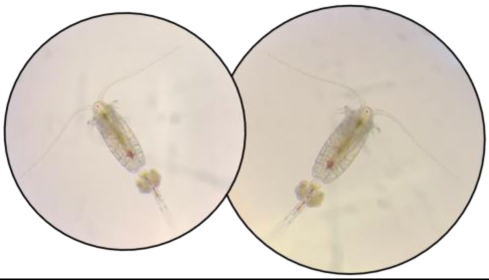
DEPARTMENT OF BIOLOGY AND ENVIRONMENTAL SCIENCES

Effects of marine contaminant mixtures on the copepod genus *Pseudocalanus*

How ambient contaminant mixtures affect mortality, food intake and fecal pellet production



Elisabeth Fenske

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Supervisor:	Ingela Dahllöf, Christina Jönander, Jenny Egardt
Examiner:	Thomas Backhaus
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(Pictures: Elisabeth Fenske)

Abstract

Chemicals are present in almost every part of human society and their benefits to our everyday life are undeniable. Besides those benefits, chemicals are known to cause harm to both human health and the environment. As a consequence of runoff, discharge or leachates, a huge amount of chemicals produced and used will finally be released into the environment. Thus, a broad variety of different chemicals end up in the marine ecosystems, where organisms are exposed to a mixture of compounds.

Figure 1: Community of zoo- and phytoplankton (picture: Elisabeth Fenske)

Zooplankton (Figure 1) play an important role in marine ecosystems and represent several functions. Being grazers and also grazed upon, zooplankton connect the producers at the base of the food web with consumers at higher trophic levels. Furthermore, zooplankton fuels the biological carbon pump by producing fecal pellets that act as food source deeper in the water column. The most abundant group of zooplankton are copepods. Therefore, understanding impacts of different stressors on copepod species is crucial and a lot of ecotoxicological studies focus on those zooplankton group. The genus *Pseudocalanus* is among the numerically most dominant copepod genera in several marine ecosystems, including the Swedish and Norwegian west coast.

The focus of this study was to determine changes of the copepod genus *Pseudocalanus* (Figure 2) and to understand how marine contaminant mixtures from the west coast of Sweden affect those organisms. Six chemical extracts derived from water samples at six locations around Stenungsund were used. The municipality Stenungsund and the surrounding area contains agriculture, industry, harbours and hosts both thousands of inhabitants and tourists, which represents a wide variety of potential chemical sources and pathways for those compounds to reach the marine environment. Even though the predicted toxicity of the six chemical extracts containing polar organic compounds did not raise any concern, the experimental approach was set-up to prove the theoretical calculations.

Figure 2: Pseudocalanus females (picture: Elisabeth Fenske)

The results of this study are both unexpected and worrying. Significant increase in mortality was detected in concentrations five times higher than ambient environmental conditions (5x MEC). Furthermore, food intake and fecal pellet production were significantly reduced, down to approximately 20 % and 40 % in the 5x MEC form site M6 and even the ambient environmental concentration (1x MEC) indicated reduction (Figure 13 A, B). Both replicability and reliability of those results were proven, and I therefore suggest further research on different copepod species, younger life stages and additional endpoints. My results raise concern, especially when considering that the chemicals the copepods were exposed to, do not represent the entire ambient contaminant situation. Compounds like heavy metals and nonpolar organic compounds were not sampled, which results in an underestimation of my findings. I therefore suggest further investigations in this and other related locations as well as improved sampling techniques which represent the entire environmental contaminant mixture to ensure a clear reflection of the environmental situation and to avoid underestimation of effects

Abstract for the public audience

We humans are surrounded by huge amounts and various types of chemicals every day! Can you think about one example?

Even though it might not be intuitive, chemicals are used in personal care products, in detergents, kitchen utensils, food packaging, electronical devices and even road markings. These are just some examples to illustrate the ubiquitous presence of various chemical compounds. Chemicals make human life and welfare easier, along with bringing relief and benefits. Unfortunately, chemicals are also known to cause harm for both the environment and human health. A huge amount of chemicals does not stay where they were initially produced or used, but are transported, and eventually reach the marine environment. Here, they harm plants and animals and ultimately us humans. The realistic situation in nature is therefore the encounter of not only one, but multiple chemicals, which are often called chemical mixtures. Besides exposure to several chemicals, marine organisms often experience further stressors like changes in temperature, plastics, competition for food or changes in pH.

Figure 3: Plankton community (sampled during sampling event in spring 2022 (pictures: Elisabeth Fenske)

Plankton communities (Figure 3) are small organisms in aquatic ecosystems, which drift in the water column and cannot swim against the currents. Plankton can be divided into zoo- and phytoplankton. While phytoplankton represent the base of the food web using the sunlight as energy source to produce nutrients, zooplankton are small animal-like organisms that act as link between the phytoplankton they feed on and the organisms that they are fed upon.

Zooplankton play an important role for the function of the marine ecosystem (Figure 4). They act, for instance, as food source for fish or whales and transport nutrients in form of fecal pellets deeper in the water column. Because of this close connection of the entire food web, changes within zooplankton communities can impact the whole food web and ecosystem. For instance, a reduction in zooplankton can lead to less food for fish larvae, which in consequence reduces the number of adult fish that can be fished by us humans.

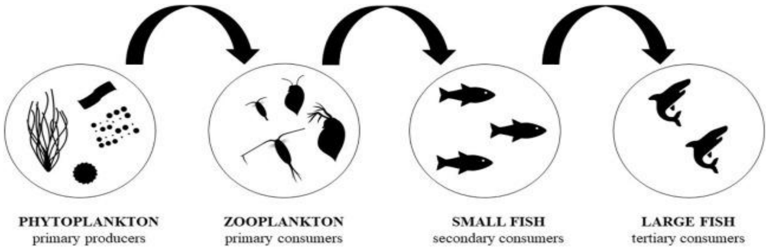


Figure 4: Illustration of the marine food web (from Lomartire et al.,

In this study, I focus on six different chemical mixtures and if and in what way they impact zooplankton. The contaminant mixtures derived from six locations around Stenungsund, where water was sampled at six locations. The zooplankton community was caught close to Lysekil, which is located north of Stenungsund. I decided to work with the most dominant copepod, which was *Pseudocalanus* (Figure 5)

Figure 5: The plankton workshop: from sampling of plankton communities (left) to Pseudocalanus females (right), (pictures: Elisabeth Fenske

The theoretical toxicity of the compounds in the mixtures had been calculated before I started my experiments and no harm for copepods was predicted. Therefore, I started the experiments with the assumption that the marine contaminant mixtures would not cause any harm to the *Pseudocalanus* females. What I found after the experiments was therefore very unexpected! The survival was highly reduced, partly only 10% of *Pseudocalanus* females survived the exposure to the chemical mixtures. Furthermore, the feeding and excretion of the copepods was highly reduced after exposure to each of the contaminant mixtures.

With the assumption that the contaminant mixtures would not cause any harm to the *Pseudocalanus* females, those pronounced results were both surprising and worrying at the same time. They illustrate that experimental testing should not be neglected and prediction of toxicity alone is not reliable enough. My results also suggest strong possible changes for the entire ecosystem in various ways. The low survival leads to fewer copepods available as food for fish or other animals feeding on copepods. The reduction in fecal pellets leads to less food for organisms deeper in the water that rely on those nutrients. The reduction in food consumption might lead to smaller copepods with less nutrient content, which means that organisms that feed on copepods must consume more food to receive the same nutrient content. For us humans that might also lead to lower numbers of fish to catch, like herring or cod.

My study indicates strongly that the contaminants present in the water around Stenungsund are more harmful for zooplankton than predicted and expected! More research is therefore needed to study the effects of similar contaminant mixtures on different species but also different levels in the food web. Furthermore, related locations on the west coast of Sweden should be studied regarding their contaminant mixture conditions.

Populärvetenskaplig sammanfattning

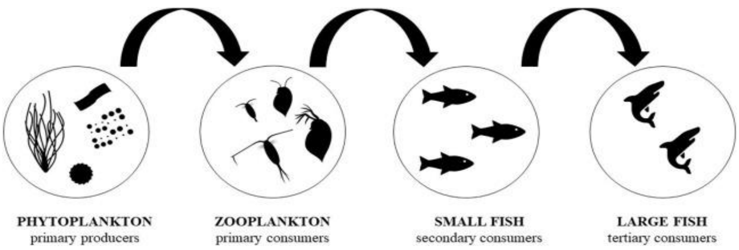
Kemikalier är allestädes närvarande och omger oss människor i alla delar av våra liv! Kan du själv tänka på ett exempel i din vardag?

