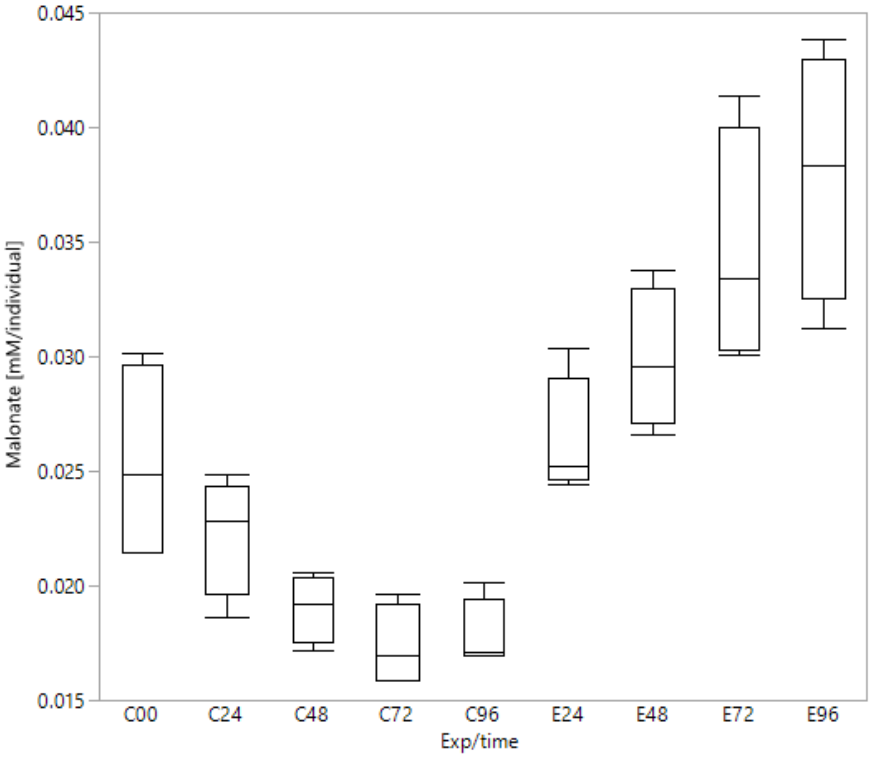


Figure 10. Change in concentration of malonate in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is <0.0001. Results of Dunnett's test show that significantly different pairs are E96, E72, C96 and C72. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

The methionine concentrations show statistical significant difference between the treatments (Fig. 11, $p<0.0001$). Concentration of methionine also decreased in all of the samples in comparison to the control sample C00. Concentration of exposed samples was slowly increasing over time but this tendency is stronger in control samples. Those changes result in 44% decrease in concentration in exposed samples after 24h, 24% after 48h, 47% after 72h and 41% after 96h as compared to control groups . Dunnett's test results show that pairs of means that are significantly different from the control (C00) are C48 ($p=0.0023$), E96 ($p=0.0005$), C24 ($p=0.0004$), E48 ($p<0.0001$), E72 ($p<0.0001$) and E24 ($p<0.0001$).

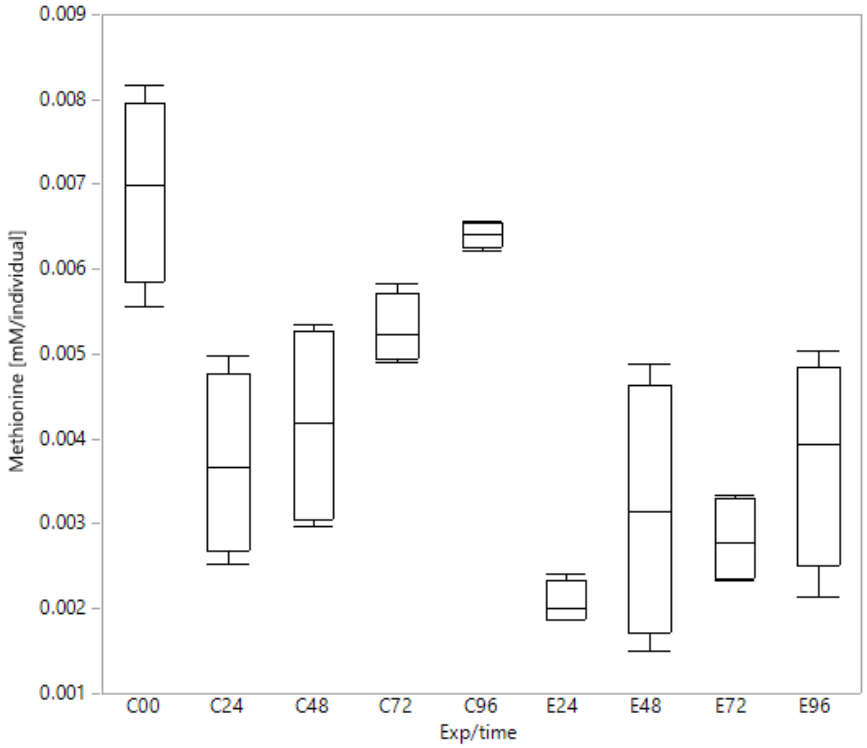


Figure 11. Change in concentration of methionine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is <0.0001. Results of Dunnett's test show that significantly different pairs are C48, E96, C24, E48, E72 and E24. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

Results of o-acetylcholine concentration show statistical significant difference between the treatments (p=0.0193). Concentrations of o-acetylcholine were upregulated in all of the samples. The biggest change in concentration between the treatment groups (control and treatment) can be observed after 48h where it decreased about 22%, where the rest of the changes are below 15%. Dunnett's test results show that pairs of means that are significantly different from the control (C00) are C24 (p=0.0074), E72 (p=0.0359) and E24 (p=0.0437).

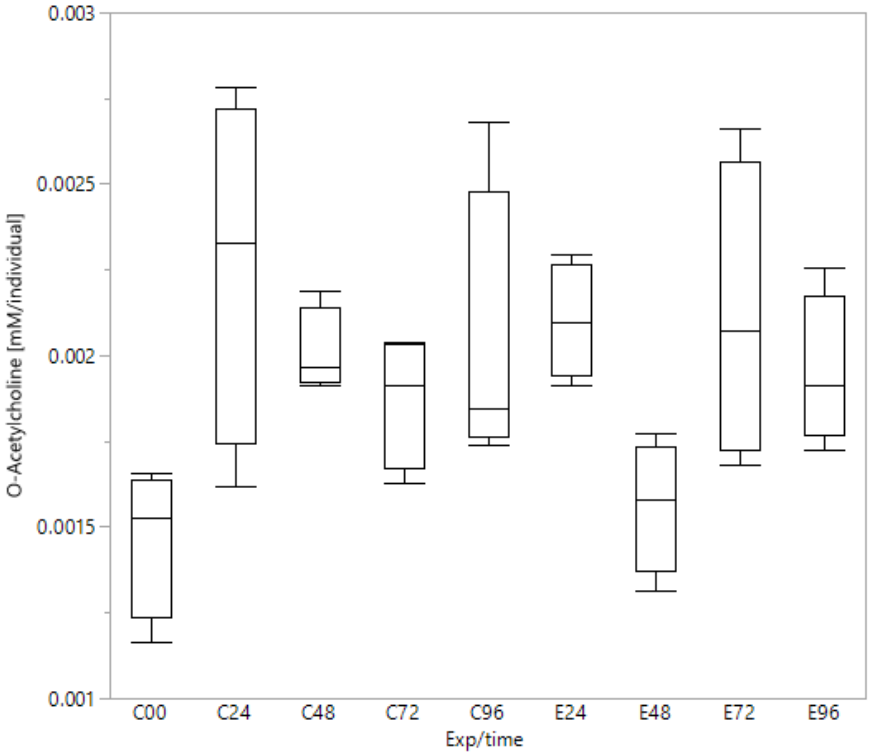


Figure 12. Change in concentration of o-acetylcholine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is 0.0193. Results of Dunnett's test show that significantly different pairs are C24, E72, and E24. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

Results of phenylalanine concentration show statistical significant difference between the treatments (p=0.0154). Concentrations of phenylalanine decreased in both control and exposed samples. In the control samples, the concentration was slowly decreasing but after 96h there is an increase. In the exposed samples concentrations seems to be relatively unchanging, but there is also upregulation after 96h of exposure. Difference in the concentration between the control and exposed samples shows a 19% decrease after 24h and 18% decrease after 96h while differences after 48h and 72h are below 15%. Dunnett's test results show that pairs of means that are significantly different from the control (C00) are E48 (p=0.0126), E72 (p=0.0109) and E24 (p=0.0055).

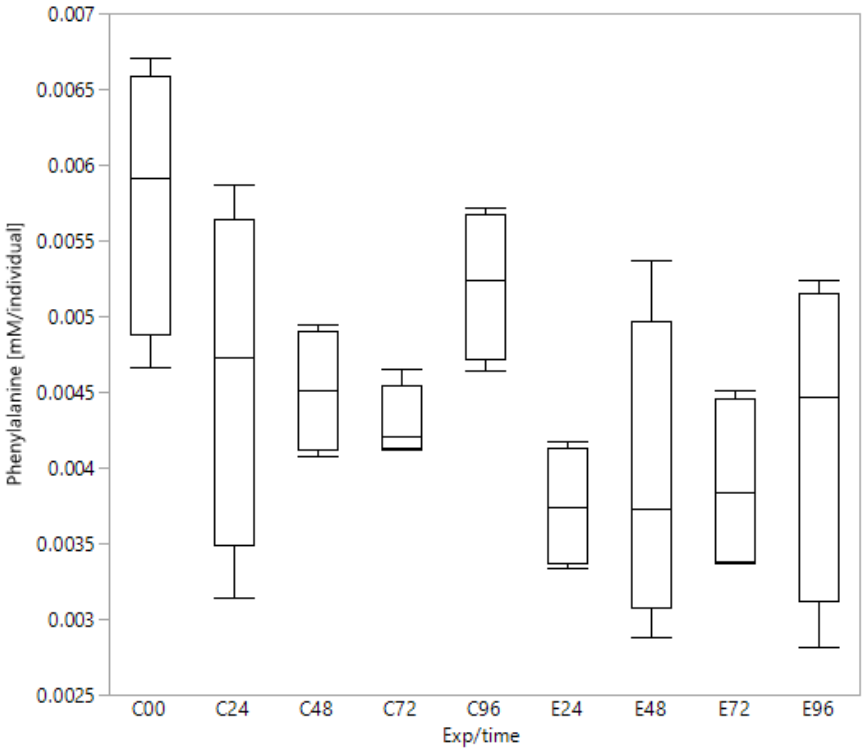


Figure 13. Change in concentration of phenylalanine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is 0.0154. Results of Dunnett's test show that significantly different pairs are E48, E72 and E24. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

Results of proline concentration show statistical significant difference between the treatments ($p<0.0001$). Concentration of proline is reduced in all of the samples. In the controls, the concentration is decreasing, but after 96h it is slightly upregulated compared to the rest of the samples. There is a 68% decrease in the concentration after 24h in the exposed sample compared to the control sample, 62% after 48h, 46% after 72h and 55% after 96h. Dunnett's test shows that almost all of pairs of means are significantly different from the control (C00), besides C24. Significant changes are C48 ($p=0.0013$), C96 ($p=0.0009$), C72 ($p<0.0001$), E96 ($p<0.0001$), E24 ($p<0.0001$) and E72 ($p<0.0001$).

