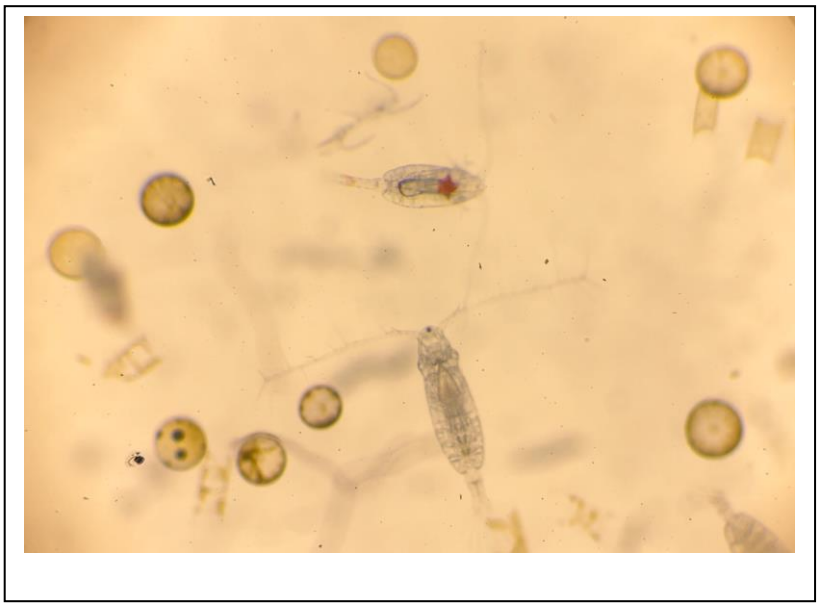
UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

TOXICITY DRIVERS IN CHEMICAL MIXTURES FROM THE SWEDISH WEST COAST – EFFECTS ON PELAGIC COMMUNITIES



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(Picture: Helena Hartmann)

Abbreviations

AWCD	Average well color development
EC50	Effect concentration 50 %
FU	Fluorescent unit
DOC	Dissolved organic carbon
DIC	Dissolved inorganic carbon
IC50	Inhibition concentration 50 %
LC50	Lethal concentration 50 %
MEC	Measured environmental concentration
MoA	Mode of action
nMDS	Non-metric multi-dimensional scaling
PICT	Pollutant induced community tolerance
PPC	Particles per copepod
SD	Standard deviation
TU	Toxic unit

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Abstract

Human society benefits from a range of ecosystem services provided by the oceans, such as oxygen production, carbon storage, and fishing. However, these services are relying on the ecosystem's functional and structural integrity, which are correlated to its biodiversity. The increasing amount of chemicals that pollute the marine environment, particularly coastal areas, threaten to impact this integrity. A monitoring study conducted at the Swedish west coast showed high levels of chemical contamination at all sampled sites, but mixture toxicity prediction regarding copepods and actual chemical mixture testing on the copepod genus *Pseudocalanus* regarding the chemical mixtures produced contradicting results. The toxicity prediction resulted in an underestimation of the mixtures toxicity, raising the concern that the environmentally present concentration levels negatively impact the natural copepod communities. Therefore, I tried identifying toxicity drivers in the chemical mixtures present at the Swedish west coast by ranking the detected chemicals according to their contributory toxicity by using ecotoxicological data from US EPA for three different organism groups. The considered groups were mesozooplankton, microalgae, and bacteria, including cyanobacteria. The highest-ranking chemicals in toxicity for mesozooplankton and microalgae, formetanate hydrochloride and Spinosyn A, respectively, were selected for further ecotoxicological testing on natural copepod, algal and bacterial communities. The results of my study showed that neither Spinosyn A nor formetanate were the toxicity drivers in the mixtures as environmental concentrations caused no significant effects in all selected endpoints. Furthermore, the calculated contributory toxicity to the overall mixture toxicity seemed to be accurate regarding formetanate. However, the success of the toxicity driver identification was heavily impacted by the low amount of ecotoxicological data available, excluding the majority of detected chemicals. Therefore, this study further highlights the need for additional data collection when it comes to chemicals present in the environment, especially regarding marine organisms.

Introduction

The pelagic food web

The pelagic food web is a complex net of interactions between twelve organism groups that are responsible for the transformation and exchange of organic and inorganic matter (Zohary *et al*., 2014). The organism groups can be divided into four major trophic levels: decomposers, primary producers, primary consumers, and secondary consumers. Both, bacteria and phytoplankton are playing an essential role in element cycling in the water column (Kirchman *et al*., 1982) but the whole pelagic food web is responsible for the exchange of CO_2 between the atmosphere and deeper water levels (Steinberg & Landry, 2017). Carbon is present in the water column as particulate organic carbon, dissolved organic carbon (DOC), and dissolved inorganic carbon (DIC). One of the key players in the carbon exchange cycle is the "microbial loop", which is partially made up of bacteria. DOC is incorporated into the food web by heterotrophic bacteria while DIC is used for energy production by other groups of bacteria and archaea (Pomeroy *et al*., 2007). Subsequently, the fixed carbon and bacteria are then taken up by other organisms as a source of food, moving the carbon up along the trophic levels (Sherr & Sherr, 1988). Furthermore, phytoplankton, namely diatoms, dinoflagellates, and nanoplankton ranging from 2-20 µm (Platt and Li, 1986), introduce carbon into the cycle as primary producers through photosynthesis. Marine zooplankton, which can be herbivorous, omnivorous, and even carnivorous, occupies multiple trophic levels (Steinberg & Landry, 2017). For instance, nanozooplankton graze on bacteria and cyanobacteria (Fenchel, 1983) while microzooplankton consumes phytoplankton such as dinoflagellates. The majority of mesozooplankton (0.2-20 mm) is made up by copepods that live on an omnivorous diet (Turner, 2004). Zooplankton, which acts as an important link between primary producers and higher trophic levels, is then consumed by secondary consumers such as larger invertebrates and small vertebrates. The large number of interactions throughout the food web makes it susceptible to interference caused by stressors. This can either occur directly or indirectly and can sometimes lead to chain reactions within an ecosystem.

Community ecotoxicology

Large amounts of ecotoxicological data are solely based on single species testing. While this is beneficial when it comes to understanding the direct effects of a chemical on one species or organism group and to compare chemicals and their respective toxicity, a lot of information is lost when it comes to the actual impact of that chemical on ecosystems. Community ecotoxicology on the other hand aims to involve more biological organization levels compared to single species testing, to incorporate the structural and functional complexity of an ecosystem. When taken to

some of the highest organizational levels, communities and ecosystems, both biotic and abiotic factors must additionally be considered when it comes to assessing the possible effects of a chemical (Cairns, 1983). A way to incorporate both factors is to use multispecies models such as field studies, micro- or mesocosms. Using these experimental set ups makes it possible to additionally include and assess possible effects on important dynamics that are essential for an ecosystem's functionality, such as predator-prey interactions, and is, therefore, a powerful tool that should be implemented in ecotoxicological testing and considered in environmental and chemical risk assessments (Boxall *et al*., 2002).

In reality, ecosystems are exposed to multiple stressors and pollutants that continuously interact with each other, compared to single substance exposure. However, the long-term damage these stressors can have on an ecosystem is controlled by its resilience and its stability. While ecological resilience describes a system's ability to absorb disturbances before alterations of the system occur (Holling, 1996), stability measures the ability of a system to recover to its original state after a disturbance (Holling, 1973). Moreover, an ecosystem's stability is closely connected to its diversity. For example, a higher diversity ensures better ecosystem functioning by reducing temporal variability of biomass production (Isbell *et al*., 2015). Additionally, the exposure of a community to contaminants can lead to a shift in community composition as a result of *pollutant induced community tolerance* (PICT), where sensitive individuals or species are being replaced by more tolerant ones (Blanck, 2002). PICT has been observed in specific marine communities, such as periphyton (Blanck & Wängberg, 1988; McClellan *et al*., 2008) and benthic nematodes (Millward & Grant, 1995), and it has additionally been connected to the exposure to specific pollutants, such as pesticides (Zabaloy *et al*., 2010). The sensitivity of different species to one chemical varies strongly and is usually considered in ecotoxicology regarding surface waters by using species sensitivity distributions. This tool is used to establish safe environmental limits for a chemical or a chemical group, such as pesticides, where the baseline hazardous concentration is set to a limit where at most 5 % of all present species would potentially be affected (Rizzi *et al*., 2021). It is based on the concept that the species sensitivity follows a symmetrical log scale distribution (Kooijman, 1987). However, this approach opens up the possibility that important keystone species are included in the 5 % of affected species, which could affect the ecosystem's functionality and should, consequently, be used with caution.

On these grounds it is important to incorporate the community aspect into the assessment when it comes to establishing a safe ecological threshold ensuring upkeep of biodiversity and ecosystem functionality.

Chemical mixtures and their presence on the Swedish west coast

Aquatic ecosystems are facing an increasing number of stressors, either due to natural causes or due to human activities and interactions. In particular, coastal areas are consistently affected by pollutants originating from agricultural and industrial run off and urban sewage (Vikas & Dwarakish, 2015; Zhou *et al*., 2022) and are, consequently, being altered by anthropogenic activity (Stauber *et al*., 2016). Ecotoxicology mainly focuses on the testing of single substances on species (Løkke *et al*., 2013), leading to difficulties when it comes to assessing the risk that chemical mixtures pose to the exposed ecosystem. Environmental monitoring, which is frequently used for risk assessments, is an important tool to assess stressors in an ecosystem. Monitoring of chemical mixtures on the Swedish west coast, ranging from *Skalkorgarna* over *Stenungsund* to *Fiskebäckskil* (Figure 1), showed the presence of a total of 172 different organic chemicals in the coastal waters, putting all six monitoring sites at risk from chemical contamination (Gustavsson *et al*., 2017). The monitoring sites are situated near wastewater treatment plants, harbors, chemical industry sites, and urban areas, leading to multiple different pollution sources for the coastal environment. Hence, a mixture toxicity prediction was performed based on concentration addition with ecotoxicological data from US EPA on marine zooplankton, which was extended with QSAR modelling for environmentally present chemicals without toxicological data (Jönander *et al*., 2022). Given that QSAR modelling is based on *Daphnia magna*, a freshwater species, an assessment factor was used. The predictions resulted in toxic units ranging from 0.002-0.01, indicating no risk for zooplankton on the Swedish west coast at measured environmental concentration (MEC).

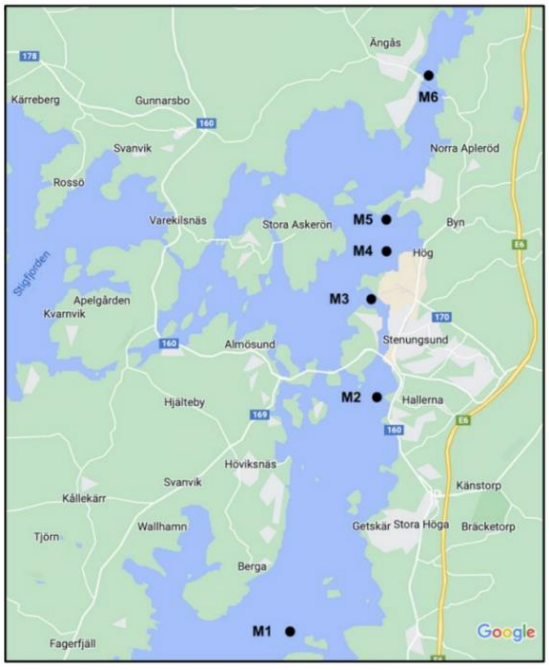

Figure 1*: Sampling sites for the six chemical mixtures collected in the Stenungsund study (from Elisabeth Fenske, 2022).*

In a separate study, copepods of the genus *Pseudocalanus* were exposed to the chemical mixture extracts from the six sites (Figure 1), resulting in significant increase in mortality and a reduction in feeding rate and fecal pellet production when exposed to five and ten times measured environmental concentrations (Elisabeth Fenske, 2022). Regarding the results, the chemical extracts from site M3 and M4 appeared to have the highest toxicity to *Pseudocalanus*. These results contradicted the toxicity prediction, where the highest calculated toxic unit for the chemical mixtures was 0.01 for site M5 (Jönander *et al*., 2022). According to this prediction, an increase of environmental concentrations by a factor of five or ten would still result in a toxic unit < 1, indicating safe environmental levels. This causes the concern whether current environmental concentration levels are already negatively affecting copepod communities in Swedish coastal waters.