Jag förstår att det inte alls är uppenbart och intuitivt, men kemikalier finns i matförpackningar, kosmetika, köksredskap, rengörings- och tvättmedel, leksaker och pysseltillbehör samt elektronik och även på vägmarkeringarna på trottoaren eller motorvägen. Även om jag bara nämner några exempel blir det tydligt vilken stor del kemikalier upptar och vilken berikning de ger till vår vardag. Tyvärr har forskning visat att kemikalier inte bara har positiva bidrag men också hotar naturen, djur och oss människor. Stora mängder av kemikalierna stannar inte där de produceras, eller används, utan transporteras vidare och når till slut akvatiska ekosystem, sjöar, älvar och oceaner. Då det finns enormt många olika kemikalier, är den realistiska situationen i miljön en blandning av flera komponenter, en så kallad cocktail av kemikalier, som organismer utsätts för. Om detta inte vore nog, tillkommer även flera andra faktorer som till exempel temperaturhöjning, konkurrens om mat, förändringarna i pH värden av vattnet, eller plaster som påverkar naturen.

Figur 1: Planktonsamhällen består av zooplankton och fytoplankton (foton: Elisabeth Fenske)

Planktonsamhällen finns i både salt-och sötvatten och de består av små organismer som flyter i vattnet utan att kunna simma mot strömmen (Figur 1). Samhällen består av både zooplankton, som man också kallar för djurplankton, och fytoplankton, som också kallas för växtplankton. Fytoplankton representerar basen av näringskedjan då de producerar organiskt material, eller biomassa, med hjälp av solljus som energi. Zooplankton däremot representerar länken mellan fytoplankton som de äter av och större organismer de blir ätna av.

Zooplankton spelar en betydande roll för marina ekosystemen och deras funktion (Figur 2). De agerar till exempel som födokälla till flera fiskarter och valar och transporterar näring i form av avföringpellets djupare i vattnet. Den täta kopplingen mellan flera komponenter i näringskedjan förtydligar att förändringar i planktonsamhällen kan påverkar hela näringskedjan och därmed ekosystemet. Till exempel kan en minskning av zooplanktonsamhället leda till att fiskyngel har tillgång till mindre föda, vilket i sin tur också kan leda till färre fullvuxna fiskar som vi människor kan fiska och äta.



Figur 2: Illustration av näringskedjan i havet (från Lomartire et al., 2021b)

Denna studie fokuserar på om och hur sex olika marina kemikalieblandningar påverkar zooplankton. Blandningarna kommer från sex olika platser nära Stenungsund på Sveriges västkust och zooplanktonsamhällen håvades bara några mil norrut, i Gullmarsfjorden nära Lysekil. För att fokuserar på hur kemikalier påverkar en art i planktonsamhället använde jag den mest förekommande hoppkräftan i samhället vilket var *Pseudocalanus* (Figur 3).

Figur 3: Plankton förberedelser: hur man kommer från ett planktonsamhälle till Pseudocalanus honor (foton: Elisabeth Fenske)

Den teoretiska toxiciteten av de sex kemikalieblandningarna beräknades innan experimentet startade. Beräkningarna kom fram till att det inte finns risk för zooplanktonsamhällen från någon av de sex blandningarna. Detta gjorde att jag inte räknade med några effekter på *Pseudocalanus* heller, och att jag ville använda mina resultat för att bekräfta de teoretiska beräkningarna. Mina resultat visar tvärtemot oförväntat hög toxicitet av alla de studerade kemikalieblandningarna. Antalet överlevande hoppkräftor minskade kraftigt, i vissa fall överlevde bara 10 % exponeringen till blandningarna. Dessutom minskade födointag och antal avföringspellets enormt efter exponeringen till alla sex kemikalieblandningarna.

Utgående från att ingen av de marina kemikalieblandningarna skulle ge en effekt på mina hoppkräftor, är resultaten både enormt oväntade och samtidigt skrämmande. De visar tydligt att teoretiska beräkningar kan ha sina svagheter och att experimentella försök är nödvändiga för att kontrollera hur organismer reagerar i sina naturliga miljöer. Toxicitet som bara grundas i förutsägelse är därför inte tillräcklig. Resultaten pekar på att på förändringar av hela näringskedjan och även skiftningar av ekosystemet kan förekomma. Det blir tydligast när man sätter mina resultat i sambandet med hoppkräftans funktioner. Till exempel betyder en minskning av antalet hoppkräftor i havet en minskning av föda för små fiskar och fiskyngel som betar på dem. Detta leder i sin tur till färre vuxna fiskar och därmed också färre fiskar som människor kan äta. Samtidigt betyder en minskning i antal producerade avföringspellets att mindre näring når djupare vattennivåer där organismer är beroende av den näringen. Dessutom betyder en minskning av födointag hos hoppkräftorna att de inte innehåller samma näring och är kanske även mindre då de inte betar lika mycket. Detta leder till att fiskar som äter hoppkräftor behöver äta mer för att få samma näring.

Mina studier tyder tydligt på att de marina kemikalieblandningarna i vattnet runt Stenungsund är farligare och mer oroväckande än beräknat! Därför behövs det mer forskning på både liknande ställen på västkusten samt på flera planktonarter och även andra organismer från olika nivåer i näringskedjan.

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Introduction

The marine environment provides various benefits, improving human lifestyle and welfare. Collectively, those benefits are classified as ecosystem services, which includes for instance fish harvest, water supply, services as tourism or transport and cultural benefits (Barbier, 2017). As those services depend on the functionality of the aquatic system, their protection and preservation are important to maintain their functions (Lomartire, Marques, & Gonçalves, 2021a, 2021b). Nevertheless, anthropogenic activities and impacts are threatening the marine ecosystems in various ways, resulting in altered functioning and structure of those systems globally.

Chemical pollution in the environment represents only one of many anthropogenic stressors impacting aquatic systems worldwide. Even though benefits of chemicals to everyday life are undeniable, anthropogenic chemical pollution is also a threat to both environment and human health (Naidu et al., 2021). Starting with the industrial revolution, the amount of chemicals produced have increased and both production and sales are projected to increase further in the following years (UNEP, 2019). Correspondingly, huge amounts of those chemicals are dispersed and released into the environment. The sources of those chemicals are wide ranging, including industry or agriculture, which consequently leads to various compounds like pharmaceuticals, pesticides or personal care products (Aryal et al., 2020). Very often, those chemicals enter various water bodies such as lakes, rivers and oceans through for example industrial discharge, runoff from agricultural land, leachates from landfills or effluent from wastewater (Jurado et al., 2012; Lomartire et al., 2021a). Thus, marine organisms are exposed to several contaminants simultaneously and could therefore experience mixture effects (Jartun & Pettersen, 2010; Tornero & Hanke, 2016). Understanding the effects of contaminant mixtures is therefore crucial to obtain a more realistic view of the effects of contaminants in the marine environment, how chemicals change the balance and alter ecosystem services (Barbier, 2017; Jurado et al., 2012).

Zooplankton play a key role in the dynamics, functions and productivity of marine food webs (Figure 6) (Banse, 1995). Acting both as grazer, feeding on bacteria or algae, and as a food source for various organisms including herring and capelin, zooplankton converts energy from lower trophic levels, such as phytoplankton, to higher trophic levels and therefore represent a crucial link between primary producers and consumers (Dalpadado, Bogstad, Gjøs1ter, Mehl, & Skjoldal, 2002). This complex relationship leads to a highly efficient nutrient cycle, highlighting the role of zooplankton with regard to predator and prey populations (Lomartire et al., 2021b). Fluctuations in planktonic biomass can therefore have drastic impacts on food webs structures or (commercial) fish populations, which consequently leads to alterations in seafood provision as one ecosystem service (Costanza et al., 2014; Dalpadado et al., 2002). Furthermore, fecal pellet production and sloppy feeding of zooplankton is essential for the biological carbon pump, transporting nutrients from the eutrophic zone deeper in the water column and to lower trophic levels (Turner, 2015; Turner & Ferrante, 1979). Zooplankton species are often used for ecotoxicology studies due to their sensitivity to toxicants as well as their importance in the aquatic food web. Studying and assessing the effects of chemical pollutants on zooplankton is therefore informative for possible impacts on the entire ecosystem (Hanazato, 2001). Copepods represent the most abundant group of zooplankton. Therefore, understanding how copepods respond to contaminants and chemical mixtures is highly important (Thompson, Dinofrio, & Alder, 2013). Metal mixtures and other chemical mixtures such as scrubber water and marine low-sulphur fuels have been shown to affect different copepod species, resulting in decreased survival, changes in community structure or alterations in development of early life stages (Filimonova et al., 2018; C. Jönander & Dahllöf, 2020; Marja Koski, Stedmon, & Trapp, 2017; Thor, Granberg, Winnes, & Magnusson, 2021).