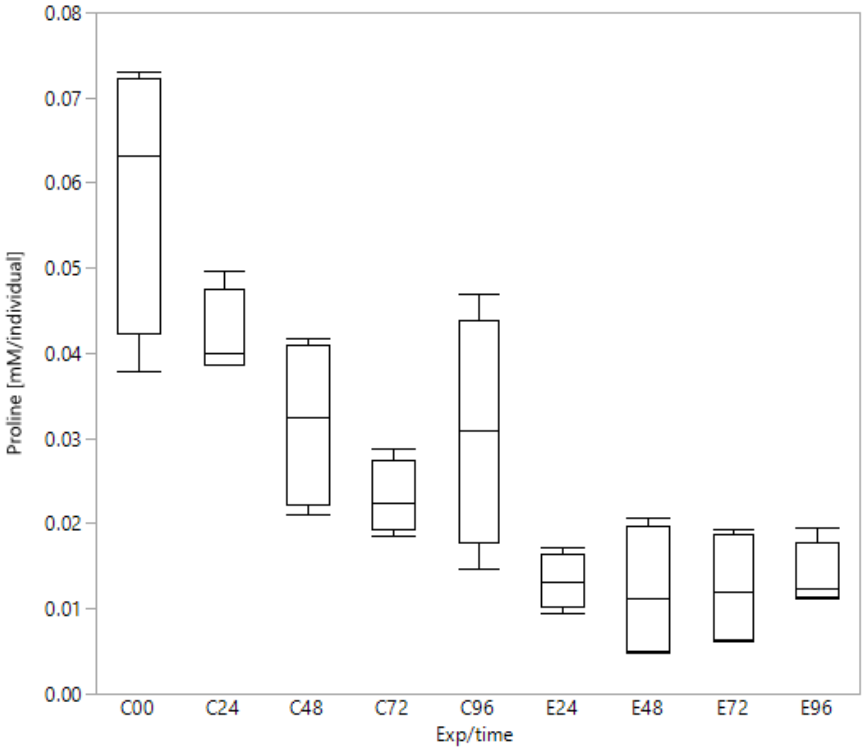


Figure 14. Change in concentration of proline in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is <0.0001. Results of Dunnett's test show that significantly different pairs are C48, C96, C72, E96, E24 and E72. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

Results of sarcosine concentration show statistical significant difference between the treatments ($p<0.0001$). In case of sarcosine difference in concentration is significantly induced in case of exposed samples but not in the control samples as compared to control C00. Exposed samples present decreased concentrations of the analyzed metabolite (46% after 96h, 32% after 72h, 30% after 48h and 21% after 24h), whereas in control samples concentration seems to stay on a similar level without significant influence of time. Dunnett's test results show that pairs of means that are significantly different from the control (C00) are E24 ($p=0.0021$), E48 ($p<0.0001$), E72 ($p<0.0001$) and E96 ($p<0.0001$).

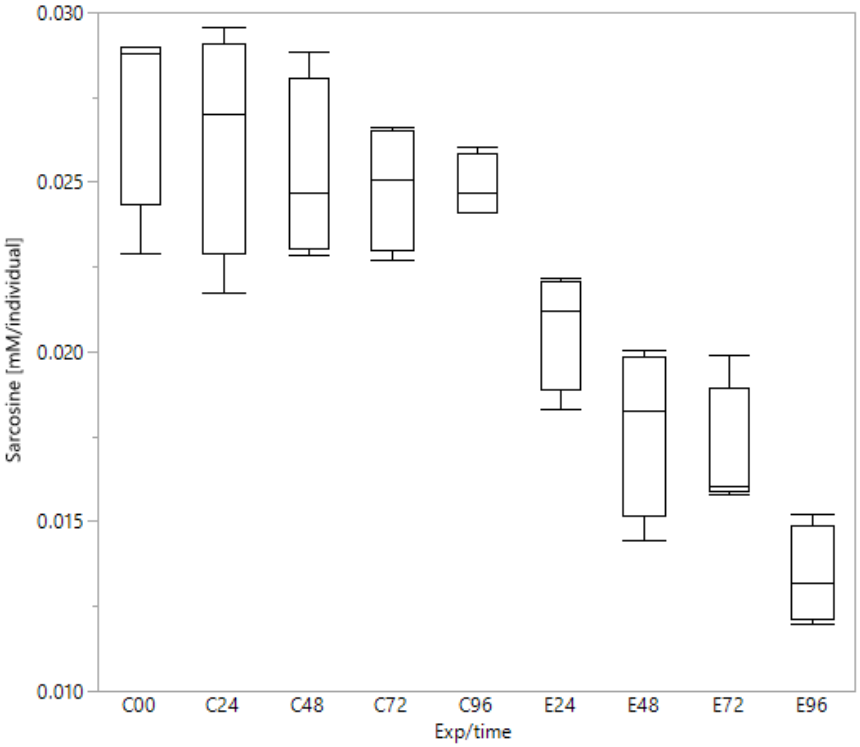


Figure 15. Change in concentration of sarcosine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is <0.0001. Results of Dunnett's test show that significantly different pairs are E24, E48, E72 and E96. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

Results of taurine concentrations show statistical significant difference between the treatments (p=0.0062). Concentration of taurine is also decreased in all of the samples compared to the control C00. One of the control samples after 48h seems to give a much lower concentration of the analyzed metabolite resulting in a much bigger range of the results for this time exposure than in the other samples. Exposed samples continue decreasing in concentrations, which are slightly lower than in the corresponding controls (30% decrease after 96h in the samples exposed to WAF medium, and below 15% in the rest of the samples). Dunnett's test results show that pairs of means that are significantly different from the control (C00) are E72 (p=0.0280), E48 (p=0.0075) and E96 (p=0.0022).

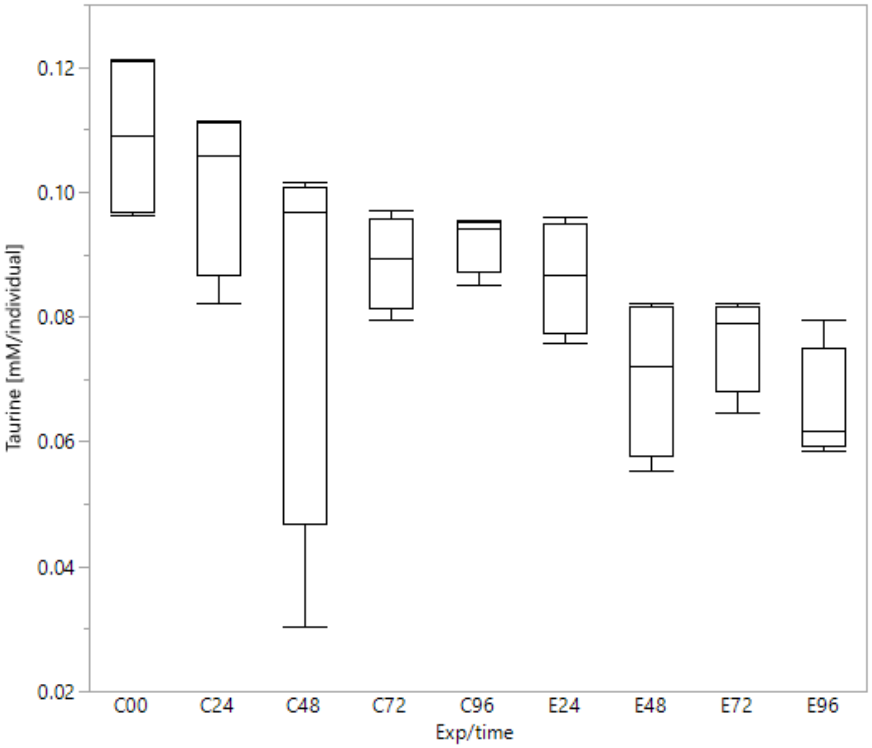


Figure 16. Change in concentration of taurine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is 0.0062. Results of Dunnett's test show that significantly different pairs are E72, E48 and E96. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

Results of threonine concentration show statistical significant difference between the treatments (p=0.0016). As in the case of the previously described metabolite, there is much lower concentration in one of the control samples after 72h exposure which results in much broader range of the concentration than in other samples. Here the decrease in the concentration was between 16-23% among the samples. Dunnett's test results show that pairs of means that are significantly different from the control (C00) are C48 (p=0.0234), C72 (p=0.0036), E96 (p=0.0030), E48 (p=0.0015), E24 (p=0.0014) and E72 (p=0.0003).

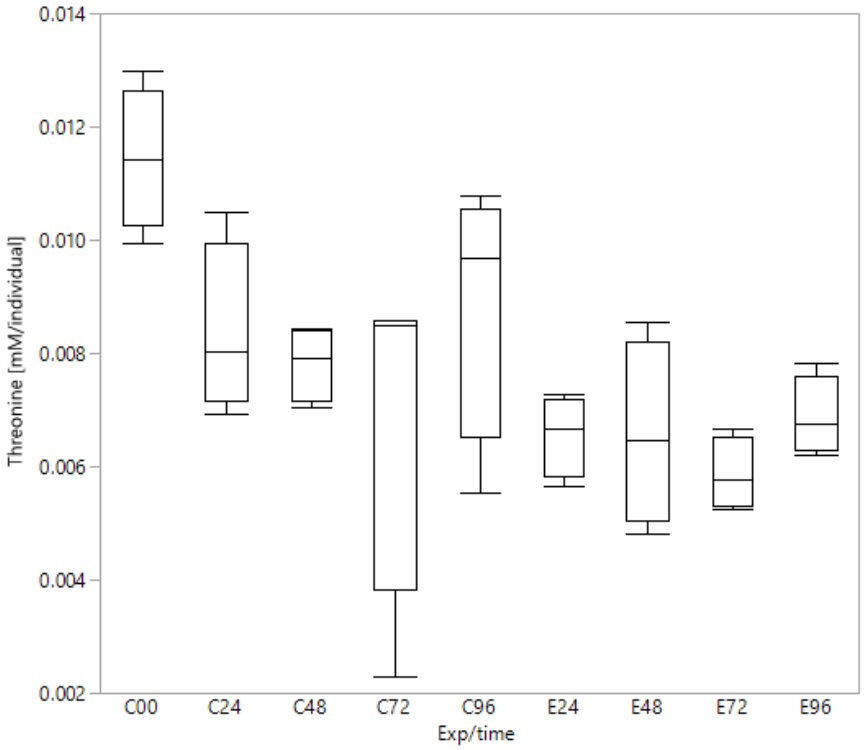


Figure 17. Change in concentration of threonine in Calanus finmarchicus (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is 0.0016. Results of Dunnett's test show that significantly different pairs are C48, C72, E96, E48, E24 and E72. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

Tyrosine concentrations show statistical significant difference between the treatments ($p<0.0001$). Concentration of tyrosine decreased in all of the samples in comparison to the control C00. There is also a slight increase in concentrations of both the control and exposed samples after 96h. Differences in concentrations among the samples are also at low level, between 22% (48h) to 34% (24h). Dunnett's test results show that pairs of means that are significantly different from the control (C00) are C24 ($p=0.0303$), C72 ($p=0.0076$), E48 ($p=0.0006$), E96 ($p=0.0005$), E72 ($p<0.0001$) and E24 ($p<0.0001$).