Environmental chemical mixtures that pose a risk to the exposed ecosystem can be difficult to deal with regarding efficient and successful impact mitigation. A favorable method is to identify toxicity drivers in the mixtures, to then facilitate targeted regulations which enable the reduction of these specific substances. The specific reduction of the environmental concentrations of the toxicity drivers would generate a solid and feasible approach, which is a needed fundamental measure when it comes to handling the increasing amount of chemicals in our marine waters.

Thus, the aim of this study was to test the method of using toxicity calculations, based on ecotoxicological data from US EPA, to identify toxicity drivers that are present in the chemical mixtures on the Swedish west coast. For this, toxic units will be used to predict the contributory toxicity of individual compounds in the mixture in relation to their measured environmental concentration. Additionally, to increase environmental relevance, their effects on native pelagic communities will be tested to create further understanding on safe environmental thresholds regarding marine environments and to generate ecotoxicological data for marine organisms. The selected substances will be tested as single substances and, furthermore, as a mixture to incorporate possible interactions. Bacterial, algal and copepod communities were selected for this study to incorporate effects on the microbial loop as well as on primary producers and consumers.

Materials and Methods

Toxic unit ranking

To identify toxicity drivers, chemicals present at site M3 and M4 from the 2017 Stenungsund study were ranked according to their toxic units (TU) as the chemical extracts from these sites showed

the highest toxicity to *Pseudocalanus*. TU indicates a chemicals contribution to the overall toxicity in a chemical mixture based on concentration addition (Junghans *et al*., 2006; Steen *et al*., 1999).

Three separate TU rankings were carried out for the following organism groups: mesozooplankton, microalgae, and bacteria. The calculation of the TU is based on the equation:

$$TU_{chemical} = \frac{MEC_{chemical}}{Mean\ EC50_{chemical}}$$

where the TU of chemical X is based on the measured environmental concentration of chemical X at site Y, divided by the calculated mean concentration that causes a 50 % effect, the so-called effect concentration 50 (EC50). Mean EC50 was calculated by using ecotoxicological data from the US EPA ECOTOX database (US EPA, 08.09.2022) for 'aquatic crustaceans', 'other invertebrates', and 'algae'.

To estimate the toxicity of different chemicals to marine mesozooplankton, ecotoxicological data for freshwater organisms was included because the majority of available ecotoxicological data is based on freshwater *Daphnia*. Ecotoxicological data of protists, flagellates, protozoa, and chromista was excluded, as well as the data for organisms with planktonic larvae stages. Cyanobacteria was included in the TU calculations for both algae and bacteria. The TU is based on the endpoints EC50, lethal concentration 50 (LC50), and inhibition concentration 50 (IC50) and only includes studies < 48 h (Table 8, appendix). Chemicals that had no chemical abstracts service (CAS) number were excluded.

Spinosyn A pilot study

Sampling

A natural algae community was sampled in October 2022, 50 m offshore at Kristineberg, Fiskebäckskil, Sweden near the sampling station *Släggö* (Figure 2). The algae were sampled with a 1.8 L Niskin bottle from the surface water and filtered through a 63 µm mesh to remove microzooplankton and larger particles. The algae were kept in a thermoconstant room at 15 °C with a light:dark cycle of 11.5:12.5 h to imitate natural circumstances.

Figure 2*: Sampling site on the Swedish west coast near Släggö, Lysekil for algae, bacterial, and zooplankton communities for all experiments (modified from https://www.google.com/maps; accessed May, 2023).*

Experimental set up

The sampled algae were exposed to spinosyn A in 320 ml Pyrex glass bottles for 48 h. The experiment consisted of four exposures, based on the highest MECs from the Stenungsund study and the exposure concentrations in the *Pseudocalanus* study (Table 1), and a methanol control. Spinosyn A was dissolved in methanol due to its low solubility in water, the solution was then added to the Pyrex bottles and left to evaporate under a fume hood until no liquid was present anymore. All handling of Spinosyn A until exposure start was done with minimal light exposure to avoid photodegradation. Following the evaporation, the bottles were topped up with the sampled sea water containing the algae community. To ensure an even distribution of the chemical throughout the bottles during the exposure period and to prevent the algae from settling, a plankton wheel was used. Five T0 samples were taken randomly between the filling of the exposure bottles, and the collected sea water containing the algae was gently mixed in between each step. The number of particles at T0 and T48 was measured with a particle counter to represent biomass. To reduce background noise in the machine, deep sea water was used that was previously filtered over a 1µm GFF filter. Additionally, fluorescence was measured with a fluorometer, to use fluorescence units (FU) as a proxy for photosynthetic efficiency. The measurement was performed three times per sample and FU per particle was compared between treatments. The median was calculated and

used for statistical testing and visualization to account for the high variation per sample between measurements.

Formetanate hydrochloride pilot study

Sampling and copepod keeping

A natural copepod community was sampled via horizontal dragging from 40 m depth with a 200 µm cod-end plankton net near *Släggö*, *Lysekil*, Sweden in November 2022 (Figure 2). The sampled community was kept in two 30 L containers in deep sea water that was being aerated with air pumps. The containers were kept in a thermoconstant room at 12 °C with a light:dark cycle of 8:16 h to imitate natural circumstances. The zooplankton was fed with 5000 cells/ml of *Rhodomonas salina* and left undisturbed for 24 h to acclimate.

Exposure

The sampled zooplankton was exposed to formetanate hydrochloride in 620 ml Pyrex glass bottles for 48 h in a closed system. The experiment consisted of four different exposures, analogue to the Spinosyn A pilot study, and a methanol control (Table 1). *R. salina* was additionally exposed to the same concentrations in 320 ml Pyrex glass bottles, to ensure a more accurate feeding rate. The formetanate hydrochloride was initially dissolved in methanol, the solution was then added to the Pyrex bottles and left to fully evaporate before filling them with filtered sea water. Copepods were concentrated from the 30 L containers over a 200 µm mesh with a glass beaker and the copepods collected on the mesh were frequently rinsed into a 1 L Pyrex glass bottle with aerated deep-sea water. The concentrated community remained in the Pyrex bottle until the exposure start, where approximately 100 copepods were added to each exposure bottle with a wide-mouth pipette and fed with 600 µgCarbon/L, resulting in 8300 cells/ml *R. salina*. Five starting samples were taken in between from the sampled copepods to later determine the initial number of copepods per replicate and, additionally, five T0 samples for *R. salina* were taken from 620 ml Pyrex bottles as well as three T0 samples from 320 ml Pyrex bottles, to determine the actual added particle number of the algae. A plankton wheel was used for the exposure duration to mimic oceanic movement and to prohibit the settling of copepods or the toxicant. After 48 h the copepods were filtered over a 200 µm mesh and transferred to scintillation vials with ethanol. The number of copepods was determined using a stereomicroscope. Copepods that were either fully intact or only showed minor damage (e.g., missing antenna segments) were considered to have survived the exposure. The exposure water was caught in a separate container and refiltered over a 60 µm mesh to collect any eggs laid during the exposure period. The eggs were left in closed petri dishes filled with aeriated sea water to incubate for 48 h in the thermoconstant room under the same temperature and light

conditions. After 48 h, acidified lugol solution was added to the petri dishes to stop the incubation process. During the filtering process some copepods were accidentally flushed through the 200 µm mesh and remained in the petri dish during the incubation time. Both total egg production and hatching success were determined by counting the number of unhatched eggs and number of hatched nauplii. Feeding rate was calculated by subtracting the measured number of particles at T 48 h from the calculated mean of the T0 *R. salina* particle numbers.

Biolog Ecoplates

The effect of formetanate hydrochloride on the bacterial community was tested with Biolog® Ecoplates™ by using the bacterial communities' physiological fingerprint as a proxy for diversity (Figure 3). For this purpose, the bacterial community connected to the zooplankton was used by filtering the water from the barrel containing the sampled copepods over a 20 µm mesh. Exposure concentrations were analogue to the formetanate hydrochloride exposure to the copepod community (Table 1). The filtered water was then added to the Ecoplates along with the toxicant, resulting in an exposure volume of 150 µl. The color development was monitored over 96 h with a plate reader and, subsequently, the calculated slopes resulting from the growth curve were compared between treatments. For the slopes, the average well color development (AWCD) was calculated for each reading and, additionally, the 31 carbon sources were grouped into six main sources and the average well color development (AWCD) calculated in accordance with Xu&Ge, 2015.

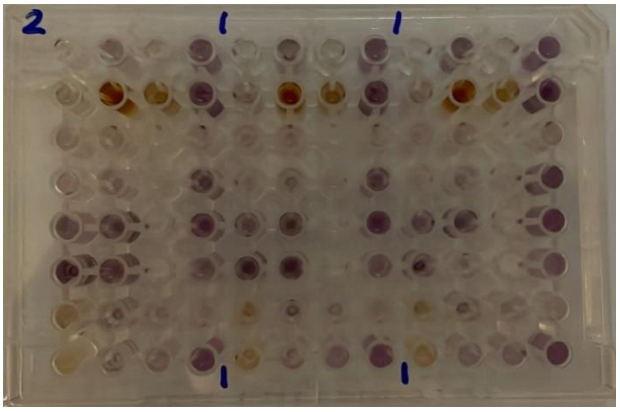

Figure 3*: Biolog EcoPlate showing a bacterial community's ability to utilize 31 different carbon sources, with tetrazolium redox dye acting as an indicator. Utilization of the carbon source results in purple coloring of the well, the resulting reaction pattern can then be used to detect changes in the community.*

Mixture toxicity study

The experiment was split up into two sub-experiments, a concentration range finding study for the individual chemicals and a mixture toxicity study. The additional concentration range finding was

performed to incorporate the changes in copepod and bacterial communities in spring compared to autumn.

Sampling and copepod keeping

The sampling for the mixture toxicity study was performed analogue to the previous formetanate study. Natural copepod communities were sampled twice, once for each experiment, at the end of February and beginning of March 2023, with one week in between sampling. Copepods were kept in aerated water in a thermoconstant room with a light:dark cycle of 9:15 h and a temperature of 7 °C to acclimate for 24 h. In the concentration range study, copepods were fed with 5000 cells/ml of *R. salina*. To ensure the produced eggs are not a result of leftover nutrient excess that resulted from feeding prior to the chemical exposure, this was not done with the community sampled for the mixture toxicity study.

Exposure

The exposure conditions and experimental set up were analogue to the prior formetanate pilot study. In the concentration range finding four spinosyn A and five formetanate concentrations were used in addition to the methanol controls (Table 1). The mixture study consisted of two mixture concentrations and two positive controls, one each for spinosyn A and formetanate (Table 1). *R. salina* was exposed to the same concentrations as a growth control. For the concentration range finding the copepods from the 30 L containers were concentrated by prefiltering them over a 200 µm mesh, for the mixture toxicity study a 250 µm mesh was being used to reduce the number of copepods flushed into the incubation dishes. After the 48 h exposure, the copepods were filtered over a 200 µm mesh in both sub-experiments. For the incubation period in the mixture toxicity study any copepods that were accidentally flushed into the petri dishes were removed manually with a pipette over a microscope to avoid subsequent egg laying and grazing.