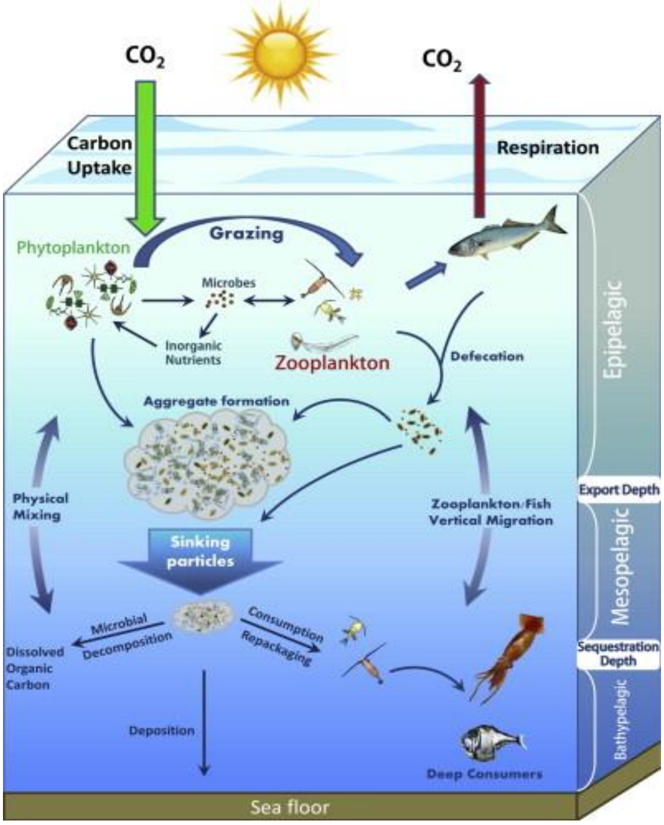

Figure 6: The role of plankton and the biological carbon pump (from Turner 2015)

The calanoid copepod genus *Pseudocalanus* is among the most numerically dominant copepods and a key species in various marine environments, including the North Pacific and the Baltic Sea (Otto et al., 2020; Questel, Blanco-Bercial, Hopcroft, & Bucklin, 2016). The differentiation between the seven globally co-occurring species is difficult, as adult stages only display subtle differences in size and morphology. Even though DNA-sequencing can be used to distinguish between species, co-existing species are often treated as a complex and reported as *Pseudocalanus* spp. (Cleary, Durbin, Rynearson, & Bailey, 2016; Frost, 1989; Questel et al., 2016). They are filter-feeders with a wide range of prey including diatoms, dinoflagellates and microscopic green algae (Cleary et al., 2016). *Pseudocalanus* species act as an important food source for several organisms at different trophic levels, including Atlantic herring, European sprat and Atlantic cod larvae (Möllmann, Kornilovs, & Sidrevics, 2000; Voss, Köster, & Dickmann, 2003). Ecotoxicological studies on the genus *Pseudocalanus* are rare and often focus on single compound exposure. For instance, the pesticide emamectin benzoate has been shown to affect mobility of three *Pseudocalanus elongatus* life stages negatively, with EC_{50} values in the range of 0.12 – 0.45 µg/L. Similar results were also found for another aquacultural pesticide (Kate J. Willis & Ling, 2003; Kate J Willis & Ling, 2004).

This study aims to start filling the knowledge gap of mixture toxicity by exposing copepods of the genus *Pseudocalanus* to several mixtures of contaminants that derived from water samples around Stenungsund, a municipality north of Gothenburg (Figure 7). Located on the west coast of Sweden, Stenungsund houses ten thousand inhabitants and tourists. The surrounding area also contains harbours, agriculture and industry as well as hosting the largest national chemical cluster. Consequently, the sources of chemical compounds are diverse and include for example agriculture, industry, shipping and wastewater treatment plants.

Figure 7: Pseudocalanus females (pictures: Elisabeth Fenske)

The concept of toxic units (TU) was implemented in 1970 and has ever since been used in ecotoxicological studies to predict the potential toxicity of single compounds and chemical mixtures (Equation 1). TUs are calculated using the measured concentration in the environment and the EC_{50} value for the concerned species (Junghans, Backhaus, Faust, Scholze, & Grimme, 2006; Sprague, 1970). Values below 1 are considered to be no reason for concern, because the measured environmental concentration is lower than the concentration causing effects in 50% of the exposed species.

$$\boxed{TU = \frac{conc}{EC\ 50}}$$

Equation 1: Toxic Units (TUs) were calculated using the measured concentration and the EC_{50} for Daphnia

Chemical mixtures from six locations in the Stenungsund area, consisting exclusively of polar organic compounds, were sampled (Figure 9). The values for the toxic units (TUs) for all six chemical mixtures range between 0.01 and 0.006 (C. Jönander, Egardt, J., Carmona, E., Spilsbury, F., Dahllöf, I. , 2022), which is far below the threshold to cause any concern.

Table 1: Information about chemical extracts from all sampling sites (Jönander, C., Egardt, J., Carmona, E., Spilsburz, F., Dahllöf, I., 2022)

Parameter	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**
Number detected chemicals	74	78	75	68	80	62
Toxic Units (Based on ECOSAR lowest EC_{50})	0.004	0.006	0.006	0.002	0.01	0.002

Even though the predicted toxicity does not cause any reason for concern, I aimed to study the cumulative effects of those compounds in concentrations including the actual measured environmental concentration (MEC) on several endpoints as well as the food source of *Pseudocalanus* copepods. This strategy was chosen to prove the theoretically predicted toxicity with effects experimentally obtained after actual exposure. According to the calculated TU values I did not expect any effects and hypothesize therefore no toxicity for these studies of *Pseudocalanus* females.

Methods

1.1. Sampling and isolation of copepods

Copepods were collected one week before each experiment in the Gullmar fjord near Lysekil, Sweden from a depth of 30 m in March, April and May 2022 (Figure 8). The copepods were sampled using vertical tows with a non-filtering cod-end (200µm) net. The copepods were diluted into two 30 L buckets with filtered sea water (FSW, 33 psu) on return to the research station, connected to light airflow and placed in a thermoconstant room for acclimation to the experimental conditions. The thermoconstant room was adjusted as well as possible to the respective ambient conditions at the time of sampling, resulting in 8°C and a 12:12 h light-dark cycle for the first experiment and 10°C and a light-dark cycle of 16:8 h for the second and third experiment. The copepods were acclimated for one week respectively and fed with *Rhodomonas salina* at a daily concentration of 200 µg carbon/L. Adult *Pseudocalanus* females were isolated from the mixed communities, acclimated under the same conditions as the mixed communities and fed with *R. salina* three days a week at a concentration of 400 µg carbon/L.

Figure 8: Map of the copepod sampling sites in Gullmar fjord (modified from Svansson, 1984). Sampling sites are marked in red (58°15.590´N 11°26.140 ´E and 58°15.996´N 11°28.569´E).

1.2. Cultivation of Rhodomonas salina

The alga *Rhodomonas salina* was used to feed the zooplankton prior to and during the experiments, but potential impact from the chemical exposure on the alga itself was also studied. The algaculture was retrieved from the Gothenburg University Marine Algal Culture Collection (GUMACC). F2*2 medium (Guillard, 1975; Guillard & Ryther, 1962) was used for algal cultivation, and cultures were grown with light bubbling to prevent sedimentation of algal cells. Every second day, one third of the culture was exchanges with newly prepared medium to obtain a density between 500 000 and 1 000 000 cells/ml.

1.3. The chemical mixtures

The chemical mixtures used for exposure derived from water samples at six different locations around the area of Stenungsund on the west coast of Sweden (Figure 9).