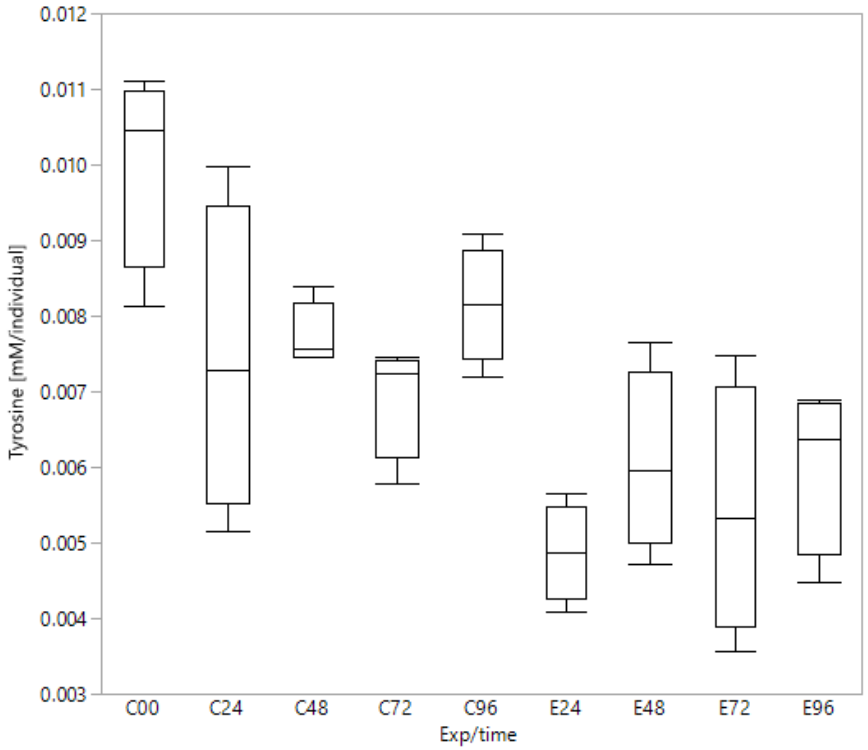


Figure 18. Change in concentration of tyrosine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is <0.0001. Results of Dunnett's test show that significantly different pairs are C24, C72, E48, E96, E72 and E24. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

Results of sn-glycero-3-phosphocholine concentration show statistical significant difference between the treatments (p=0.0437). Observed changes in the concentration of sn-glycero-3-phosphocholine are the smallest among detected metabolites. Here concentration decreased over time in both, control and exposed samples. Here changes among the samples in the same time exposure are also on a low level (1-16%). Dunnett's test results show that pairs of means that are significantly different from the control (C00) are E72 (p=0.0360), C72 (p=0.0331) and E96 (p=0.0163).

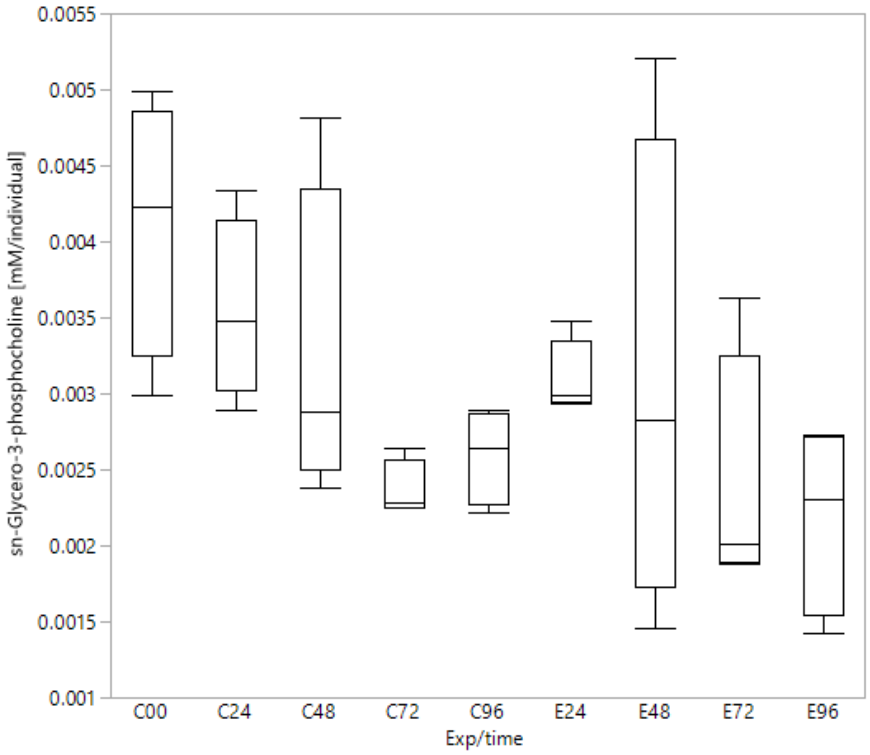


Figure 19. Change in concentration of syn-glycero-3-phosphocholine in *Calanus finmarchicus* (n=100 per treatment) after 24, 48, 72 and 96h exposure in control samples and in samples of oil WAF. P-value of ANOVA is 0.0437. Results of Dunnett's test show that significantly different pairs are E72, C72 and E96. Samples marked as C00 are the controls taken at the beginning of the experiment. Each of the bars on the graph consists of 4 groups (4 bottles) with 25 animals in each of them giving in total 100 animals per each point on the graph (treatment). Bars have marked median and whiskers present minimum and maximum values. Controls are marked as C and exposed samples are marked with E each with corresponding time of exposure.

4. Discussion

4.1. WAF toxicity

BTEX (benzene, toluene, ethylbenzene and three xylene isomers) has significant shares in the composition of generated WAF, whereas for semi-volatile compounds – naphthalene is the dominating compound (Table 3). Most of the detected compounds are volatile, and naphthalene accounts for only 1.86% of the total petroleum hydrocarbons in the WAF. During the exposure bottles were sealed with lids with Teflon lining to prevent evaporation and uncontrolled loss of volatile compounds to test their influence. However, in a real-life oil spill, these compounds would rather escape the marine environment by evaporation.

Table 3. Concentrations of major compounds detected in the WAF of naphthenic North Atlantic oil (µg/L). Besides naphthalene which is semi-volatile compound, all of the presented chemical compounds are volatile compounds.

Compound	conc. in WAF medium [µg/L]	Class
Toluene	1611.76	VOC
m-Xylene	1363.21	VOC
Benzene	918.02	VOC
Cyclohexane	735.44	VOC
o-Xylene	481.10	VOC
Ethylbenzene	400.61	VOC
Methylcyclopentane	322.42	VOC
Cyclopentane	361.12	VOC
Methylcyclohexane	322.42	VOC
Isopentane	277.960	VOC
1,2,4-Trimethylbenzene	185.90	VOC
1-Methyl-3-ethylbenzene	162.69	VOC
Naphthalene	137.84	SVOC
p-Xylene	128.50	VOC

A previous study on *C. finmarchicus* shows that a concentration as low as 0.08 mg oil/L distorts feeding activity as observed through algae uptake, gut filling and fecal pellet production (Hansen et al. 2017). Furthermore, that study shows that the reduction in metabolite concentrations between two types of oil were similar and suggests that responses were non-oil type specific. Finally, the oil-microdroplets seems to contribute to a starvation-type response, which is reflected as a reduction of metabolites concentration (B. A. Hansen 2017).

4.2. Metabolome profile

Most of the identified metabolites show statistically significant change in the concentration over time during the experiment in both the exposed and the control groups. These changes could be explained by both exposure to the WAF medium and to the starvation effect. In this experiment, starvation seems to result in changes in concentration of many amino acids, their precursors and betaine. This can be concluded by comparing the control groups with the C00 group. Both the control groups and the exposed groups did not receive food during the experiment and were thus starving. From the presented results, only malonate shows very strong and clear induction (100% after 72h and 113% after 96h exposure as compared to control groups) in the exposed specimens. On the other hand, malonate concentrations decreased in the control samples over time, suggesting an effect induced by the starvation (Figure 9).

Malonate is a competitive inhibitor of the succinate dehydrogenase enzyme (also called respiratory complex II) found in the inner mitochondrial membrane of eukaryotes and many bacterial cells. Thus, malonate competes with the usual substrate of the enzyme – succinate (Potter 1951). Malonate decreases cellular respiration which is a key process that take place in the cells of organisms to convert biochemical energy from nutrients into adenosine triphosphate (ATP) which serves as "molecular unit of currency" of intracellular energy transfer (Knowles 1980). Changes observed in malonate concentrations between the exposure groups suggest induction of malonate by oil exposure what may possibly results in cellular respiration processes in cells of *C. finmarchicus*. Such induction of concentration after the exposure to oil suggests that malonate may be a good biomarker of oil exposure in adult *Calanus finmarchicus*.

The next strongest changes between the treatment groups were found for proline with a high downregulation of 68% after 24h and 62% after 48h exposure to WAF medium in comparison to the respective control samples. This reduction was lower in the case of the 72h exposure (46%) and the 96h exposure (55%). However, besides the control sample after 24h, all of the samples present statistically significant change in the concentrations. The concentration of proline was downregulated in the control samples and for the exposure samples a similar reduction can be observed. This suggests that both, starvation and the WAF medium exposure are causing downregulation of the proline concentration in *Calanus finmarchicus*. Proline is a non-essential amino acid, and can be synthesized from the non-essential amino acid L-glutamate. In general, proline is considered a constituent of many proteins. One example is collagen where proline can be found in high concentrations, and it has been shown that proline constitutes almost one third of collagen. Collagen is the major supportive protein in tissues such as skin, tendons, bones and connective tissues (services 2018). In addition, it promotes its health and healing process. A study on fish amino acid nutrition suggests that proline is considered to be dispensable amino acid and promotes feed intake (P. M. Li 2009).

Concentrations of glutamine were strongly reduced (63%) after 96h exposure to WAF medium, 60% after 24h and about 50% after 48h and 72h exposure. These changes are similar to the effects observed in the control samples. This can indicate that starvation results in upregulation of the metabolite over time. Exposure to the WAF medium seems to reduce or impair this effect, as seen in Figure 4. Nevertheless, the result also indicates that the exposure to WAF medium seems to decrease metabolite concentration in the first 48h after the oil exposure. Changes in glutamine concentrations in exposed samples are similar to the effect of the starvation observed in the control samples as the concentrations of the metabolite increased.

Glutamine is considered as a conditionally essential α-amino acid, which means that organisms usually can synthesize sufficient amounts of it. However, in some cases of stress, glutamine demand increases, and this amino acid must be obtained from the diet. Glutamine serves as respiratory fuel for rapidly proliferating cells such as enterocytes and lymphocytes, and important precursor of nucleic acids, nucleotides, amino sugars and proteins (Lacey 1990). It is also a key component in the nitrogen metabolism (Zielke 1984). According to the research on the biochemical composition of copepods, glutamine/glutamic acid, leucine, alanine and glycine were amongst most abundant protein-bound amino acids (van der Meeren 2008). Glutamine is crucial to immune response in fish. It serves as a main energy source for leukocytes and an important modulator of cytokine and NO production (P. M. Li 2009).

The rest of the presented results do not show changes in concentrations between the treatment groups (control and exposed) higher than 50%.

Betaine is an oxidative metabolite of choline and is also involved in methylation reactions and detoxification of homocysteine. Betaines serve as osmolytes which can be synthesized or taken up from the environment for protection against osmotic stress, drought, high salinity or temperature. Betaine is also an important compound in sulfur-amino acid metabolism and participates in the synthesis of methionine from homocysteine (Kim 2002). In the present experiment betaine concentration differences between control and exposed treatments were very small (22% and below), which suggests that starvation had stronger influence than the oil exposure (Figure 4).

Glycylproline is an end-product of collagen metabolism that in consequence is cleaved by prolidase. The arising molecules of proline are recycled into collagen or other proteins (National Institute of Health 2018). In the presented results (Figure 6) the changes in the concentrations of glycylproline are relatively small (the largest change - 40% - after 72h of exposure to WAF medium) and is slowly decreasing over time in both, control and exposed treatments. This result demonstrates starvation's effect.