The bacterial community was exposed in the concentration range finding study but not the mixture study.

Table 1: Experimental set up for all performed studies stating test organisms, test substances and their concentrations, the number of replicates, and the selected endpoints.

Study	Test organisms	Test substances	Tested concentrations [ng/L]	Replicates	Selected Endpoints
Spinosyn A pilot study	Natural algae community	Spinosyn A	47 235 470	5	Growth Fluorescence

Formetanate hydrochloride pilot study	Natural copepod community R. salina Natural bacterial community	Formetanate hydrochloride	19 95 190	5 for ZP 3 for Algae 3 for Bacteria	Feeding rate Mortality Hatching Egg production Diversity bacterial community
Concentration range finding	Natural copepod community R. salina Natural bacterial community	Spinosyn A Formetanate hydrochloride	Spinosyn: - 47 - 470 - 4,700 - 47,000 Formetanate - 19 - 190 - 1,900 - 19,000 - 190,000	3	Feeding rate Mortality Hatching Egg production Diversity bacterial community
Mixture toxicity study	Natural copepod community R. salina	Spinosyn A Formetanate hydrochloride	Spinosyn: - 470 - 4,700 Formetanate: - 190 - 1,900 Mixture: - 470 ng/L Spinosyn A, 190 ng/L formetanate hydrochloride - 4,700 ng/L Spinosyn A, 1,900 ng/L formetanate hydrochloride	5	Feeding rate Mortality Hatching Egg production Diversity

Data analysis

All endpoints except copepod and bacterial community composition were analyzed with R 4.1.3 (R Core Team, 2022) and Rstudio (R Core Team, 2022). Used tests from secondary packages are given in brackets at the corresponding analytical steps. To test for differences regarding the mean between treatments generalized linear models were fitted with treatment as the predictor. Before performing an ANOVA with the fitted models, a Shapiro-Wilk test was done to ensure that the residuals of the sample data follow a normal distribution. Additionally, the variances were tested

for homogeneity (levene.Test()) (Fox & Weisberg, 2019) (car package). Whenever the sample data did not fit these criteria, a Kruskal-Wallis test was performed instead to test for differences between the treatments and the controls. Whenever a significant difference between treatments or the indication of a trend was found, a post-hoc test was performed. For parametric data a Tukey's range taste was used, whereas Dunn's test (dunnTest()) (Ogle *et al*., 2022) (FSA package) was used for non-parametric data. To compare the means of the individual controls in the chemical mixture study, a T-Test was performed. Pearson's product moment correlation coefficient was used to test for possible correlation between two or more variables. The data was visualized with ggplot2 (Wickham, 2016) (ggplot2 package), viridis (Garnier *et al*., 2021) (viridis package), and Ggally (Schloerke *et al*., 2021) (Ggally package).

The resulting slopes from the Biolog® Ecoplates™ were compared and analyzed with PRIMER 7 (Version 7.0.21). A non-metric multi-dimensional scaling (nMDS) analysis was done based on the euclidean distance and tested using ANOSIM. The copepod community was square root transformed to reduce the impact of the most abundant taxa and standardized to account for difference in total number of copepods. The nMDS was based on the Bray-Curtis similarity indices and analyzed with an ANOSIM.

Results

Toxic unit ranking

Overall, only a small percentage of chemicals found at the sites could be used for the toxic unit ranking due to missing ecotoxicological data (Table 2) (Table 9, appendix). For zooplankton, 20 % (site M3) and 17 % (site M4) of all present measured chemicals were used for the ranking, while the amount of included chemicals for algae was slightly reduced with ~15 % for both sites. Only 2 chemicals were available for ranking regarding bacteria, but the available ecotoxicological data's EC50s were based on biofilm communities containing bacteria, not sole bacteria species.

Table 2: Overview of the number of chemicals present at the environmental sites (#Chem) before and after criteria filtering (Table 8, appendix), and the number of those chemicals for which there are ecotoxicological entries in the US EPA exotox data base before and after criteria filtering.

Site	Organism group	#Chem Stenungsund study total	#Chem Stenungsund study after filtering	#Chem US EPA data total	#Chem US EPA data after filtering
M3	ZP	75	72	26	15
M3	Algae	75	72	26	11
M3	Bacteria	75	72	26	2

M4	ZP	68	65	21	12
M4	Algae	68	65	21	10
M4	Bacteria	68	65	21	2

The ranking for zooplankton (Figure 4) showed that two of the substances present at sites M3 and M4, formetanate and 2-4-Dinitrophenol, gave a TU that was a) 33.5x and 14.9x higher and b) 22.9x and 5.45x higher, respectively, compared to the chemicals ranked on third place (Figure 4). However, 2-4-Dinitrophenol had to be excluded from toxicity testing due to its chemical properties making it unsafe to handle. The ranking for algae showed spinosyn A as the substance with the highest TU, with an a) 2.5x and b) 13.8x higher TU compared to 2-4-Dinitrophenol, which was ranked second highest (Figure 5).

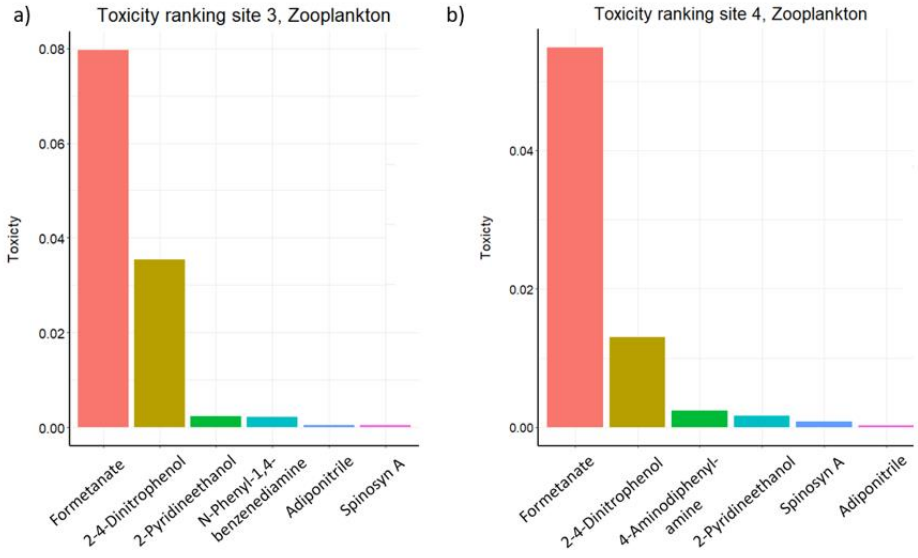

***Figure 4**: Toxic unit ranking based on the ecotoxicological data from US EPA for the chemicals present at a) M3 and b) M4 for the organism group zooplankton.*

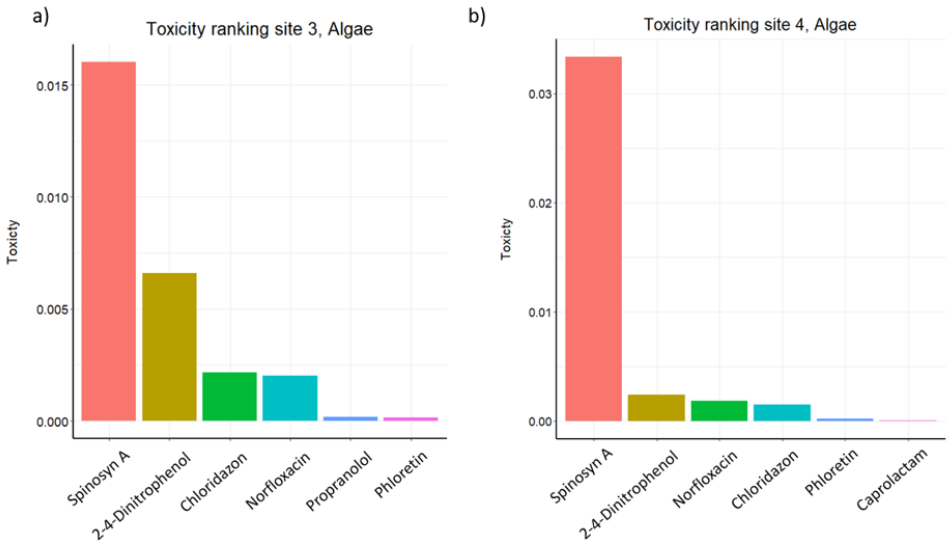

***Figure 5:** Toxic unit ranking based on the ecotoxicological data from US EPA for the chemicals present at a) M3 and b) M4 for the organism group algae.*

Study design

Spinosyn A was selected for a pilot study in October 2022, to study possible effects on natural marine algae communities due to its comparatively high TU regarding microalgae (Figure 5). It is used as a neurotoxic insecticide that is produced via isolation from the soil bacterium *Saccharopolyspora spinosa* and functions by disrupting nicotinic acetylcholine receptors (Elston, 2018). Additionally, testing on natural copepod and bacterial communities and a lab grown *R. salina* culture was done with the acaricide formetanate hydrochloride in November 2022. Formetanate hydrochloride affects the nervous system via overstimulation through inhibiting acetylcholinesterase, leading to cholinergic poisoning (Farooqui, 2013).

Due to their similar MoA, an additional mixture toxicity study was performed at the end of February 2023, with Spinosyn A and formetanate hydrochloride to consider possible interactive effects.

Selection of organisms

Mesozooplankton and microphytoplankton were selected as study organisms since formetanate hydrochloride and Spinosyn A were ranked first, respectively, for their specific organism groups, to test for possible effects on both primary consumers and primary producers. The effect of pesticides on non-target organisms can occur, e.g., a change in bacterial community composition was detected when soil bacteria was exposed to pirimicarb (Widenfalk *et al*., 2008), an insecticide that inhibits cholinesterase. To include possible effects on non-target organisms, bacteria was additionally selected as third study organism, as they are a crucial constituent in the food web. While bacteria do not possess the target site needed for either Spinosyn A's nor formetanate hydrochloride's primary mode of action, other, yet unidentified, mode of actions could influence bacterial communities and should therefore be considered.

Spinosyn A pilot study

No significant differences between the treatments and the methanol controls were observed regarding biomass, growth, median FU, and FU per particle (Table 3). All treatments showed an increase in biomass after 48 h with relatively low standard deviation (SD) compared to the starting samples (Figure 6a). Overall, algae exposed to 1xMEC showed the lowest average growth in combination with the highest SD (Figure 6b). The median FU per particle was declining with increasing exposure concentration (Figure 7b), however, due to the high SD, there is no statistical significance. This trend was not observed with the median FU (Figure 7a), where all treatments showed similar fluorescence, compared to the control with 10xMEC showing the highest SD.

Fluorescence was significantly increased in all treatments and controls compared to the start samples (Figure 7a) (Table 3) but start samples had a higher fluorescence per particle compared to all other treatments and controls (Figure 7b).