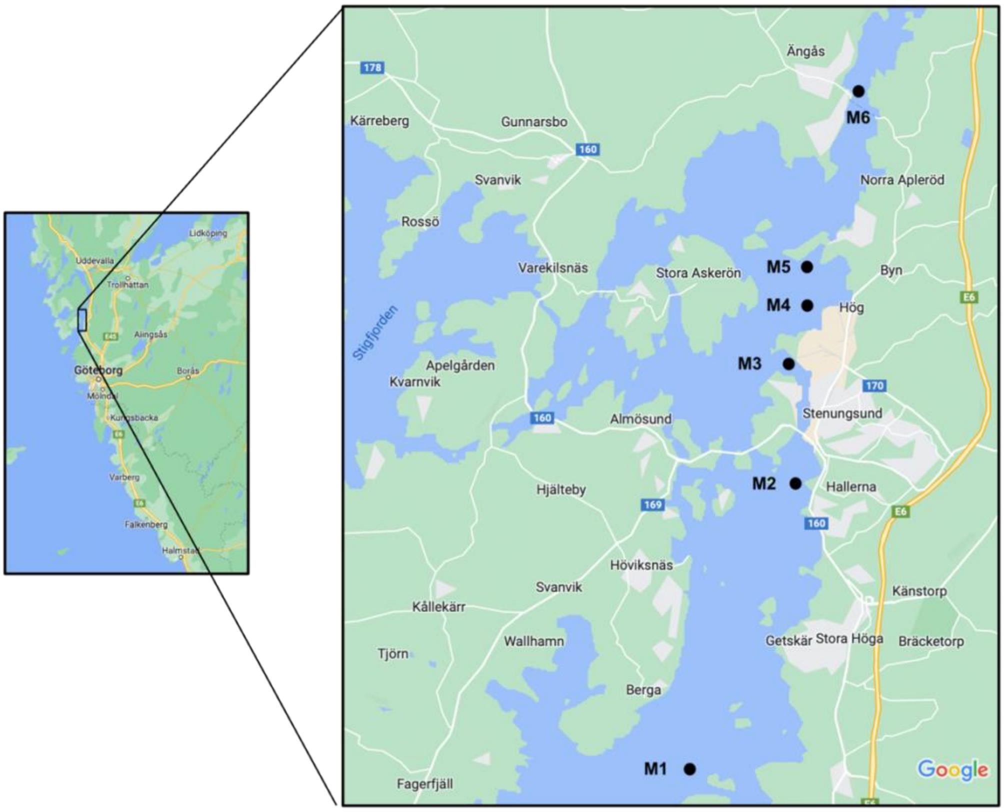

Figure 9: Location of the six sampling sites representing the six chemical mixtures used for exposure (modified from https://www.google.com/maps; accessed July 1, 2022)

The sampling itself took place in October 2020 as part of a separate study in Hakefjorden and Askeröfjorden ranging from Svanesund as northernmost location to Jörlanda in the south. Depending on their location, the sampling sites might experience different inflow pathways and can therefore be dominated by different compounds. Solid phase extraction was used to obtain chemical extracts from all six sites, 1000 times more concentrated than the measured environmental concentration (MEC). The chemical extracts were eluted in methanol and frozen until further use. The chemical composition differed between 62 and 80 compounds depending on site and also the toxic units (TUs) differed in a range from 0.01 and 0.006 between the six extracts (Table 1).

1.4. Experimental Setup and Exposure

The study consists of three experiment parts. During the first experiment I studied the impact of three concentrations of the chemical mixture from sampling site M3. During the second experiment, I focussed on the effects of four concentrations of the chemical mixture from sampling site M6 and during the final experiment, I determined the impacts of one concentration from every sampling site (Table 2). The studied species were the same in all experiments, *Pseudocalanus*. Copepods were fed with *R. salina* three days a week (Monday, Wednesday, Friday) in a concentration of 400 µg carbon/L.

All experiments were conducted in 148 ml fluorinated HDPE bottles. Methanol was used as a solvent for the working solutions that were prepared from the extract at 100 times higher concentrations than the actual exposure concentrations, to ensure the same addition of solvent in each bottle (1.48 ml). Working solutions were prepared one day before each experimental start to ensure sufficient mixing. The same volume of solvent was added to each control.

Table 2: Overview about experimental design

	Experiment 1	**Experiment 2**	**Experiment 3**
Site	M3	M6	M1, M2, M3, M4, M5, M6
Treatment	0.1x MEC, 1x MEC, 10x MEC	0.1x MEC, 1x MEC, 5x MEC, 10x MEC	5x MEC
Chemical analysis	Yes, one per treatment after exposure	No	Yes, one per treatment before exposure

Chemical mixture working solution, or methanol for the control, was added to each experimental bottle, which was then placed into a fume hood. As the bottles were opened the methanol evaporated from all samples, while the chemical extract remained. When dry, 100 ml of FSW was added to each sample to dissolve the chemicals, and all bottles were moved to the thermoconstant room. The density of the *R. salina* culture was determined using a Beckman Coulter Multisizer 3 Particle Counter. The required amount of algaculture to obtain 250 µg carbon/L in each experiment bottles was calculated and added to each of them. 15 adult *Pseudocalanus* females were added to each copepod bottle. Every experimental bottle was then top filled with FSW and a Teflon® slide was placed on top of each bottle before closing the lids to avoid air bubbles. The bottles were taped on a plankton wheel (for slow rotation) and kept in the thermoconstant room under dark conditions for 46 h (Figure 10). The conditions were chosen to mimic the movement in the ocean as well as possible and limit the growth of R.salina due to the absent of light.

Figure 10: Plankton wheel used for the three experiments (picture: Elisabeth Fenske)

The *Pseudocalanus* females were collected in petri dishes after exposure for 46 h by carefully emptying each copepod bottle through a 200 µm mesh. While the copepods were collected in the 200 µm mesh and placed in petri dishes, the remaining water from each replicate was poured through a 25 µm mesh to collect the fecal pellets in a second petri dish. The remaining liquid of each replicate was saved and used for algal density measurements and chemical analysis. Acidified Lugol´s solution was added to each petri dish containing fecal pellets in a ratio of (1 ml : 60 ml) to fix the samples until counting. All petri dishes and bottles were kept in the thermoconstant room until further use.

1.5. Determination of mortality and counting of fecal pellets

Immediately after the exposure, the immobility of the adult *Pseudocalanus* females was determined visually using a stereo microscope. According to the OECD guideline 202 for testing of chemicals, adults *Daphnia* sp. were considered immobile, when not moving for 15 seconds after gentle agitation (OECD, 1984). The same methodology was used for mortality testing on *Pseudocalanus* females. The number of alive (mobile) and dead (immobile) *Pseudcalanus* females as well as the total number of individuals present in the petri dishes after exposure was counted in a randomized order to minimize biased counting. The mortality rate was calculated by dividing the number of dead females by the total number females in each experiment sample.

The number of fecal pellets was determined using a stereo microscope by placing the petri dish into a prepared lid with a square pattern to facilitate the counting. The number of fecal pellets with a minimum size of 0.24 mm were counted. All samples were counted blind in a randomised order.

1.6. Food intake and algal density

Each bottle was sampled at the end of the exposure and measured on the Beckman Coulter Multisizer 3 Particle Counter to determine the copepod feeding rate. The detected size was set according to the size range that was measured to determine the required food amount right before the experiment started. For all three experiments the size of algal cells varied between 4.6 µm and 11.9 µm.

The initial algal concentration was measured in separately prepared bottles (n=3), which represent the `start´ concentration. After exposure for 46 h, the *R. salina* concentration was measured in all remaining copepod bottles (n=4) and algal control bottles without copepods (n=3). Then, the density difference over time (46 h) was calculated in both algal and copepod bottles by subtracting the mean of the start bottles from each treatment. The actual food intake was calculated by dividing the density differences in the copepod treatments by the corresponding differences in the algal bottles (Equation 2).

$$food\ intake = \frac{average\ cell\ concentration\ `start` - cell\ concentration\ copepod\ bottle}{average\ cell\ concentration\ `start` - average\ cell\ concentration\ algae\ bottle}$$

Equation 2: Calculation of food intake of Pseudocalanus females in one copepod bottle over time (46h)

To obtain the food intake in cells/ml/copepod/day (change in cells/ml caused by each female each day), the obtained food intake value was divided by the total number of *Pseudocalanus* females present in each treatment after exposure and adjusted per 24 h.

1.7. Statistical analysis

Effects on adult mortality, food intake, algal density and fecal pellet production were analysed using the software R (version 4.1.3). To detect differences between treatments and control Analysis of Variance (ANOVA) and Dunnett´s post hoc test were used. To determine homogeneity of variance, Levene´s test was used. Normality of residuals were checked using Shapiro-Wilks test. In case any assumption was violated, the data was transformed using square root or log10. If the assumption for homogeneity of variance and normality of residuals were still not fulfilled, a Kruskal-Wallis test and a Dunn’s post hoc test were used instead. For the endpoint food intake in the second experiment (sampling site M6) the assumptions were not fulfilled after data transformation and thus, a Kruskal-Wallis test and a Dunn´s post hoc test were used.

1.8. Chemical analyses

Confirmation of exposure concentrations of the chemicals in the mixture was done by solid phase extraction, where the extracts were sent to the Helmholtz Centre for sustainable research (UFZ), Germany for analysis. For the first experiment the water of one control, one 1x MEC treatment and one 10x MEC treatments were used after 46 h exposure. This handling ensures the detection of potential changes in the chemical mixture between the different treatments due to exposure conditions and organisms. For the third experiment, samples for chemical analysis were prepared before exposure to detect possible changes of the chemical mixture due to their storage under freezing conditions. By analysing one concentration of all sampling sites, it is possible to detect alterations in the various chemical mixtures.