Isoleucine is an essential α-amino acid in humans and is used in biosynthesis of proteins. Isoleucine assists in wound healing, detoxification of nitrogenous wastes, and stimulating immune system functioning as well

as stimulating and promoting several hormones. It is also a key contributor in hemoglobin formation and regulation of sugar level in blood and is concentrated in muscle tissues (Institute 2018). The concentration of isoleucine decreased after 24h of the exposure in control samples but after 96h of exposure, the concentration returns to the starting concentration of C00. On the other hand, in the samples exposed to WAF, the decrease in concentration is stronger after 24h (20% stronger than in C24) and the increase is much slower than in the control samples. This suggests that the oil medium affects isoleucine concentration.

Leucine is an essential amino used in biosynthesis of proteins. Leucine is considered to reduce degradation of muscle tissue in aged rats through increasing synthesis of muscle proteins (Combaret 2005). Leucine takes part in cell signaling in fish (P. M. Li 2009) and is functional amino acid in stimulating muscle protein synthesis in mammals (Nakashima 2007). The decrease in leucine concentration after the exposure to WAF of oil suggests low alteration (max 19% change in concentration between the treatments after 24h) of oil in energy production by downregulation of synthesis of muscle protein (Wilkinson 2013). Decreased concentrations in both treatment groups (control and exposed) suggest an effect of starvation.

Lysine is an α-amino acid, precursor of carnitine compounds and takes part in biosynthesis of fatty acids (Dall 1987). The results show that exposure to the medium of WAF of oil results in a low (max - 30% after 72h exposure) downregulation of pathways involved in lysine synthesis. Both, lysine and methionine can serve as tissue amino acids reserves stored for periods with limited access to food like molting (Maity 2012). The study of Kitabayashi et al. indicates that methionine has a crucial role in maintaining high growth of Kuruma shrimp (*Penaeus japonicas*) (Kitabayashi 1971).

Methionine is an essential amino acid in humans and is crucial in processes such as of angiogenesis, protein synthesis, methylation of DNA and polyamide synthesis (Cavuoto 2012). Methionine is also the substrate of amino acids such as cysteine and taurine and is crucial in the metabolism and health of many species. Changes in both methionine and lysine concentrations in *C. finmarchicus* does not seem to be highly influenced by the oil exposure. Concentrations are modified in the same way in both control and exposed treatments. The concentration of methionine decreased as compared to the control C00 after 24h of exposure (44% as compared to C24) but are slowly increasing over time indicating incorporation of these amino acids in energy production. Low induction of methionine concentrations after oil exposure suggest an effect of starvation.

O-Acetylcholine is a neurotransmitter used in signaling to other cells as neurons, muscle and gland cells and is synthesized in certain neurons from choline and acetyl-CoA (Tiwari 2013). In the experiment concentration of o-acetylcholine increased in the 24h in both treatment groups (control and exposed). In the next hours of the exposure, the concentration of metabolite decreases in the control samples, but in exposed

samples it seems to stay on the similar lever with the exception of the sample after 48h where the biggest decrease in concentration (as compared to C48) is observed (22%). Low induction of o-Acetylcholine concentrations after oil exposure suggest an effect of starvation.

Phenylalanine is an amino acid that can be obtained in the process of hydrolysis of common proteins. It can be found in high concentrations in hemoglobin and serves as one of the essential amino acids for fowls and mammals, whereas microorganisms synthesize phenylalanine from glucose and pyruvic acid. In addition, phenylalanine is tyrosine precursor (Go 2015). Changes in concentration between the treatment groups (control and exposed) are low (max - 19% after 24h exposure). Low induction of phenylalanine concentrations after oil exposure suggest an effect of starvation.

Sarcosine is an amino acid derivative, an intermediate and byproduct of glycine synthesis and degradation. It is found in muscles and other body tissues. Sarcosine is formed through metabolism of both choline and methionine. It is quickly degraded to glycine which is an important component of proteins and is main metabolic source for living cell-components such as glutathione, creatine, purines and serine (Mudd 1980). Concentrations of sarcosine in control samples are not influenced by the time of the exposure, whereas concentrations of exposed samples significantly decreased over time (46% after 96h). This modulation of sarcosine concentrations after oil exposure indicate the effect of oil on this metabolite.

Taurine is an amino sulfonic acid, a building block of proteins widely distributed in animal tissues. It is a major constituent of bile and takes part in conjugation of bile acids, antioxidation, osmoregulation and membrane stabilization. Taurine modulates calcium signaling but also the development and function of skeletal muscles, the retina and the central nervous system (Panda 2018). Concentration of taurine is decreasing in all of the samples. The biggest change between the treatment groups (control and exposed) is after 96h (30%). Such modulation of taurine may suggest effect of starvation which is slightly increased by the exposure to oil.

Threonine is an proteinogenic amino acid used in the process of biosynthesis of proteins and essential amino acid in humans supporting immune system (services 2018). Changes in threonine concentrations does not seem to be influenced much by the oil exposure, where decrease in metabolite concentration is in the range 16-23% between the treatment groups (control and exposed). This suggest the effect of starvation.

Tyrosine is a non-essential amino acid used by cells to synthesize proteins. It occurs in proteins that take part in signal transduction processes. Tyrosine is a receiver of phosphate groups transferred by protein kinases. Tyrosine is an important contributor in biosynthetic cascade leading to dopamine biosynthesis, where phenylalanine is converted by enzyme tryptophan-phenylalanine hydroxylase to tyrosine (McCoole 2012). Changes in concentrations of tyrosine are not highly affected by the exposure to oil. Decrease after

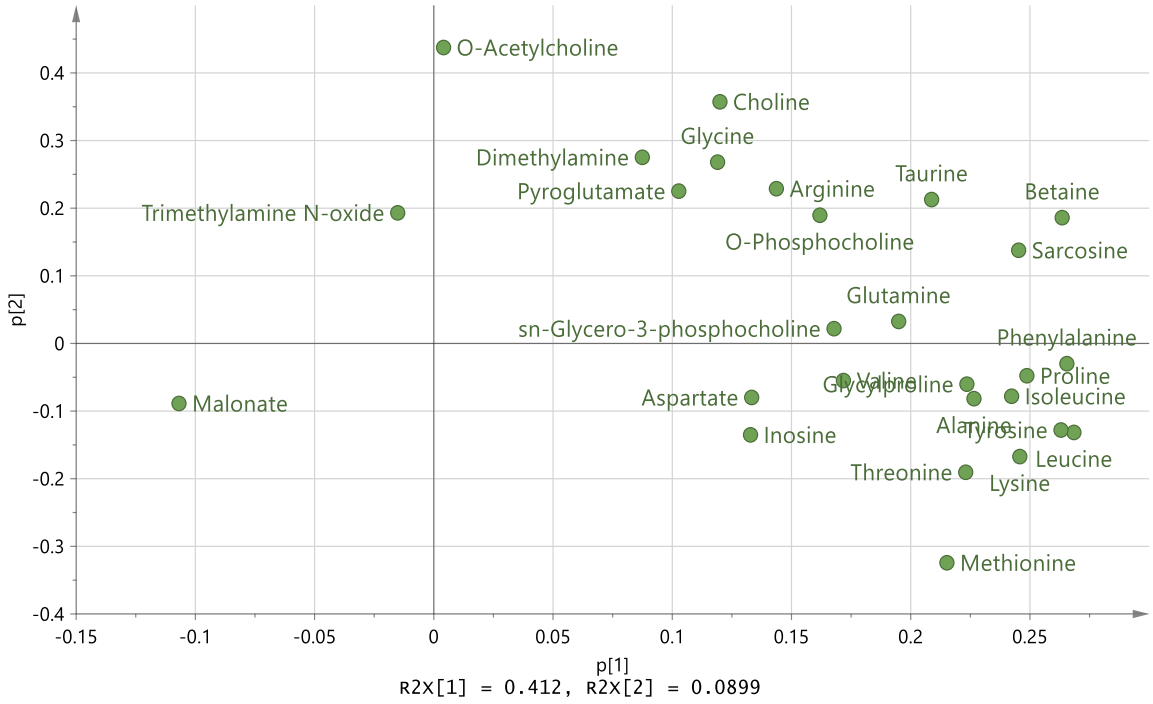
the exposure to oil is in the range of 22-34% compared to the corresponding control samples what suggests effect of starvation.

Sn-Glycero-3-phosphocholine is a phospholipid and precursor to choline biosynthesis but also an intermediate in phosphatidylcholine metabolism which is a major component of biological membranes. Studies show that it boosts brain transduction mechanisms and decreases age-dependent structural changes in brains of rats (Traini 2013). Changes in sn-Glycero-3-phosphocholine concentrations also does not seem to be influenced by the oil exposure, where decrease in metabolite concentration is in the range up to 16%. Small changes in the metabolite concentrations between the treatment groups suggests that the effect is not significantly influenced by the oil exposure.

Starved organisms rely on the glycerophospholipid metabolism and energy production through protein-based catabolism (Maity 2012). It is suggested that during starvation the muscle proteins are progressively hydrolyzed, but muscles seem to maintain its amino acid composition. The freed amino acids become available for energy production. Proline may be used as energy, but the ability to synthesize it seems to be limited by the exposure to WAF of oil as demonstrated by the decreased and stable concentration over time in the exposed samples (Fig.14).

Deficiency of protein or amino acids may impair immune function and increase the susceptibility of both, animals and humans to disease (P. Y. Li 2007). The similarities in metabolome changes in *Calanus finmarchicus* suggest that WAF of oil has an analogous mode of action to starvation and in addition may be impairing energy production and ionoregulation.

Crustaceans have limited capacity for lipid biosynthesis, so they have to depend on external sources to supplement lipid reserves (Maity 2012). These reserves are exhausted during food shortage. During starvation periods, crustaceans use glycogen, rapid utilization of glycerides and degradation of proteins and structural lipids as an energy source, which results in depletion of lipid reserves (Cherel 1992). This experiment shows how both, exposure to WAF of oil and starvation influence metabolome profile of *C. finmarchicus* during acute oil exposure what can be observed through protein degradation and loss of amino acids and osmolytes where malonate seems to be a promising indicator of the oil exposure.



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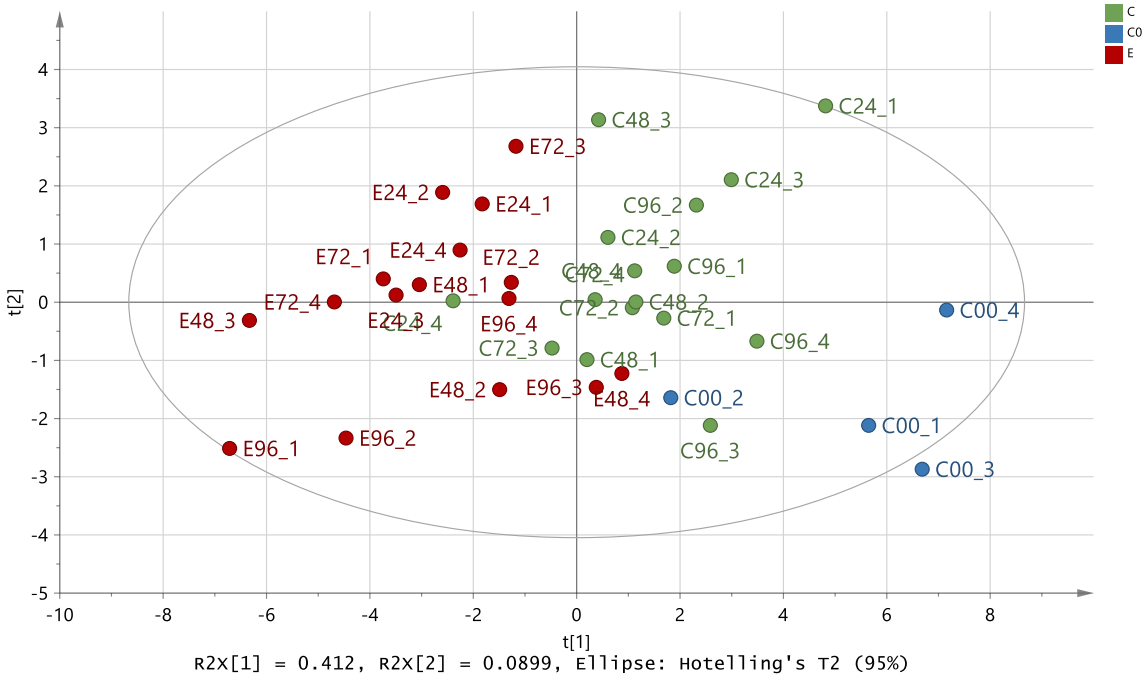

B

Figure 20. Principal component analysis (PCA), loading plot (A) and score plot (B) of *C. finmarchicus* metabolites (n=27 metabolites) and variables (n=32; four samples of control 0, sixteen control samples, sixteen exposed samples). PC1 = 0.412, PC2 = 0.0899.