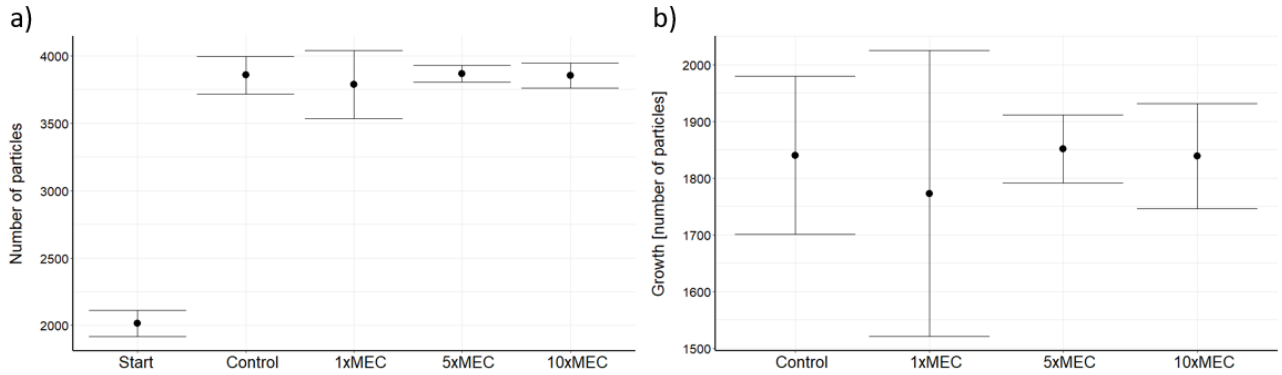

Figure 6: *a) Biomass as number of particles in natural algal communities at the start (t0) and after 48 h exposure to Spinosyn A and b) algal growth in number of particles in relation to number of particles from the start samples, after 48 h exposure to Spinosyn A, n = 5.*

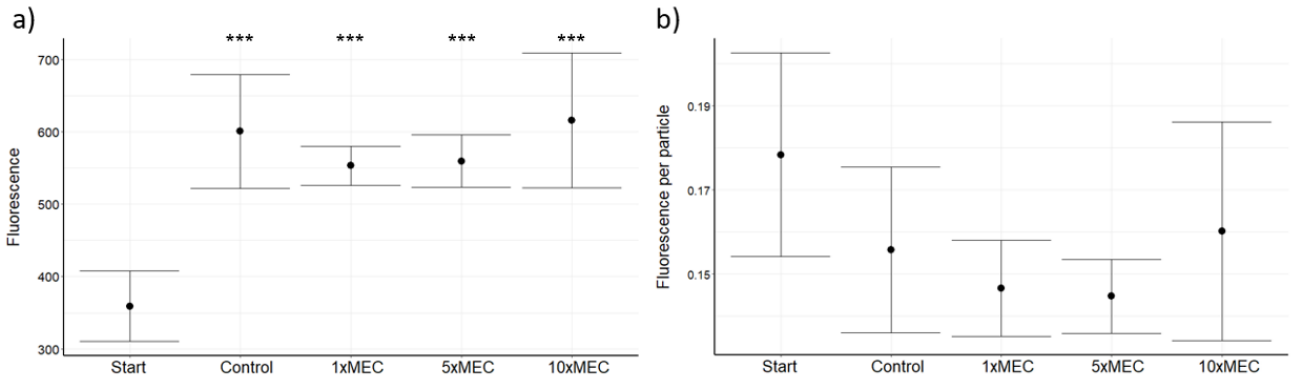

Figure 7: *a) Photosynthetic efficiency as median fluorescence of natural algal communities at the start (t0) and after 48 h exposure to Spinosyn A and b) median fluorescence per particle at the start (t0) and after 48 h exposure to Spinosyn A, n = 5.*

Table 3: Statistical test results regarding the selected endpoints from the 48 h Spinosyn A pilot study with natural algae communities with and without starting samples, treatments were compared to methanol controls.

Endpoint	Statistical test	Df	f-value / chi-squared	p-value
Biomass	ANOVA	3,16	0.2148	0.8847
Growth	ANOVA	3, 16	0.2148	0.8847
Fluorescence	ANOVA	3, 16	0.8914	0.4668
Fluorescence/particle	ANOVA	3, 16	0.6804	0.5768
Fluorescence including starts	ANOVA	4, 20	11.075	<0.0005***
Fluorescence/particle including starts	ANOVA	4, 20	1.9549	0.1405

Formetanate hydrochloride pilot study

The copepods acting as controls had, on average, a feeding rate of 123 particles per copepod (ppc) compared to 149 ppc in the 1xMEC exposure. Feeding rate slightly increased in the lowest exposure (1xMEC) and then showed a decrease with increasing toxicant exposure (Figure 8). Standard deviation was lowest in the controls with ±7.9 and highest in the 1xMEC with ±39.1.

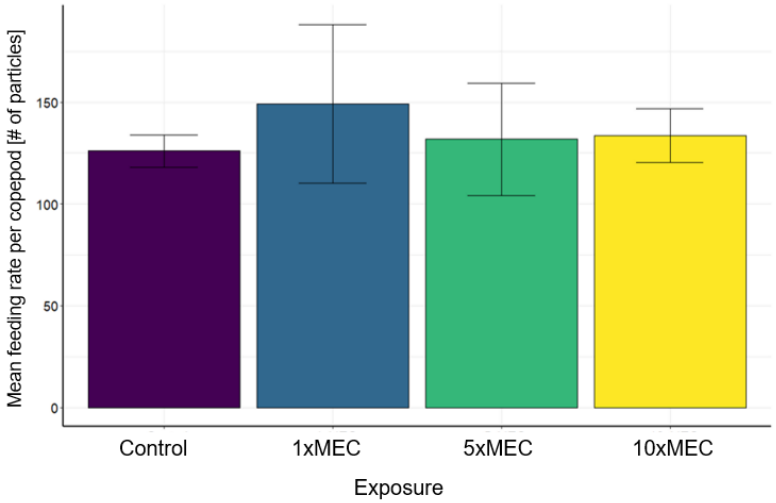

Figure 8: *Mean feeding rate per copepod and standard deviation after 48 h exposure to formetanate hydrochloride in the pilot study, n = 5.*

The controls had a mortality of 11.7 ±7.2 %. The formetanate exposure caused a non-significant increase in mortality in all toxicant exposures with the copepods exposed to the 1xMEC experiencing the highest mortality and SD with 26.8 ±26.2 % (Table 4). Both the 5xMEC and 10xMEC showed mortality similar to the 1xMEC with decreasing SD (Figure 9).

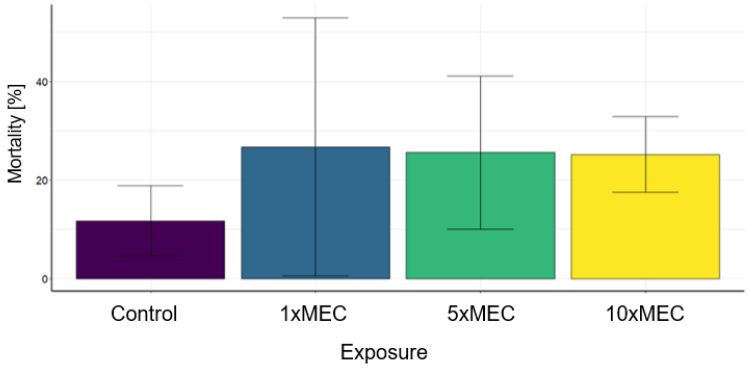

Figure 9: *Mean mortality during the experiment compared to the start samples and standard deviation after 48 h exposure to formetanate hydrochloride in the pilot study, n = 5.*

Alive copepods were accidentally flushed into the petri dishes used for the reproduction endpoints and remained there for the full incubation period. Neither the mean total egg production (Figure 10a) nor the mean hatching success (Figure 10b) showed any significant differences between treatments and controls. The controls and the 5xMEC exposure showed the lowest number of total egg production with 59 eggs, with the 1xMEC and the 10xMEC exposure showing a mean of 85

eggs and 74 eggs, respectively. The deviation was relatively high across all treatments. Hatching showed a slight decrease from 84 % to 77 % with increasing exposure. To account for the copepods that were in the petri dishes during the incubation, a correlation test was done. Egg production showed a significant positive correlation to the number of copepods in the petri dish (Table 4). Hatching success was not significantly affected by the presence of copepods (Table 4).

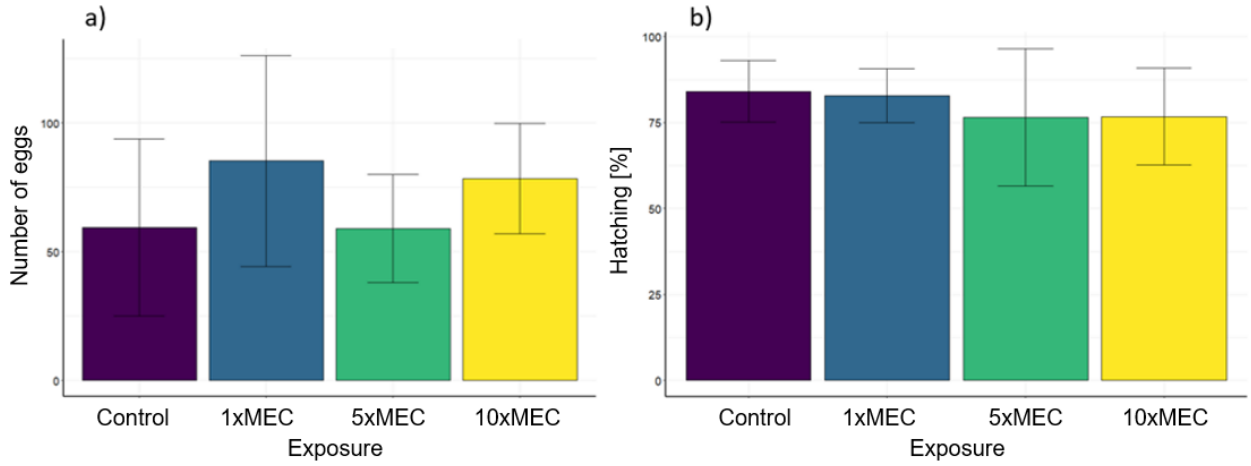

Figure 10: a) *Mean total egg production (number of eggs) and* ***b)*** *mean hatching success [%] of natural copepod communities and standard deviation after 48 h exposure to formetanate hydrochloride in the pilot study, n = 5.*

Table 4: Statistical test results regarding the selected endpoints from the 48 h formetanate hydrochloride pilot study exposure, treatments were compared to methanol controls. Additionally, correlation testing between selected endpoints and the number of copepods present in the petri dishes during incubation was performed.

Endpoint	Statistical test	Df	f-value / chi-squared / t-value	p-value
Feeding rate	ANOVA	3, 16	1.2752	0.3165
Feeding rate per copepod	Kruskal-Wallis	3	4.2343	0.2373
Mortality	ANOVA	3, 16	0.7759	0.5244
Hatching	ANOVA	3, 16	0.3394	0.7971
Egg production	ANOVA	3, 16	0.7571	0.5343
Egg Production	Cor.test	18	3.9895	0.0008597
Hatching	Cor.test	18	-1.0305	0.3164

Bacterial community composition showed no dissimilarity between all treatments after 96 h formetanate exposure (R = -0.05, p = 0.785), the nMDS fitting resulted in a stress level of 0.1 (Figure 11). All treated communities including the communities acting as controls showed high biological variation.