Collection of chemicals was done using a solid phase extraction kit (Macherey-Nagel). The kit for solid phase extraction (CHROMABOND® HR-X polypropylene columns) was used in combination with a vacuum aperture equipped with a manifold lid to run several samples simultaneously was used. The columns, containing the solid phase material, were fastened on stopcocks to regulate the flow through the column. To activate the solid phase filters, 5ml of ethyl-acetate followed by 5 ml methanol and 5 ml LC-MS grade (liquid chromatography-mass spectrometry) water were pipetted into the column and drawn through the filter by adjusting the vacuum in the aperture. After that, the bottles were weight (without lid and Teflon® slide) and the liquid drawn through the corresponding solid phase filter column.

Then, the empty bottles were weighed again, and the columns wrapped in aluminium foil and stored in the freezer. The difference between the weight of the bottles is later needed to determine the exact volume that had been added to each solid phase filter. The results of the chemical analysis are not part of this study.

1.9. Relation between calculated toxicity and observed effects

To investigate possible linear relations between the calculated toxicity and the observed effects, linear regressions of the three endpoints mortality, food intake as wells as fecal pallet production and the precalculated summed toxic unit values were performed. For this, the Spearman´s rank correlation coefficient was calculated using the software R (version 4.1.3).

Results

1. Concentration range experiments (M3 and M6)

1.1. Mortality

Mortality was significantly increased to 90% ($p<0.001$) after exposure to 10x MEC of the chemical mixtures from site M3 (Figure 11, A). A slight but not significant increase in mortality could also be detected for the 1x MEC treatment ($p=0.2$). After exposure to the chemical mixture from sampling site M6 (Figure 11, B) the mortality was significantly increased in both the 5x and the 10x MEC treatments, which resulted in 42% respectively 86% reduced survival of *Pseudocalanus* females. Exposure to 0.1x and 1x MEC from either site did not cause significant differences compared to the controls ($p=0.4$ for M3 and $p=0,96$ for M6).

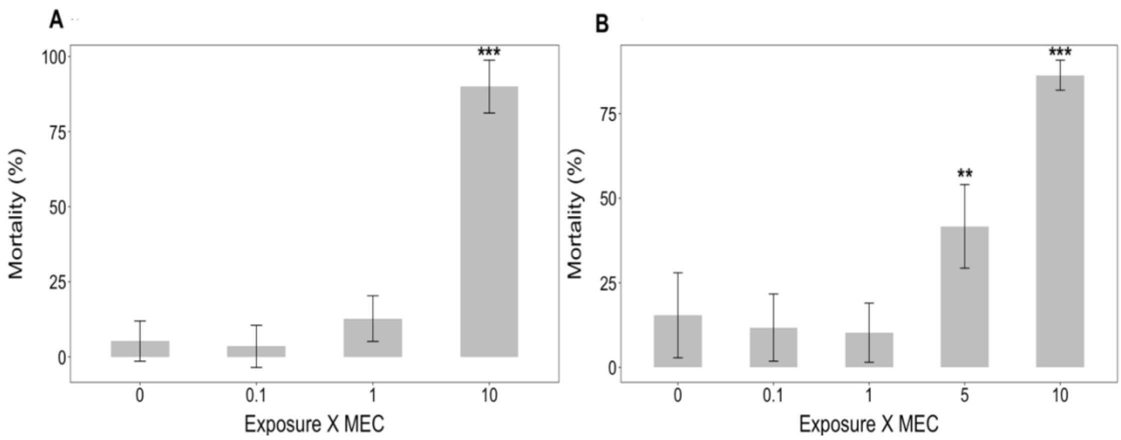

Figure 11: Mortality (%) of Pseudocalanus females after exposure to the chemical mixtures from sampling site M3 (A) and M6 (B) according to the different exposure treatments (mean± SD of the four replicates per treatment) in times Measured Environmental Concentration (MEC)

1.2. Food Intake

Food intake of *Pseudocalanus* females was similarly impacted by the chemical mixtures from the two sampling sites M3 and M6 (Figure 12). The food intake in the highest treatment from site M3 (Figure 12, A) was significantly reduced to zero ($p<0.001$). The exposure to the chemical mixture from M6 (Figure 12, B) resulted in significantly reduced intake of *R.salina* in the two highest treatments (5x MEC and 10x MEC). The food intake was decreased by approximately 80% in the 5x MEC treatment ($p=0.03$) compared to the controls. The 10x MEC treatment showed no detectable food intake ($p=0.002$), similarly to the highest treatment from site M3. After exposure to 0.1x MEC of both chemical mixtures (M3 and M6) the food intake was slightly larger than in the higher treatments, about 6% in the M3 treatment ($p=0.9$). The 1x MEC, which represents the actual chemical concentration in the environment, showed non-significant reduction in food intake ($p=0.3$) up to approximately 36% in the M6 treatment compared to the control.

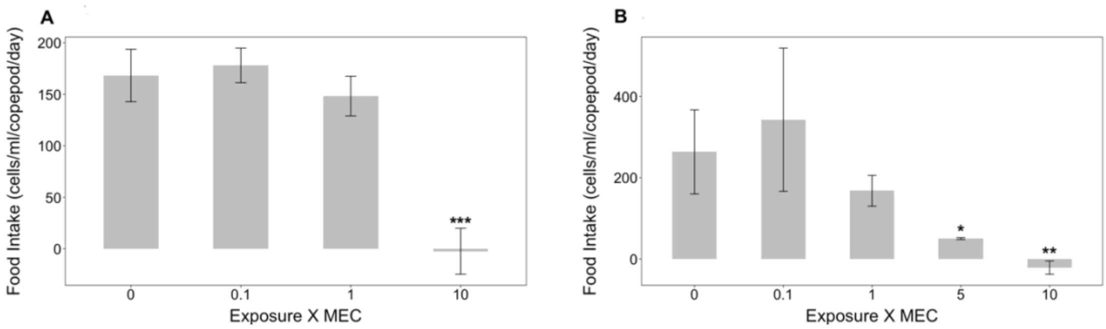

Figure 12: Food Intake of Pseudocalanus females (change in cells/ml caused by each copepod/day) after exposure to the chemical mixture from sampling site M3 (A) and M6 (B) according to the exposure treatments (mean ± SD of the four replicates per treatment) in times Measured Environmental Concentration (MEC)

1.3. Fecal Pellets

The number of fecal pellets produced per *Pseudocalanus* female was impacted by exposure to the chemical mixtures (Figure 13). Both chemical extracts showed a similar overall trend in production of fecal pellets, with an increase in the lowest treatment (0.1x MEC) followed by a decrease in the 1x MEC treatments and drastic reduction in the highest treatments. Significant reduction in produced fecal pellets were detected for the 10x MEC treatment in both chemical mixtures (M3 and M6). Fecal pellet production was significantly reduced to almost 20% compared to the control after exposure to the M3 (Figure 13, A) mixture ($p<0.001$). The results were even more pronounced for site M6 (Figure 13, B), where the number of produced fecal pellets were reduced to 5% ($p<0.001$). The two lowest treatments from sampling site M3 show increased numbers of produced fecal pellets compared to the control, 18% for the 0.1x MEC and 13% for the 1x MEC, but the numbers barely differ between both treatments. The results from sampling site M6 represented the food intake results very well and showed the same trend for the treatments. The 0.1x MEC treatment showed an increase by 47% compared to the control ($p=0.6$), while the higher treatments showed reduced number of fecal pellets. The number of produced fecal pellets declined with approximately 62% after exposure to 5x MEC ($p=0.05$).

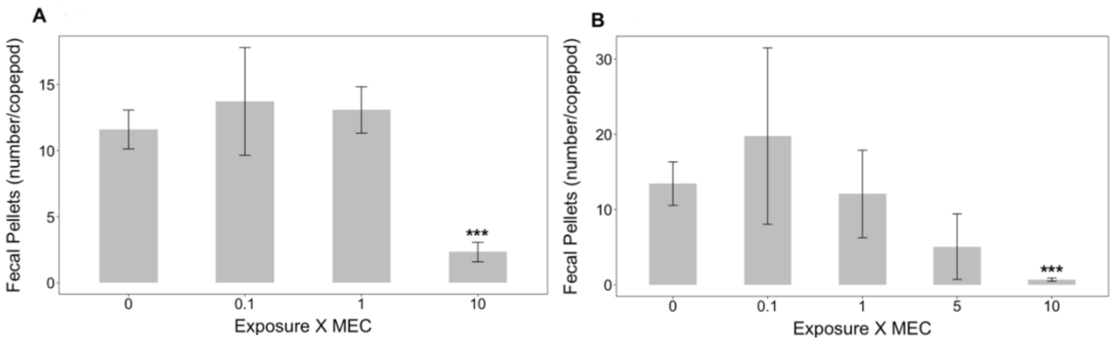

Figure 13: Fecal Pellet produced of Pseudocalanus females (number of pellets/copepod) after exposure to the chemical mixture from sampling site M3 (A) and M6 (B) according to the exposure treatments (mean ± SD of the four replicates per treatment) in times Measured Environmental Concentration (MEC)

2. Comparison between M3 and M6

Food intake was stronger impacted by exposure to the chemical extract from site M6 compared to site M3 (Figure 14, A). The same trend could be detected for the fecal pellet production, compounds from M6 affect the production of fecal pellets stronger compared to the extracts from site M3 (Figure 14, B). Survival was more affected by the extract from site M3, especially in the highest treatment where survival was reduced to 11% (Figure 14, C).