Principal component analysis resulted in one significant component explaining 41,2% of variation in the model. However, for visualization purposes, the second component was calculated. PC2 explained about 9% of variation in the model. Loading plot shows how that most of the analyzed metabolites group on the right side of the plot presenting the effect of starvation, whereas metabolites representing the effect of oil exposure are grouped on the left side of the plot (Fig. 20). The score plot visualizes clustering of the treatment groups. Samples of control group of time 0, controls and exposed samples are relatively well clustered where exposure resulted in a metabolic shift compared to control treatment.

4.3. Experimental design -implications

The exposure medium was prevented from evaporation of volatile and semi-volatile compounds, which in natural conditions would possibly evaporate and the oil would undergo weathering processes. Thus, the experimental setup presents the worst-case scenario of the effects of oil. However studies show that weathering processes like emulsification, evaporation and others are limited by ice coverage and wave damping (Brandvik 2009). Study conducted by Brandvik shows that evaporative loss for the open water was 30% while 19% for 90% ice coverage what is mainly determined by the oil film thickness. Ice limits oil spreading thus increasing the film thickness what reduces evaporative loss.

Moreover, during this experiment test organisms were not fed, so the presented effects show the influence of both oil and starvation. However, it is still possible to distinguish changes between these two effects. The effects of oil exposure are manifested by the high upregulation of malonate in the samples exposed to crude oil WAF while in the control samples concentration of this metabolite decreased over time. On the other hand, it is still not clear if this effect would be seen in nature, in a real case scenario. Study conducted on cod larvae (Hansen et al. 2016) suggests that acute oil exposure results in effects similar to complete food deprivation. Effects induced by the oil exposure resulted in protein and cellular degradation, loss of amino acids and glucose and were observed though decreased food uptake and reduced growth.

Studies on the sensitivity to water-soluble fractions of fresh and weathered oil on *C. finmarchicus* shows interesting results where the authors conclude that the adult males of this copepod turned out to be most sensitive (Jager 2016). In addition, it has been observed that larvae were equally susceptible to oil toxicity as late copepodites and adult females (Jager 2016). What is more, study on cod larvae comparing results from chemically and mechanically dispersed oils shows that toxicity did not appear to differ much between these oil treatments (B. H. Hansen 2016).

5. Conclusion

Presented results of the metabolome profile of *Calanus finmarchicus* show that the effect of acute oil exposure can be successfully measured using ^{1}H NMR spectroscopy. This type of experiment is conducted to establish and prepare for the possible severe effects of marine oil pollution. Since changes in the metabolome profile were successfully detected, it can be concluded that presented changes, in for example malonate, seem to be a promising biomarker of oil exposure in a model zooplankton organism for cold climate, such as *Calanus finmarchicus*.

A more in-depth analysis would be required to confirm these findings. It would be also interesting to investigate changes in malonate concentration on other stages of copepods as well as in actually oil contaminated environments. This can help to better understand mechanisms of PAHs toxicity in *Calanus finmarchicus.*

On the other hand, NMR analysis has some limitations as low sensitivity compared to analytical techniques taking into account liquid chromatography-mass spectrometry (LC-MS) (Wang 2011). Combination of NMR with LC-MS analysis could increase precision and help elucidate more in-depth analysis what can allow better understanding of the mechanisms of oil toxicity in *Calanus finmarchicus*. Since ^{1}H NMR spectroscopy is considered as not optimal for analysis of lipids, it is suggested to include LC-MS analysis of lipids in future studies.

It should be noted that standardized laboratory experiments using WAF of oil as an exposure medium represent a worst-case scenario of the severe effects of oil since it does not take into consideration processes such as weathering of oil which take place in case of an oil spill in the aquatic environment. However, these results still point to *Calanus finmarchicus* as a possible bioindicator for oil contamination in the cold climate.

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Appendix A: Oil and WAF profile - VOC

Table 4&5. Concentrations of volatile organic compounds found in oil and WAF (g/kg). The crude oil samples were run in triplicates, while WAF – in duplicates.

Oil profile - VOC			
Compound	**[g/kg]**	**Compound**	**[g/kg]**
Isopentane	1.84±0.07	Ethylbenzene	1.57±0.01
n-C5 (Pentane)	0.54±0.02	m-Xylene	5.38±0.06
Cyclopentane	1.28±0.09	p-Xylene	1.73±0.02
2-methylpentane	2.16±0.09	o-Xylene	1.52±0.01
3-Methylpentane	1.41±0.04	n-C9 (Nonane)	0
n-C6 (Hexane)	0.30±0.01	Propylbenzene	0.52±0.01
Methylcyclopentane	6.36±0.08	1-Methyl-3-ethylbenzene	1.58±0.02
Benzene	0.5	1-Methyl-4-ethylbenzene	0.64±0.01
Cyclohexane	9.38±0.2	1.3.5-Trimethylbenzene	1.12±0.01
2.3-Dimethylpentane	0.96±0.02	1-Methyl-2-ethylbenzene	0.62±0.01
3-methylhexane	1.11±0.04	1.2.4-Trimethylbenzene	2.32±0.03
n-C7 (Heptane)	0.03	n-C10 (Decane)	0.05±0.09
Methylcyclohexane	20.31±0.32	1.2.3-Trimethylbenzene	0.80±0.01
Toluene	2.90±0.03	n-Butylbenzene	0.18±0.01
2.4 diethylhexane	0.09	1.2.4.5-Tetramethylbenzene	0.28±0.01
2-Methylheptane	0.03±0.02	n-Pentylbenzene	0
n-C8 (Octane)	0	C4-Benzenes	4.53±0.07
		C5-Benzenes	4.43±0.08
Sum BTEX	**13.61±0.14**	Sum C3-benzene	7.60±0.09

WAF - VOC			
Compound	**[µg/L]**	**Compound**	**[µg/L]**
Isopentane	277.96±14.66	Ethylbenzene	400.61±9.88
n-C5 (Pentane)	38.45±2.64	m-Xylene	1363.21±92.12
Cyclopentane	361.12±29.10	p-Xylene	128.50±16.49
2-methylpentane	36.45±1.03	o-Xylene	481.10±31.97
3-Methylpentane	28.32±5.77	n-C9 (Nonane)	0
n-C6 (Hexane)	1.85±0.29	Propylbenzene	41.63±4.55
Methylcyclopentane	375.55±43.97	1-Methyl-3-ethylbenzene	162.69±19.25
Benzene	918.02±96.55	1-Methyl-4-ethylbenzene	54.65±6.66
Cyclohexane	735.44±9.21	1.3.5-Trimethylbenzene	84.42±10.34
2.3-Dimethylpentane	21.66±4.06	1-Methyl-2-ethylbenzene	74.07±9.03
3-methylhexane	2.04±0.25	1.2.4-Trimethylbenzene	185.90±25.15
n-C7 (Heptane)	0	n-C10 (Decane)	0
Methylcyclohexane	322.42±30.62	1.2.3-Trimethylbenzene	86.64±12.14

Toluene	1611.76±268.06	n-Butylbenzene	1.97±0.35
2.4 diethylhexane	0.14±0.14	1.2.4.5-Tetramethylbenzene	6.96±0.92
2-Methylheptane	0	n-Pentylbenzene	4.68±0.44
n-C8 (Octane)	0	C4-Benzenes	102.26±14.19
Sum BTEX	**4903.02±534.12**	C5-Benzenes	14.88±1.40

Appendix B: Oil and WAF profile - SVOC

Tables 6&7. Concentrations of semi-volatile organic compounds found in oil and WAF (g/kg). SVOC analyses were performed on a single crude oil sample and on three different WAF samples: WAF, WAF exposure medium and WAF after the 96h.

Oil profile - SVOC			
Compound	**[g/kg]**	**Compound**	**[g/kg]**
Decalin	0.985	C3-dibenzothiophenes	0.099
C1-decalins	1.679	C4-dibenzothiophenes	0.055
C2-decalins	1.291	Fluoranthene	0.013
C3-decalins	0.904	Pyrene	0.017
C4-decalins	0.646	C1-fluoranthrenes/pyrenes	0.135
Benzo(b)thiophene	0.000	C2-fluoranthenes/pyrenes	0.152
Naphthalene	0.953	C3-fluoranthenes/pyrenes	0.131
C1-naphthalenes	1.890	Benz(a)anthracene	0.000
C2-naphthalenes	2.247	Chrysene	0.014
C3-naphthalenes	1.613	C1-chrysenes	0.076
C4-naphthalenes	0.846	C2-chrysenes	0.084
Biphenyl	0.280	C3-chrysenes	0.049
Acenaphthylene	0.019	C4-chrysenes	0.000
Acenaphthene	0.031	Benzo(b)fluoranthene	0.006
Dibenzofuran	0.043	Benzo(k)fluoranthene	0.000
Fluorene	0.128	Benzo(e)pyrene	0.007
C1-fluorenes	0.319	Benzo(a)pyrene	0.000
C2-fluorenes	0.406	Perylene	0.004
C3-fluorenes	0.299	Indeno(1.2.3-c.d)pyrene	0.001
Phenanthrene	0.203	Dibenz(a.h)anthracene	0.000
Anthracene	0.000	Benzo(g.h.i)perylene	0.003
C1-phenanthrenes/anthracenes	0.447	Phenol	0.000
C2-phenanthrenes/anthracenes	0.495	C1-Phenols (o- og p-cresol)	0.000
C3-phenanthrenes/anthracenes	0.490	C2-Phenols	0.000
C4-phenanthrenes/anthracenes	0.285	C3-Phenols	0.000
Dibenzothiophene	0.026	C4-Phenols	0.000
C1-dibenzothiophenes	0.094	C5-Phenols	0.000
C2-dibenzothiophenes	0.124	30 ab hopane	0.180