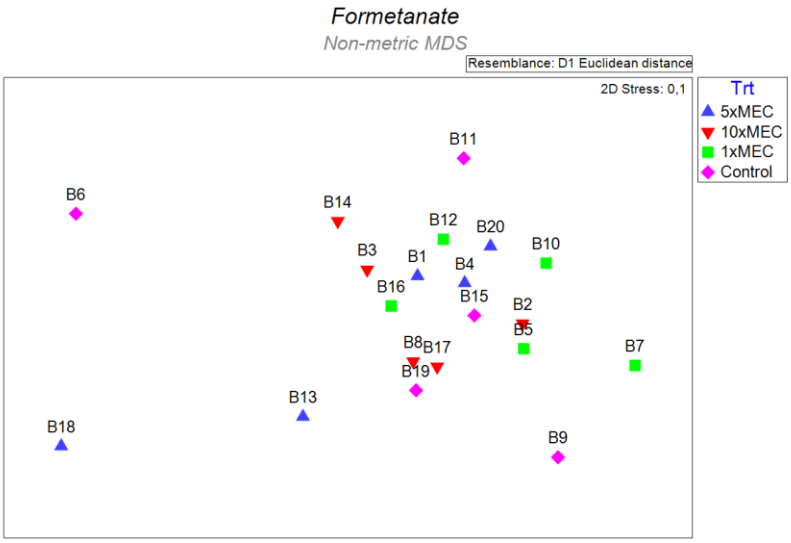

Figure 11: *nMDS-analysis of the calculated slopes from the exposed bacteria based on euclidean distance, with the 31 substrates grouped into six main sources, 96 h exposure to formetanate hydrochloride in the pilot study, n = 5.*

Mixture toxicity study

Concentration range finding

The Spinosyn A and formetanate hydrochloride controls were tested for differences in means for the selected endpoints. There were no significant differences between the respective controls for the endpoints feeding rate per copepod, mortality, egg production and hatching success (Table 5), but the controls were not pooled due to $p < 0.6$. Further statistical testing was done with the respective controls.

Table 5: Statistical test results comparing the means of the controls from the concentration range finding.

Endpoint	Statistical test	Df	t-value	p-value
Feeding rate	t.test	4	1.7824	0.1493
Feeding rate per Copepod	t.test	4	0.80368	0.4666
Mortality	t.test	4	0.83245	0.452
Egg production	t.test	4	-0.57585	0.5956
Hatching success	t.test	4	-0.71596	0.5136

The copepods acting as controls from the concentration range finding showed an average feeding rate of 50 ±0.9 and 46 ±7.9 ppc for formetanate and Spinosyn A, respectively. Feeding rate in the formetanate exposure slightly decreased with 100xMEC and 1,000xMEC treatment and was significantly impacted by the 10,000xMEC exposure with 8.5 ±3.4 ppc (Table 6). No change in feeding rate was observed in the 1xMEC and 10xMEC exposure (Figure 12). Feeding rate in the

Spinosyn A exposure was increased in the 10xMEC with 58 ±11.6 ppc and decreased in the 1,000xMEC with 37 ±4 ppc compared to controls with 46 ±8 ppc. Standard deviation was highest with the 10xMEC treatment for both formetanate and Spinosyn A. A significant decrease in feeding rate between the 10xMEC and 1,000xMEC Spinosyn A exposure was observed (Table 6) (Figure 13).

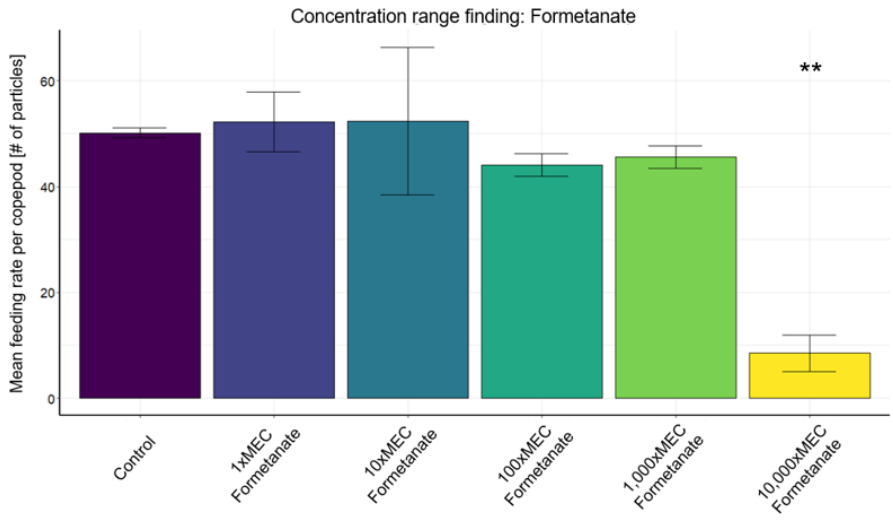

Figure 12: *Mean feeding rate per copepod and standard deviation after 48 h of exposure to formetanate hydrochloride in the concentration range finding study, n = 3.*

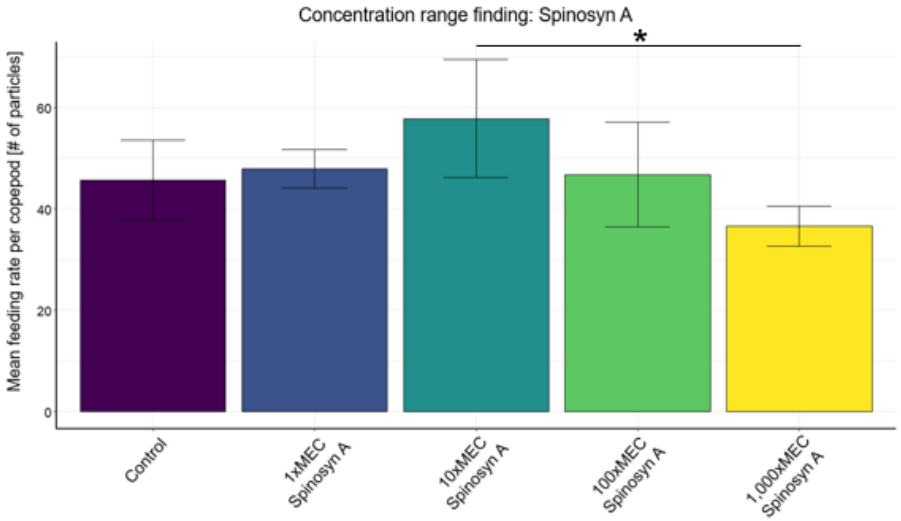

Figure 13: *Mean feeding rate per copepod and standard deviation after 48 h of exposure to Spinosyn A in the concentration range finding study, n = 3.*

The Spinosyn A controls had the highest mortality with 20.6 ±13.4 %. There was no difference between treatments regarding mortality (Table 6), with all Spinosyn A and formetanate treatments expressing relatively low mortality (6-16 %) and high variation apart from the 1x and 100xMEC Spinosyn A exposure and the 10,000xMEC formetanate exposure (Figure 14&15).

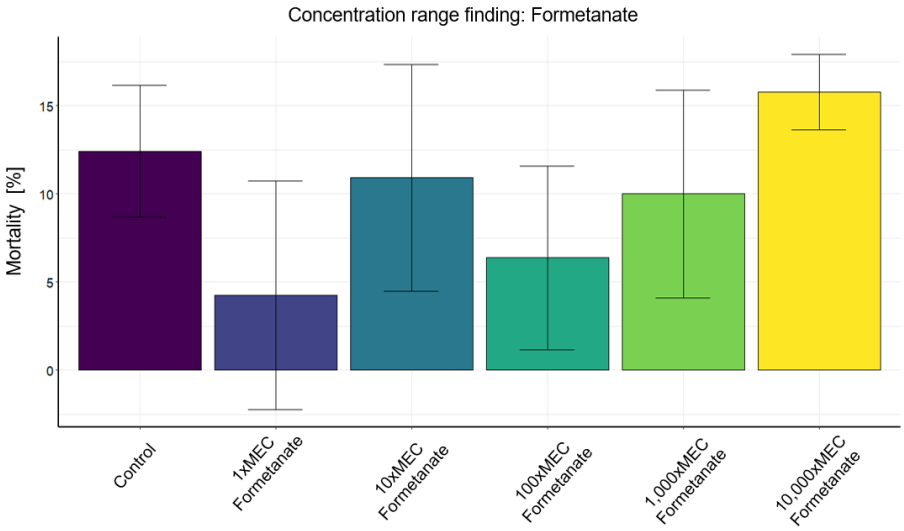

Figure 14: *Mean mortality compared to the start samples including standard deviation after 48 h exposure to formetanate hydrochloride in the concentration range finding study, n = 3.*

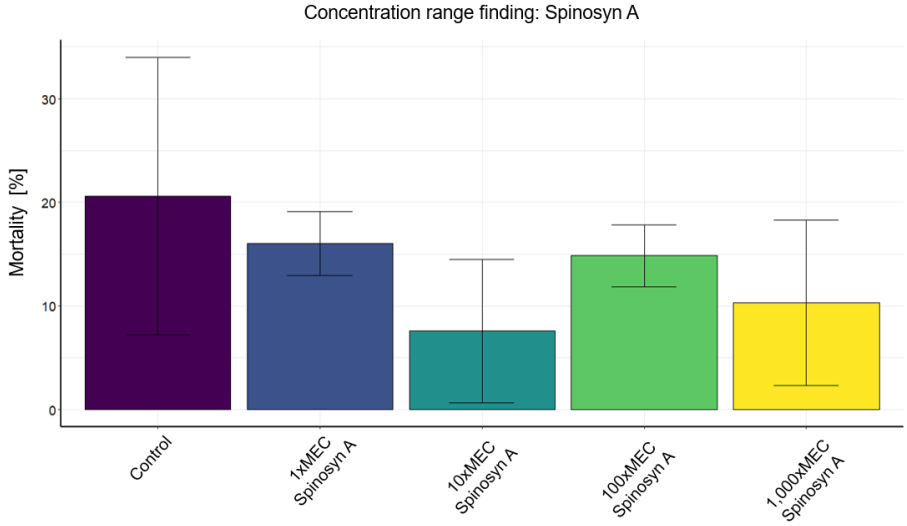

Figure 15: *Mean mortality compared to the start samples including standard deviation after 48 h exposure to Spinosyn A in the concentration range finding study, n = 3.*

One of the three replicates of the 100xMEC formetanate exposure was excluded for the statistical analysis regarding hatching success due ethanol contamination at the start of the incubation period. The sample was included regarding egg production. Controls showed a hatching success of 59 ±24.8 % and 72 ±5.9 % for formetanate and Spinosyn A, respectively. The 100xMEC formetanate exposure showed impacted hatching success (Table 6) compared to the control and the lowest formetanate exposure. However, hatching success was not impacted by the higher formetanate exposures (Figure 16). A significant decrease in hatching success was observed with the 10x, 100x, and 1,000xMEC Spinosyn A exposure compared to controls (Figure 17) (Table 6).