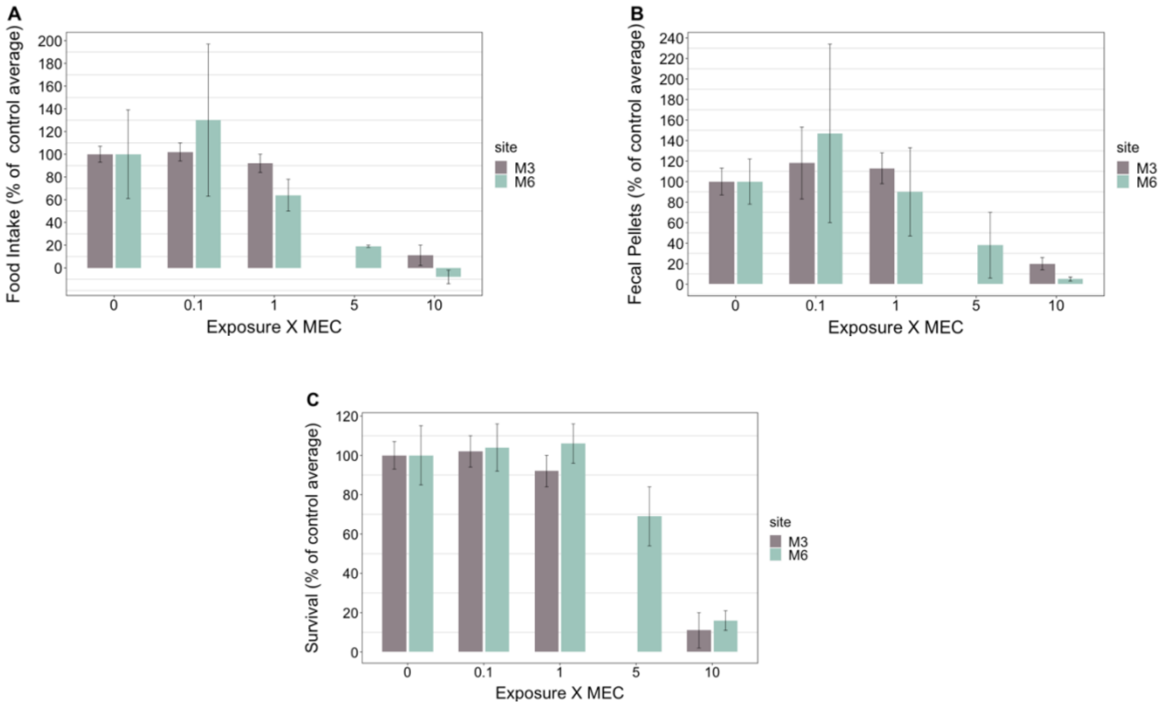

Figure 14: Comparison of the Food Intake, Fecal Pellet Production and Survival (% of control average) according to the exposure treatments (mean ± SD of the four replicates per treatment) in times Measured Environmental Concentration (MEC) of the chemical mixtures from sampling site M3 (grey) and M6 (green)

3. Single compound comparison

Based on the two previous concentration range experiments with site M3 and M6, the exposure treatment for the comparison of all sites was chosen to be 5x MEC (Figure 15).

Mortality was significantly increased after exposure to chemical mixtures from sampling site M3 ($p<0.001$) and M4 ($p=0.002$), where mortality increased with 55% and 49% compared to the control (Figure 15, A). Increased adult mortality was detected for all the remaining chemical mixtures as well, but changes were not significant ($p=0.1$ for M6; $p=0.2$ for M1; $p=0.7$ for M2; $p=0.98$ for M5). Food intake was significantly reduced after exposure to all chemical mixtures, with the highest reduction for the mixtures from M2 and M6 where the food intake was reduced to approximately one third (Figure 15, B). Similar to the reduced food intake, the number of produced fecal pellets decreased after exposure to the mixtures from all sites (Figure 15, C). The highest impact on all three endpoints, mortality, fecal pellets and food intake, could be detected for the chemical mixtures from sampling site M3, M4 and M6 (Figure 15, A, B, C). Density of *R.salina* (Figure 15, D) was not impacted significantly in any of the chemical mixtures compared to the control (p-values between 0.6 and 1)

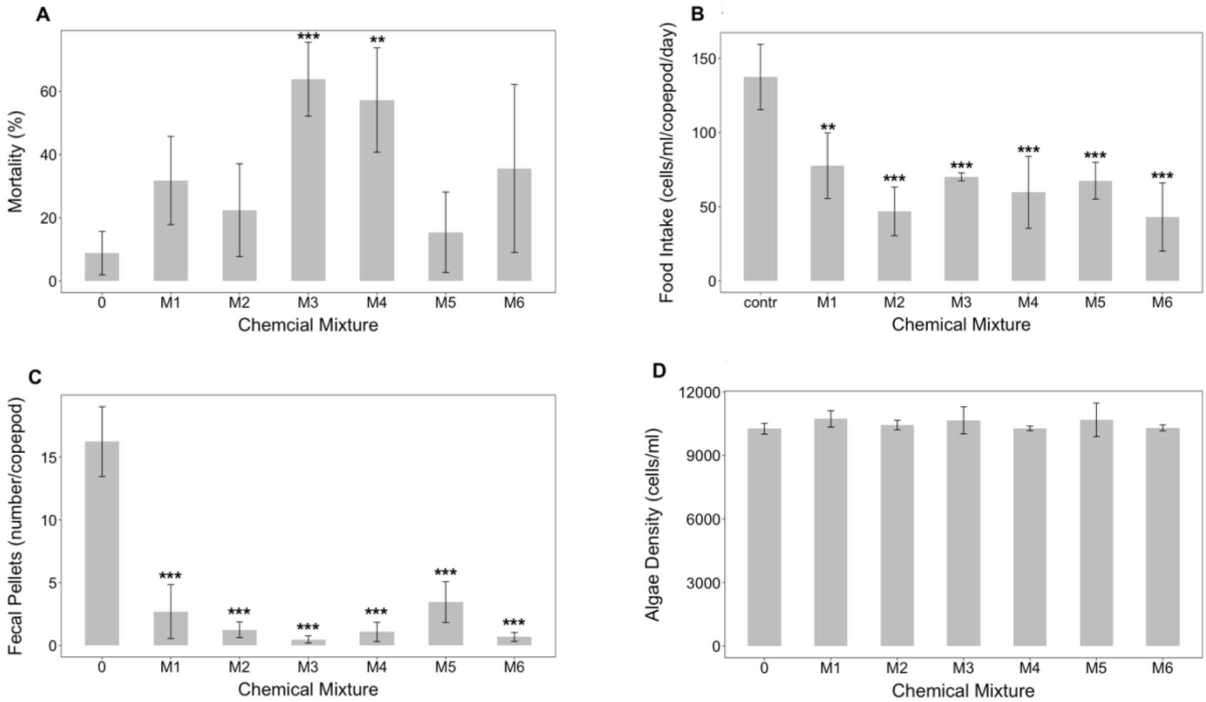

*Figure 15: Impacts on Mortality, Food Intake, Fecal Pellet Production and Algal Density after exposure to 5x Measured Environmental Concentration (MEC) of the chemical extracts from all sampling sites (mean ± SD of the four replicates). Significance between control and treatments is marked with *** (p<0.001) and **(0.001<p<0.01)*

4. Relation between calculated toxicity (∑TUs) and observed effects

No linear relation between the ∑TUs and the observed effects for any of the three endpoints was detected, indicated with a p-value of 0.91 for mortality, 0.73 for food intake and 0.82 for the fecal pellet production (Figure 16). The Spearman´s rank correlation coefficient ρ was close to zero in all three calculations, ranging between -0.109 for the relations between (∑TUs) and fecal pellet production and 0.055 for the relation between (∑TUs) and mortality.