WAF SVOC			
Compound	**WAF [µg/L]**	**WAF exposure medium [µg/L]**	**WAF after 96h [µg/L]**
Decalin	0.565	0.474	0.023
C1-decalins	0.240	0.232	0
C2-decalins	0	0	0
C3-decalins	0	0	0
C4-decalins	0	0	0
Benzo(b)thiophene	0	0	0
Naphthalene	137.840	110.554	12.338
C1-naphthalenes	71.630	57.315	4.160
C2-naphthalenes	36.228	32.999	2.767
C3-naphthalenes	7.804	6.549	0.631
C4-naphthalenes	0	0	0
Biphenyl	4.075	0	0.884
Acenaphthylene	0.062	0.054	0.004
Acenaphthene	0.779	0.674	0.064
Dibenzofuran	1.149	0.978	0.095
Fluorene	2.505	2.183	0.215
C1-fluorenes	1.692	1.473	0.130
C2-fluorenes	0.836	0.787	0.059
C3-fluorenes	0	0	0
Phenanthrene	1.591	1.402	0.124
Anthracene	0	0	0
C1-phenanthrenes/anthracenes	1.111	0.957	0.065
C2-phenanthrenes/anthracenes	0.392	0.316	0
C3-phenanthrenes/anthracenes	0	0	0
C4-phenanthrenes/anthracenes	0	0	0
Dibenzothiophene	0.460	0.286	0.022
C1-dibenzothiophenes	0.852	0.850	0.087
C2-dibenzothiophenes	0	0	0
C3-dibenzothiophenes	0	0	0
C4-dibenzothiophenes	0	0	0
Fluoranthene	0.023	0.019	0.003
Pyrene	0.024	0.019	0.002
C1-fluoranthrenes/pyrenes	0.070	0.064	0.004
C2-fluoranthenes/pyrenes	0	0	0
C3-fluoranthenes/pyrenes	0	0	0
Benz(a)anthracene	0.003	0.002	0.000
Chrysene	0.014	0.011	0.001

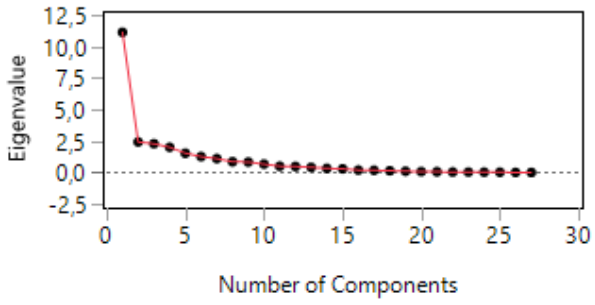
C1-chrysenes	0	0	0
C2-chrysenes	0	0	0
C3-chrysenes	0	0	0
C4-chrysenes	0	0	0
Benzo(b)fluoranthene	0	0	0
Benzo(k)fluoranthene	0	0	0
Benzo(e)pyrene	0	0	0
Benzo(a)pyrene	0	0	0
Perylene	0	0	0
Indeno(1.2.3-c.d)pyrene	0	0	0
Dibenz(a.h)anthracene	0	0	0
Benzo(g.h.i)perylene	0	0	0
Phenol	0	0	0
C1-Phenols (o- og p-cresol)	0	0	0
C2-Phenols	0	0	0
C3-Phenols	0	0	0
C4-Phenols	0	0	0
C5-Phenols	0	0	0
30 ab hopane	0	0	0
TEM	3088.963	2786.592	198.625

Appendix C: Results from statistical analyses

Table 8. Principal components / factor analysis (on correlations)

Number	Eigenvalue	Percent	Cum Percent
1	11,1315	41,228	41,228
2	2,4283	8,994	50,222
3	2,2878	8,473	58,695
4	2,0048	7,425	66,120
5	1,5312	5,671	71,791
6	1,2655	4,687	76,478
7	1,1019	4,081	80,559
8	0,8562	3,171	83,731
9	0,8309	3,077	86,808
10	0,6675	2,472	89,280
11	0,5000	1,852	91,132
12	0,4808	1,781	92,913
13	0,4131	1,530	94,443
14	0,3297	1,221	95,664
15	0,2897	1,073	96,737
16	0,1932	0,716	97,452
17	0,1804	0,668	98,120
18	0,1376	0,510	98,630
19	0,1009	0,374	99,004
20	0,0733	0,272	99,276
21	0,0608	0,225	99,501
22	0,0394	0,146	99,647
23	0,0364	0,135	99,781
24	0,0270	0,100	99,882
25	0,0218	0,081	99,962
26	0,0062	0,023	99,985
27	0,0040	0,015	100,000

Figure 21. Scree Plot



In this section can be found results from statistical analysis of the metabolites concentrations.

- Additional information on the analysis of betaine concentrations

Summary of Fit

Rsquare	0.542138
Adj Rsquare	0.406475
Root Mean Square Error	0.015867
Mean of Response	0.178863
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00804905	0.001006	3.9962	0.0031*
Error	27	0.00679781	0.000252		
C. Total	35	0.01484685			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.198278	0.00793	0.18200	0.21456
C24	4	0.194604	0.00793	0.17833	0.21088
C48	4	0.184610	0.00793	0.16833	0.20089
C72	4	0.178389	0.00793	0.16211	0.19467
C96	4	0.196842	0.00793	0.18056	0.21312
E24	4	0.172851	0.00793	0.15657	0.18913
E48	4	0.165253	0.00793	0.14897	0.18153
E72	4	0.164835	0.00793	0.14856	0.18111
E96	4	0.154107	0.00793	0.13783	0.17039

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.1982783	0.0199166	0.0099583	0.1665866	0.22997
C24	4	0.1946037	0.0245501	0.012275	0.1555391	0.2336683
C48	4	0.1846097	0.0084561	0.004228	0.1711542	0.1980652
C72	4	0.1783891	0.0098575	0.0049288	0.1627036	0.1940746
C96	4	0.1968419	0.007701	0.0038505	0.1845878	0.209096
E24	4	0.1728506	0.0148806	0.0074403	0.1491723	0.1965289
E48	4	0.1652525	0.0164924	0.0082462	0.1390094	0.1914956
E72	4	0.1648345	0.0132024	0.0066012	0.1438265	0.1858425
E96	4	0.1541071	0.0192572	0.0096286	0.1234645	0.1847497

Means Comparisons

Comparisons with a control using Dunnett's Method

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-0.03	1.0000
C96	-0.03	1.0000
C24	-0.03	0.9999
C48	-0.02	0.7504
C72	-0.01	0.3789
E24	-0.01	0.1621
E48	0.001	0.0393*

Level	Abs(Dif)-LSD	p-Value
E72	0.002	0.0361*
E96	0.012	0.0035*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of glutamine concentrations

Summary of Fit

Rsquare	0.884856
Adj Rsquare	0.850739
Root Mean Square Error	0.002436
Mean of Response	0.015049
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00123123	0.000154	25.9360	<.0001*
Error	27	0.00016022	5.934e-6		
C. Total	35	0.00139145			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.015077	0.00122	0.01258	0.01758
C24	4	0.017564	0.00122	0.01506	0.02006
C48	4	0.018500	0.00122	0.01600	0.02100
C72	4	0.022023	0.00122	0.01952	0.02452
C96	4	0.025052	0.00122	0.02255	0.02755
E24	4	0.007199	0.00122	0.00470	0.00970
E48	4	0.009282	0.00122	0.00678	0.01178
E72	4	0.011434	0.00122	0.00894	0.01393
E96	4	0.009310	0.00122	0.00681	0.01181

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.0150765	0.002546	0.001273	0.0110252	0.0191278
C24	4	0.0175636	0.0030559	0.0015279	0.012701	0.0224262
C48	4	0.0185003	0.0019817	0.0009908	0.015347	0.0216536
C72	4	0.0220229	0.0027748	0.0013874	0.0176075	0.0264383
C96	4	0.0250515	0.0034125	0.0017062	0.0196215	0.0304815
E24	4	0.0071991	0.0005548	0.0002774	0.0063162	0.008082
E48	4	0.0092815	0.0023524	0.0011762	0.0055384	0.0130246
E72	4	0.0114342	0.0028099	0.0014049	0.0069631	0.0159053
E96	4	0.00931	0.0007596	0.0003798	0.0081014	0.0105186

Means Comparisons

Comparisons with a control using Dunnett's Method

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C96	0.005	<.0001*
C72	0.002	0.0028*
C48	-1e-3	0.2678

Level	Abs(Dif)-LSD	p-Value
C24	-2e-3	0.5929
C00	-5e-3	1.0000
E72	-1e-3	0.2145
E96	0.001	0.0153*
E48	0.001	0.0147*
E24	0.003	0.0007*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of glycylproline concentrations

Summary of Fit

Rsquare	0.509648
Adj Rsquare	0.364358
Root Mean Square Error	0.003208
Mean of Response	0.010971
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00028889	0.000036	3.5078	0.0067*
Error	27	0.00027795	0.000010		
C. Total	35	0.00056683			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.016825	0.00160	0.01353	0.02012
C24	4	0.013471	0.00160	0.01018	0.01676
C48	4	0.011109	0.00160	0.00782	0.01440
C72	4	0.012470	0.00160	0.00918	0.01576
C96	4	0.010722	0.00160	0.00743	0.01401
E24	4	0.009403	0.00160	0.00611	0.01269
E48	4	0.010009	0.00160	0.00672	0.01330
E72	4	0.007435	0.00160	0.00414	0.01073
E96	4	0.007298	0.00160	0.00401	0.01059

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.0168245	0.0023112	0.0011556	0.0131468	0.0205022
C24	4	0.013471	0.0048485	0.0024243	0.0057559	0.0211861
C48	4	0.0111093	0.0015871	0.0007936	0.0085838	0.0136348
C72	4	0.0124697	0.0022259	0.0011129	0.0089279	0.0160115
C96	4	0.0107217	0.0050853	0.0025426	0.0026299	0.0188135
E24	4	0.0094031	0.0030916	0.0015458	0.0044837	0.0143225
E48	4	0.0100092	0.0040388	0.0020194	0.0035826	0.0164358
E72	4	0.0074347	0.0015671	0.0007835	0.0049411	0.0099283
E96	4	0.0072979	0.001463	0.0007315	0.0049699	0.0096259

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-0.01	1.0000
C24	-3e-3	0.5689
C72	-2e-3	0.3002
C48	-7e-4	0.0983
C96	-3e-4	0.0687
E48	4e-4	0.0343*
E24	0.001	0.0184*
E72	0.003	0.0021*
E96	0.003	0.0018*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of isoleucine concentrations

Summary of Fit

Rsquare	0.522685
Adj Rsquare	0.381258
Root Mean Square Error	0.001243
Mean of Response	0.007326
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00004571	5.7135e-6	3.6958	0.0049*
Error	27	0.00004174	1.5459e-6		
C. Total	35	0.00008745			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.009128	0.00062	0.00785	0.01040
C24	4	0.007167	0.00062	0.00589	0.00844
C48	4	0.007456	0.00062	0.00618	0.00873
C72	4	0.008189	0.00062	0.00691	0.00946
C96	4	0.008898	0.00062	0.00762	0.01017
E24	4	0.005711	0.00062	0.00444	0.00699
E48	4	0.006680	0.00062	0.00540	0.00796
E72	4	0.006321	0.00062	0.00505	0.00760
E96	4	0.006382	0.00062	0.00511	0.00766

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.0091276	0.0018312	0.0009156	0.0062138	0.0120414
C24	4	0.0071668	0.0011411	0.0005705	0.0053511	0.0089825
C48	4	0.0074556	0.0007084	0.0003542	0.0063283	0.0085829
C72	4	0.008189	0.0012293	0.0006146	0.0062329	0.0101451
C96	4	0.0088977	0.0002496	0.0001248	0.0085005	0.0092949
E24	4	0.0057114	0.0005826	0.0002913	0.0047844	0.0066384
E48	4	0.0066804	0.001887	0.0009435	0.0036778	0.009683
E72	4	0.0063213	0.0012741	0.0006371	0.0042939	0.0083487
E96	4	0.0063821	0.0012882	0.0006441	0.0043322	0.008432

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-2e-3	1.0000
C96	-2e-3	1.0000
C72	-2e-3	0.8444
C48	-8e-4	0.3091
C24	-5e-4	0.1735
E48	-5e-5	0.0561
E96	3e-4	0.0260*
E72	3e-4	0.0221*
E24	0.001	0.0040*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of leucine concentrations