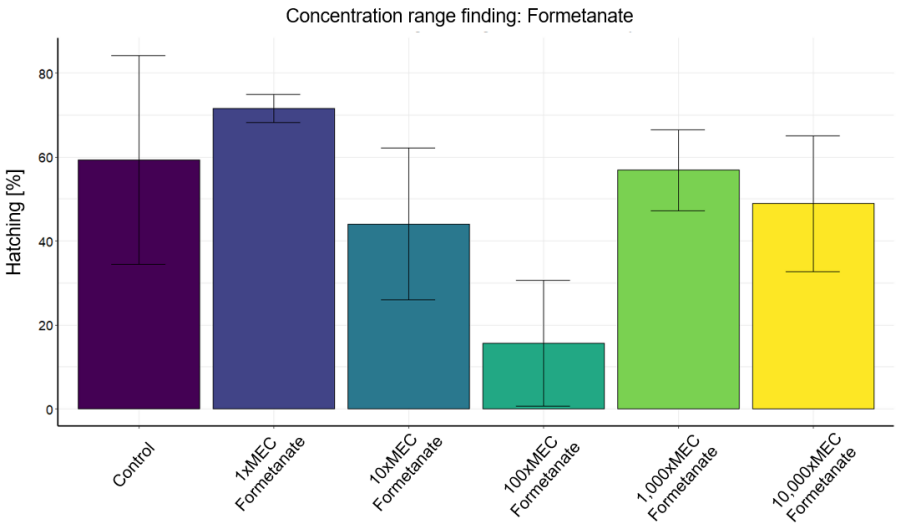

Figure 16: *Mean hatching success [%] including standard deviation after 48 h exposure to formetanate hydrochloride in the concentration range finding study, n = 3, 100xMEC formetanate n = 2.*

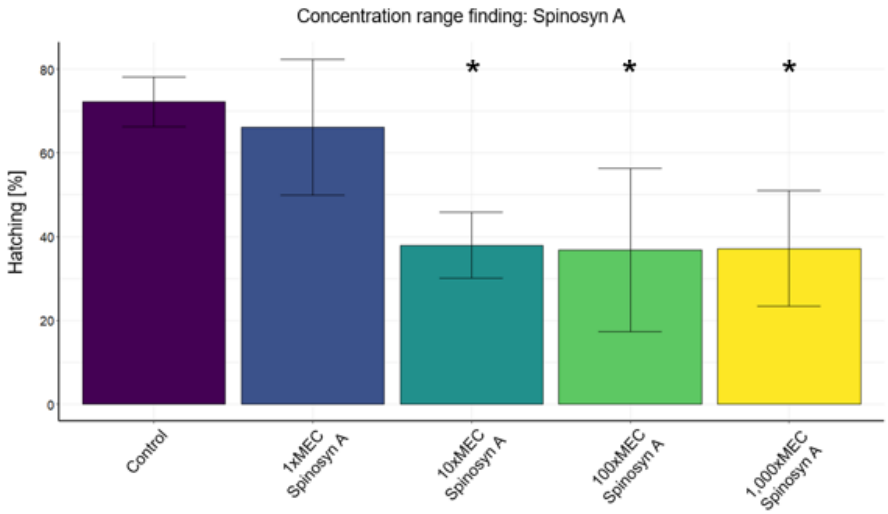

Figure 17: *Mean hatching success [%] including standard deviation after 48 h exposure to Spinosyn A in the concentration range finding study, n = 3.*

Total egg production showed no difference between controls and treatments for both formetanate and Spinosyn A (Table 6), with a relatively low production overall. Both controls showed very high SD, in addition to the 1xMEC formetanate and the 100xMEC formetanate exposure. Egg production in the formetanate exposure, including controls, ranged from 17 to 25 eggs (Figure 18), whereas egg production in the Spinosyn A exposure, including controls, ranged from 23 to 35 eggs (Figure 19).

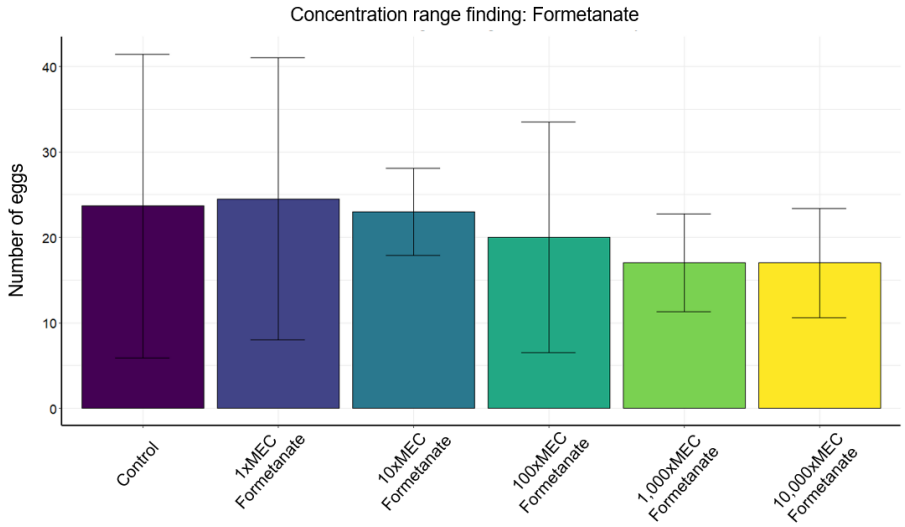

Figure 18: *Mean total egg production including standard deviation after 48 h exposure to formetanate in the concentration range finding study, n = 3.*

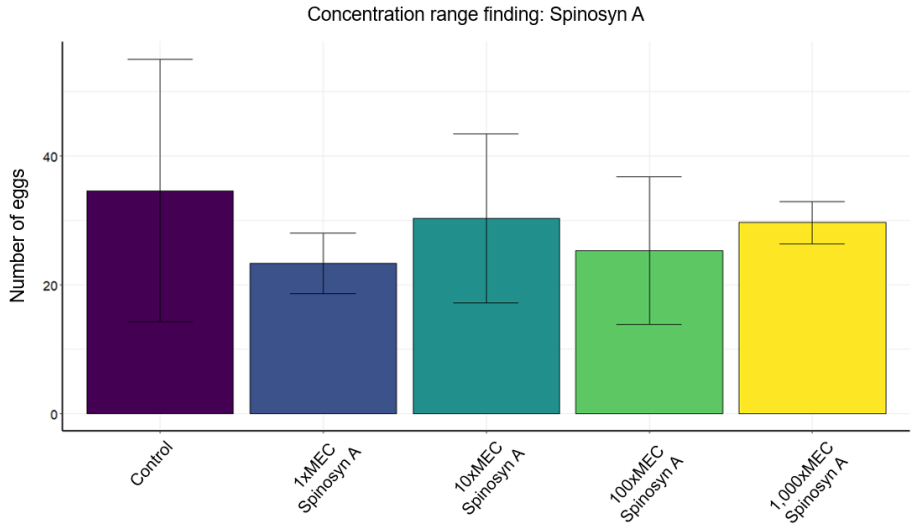

Figure 19: *Mean total egg production including standard deviation after 48 h exposure to Spinosyn A in the concentration range finding study, n = 3.*

Table 6: Statistical test results regarding the selected endpoints from the concentration range finding study, treatments were compared to methanol controls. Additionally, correlation testing between selected endpoints and the number of copepods present in the petri dishes during incubation was performed.

Endpoint	Chemical	Statistical test	Df	f-value / chi-squared / t-value	p-value
Feeding rate	Formetanate	ANOVA	5, 12	14.761	<0.0005 ***
Feeding rate per copepod	Formetanate	Kruskal-Wallis	5	17	<0.005**
Mortality	Formetanate	ANOVA	5, 12	1.2644	0.3404
Hatching	Formetanate	ANOVA	5, 11	2.351	0.1103
Egg production	Formetanate	ANOVA	5, 11	0.1459	0.9772

Hatching	Formetanate	Cor.test	15	-3.6754	<0.005**
Egg production	Formetanate	Cor.test	15	-1.3527	0.1962
Algae growth	Formetanate	Kruskal-Wallis	5	2.7778	0.7342
Feeding rate	Spinosyn A	ANOVA	4, 10	2.9111	0.07768
Feeding rate per copepod	Spinosyn A	Kruskal-Wallis	4	14	<0.05*
Mortality	Spinosyn A	ANOVA	4, 10	0.8359	0.5326
Hatching	Spinosyn A	ANOVA	4, 10	3.3191	0.056
Egg production	Spinosyn A	ANOVA	4, 10	0.2641	0.8944
Hatching	Spinosyn A	Cor.test	13	-0.30848	0.7626
Egg production	Spinosyn A	Cor.test	13	-0.14733	0.8851
Algae growth	Spinosyn A	ANOVA	4, 10	0.4126	0.796

There was no difference in carbon usage between the bacterial communities regarding exposure (R = -0.025, p = 0.583). The nMDS was done with only two selected carbon groups, carboxylic acids and carbohydrates (Figure 20). The remaining carbon groups were excluded due to minimal or no color development. One outlier was detected in the Spinosyn A 10xMEC exposure, where no carbon source utilization took place.

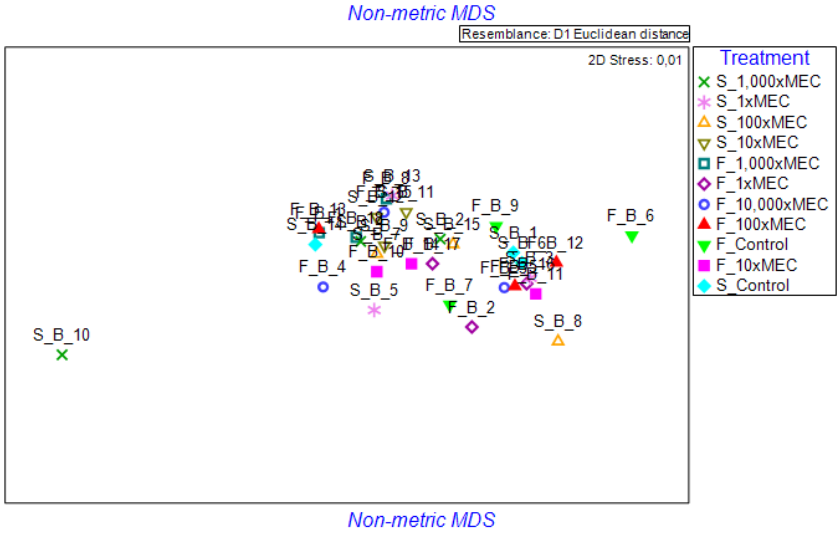

Figure 20: *nMDS-analysis of the calculated slopes from the exposed bacteria based on euclidean distance, from the carboxylic acid and carbohydrate sources, 96 h exposure to formetanate hydrochloride and Spinosyn A, concentration range finding, n = 3. The formetanate hydrochloride treatments were abbreviated with F and their corresponding concentration, the abbreviation S was used for Spinosyn A treated samples.*

Mixture study

Feeding rate per copepod was not affected by the different treatments (Table 7). Controls expressed a feeding rate of 130 ±12 ppc. The lowest feeding rate and highest SD was observed with the 100xMEC Spinosyn A exposure with 117 ±20.1 ppc and the highest observed rate of 139 ±10.7 ppc was in the 100x mixture exposure. Overall, average feeding rate per copepod differed only minimally between all treatments and controls (Figure 21).

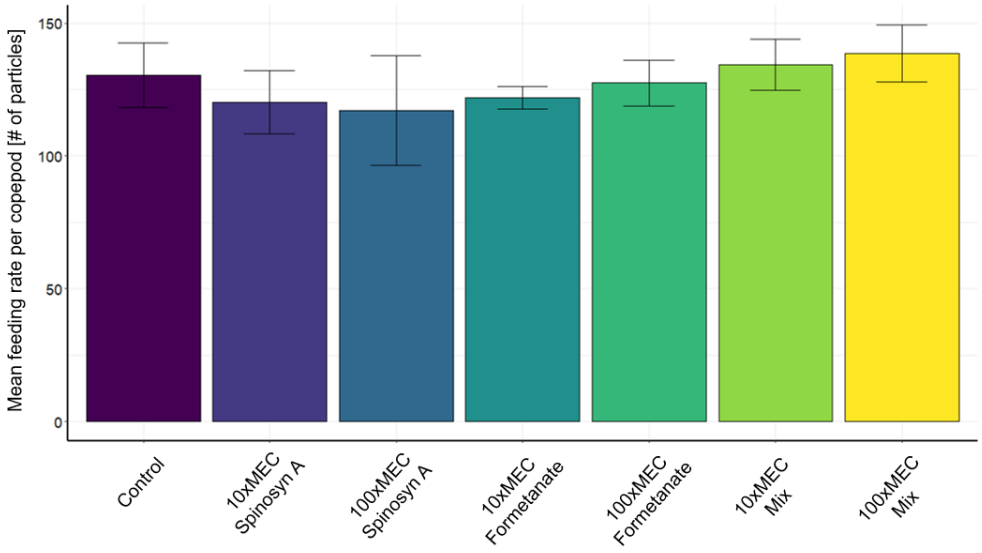

Figure 21: *Mean feeding rate per copepod and standard deviation after 48 h of exposure to formetanate, Spinosyn A, and mixture concentrations, n = 5.*

Hatching success showed large amounts of variation within treatment for all treatments. Controls had the lowest hatching rate with 6.1 ±8.7 %, followed by the 10xMEC formetanate exposure. Exposure to 100xMEC Spinosyn A showed the highest hatching success and lowest SD with 32 ±11.7 % (Figure 22).