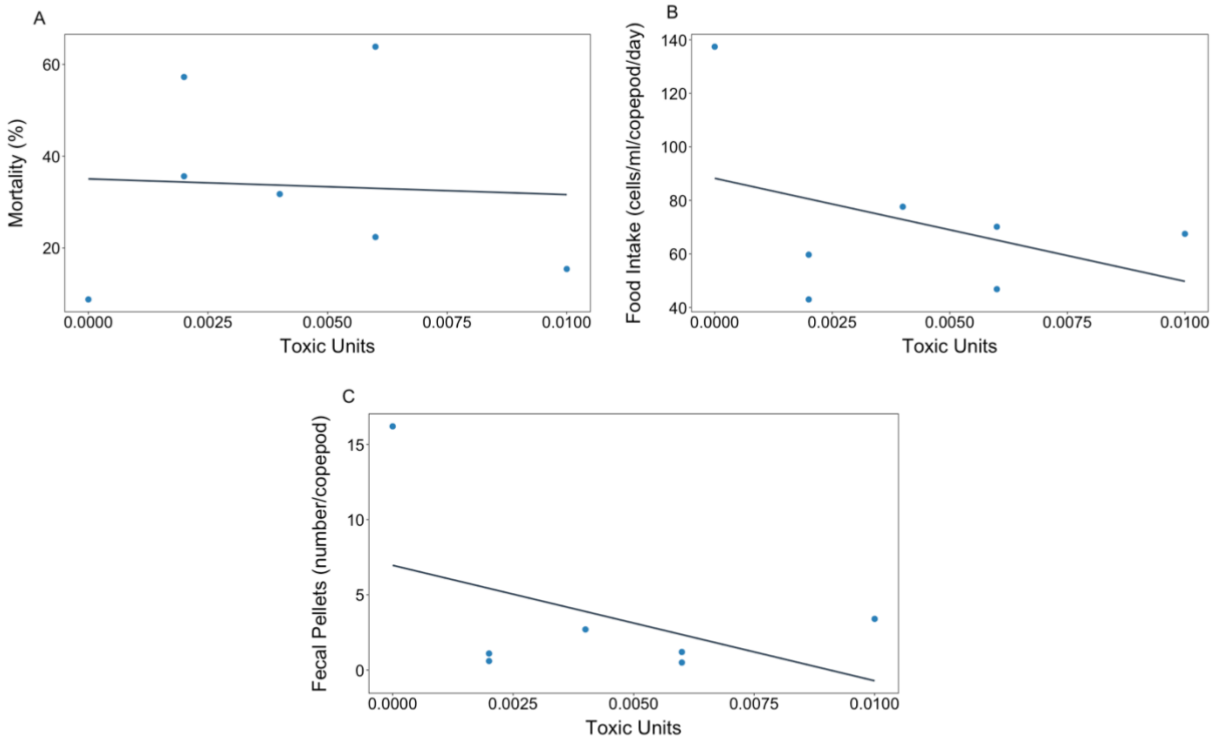

Figure 16: Relation between calculated toxicity (∑TUs) and observed effects on mortality (A), food intake (B) and fecal pellet production (C) after exposure to 5x Measured Environmental Concentration (MEC) of the chemical extracts from all sampling sites. (Each dot represents the mean of the four replicates per sampling site plotted against the calculated sum of TUs, black line represents the linear regression line)

Discussion and Outlook

No effects on *Pseudocalanus* survival, food intake and fecal pellet production were expected to be seen due to the low predicted TU values for all chemical mixtures, even at 10 X MEC. Even though the extracts did not contain heavy metals and nonpolar organic compounds, and therefore do not represent the entire ambient conditions, no effects were hypothesised as even the highest TU value (for site M5) did not exceed 0.1 at 10x the environmental concentration. However, impacts on all those three endpoints could be detected during my short-term study with acute exposure for 46h. Because of that, the hypothesis of my study has to be rejected.

The chemical extracts investigated in my study derived from one sampling event for each sampling location, the obtained extracts did not represent all present compounds, and the calculation for predicted TUs was done using the lowest EC_{50} values for *Daphnia*, which all together demonstrate an underestimation of the actual hazard for the environment (Table 1). Despite this underestimation, the results on one single copepod genus were already significant and become even more pronounced when considering the short exposure time of less than two days. My results contribute to the monitoring study around Stenungsund in an alarming way, especially considering even stronger impacts on organisms encountering the ambient conditions.

1. Mortality

The mortality for the control in this study was 8.8%. According to the OECD guideline used for mortality testing, results are valid when not more than 10% of *Pseudocalanus* females are immobile after exposure (OECD, 1984).

Adult mortality of *Pseudeocalanus* females was mainly affected at high concentrations of chemical extracts and highest for the 5x MEC treatments from mixture M3 and M4. For the chemical mixture from site M3 even the ambient environmental concentration gave increased adult mortality after exposure for 46 h, which suggests a high toxicity of that mixture. Within the range of the calculated toxic units, the chemical mixture from site M5 would suggest the strongest effects of all mixtures. Instead, effects on mortality are most pronounced after exposure to the lowest TU value (M4) and lowest after exposure to the highest TU value (M5). Increased mortality due to exposure to mixtures of compounds of zooplankton species could also be detected in *Daphnia magna*, where mortality was significantly increased to more than 60 % after exposure to a pharmaceutical mixture for 6 days (Flaherty & Dodson, 2005). Scrubber discharge water, a mixture related to shipping and harbours, also showed increased mortality of adult *Acatia tonsa*, resulting in almost 100% mortality after exposure to 30% scrubber water for 24h (Marja Koski et al., 2017)

Early life stages of various zooplankton in general are more vulnerable than the adult counterparts (Almeda, Wambaugh, Chai, et al., 2013). Juvenile copepods are more sensitive and show higher mortality, which could also be shown under high quality and quantity conditions of food (Ketil & Mark, 2004; M. Koski & Breteler, 2003). Higher sensitivity to chemical compounds could furthermore be detected when exposing both nauplii and adult females of the copepod species *Tigriopus japonicus* to the antimicrobial agent Triclosan, where the NOEC value (no observed effect concentration) for adult females was 15 times higher compared to the NOEC for the nauplii (Park, Han, Lee, Seo, & Lee, 2017). Those results suggest that younger life stages of *Pseudocalanus* and other copepod species around Stenungsund might be even more vulnerable than the adult females exposed in my study.

2. Food intake and fecal pellets

Food intake is often calculated theoretically using the number of produced fecal pellets as a proxy for ingestion (Jensen, Nielsen, & Dahllöf, 2008). In my study both ingestion and excretion were measured experimentally, which increases the reliance of the outcome of those endpoints. Both food intake and fecal pellet production show consistent trends resulting in significantly reduced intake of *R.salina* and significant reduction in numbers of produced fecal pellets in the 5x MEC treatments from every chemical extract (Figure 15). The density of *R.salina* was not impacted under those exposure conditions in any of the treatments and chemical extracts, which indicates that the driver for reduction in food intake and fecal pellet production was not alteration in food availability (Figure, Figure). The good correlation between food intake and fecal pellet production could also be detected in previous studies (Seuthe, Darnis, Riser, Wassmann, & Fortier, 2007). In my study the results for both endpoints are highly reliable because they were determined independently and obtained experimentally instead of grounded on calculations. The consistency of both endpoints confirms the reliance of my results and confirm, that both foraging and excretion are impacted by the exposure to chemical extracts. Similar effects on food intake and fecal pellet production could be detected after exposure to other chemicals like the PAH pyrene (Jensen et al., 2008) or crude oil (Almeda, Wambaugh, Wang, et al., 2013; Hansen et al., 2017)

Interestingly, both food intake and production of fecal pellets increased slightly for the lowest treatments (0.1x MEC) after exposure to the chemical mixtures from sampling site M3 and M6 (Figure 13, Figure 13). My findings of positive effect after exposure to low concentrations of chemicals go hand in hand with results obtained in a study where copepod communities were exposed to low levels of the water-soluble fraction of a ultra-low sulphur fuel oil (C. Jönander & Dahllöf, 2020). Another study showed the increased production of fecal pellets of the two copepod species *Calanus finmarchicus* and *Calanus glacialis* after exposure to low concentrations of the PAH pyrene (Jensen et al., 2008). Other stressors like ocean acidification have also been shown to have beneficial effects in low levels (Thor & Oliva, 2015). Those seemingly beneficial effects of low-level stressors can be explained with the phenomenon of hormesis, which describes the stimulating impact of a low-level stressor that is toxic in higher levels (Sebastiano, Messina, Marasco, & Costantini, 2022).