Summary of Fit

Rsquare	0.583783
Adj Rsquare	0.46046
Root Mean Square Error	0.002059
Mean of Response	0.01379
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00016050	0.000020	4.7338	0.0010*
Error	27	0.00011443	4.238e-6		
C. Total	35	0.00027494			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.019110	0.00103	0.01700	0.02122
C24	4	0.014081	0.00103	0.01197	0.01619
C48	4	0.013560	0.00103	0.01145	0.01567
C72	4	0.013747	0.00103	0.01163	0.01586
C96	4	0.014704	0.00103	0.01259	0.01682
E24	4	0.011353	0.00103	0.00924	0.01346
E48	4	0.012502	0.00103	0.01039	0.01461
E72	4	0.012350	0.00103	0.01024	0.01446
E96	4	0.012707	0.00103	0.01060	0.01482

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.0191102	0.0024956	0.0012478	0.0151391	0.0230813
C24	4	0.0140809	0.0017589	0.0008795	0.011282	0.0168798
C48	4	0.0135603	0.0009771	0.0004886	0.0120055	0.0151151
C72	4	0.0137465	0.0011844	0.0005922	0.0118618	0.0156312
C96	4	0.0147041	0.0013137	0.0006569	0.0126137	0.0167945
E24	4	0.0113525	0.0010635	0.0005318	0.0096602	0.0130448
E48	4	0.012502	0.0026016	0.0013008	0.0083623	0.0166417
E72	4	0.01235	0.001816	0.000908	0.0094603	0.0152397
E96	4	0.0127072	0.0036799	0.0018399	0.0068517	0.0185627

Means Comparisons
Comparisons with a control using Dunnett's Method
Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-4e-3	1.0000
C96	3e-4	0.0325*
C24	0.001	0.0118*
C72	0.001	0.0067*
C48	0.001	0.0048*
E96	0.002	0.0011*
E48	0.002	0.0007*
E72	0.003	0.0006*
E24	0.004	<.0001*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of lysine concentrations

Summary of Fit

Rsquare	0.510474
Adj Rsquare	0.365429
Root Mean Square Error	0.004384
Mean of Response	0.016736
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00054109	0.000068	3.5194	0.0065*
Error	27	0.00051888	0.000019		
C. Total	35	0.00105997			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.025648	0.00219	0.02115	0.03015
C24	4	0.016906	0.00219	0.01241	0.02140
C48	4	0.015552	0.00219	0.01105	0.02005
C72	4	0.019241	0.00219	0.01474	0.02374
C96	4	0.018977	0.00219	0.01448	0.02347
E24	4	0.012494	0.00219	0.00800	0.01699
E48	4	0.013452	0.00219	0.00895	0.01795
E72	4	0.013389	0.00219	0.00889	0.01789
E96	4	0.014964	0.00219	0.01047	0.01946

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.0256481	0.003477	0.0017385	0.0201155	0.0311807
C24	4	0.0169062	0.0052622	0.0026311	0.0085329	0.0252795
C48	4	0.0155515	0.0059575	0.0029787	0.0060718	0.0250312
C72	4	0.0192413	0.0039235	0.0019618	0.0129981	0.0254845

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C96	4	0.0189772	0.0021533	0.0010767	0.0155508	0.0224036
E24	4	0.0124944	0.0019562	0.0009781	0.0093817	0.0156071
E48	4	0.013452	0.0033026	0.0016513	0.0081968	0.0187072
E72	4	0.0133893	0.0058121	0.0029061	0.0041409	0.0226377
E96	4	0.0149644	0.0053985	0.0026993	0.0063742	0.0235546

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-0.01	1.0000
C72	-2e-3	0.2335
C96	-2e-3	0.2005
C24	-5e-5	0.0518
C48	0.001	0.0190*
E96	0.002	0.0120*
E48	0.003	0.0036*
E72	0.003	0.0034*
E24	0.004	0.0016*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of malonate concentrations

Summary of Fit

Rsquare	0.842938
Adj Rsquare	0.7964
Root Mean Square Error	0.003464
Mean of Response	0.025612
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00173857	0.000217	18.1133	<.0001*
Error	27	0.00032394	0.000012		
C. Total	35	0.00206252			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.025327	0.00173	0.02177	0.02888
C24	4	0.022279	0.00173	0.01873	0.02583
C48	4	0.019019	0.00173	0.01547	0.02257
C72	4	0.017357	0.00173	0.01380	0.02091
C96	4	0.017824	0.00173	0.01427	0.02138
E24	4	0.026304	0.00173	0.02275	0.02986
E48	4	0.029870	0.00173	0.02632	0.03342
E72	4	0.034580	0.00173	0.03103	0.03813
E96	4	0.037947	0.00173	0.03439	0.04150

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.025327	0.0045387	0.0022694	0.0181049	0.0325491
C24	4	0.0222794	0.0026284	0.0013142	0.018097	0.0264618
C48	4	0.019019	0.0014471	0.0007235	0.0167164	0.0213216
C72	4	0.0173565	0.0017873	0.0008937	0.0145125	0.0202005
C96	4	0.0178239	0.0015662	0.0007831	0.0153318	0.020316
E24	4	0.0263036	0.002727	0.0013635	0.0219644	0.0306428
E48	4	0.0298699	0.0030389	0.0015195	0.0250343	0.0347055
E72	4	0.03458	0.0051899	0.0025949	0.0263217	0.0428383
E96	4	0.0379468	0.0053968	0.0026984	0.0293594	0.0465342

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
E96	0.006	0.0001*
E72	0.002	0.0053*
E48	-2e-3	0.3334
E24	-0.01	0.9995
C00	-0.01	1.0000
C24	-4e-3	0.7328
C48	-6e-4	0.0875
C96	0.001	0.0299*
C72	0.001	0.0191*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of methionine concentrations

Summary of Fit

Rsquare	0.777902
Adj Rsquare	0.712095
Root Mean Square Error	0.000952
Mean of Response	0.004258
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00008574	0.000011	11.8210	<.0001*
Error	27	0.00002448	9.066e-7		
C. Total	35	0.00011021			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.006931	0.00048	0.00595	0.00791
C24	4	0.003709	0.00048	0.00273	0.00469
C48	4	0.004169	0.00048	0.00319	0.00515
C72	4	0.005295	0.00048	0.00432	0.00627
C96	4	0.006401	0.00048	0.00542	0.00738
E24	4	0.002069	0.00048	0.00109	0.00305

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
E48	4	0.003171	0.00048	0.00219	0.00415
E72	4	0.002812	0.00048	0.00184	0.00379
E96	4	0.003762	0.00048	0.00279	0.00474

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.0069312	0.0010915	0.0005457	0.0051944	0.008668
C24	4	0.0037088	0.0010802	0.0005401	0.0019899	0.0054277
C48	4	0.0041686	0.0012071	0.0006036	0.0022478	0.0060894
C72	4	0.0052953	0.0004096	0.0002048	0.0046435	0.0059471
C96	4	0.0064011	0.0001505	7.526e-5	0.0061616	0.0066406
E24	4	0.0020691	0.0002551	0.0001275	0.0016633	0.0024749
E48	4	0.0031711	0.001513	0.0007565	0.0007636	0.0055786
E72	4	0.002812	0.0005218	0.0002609	0.0019817	0.0036423
E96	4	0.003762	0.0012359	0.0006179	0.0017955	0.0057285

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-2e-3	1.0000
C96	-1e-3	0.9614
C72	-3e-4	0.1178
C48	0.001	0.0023*
E96	0.001	0.0005*
C24	0.001	0.0004*
E48	0.002	<.0001*
E72	0.002	<.0001*
E24	0.003	<.0001*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of O-Acetylcholine concentrations

Summary of Fit

Rsquare	0.458786
Adj Rsquare	0.298427
Root Mean Square Error	0.000309
Mean of Response	0.001931
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	2.18402e-6	2.73e-7	2.8610	0.0193*
Error	27	2.57641e-6	9.5423e-8		
C. Total	35	4.76044e-6			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.001469	0.00015	0.00115	0.00179

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C24	4	0.002265	0.00015	0.00195	0.00258
C48	4	0.002010	0.00015	0.00169	0.00233
C72	4	0.001873	0.00015	0.00156	0.00219
C96	4	0.002029	0.00015	0.00171	0.00235
E24	4	0.002101	0.00015	0.00178	0.00242
E48	4	0.001562	0.00015	0.00124	0.00188
E72	4	0.002120	0.00015	0.00180	0.00244
E96	4	0.001951	0.00015	0.00163	0.00227

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.0014687	0.0002185	0.0001093	0.0011209	0.0018165
C24	4	0.0022648	0.0005079	0.0002539	0.0014566	0.003073
C48	4	0.0020102	0.0001231	6.1547e-5	0.0018143	0.0022061
C72	4	0.0018734	0.0001976	9.8824e-5	0.0015589	0.0021879
C96	4	0.0020292	0.000439	0.0002195	0.0013306	0.0027278
E24	4	0.0021014	0.0001671	8.3571e-5	0.0018354	0.0023674
E48	4	0.0015618	0.00019	0.000095	0.0012595	0.0018641
E72	4	0.0021204	0.0004388	0.0002194	0.0014221	0.0028187
E96	4	0.0019513	0.0002226	0.0001113	0.0015972	0.0023054

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C24	2e-4	0.0074*
E72	3e-5	0.0359*
E24	1e-5	0.0437*
C96	-6e-5	0.0893
C48	-8e-5	0.1067
E96	-1e-4	0.1804
C72	-2e-4	0.3345
E48	-5e-4	0.9992
C00	-6e-4	1.0000

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of phenylalanine concentrations

Summary of Fit

Rsquare	0.470027
Adj Rsquare	0.312998
Root Mean Square Error	0.000771
Mean of Response	0.004472
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00001425	1.7809e-6	2.9932	0.0154*
Error	27	0.00001606	5.9496e-7		

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
C. Total	35	0.00003031			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.005795	0.00039	0.00500	0.00659
C24	4	0.004621	0.00039	0.00383	0.00541
C48	4	0.004511	0.00039	0.00372	0.00530
C72	4	0.004294	0.00039	0.00350	0.00509
C96	4	0.005210	0.00039	0.00442	0.00600
E24	4	0.003745	0.00039	0.00295	0.00454
E48	4	0.003925	0.00039	0.00313	0.00472
E72	4	0.003893	0.00039	0.00310	0.00468
E96	4	0.004250	0.00039	0.00346	0.00504

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.005795	0.0008909	0.0004454	0.0043774	0.0072126
C24	4	0.0046208	0.0011331	0.0005666	0.0028178	0.0064238
C48	4	0.0045106	0.0004151	0.0002075	0.0038501	0.0051711
C72	4	0.004294	0.0002426	0.0001213	0.0039079	0.0046801
C96	4	0.0052098	0.0005072	0.0002536	0.0044027	0.0060169
E24	4	0.0037449	0.0004132	0.0002066	0.0030874	0.0044024
E48	4	0.0039254	0.0010446	0.0005223	0.0022632	0.0055876
E72	4	0.0038931	0.000597	0.0002985	0.0029431	0.0048431
E96	4	0.0042503	0.0010818	0.0005409	0.0025289	0.0059717

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-2e-3	1.0000
C96	-1e-3	0.8414
C24	-4e-4	0.2002
C48	-3e-4	0.1366
C72	-5e-5	0.0601
E96	-2e-6	0.0504
E48	3e-4	0.0126*
E72	4e-4	0.0109*
E24	0.001	0.0055*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of proline concentrations