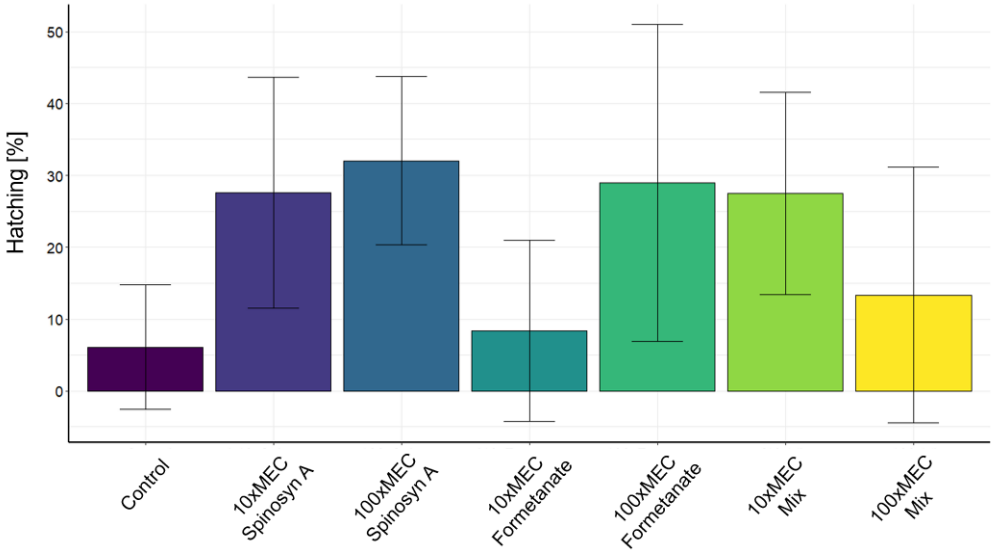

Figure 22: *Hatching success [%] and standard deviation after 48 h of exposure to formetanate, Spinosyn A, and mixture concentrations, n = 5.*

Total egg production was overall very low with most treatments showing high variation. Controls laid 11 ±9.1 eggs in total per replicate compared to 7 ±2 eggs in the 100xMEC Spinosyn A exposure and 16.4 ±15.1 eggs in the 10xMEC formetanate exposure (Figure 23).

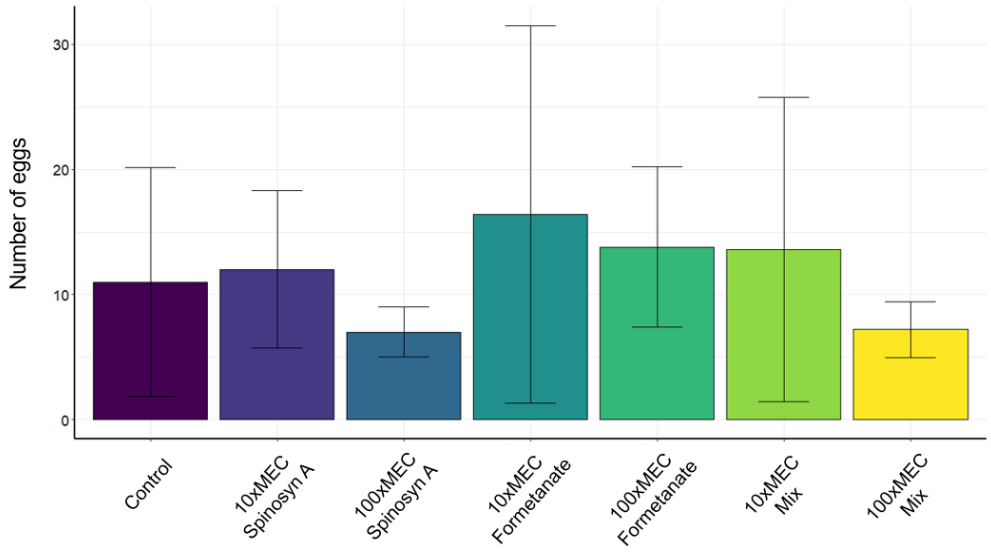

Figure 23: *Total egg production and standard deviation after 48 h of exposure to formetanate, Spinosyn A, and mixture concentrations, n = 5.*

Correlation testing was performed to confirm that manually removing all copepods from the petri dishes before the start of the incubation period had no influence on hatching, and that the time the copepods remained in the petri dishes until removal had no influence on total egg production. This was done under the assumption that the amount of stress caused to the eggs in the petri dish due to disturbance and possible heat exposure is linear to the number of copepods removed. Neither total egg production nor hatching success were influenced, thus, the results regarding the endpoints hatching and reproduction in the mixture study were not impaired (Table 7).

Table 7: Statistical test results regarding the selected endpoints from the 48 h mixture toxicity study, treatments were compared to methanol controls. Additionally, correlation testing between selected endpoints and the number of copepods that were removed from the petri dish at the start of the incubation time was performed.

Endpoint	Statistical test	Df	f-value / chi-squared / t-value	p-value
Feeding rate	ANOVA	6, 28	1.0662	0.4058
Feeding rate per copepod	ANOVA	6, 28	1.705	0.1567
Mortality				
Hatching	ANOVA	6, 28	2.032	0.09456
Egg production	ANOVA	6, 28	0.6196	0.7129
Algae growth	ANOVA	6, 15	0.7587	0.6136
Correlation between hatching	Cor.test	33	-0.43501	0.6664

and #Copepods/petri				
Correlation between eggs laid and #Copepods/petri	Cor.test	33	-0.10091	0.9202

The diversity composition testing is based solely on copepods. Cyphonautes, water fleas, barnacle larvae and nauplii, polychaetas, and arrow worms were excluded from the composition. Copepod community composition was not changed by the different exposures (R = 0.034, p = 0.266). The T0 samples (labeled none in Figure 24) taken as start communities varied slightly from the 100xMix and the 10xMix. The communities exposed to the 10xMix also slightly varied from the controls, the 100xMix and the 100xMEC Spinosyn exposure (Figure 24).

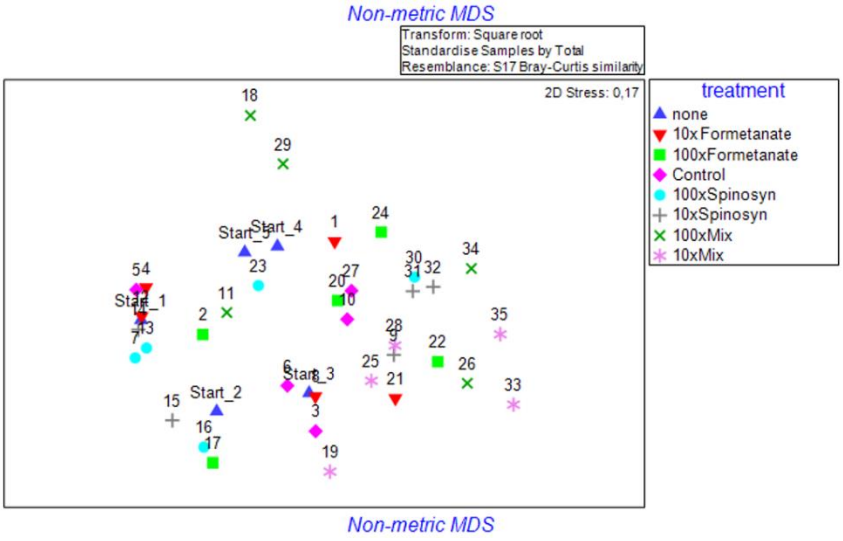

Figure 24: *nMDS-analysis of the community composition from the exposed copepods, the controls, and the starting samples (none) based on a Bray-Curtis matrix, 48 h exposure to formetanate hydrochloride, Spinosyn A, and a mixture; mixture study. The data is square root transformed and standardized, n = 5.*

Discussion

Toxic unit ranking

The calculation of a TU based on the environmental measured concentrations was not possible for most of the chemicals present at sites M3 and M4 from the Stenungsund study. This was caused by the absence of ecotoxicological data on US EPA for a majority of chemicals for the selected organism groups. The remaining number of chemicals was again reduced by applying filters to their data regarding specific endpoints (EC50, LC50, and IC50) and concentration signs, as well as the manual exclusion of some species. Furthermore, US EPA only offered ecotoxicological data for “plants” and “animals”, but not for “monera”. The only bacterial data included in the ranking

was ecotoxicological data for species belonging to cyanobacteria and "Monera Kingdom" that were included in the "plant" category. The data for "Monera Kingdom" only had "Monera" as species entry, giving no indication as to what species the EC50 was based on. Thus, the data for "Monera" was manually compared to its literature, showing that it was based on biofilms. Multiple different EC50 values were available regarding biofilms, but they all stemmed from the same study. No data on widely used marine bacteria in ecotoxicological testing, such as *vibrio fischeri* (Schiavo *et al*., 2018), was found on US EPA. Furthermore, freshwater species such as *Daphnia magna* and *Raphidocelis subcapitata* had to be included in the TU ranking due to lack of data based on marine species. For example, the TU calculation for zooplankton, formetanate, for site M3 and M4 is solely based on *Daphnia magna* as no other ecotoxicological data remained after the filtering. Studies show that sensitivity varies strongly between species, some showing marine species to be more sensitive (Pérez & Beiras, 2010; Minguez *et al*., 2014), whereas others showed a reduced sensitivity (Minguez *et al*., 2016). Therefore, it must be considered that the lack of data for most chemicals and, additionally, for marine zooplankton and algae, can lead to the toxic unit calculations being unreliable, impeding accurate toxicity predictions. For this study, the calculated and used TU is based on the mean EC50/LC50/IC50. A TU based on the median EC50/LC50/IC50 was also calculated and resulted in a TU that was 2.6 times higher than the TU based on the mean. Though, due to the ecotoxicological data only consisting of very few outliers, the mean was used to avoid an overestimation of toxicity. The number of chemicals included in the ranking could have potentially been increased by using assessment factors, in addition to the utilization of supplementary databases, and should be considered when applying this method in further studies. However, the use of assessment factors could lead to an overestimation of contributory toxicity, as their utilization is based on uncertainty and, therefore, applies the precautionary principle. Thus, it is not unlikely that the toxicity ranking would get skewed, ranking chemicals with few ecotoxicological data as higher toxicity contributors.

The toxic unit calculations for the Stenungsund study ranked the same chemical, formetanate, as chemical contributing the most to the toxicity of the mixtures to copepods on all six sites (Jönander *et al*., 2022. However, as it is also based on ecotoxicological data from US EPA, a large amount of chemicals found in the study was likely also excluded from the toxicity ranking due to missing data. The calculated toxic units regarding zooplankton were coherent to the ones calculated in this study, but a calculation for algae or bacteria was not done. This indicates that no new ecotoxicological data was added to US EPA since 2020, regarding the included chemicals for the organism group zooplankton. Additionally, modelling of LC50 for Daphnia based on ECOSAR was done, where the LC50 was adjusted with an assessment factor of 10 to accommodate for the

use of saltwater communities. For site M3 TDCPP, a chlorinated organophosphate used as flame retardant, was the highest toxicity contributing chemical (Jönander *et al*., 2022). As there was no data for TDCPP in the downloaded files from US EPA, it was not included in this study's toxicity ranking. The TU of formetanate produced by the modelling was decreased by a factor of 51 compared to the calculated TU in this study, which would be an underestimation of toxicity.