3. Replicability of experiments

While experiment one (M3) was done to obtain an overview about possible effects using three exposure treatments, the following experiment (M6) was adjusted to the obtained findings and a fourth exposure treatment was added. According to the results obtained from this experiment, the 5x MEC treatment was chosen for the final experiment, in which all six chemical extracts were studied. In that way, I obtained results for the same exposure for the chemical extract from sampling site M6 twice and can therefore discuss the reliability and replicability of my experiments and results. Mortality differed between 6.1% between the two exposure events for the M6 mixture (Figure 11, B and Figure 15 A) and is higher in the second experiment (42%) compared to the third (36%). Food intake (cells/ml/copepod/day) was significantly decreased and varies between approximately 50 and 23 cells/ml/copepod/day in both experiments (Figure 12, B and Figure 15, B). Fecal pellet production was similar in both experiments as well, and differed between 1 and 5 pellets/copepod (Figure 13, B and Figure 15, C). All three studied endpoints showed very similar results and the highest values were consistently detected in the second experiment. That means that copepod mortality was stronger impacted in the second experiment, while food intake as well as fecal pellet production were more adversely affected in the third experiment. This suggests that the *Pseudocalanus* females used in the second and third experiment responded slightly different, which seems realistic because the copepods were sampled with more than two weeks in between.

4. Sensitivity changes depending on sampling date

The differences in sensitivity between the sampling dates becomes more prominent, when considering the observation that body sizes of adult *Pseudocalanus* females decreased with the month of zooplankton sampling. Even though those findings were subjectively observed, two independently working researchers came to the same conclusion, that body size of adult females decreased with rising water temperature, from April to May. The inverse relation between temperature and body size of organisms, resulting in larger animals of the same clades in regions with lower temperature, was firstly described as Bergmann ´s rule. Supporting this rule, body size of copepods depends on water temperature as well, resulting in larger copepod species in colder regions and smaller copepods in temperate waters (Campbell et al., 2021; Evans, Hirst, Kratina, & Beaugrand, 2020). Body size does not only vary interspecific but also shifts intraspecific during seasons, resulting in slightly larger individuals during colder periods and smaller individuals of the same species during warmer seasons (El-Maghraby, 1965; Warren, Evans, Jude, & Ayers, 1986). This correlation was observed for the zooplankton samplings in my study as well. *Pseudocalanus* females were larger during the first sampling in March and smallest during the last sampling in May. Those differences between March and May correspond to an increase in water temperature during that time span from approximately 4°C to almost 13°C (data collected during the experiment). Supporting my findings of higher mortality of smaller *Pseudocalanus* females (sampled in May), higher sensitivity to crude oil pollution was also found in smaller copepods species during warmer periods As both food intake and number of produced fecal pellets were stronger reduced in the adult females from the last zooplankton sampling event compared to the females from earlier samplings, it can be suggested that smaller individuals ingest and egest less, compared to larger individuals of the same species. Mortality has been shown to follow this trend as well, resulting in higher sensitivity to crude oil in smaller species and warmer temperatures (Jiang, Huang, Chen, Zeng, & Xu, 2012). My findings do not support this correlation, as higher mortality was detected in the slightly larger *Pseudocalanus* females from sampling during April. However, mortality can also be impacted by human factors like copepod handling and might therefore not follow previous findings.

A study from 2022 monitored polycyclic aromatic hydrocarbons (PAHs) and metals in sediment, water and mussels in an area close to my study location, Grebbestad. The authors could prove the linkage between leisure boat activity near Grebbestad and increased concentrations of PAHs in water, sediment and oysters. Besides the PAHs, even several metals were monitored in the sediment in the area around Grebbestad, with highest concentrations within the harbour (Kjell Nordberg, 2022). The correlation between concentration peaks of PAHs and high season of leisure boat activity could also be seen in another study on a third location on the west coast of Sweden, Kostahavet, where both PAHs and metal ions increased within the peak season of leisure boat activity (Egardt, Mørk Larsen, Lassen, & Dahllöf, 2018). Both Grebbestad and Stenungsund are municipalities on the west coast of Sweden and have harbours, recreation and leisure boat activity in common. Even though PAHs represent only one group of anthropogenic pollution and leisure boats only one specific source for those chemicals, the results visualize the importance of this source, especially when considering all other sources and chemicals reaching the aquatic environment. The importance of leisure boat activity becomes even more pronounced when taking into account the peak of that recreation during summer season but also maintenance during the entire year (Egardt et al., 2018).

Both mentioned studies above include several metals and proved that they are present and partly highly concentrated at locations close to my sampling site, which strongly suggests that similar levels of metals are also present at my sampling location in the Gullmar fjord. The extracts *Pseudocalanus* females were exposed to, did not contain any metals, which clarify once more the need of further studies as well as the underestimation of my findings.

5. Relation between predicted toxicity (∑TUs) and observed effects

There was no linear relation between the calculated ∑TU and any of the studied endpoints (**Error! Reference source not found.**). Instead, chemical mixtures with low TU values (M4, M6) show partly high toxicity and the extract ranked to be most toxic resulted in minor effects after exposure to 5x MEC (M5).

My study was based on the calculated ∑TUs and due to the low predicted toxicity, I hypothesised no effects to be seen. A lack of experimental toxicity data for several compounds resulting in reliance on modelled toxicity data is a possible reason for the detected discrepancy. The usage of *Daphnia sp*. as model organism for the toxicity calculation due to a lack of data for copepods could be another possible cause, resulting in underestimation of the toxicity when relying predicted toxicity in form of TUs exclusively. Nevertheless, the concept of toxicity prediction using ∑TUs should not be diminished but the limitations of modelled toxicity calculations have to be taken into account.

6. Outlook

The important role of *Pseudocalanus* and zooplankton in general for the marine environment and food webs illustrates that possible changes are likely to be extensive. Combining both bottom-up and top-down control in the food webs, my results suggest both impacts on higher and lower trophic levels. A decrease in number of copepods leads directly to less food for higher trophic levels grazing on zooplankton, which includes both fish, whales and birds. Consequently, the decrease in food availability would continue higher up in the food chain and finally impact the fishing industry. On the other hand, top-down control of copepods includes the reduction in fecal pellets that are produced and feed organisms deeper in the water column. Besides that, the reduced food intake might lead to smaller copepods with lower nutritional content. This suggests that grazers must feed more to obtain the same nutrition from their food source. Often, food intake is also linked to egg production, with the assumption that higher food availability and food intake leads to higher numbers of produced eggs. Even though egg production was not determined in this study, the investigation of that endpoint would be interesting in following studies. Reduced egg production as a consequence of exposure to chemical mixtures would strengthen the impacts on the ecosystem even further.

Following my experiments, it would be necessary to gain information about the compound or compounds that drive the toxicity of the mixtures. That information can be used to determine potential major sources of those compounds and to assess better monitoring and regulations. Furthermore, that information should be communicated to the public to increase awareness. Further research should also be considering chemical mixtures in combination with other stressors, like ocean acidification, water temperature rises or the presence of invasive species. The higher sensitivity of agricultural pollutants in combination with higher temperatures could already be detected for the copepod species *Eucyclops serrulatus* (Lorenzo et al., 2015), which suggests that similar reactions are true for different zooplankton species and stressors as well.

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Appendix 1

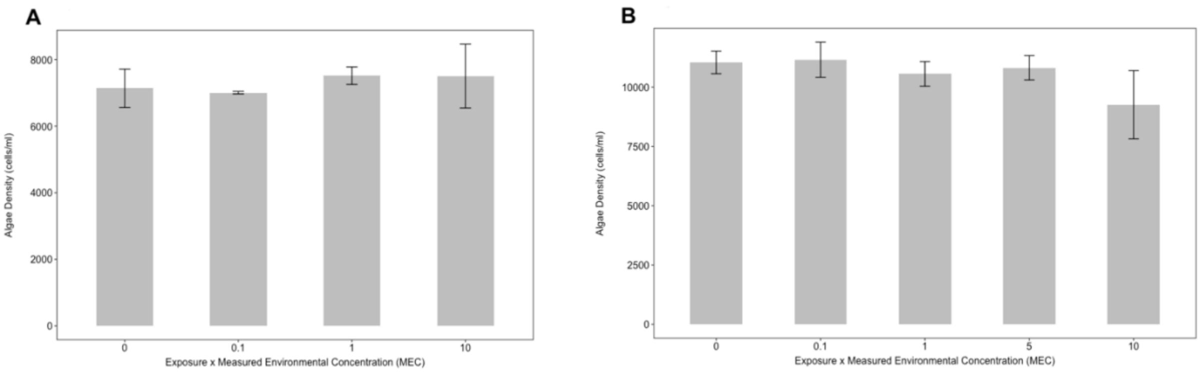

Figure 17: Density of Rhodomonas salina (cells/ml) according to the exposure treatments (mean± SD of the three replicates per treatment) in times Measured Environmental Concentration (MEC) of the chemical mixture from sampling site M3 (A) and M6 (B).