Summary of Fit

Rsquare	0.796264
Adj Rsquare	0.735898
Root Mean Square Error	0.008969
Mean of Response	0.026523
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00848894	0.001061	13.1906	<.0001*
Error	27	0.00217202	0.000080		
C. Total	35	0.01066096			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.059293	0.00448	0.05009	0.06849
C24	4	0.042093	0.00448	0.03289	0.05129
C48	4	0.031865	0.00448	0.02266	0.04107
C72	4	0.023036	0.00448	0.01383	0.03224
C96	4	0.030885	0.00448	0.02168	0.04009
E24	4	0.013292	0.00448	0.00409	0.02249
E48	4	0.012018	0.00448	0.00282	0.02122
E72	4	0.012360	0.00448	0.00316	0.02156
E96	4	0.013868	0.00448	0.00467	0.02307

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.0592933	0.0161667	0.0080833	0.0335685	0.0850181
C24	4	0.0420926	0.0052048	0.0026024	0.0338106	0.0503746
C48	4	0.0318649	0.0099781	0.004989	0.0159875	0.0477423
C72	4	0.0230356	0.0043608	0.0021804	0.0160967	0.0299745
C96	4	0.0308845	0.0135275	0.0067638	0.0093592	0.0524098
E24	4	0.0132924	0.0031746	0.0015873	0.0082409	0.0183439
E48	4	0.0120175	0.0080238	0.0040119	-0.00075	0.0247852
E72	4	0.0123595	0.0067164	0.0033582	0.0016722	0.0230468
E96	4	0.0138681	0.0037963	0.0018981	0.0078274	0.0199088

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-0.02	1.0000
C24	-8e-4	0.0655
C48	0.009	0.0013*
C96	0.01	0.0009*
C72	0.018	<.0001*
E96	0.027	<.0001*
E24	0.028	<.0001*
E72	0.029	<.0001*
E48	0.029	<.0001*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of sarcosine concentrations

Summary of Fit

Rsquare	0.847004
Adj Rsquare	0.801672
Root Mean Square Error	0.002278
Mean of Response	0.021951
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00077540	0.000097	18.6844	<.0001*
Error	27	0.00014006	5.187e-6		
C. Total	35	0.00091546			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.027379	0.00114	0.02504	0.02972
C24	4	0.026326	0.00114	0.02399	0.02866
C48	4	0.025268	0.00114	0.02293	0.02760
C72	4	0.024863	0.00114	0.02253	0.02720
C96	4	0.024884	0.00114	0.02255	0.02722
E24	4	0.020716	0.00114	0.01838	0.02305
E48	4	0.017763	0.00114	0.01543	0.02010
E72	4	0.016959	0.00114	0.01462	0.01930
E96	4	0.013397	0.00114	0.01106	0.01573

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.027379	0.0029749	0.0014875	0.0226453	0.0321127
C24	4	0.0263264	0.003323	0.0016615	0.0210388	0.031614
C48	4	0.0252681	0.0026844	0.0013422	0.0209966	0.0295396
C72	4	0.0248634	0.0018877	0.0009439	0.0218596	0.0278672
C96	4	0.0248843	0.0009344	0.0004672	0.0233975	0.0263711
E24	4	0.0207157	0.0017421	0.000871	0.0179437	0.0234877
E48	4	0.0177631	0.0024817	0.0012409	0.0138141	0.0217121
E72	4	0.0169594	0.0019618	0.0009809	0.0138378	0.020081
E96	4	0.0133969	0.0014526	0.0007263	0.0110855	0.0157083

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-5e-3	1.0000
C24	-4e-3	0.9863
C48	-2e-3	0.6868
C96	-2e-3	0.5201
C72	-2e-3	0.5114
E24	0.002	0.0021*
E48	0.005	<.0001*
E72	0.006	<.0001*
E96	0.009	<.0001*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of taurine concentrations

Summary of Fit

Rsquare	0.512561
Adj Rsquare	0.368135
Root Mean Square Error	0.014935
Mean of Response	0.085663
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00633326	0.000792	3.5489	0.0062*
Error	27	0.00602284	0.000223		
C. Total	35	0.01235610			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.108887	0.00747	0.09356	0.12421
C24	4	0.101262	0.00747	0.08594	0.11658
C48	4	0.081428	0.00747	0.06611	0.09675
C72	4	0.088850	0.00747	0.07353	0.10417
C96	4	0.092158	0.00747	0.07684	0.10748
E24	4	0.086271	0.00747	0.07095	0.10159
E48	4	0.070458	0.00747	0.05514	0.08578
E72	4	0.076236	0.00747	0.06091	0.09156
E96	4	0.065417	0.00747	0.05009	0.08074

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.1088871	0.0135968	0.0067984	0.0872515	0.1305227
C24	4	0.1012624	0.013643	0.0068215	0.0795533	0.1229715
C48	4	0.0814283	0.0340881	0.017044	0.0271865	0.1356701
C72	4	0.0888497	0.0074349	0.0037175	0.0770191	0.1006803
C96	4	0.0921576	0.0047435	0.0023718	0.0846096	0.0997056
E24	4	0.0862714	0.0091219	0.0045609	0.0717565	0.1007863
E48	4	0.0704577	0.0127316	0.0063658	0.0501989	0.0907165
E72	4	0.0762356	0.0078576	0.0039288	0.0637323	0.0887389
E96	4	0.065417	0.0094756	0.0047378	0.0503393	0.0804947

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-0.03	1.0000
C24	-0.02	0.9758
C96	-0.01	0.4966
C72	-0.01	0.3113
E24	-0.01	0.2044

Level	Abs(Dif)-LSD	p-Value
C48	-2e-3	0.0831
E72	0.003	0.0280*
E48	0.008	0.0075*
E96	0.014	0.0022*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of threonine concentrations

Summary of Fit

Rsquare	0.568346
Adj Rsquare	0.440448
Root Mean Square Error	0.001613
Mean of Response	0.007712
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00009253	0.000012	4.4438	0.0016*
Error	27	0.00007028	2.603e-6		
C. Total	35	0.00016281			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.011442	0.00081	0.00979	0.01310
C24	4	0.008381	0.00081	0.00673	0.01004
C48	4	0.007828	0.00081	0.00617	0.00948
C72	4	0.006964	0.00081	0.00531	0.00862
C96	4	0.008921	0.00081	0.00727	0.01058
E24	4	0.006555	0.00081	0.00490	0.00821
E48	4	0.006580	0.00081	0.00492	0.00823
E72	4	0.005863	0.00081	0.00421	0.00752
E96	4	0.006874	0.00081	0.00522	0.00853

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.0114418	0.0012565	0.0006282	0.0094425	0.0134411
C24	4	0.0083809	0.0015165	0.0007583	0.0059678	0.010794
C48	4	0.007828	0.0006573	0.0003287	0.0067821	0.0088739
C72	4	0.0069635	0.0031076	0.0015538	0.0020186	0.0119084
C96	4	0.0089205	0.0023207	0.0011603	0.0052278	0.0126132
E24	4	0.006555	0.0007098	0.0003549	0.0054255	0.0076845
E48	4	0.0065797	0.0016357	0.0008179	0.0039769	0.0091825
E72	4	0.0058634	0.000646	0.000323	0.0048355	0.0068913
E96	4	0.0068742	0.0006896	0.0003448	0.0057769	0.0079715

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-3e-3	1.0000
C96	-7e-4	0.1801
C24	-2e-4	0.0697
C48	4e-4	0.0234*
C72	0.001	0.0036*
E96	0.001	0.0030*
E48	0.002	0.0015*
E24	0.002	0.0014*
E72	0.002	0.0003*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of tyrosine concentrations

Summary of Fit

Rsquare	0.670896
Adj Rsquare	0.573383
Root Mean Square Error	0.001206
Mean of Response	0.006962
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00008011	0.000010	6.8801	<.0001*
Error	27	0.00003930	1.455e-6		
C. Total	35	0.00011941			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.010030	0.00060	0.00879	0.01127
C24	4	0.007421	0.00060	0.00618	0.00866
C48	4	0.007735	0.00060	0.00650	0.00897
C72	4	0.006933	0.00060	0.00570	0.00817
C96	4	0.008147	0.00060	0.00691	0.00938
E24	4	0.004874	0.00060	0.00364	0.00611
E48	4	0.006071	0.00060	0.00483	0.00731
E72	4	0.005426	0.00060	0.00419	0.00666
E96	4	0.006021	0.00060	0.00478	0.00726

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.0100301	0.0013151	0.0006576	0.0079374	0.0121228
C24	4	0.0074214	0.0020465	0.0010232	0.004165	0.0106778
C48	4	0.0077349	0.0004355	0.0002178	0.0070419	0.0084279
C72	4	0.0069331	0.0007746	0.0003873	0.0057006	0.0081656
C96	4	0.0081472	0.0007727	0.0003864	0.0069176	0.0093768
E24	4	0.0048735	0.0006425	0.0003212	0.0038511	0.0058959
E48	4	0.0060705	0.0012061	0.0006031	0.0041513	0.0079897
E72	4	0.0054264	0.0016445	0.0008222	0.0028096	0.0080432
E96	4	0.0060211	0.0011057	0.0005528	0.0042617	0.0077805

Means Comparisons
Comparisons with a control using Dunnett's Method
Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-2e-3	1.0000
C96	-5e-4	0.1811
C48	-1e-4	0.0686
C24	2e-4	0.0303*
C72	0.001	0.0076*
E48	0.002	0.0006*
E96	0.002	0.0005*
E72	0.002	<.0001*
E24	0.003	<.0001*

Positive values show pairs of means that are significantly different.

- Additional information on the analysis of sn-Glycero-3-phosphocholine concentrations

Summary of Fit

Rsquare	0.413855
Adj Rsquare	0.240183
Root Mean Square Error	0.000818
Mean of Response	0.002956
Observations (or Sum Wgts)	36

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Exp/time	8	0.00001276	1.5946e-6	2.3830	0.0437*
Error	27	0.00001807	6.6917e-7		
C. Total	35	0.00003082			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C00	4	0.004110	0.00041	0.00327	0.00495
C24	4	0.003545	0.00041	0.00271	0.00438
C48	4	0.003240	0.00041	0.00240	0.00408
C72	4	0.002364	0.00041	0.00152	0.00320
C96	4	0.002595	0.00041	0.00176	0.00343
E24	4	0.003095	0.00041	0.00226	0.00393
E48	4	0.003078	0.00041	0.00224	0.00392
E72	4	0.002385	0.00041	0.00155	0.00322
E96	4	0.002189	0.00041	0.00135	0.00303

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
C00	4	0.0041097	0.0008476	0.0004238	0.002761	0.0054584
C24	4	0.0035454	0.0006	0.0003	0.0025906	0.0045002
C48	4	0.0032395	0.0010793	0.0005396	0.0015221	0.0049569
C72	4	0.0023636	0.0001896	9.4823e-5	0.0020618	0.0026654
C96	4	0.0025954	0.0003208	0.0001604	0.0020849	0.0031059

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
E24	4	0.0030951	0.0002533	0.0001267	0.002692	0.0034982
E48	4	0.003078	0.0015731	0.0007866	0.0005748	0.0055812
E72	4	0.0023845	0.0008385	0.0004192	0.0010503	0.0037187
E96	4	0.0021888	0.0006312	0.0003156	0.0011844	0.0031932

Means Comparisons

Comparisons with a control using Dunnett's Method

Control Group = C00

Confidence Quantile

|d|	Alpha
2.83596	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
C00	-2e-3	1.0000
C24	-1e-3	0.8925
C48	-8e-4	0.5507
E24	-6e-4	0.3897
E48	-6e-4	0.3726
C96	-1e-4	0.0801
E72	8e-5	0.0360*
C72	1e-4	0.0331*
E96	3e-4	0.0163*

Positive values show pairs of means that are significantly different.