Algal growth and bacterial communities

Natural marine algae communities were not affected by Spinosyn A exposures regarding growth or photosynthetic efficiency. All treatments, including controls, showed significantly increased fluorescence compared to the start samples, which is caused by a lower number of particles in the start samples compared to the treatments and controls. This is supported by all treatments and controls showing a similar or slightly lower fluorescence per particle compared to the start samples. Additionally, testing with cultures of the marine algae *R. salina* was performed in subsequent experiments with Spinosyn A, formetanate hydrochloride and a chemical mixture. No effect on growth was observed in any of the exposures which does not align with the expected toxicity of Spinosyn A. Spinosyn A's TU for algae was 0.016 and 0.03 for site M3 and M6, respectively, thus, an increase in MEC by 100x or 1000x would result in a TU > 1. Almost all EC50 values from US EPA used for the TU calculation were based on the endpoint growth. Since the same endpoint was used in this study in all experiments, it is, therefore, unlikely that an effect occurred but was not observed. These results indicate that the predicted toxicity of Spinosyn A to marine microalgae was likely an overestimation.

Bacterial communities showed no changes in community composition after 96 h exposure to either Spinosyn A or formetanate hydrochloride. The biolog plates were kept in the dark, at room temperature to ensure carbon metabolization within the 96 h exposure period. The change in temperature compared to natural conditions can favor certain bacteria, however, due to the controls being kept under the same circumstances, this should not affect the overall effect of the chemicals on the community.

Thus, it can be concluded that neither of the two tested chemicals, nor their simultaneous presence, negatively impact pelagic algal or bacterial communities at the current measured environmental concentrations. Whether any interactions take place between Spinosyn A and formetanate and other chemicals present in the water column can not be said.

Copepod communities

Feeding rate

Average feeding rate per copepod in the controls varied between experiments. In the mixture and the formetanate pilot study around 123-130 particles per copepod (ppc) was observed compared to 46-50 ppc in the concentration range finding study. The temperature in the thermo constant room was set to represent ambient water, leading to a decrease in temperature by 5 °C in the concentration range finding and mixture study compared to the formetanate pilot study. The lower temperatures likely decreased the copepods' metabolism, leading to a reduction in feeding for the concentration range study. While the temperature remained the same for the mixture study, the copepods were starved 24 h prior to exposure, explaining the increased feeding rate compared to the concentration range study. Feeding rate was significantly impacted by the 10,000xMEC formetanate hydrochloride treatment, however, based on the calculated TU, an effect was expected at 100xMEC and 1.000xMEC. While the feeding rate showed a declining rate at these concentrations, it was not significant when compared to the controls.

Environmental stressors can lead to an increase in an organism's fitness at low exposure levels, called hormesis (Costantini *et al.*, 2010). This hormetic effect was observed in the formetanate pilot study and the Spinosyn A concentration range study, where an increase in feeding rate occurred for low chemical exposures, followed by a decrease with increasing chemical concentration. The endpoint "average feeding rate per copepod" could potentially be affected by the uneven presence of certain species and sexes in the different treatments, as they express differing feeding rates. However, as the copepods were added to the exposure samples in a random order and multiple replicates existed for each treatment, an even mix resulting in a similar starting composition for each treatment was expected.

Mortality and community composition

The controls in the formetanate pilot study and the concentration range study suffered a mortality of ~12 % while the Spinosyn A controls in the concentration range finding study had a mortality of 20.6 ±13.4 %. Control mortality can be explained by the handling prior to the experiment and the sampling procedure, and, potentially, the experimental set up itself. Given that the experimental set up was analogue in all three experiments and that copepods were handled equally in all exposures, it is likely that a pipetting error occurred in one of the Spinosyn A controls. This would explain the high mortality for the Spinosyn A controls, paired with the high variation, as

$$mortality = \frac{number\ of\ counted\ copepods\ alive}{number\ of\ copepods\ in\ start\ samples}.$$

A pipetting error would have led to a significantly smaller amount of copepods present in the control sample compared to the starting samples, leading to a false increase in mortality. Mortality was not calculated for the mixture study due to the high variance in number of copepods in the start samples.

The copepods from the mixture study were identified manually according to their genus. The genus of *Acartia* and *Oithona* were combined due to misidentification in the start samples and petri dishes. *Pseudocalanus* and *Paracalanus* were additionally grouped into one genus, to avoid further misidentification due to their morphological similarity. Copepodites made up a large portion of the sample but due to missing knowledge regarding the identification of copepodite stages, they were identified as adult copepods. As copepodites sometimes don't express the typical species or genus characteristics, this might have led to further misidentification. *Temora* and the grouped genus of *Para-* and *Pseudocalanus* made up 76-96 % of the total community composition. The data was standardized by total to adjust for the high variation in total number of copepods. Additionally, the data was square root transformed to adjust for the high unevenness caused by *Temora*, *Pseudo-* and *Paracalanus*.

There was no significant change in community composition in the mixture study, but a trend was observed that the experimental set up itself might have an impact on the composition, in combination with the exposure, as the starting community differed slightly from the 10xMix and 100x Mix. This can be explained by some species being more sensitive when it comes to handling, in addition to a decreased tolerance when exposed to chemicals. Whether the decreased tolerance is a result from the prior handling and experimental set up or due to species specific sensitivity is not known. Moreover, the copepods exposed to the 10xMix seemed to have shown a slight change in composition compared to the controls. Therefore, it would be interesting to redo the exposure with more replicates and an identification according to copepod species. Another factor that could be considered during identification is the sexing of the copepods and the differentiation between copepodite stages and copepods, to possibly associate egg production per female and to calculate a more accurate feeding rate per copepod. Copepod species identification can prove to be very time consuming and difficult, but copepod communities are an important indicator of change in aquatic systems (Chang *et al*., 2012) and should therefore be monitored. Identification software, such as ZooImage, can be a powerful tool for larger scale studies regarding community composition (Bell & Hopcroft, 2008) and further improvement of these software should be undertaken to increase accuracy.

A cause for concern could be the relatively low biodiversity observed in the natural copepod community, this can however be caused by seasonal variability causing the absence of otherwise present species (Mascart *et al*., 2015), or errors during the identification process.

Reproduction

Egg production in the formetanate pilot study and hatching in the formetanate concentration range study was significantly affected by copepods being present in the petri dishes during the incubation. Total number of eggs produced was increased when copepods were present in the petri dish due to subsequent egg laying, and hatching success was negatively impacted, possibly by copepods grazing on eggs, as no other food source was present during incubation. Copepods were manually removed from the petri dishes in the mixture study to avoid their impact on reproduction results. This was done over a microscope in the thermoconstant room via pipette to ensure the temperature of the water would not increase and negatively impact the hatching. This method should further be applied to ensure accurate data regarding egg production and hatching as the manual removal had no impact on either reproduction endpoint. Although most copepods range between 0.5-3 mm in length (Thorp & Rogers, 2011), their width is considerably shorter, leading to accidental flushing through a 200 or 250 µm net. The prefiltering over a larger sized net (250 µm compared to 200 µm) to concentrate the copepods before exposure start is unfavorable when the focus is put on community composition, as it most likely leads to a decrease in biodiversity and, therefore, limits representation. Additionally, even with the prefiltering, a considerable number of copepods were still flushed into the petri dish. Egg laying can vary highly per individual (Tester & Turner, 1990), which was observed in all experiments. The reduced number of eggs observed in the mixture study compared to the formetanate pilot study could be caused by the large amount of copepodites present in each sample, that have not reached sexual maturity and are, therefore, unable to lay eggs. While no differentiation between copepods and copepodites was made for the concentration range study when counting the number of copepods, it is likely that the equally low egg production was also caused by the presence of copepodites. Additionally, *Temora longicornis* shows reduced reproductive activities and slow development regarding copepodite stages in early spring compared to summer and early autumn (Peters *et al*., 2013). This supports the theory that copepodites were present in similar numbers in the concentration range study compared to the mixture study, and that the low egg production was likely due to natural circumstances. Furthermore, temperatures below 10 °C lead to highly reduced egg production (Uye & Shibuno, 1992), a factor that needs to be considered in the mixture and concentration range studies, where temperature was set to 7 °C.

Marine and estuarian copepods can produce eggs that are in an embryonic dormant state, delaying hatching until more favorable environmental conditions are present, so-called resting eggs, with temperature being the most common environmental driver regarding the production (Holm *et al*., 2018). The most abundant egg laying genus in the mixture study was *Temora longicornis*, a species that has an increased production of resting eggs in spring (Castellani, 2003). Additionally, species such as *Acartia tonsa* and *Centropages hamatus*, both commonly found in Swedish coastal waters and their genus present in the community used in the mixture study, also produce resting eggs (Uye, 1985). Therefore, the relatively low temperatures during the mixture exposure in spring most likely resulted in the production of resting eggs, leading to the low hatching success observed, especially in the controls. Egg production could not be correlated to individuals, as some species belonging to the genus of *Oithona* (Cornwell *et al*., 2018) and *Pseudocalanus* (Corkett & McLaren, 1969) are egg sac spawners, and the genus of *Oithona* and *Acartia*, as well as *Pseudocalanus* and *Paracalanus*, were combined during identification. In addition, egg sacs were not included regarding egg production due to their size and their attachment to the copepod. As the separation of eggs and copepods for the incubation was done with a 200 µm mesh, the egg sacs were not flushed through the mesh. It can be excluded that hatching was impacted by anoxic conditions in any experiment as the filtered sea water used for incubation was previously aeriated for 24 h. Furthermore, the petri dishes were closed with lids to minimize any debris falling into the water that could affect hatching.

The feeding with algae cultures such as *Rhodomonas baltica* showed good results regarding egg production (Zhang *et al*., 2013), making the mono diet with *Rhodomonas salina* that was applied in this study viable. In addition, a more diverse diet did not seem to prove advantageous regarding reproduction in *Temora* (Dam & Lopes, 2003). It is therefore unlikely that the used diet had a negative impact on reproduction in this study.

Conclusion

Current measured environmental concentrations and environmentally relevant concentrations of Spinosyn A and formetanate hydrochloride do not pose a threat to the native pelagic communities if present by themselves regarding selected endpoints. Therefore, it can be concluded that they are not the toxicity drivers in the chemical mixtures found on the Swedish west coast. However, it cannot be excluded that they interact with any of the other chemicals that were present in the environment, possibly in a synergistic way. Spinosyn A seemed to be more toxic than formetanate hydrochloride to copepods, causing impacted hatching and changes in feeding behavior at 10xMEC

in the concentration range finding study. However, these effects were not observed in the mixture study.

Since the Stenungsund monitoring study only focused on polar organic chemicals, unipolar and inorganic chemicals were excluded from the previous mixture toxicity prediction and my toxicity ranking. Inorganic chemicals, such as toxic metal compounds, could, therefore, additionally be present undetected in the mixtures. This could lead to an increase in toxicity of the actual chemical mixture compared to the calculated toxicity based on the detected organic chemicals in the mixture. Moreover, this could lead to the actual toxicity drivers in the mixture getting excluded from the ranking altogether, as the ranking is based on detected chemicals. Another important factor that should be considered regarding the underestimation of the mixture toxicity and the identification of toxicity drivers is the limit of detection. It is possible that additional organic chemicals were present in the environmental samples but were not detected as their concentration was below detection limits. Furthermore, it is important to keep in mind that environmental monitoring is only a snap-reading method. Environmental concentrations of chemicals can abruptly change due to anthropogenic or natural events, resulting in a significant increase.

The aim of this study was to test whether the use of basic toxicity calculations based on ecotoxicological data from US EPA is a viable method regarding the identification of toxicity drivers in environmental mixtures from monitoring studies. Unfortunately, related to the large amount of chemicals excluded due to missing ecotoxicological data overall, in addition to little to no data present for marine organisms, this was not possible. This simplified method could prove to be an easily implementable factor in chemical mixture risk assessments to pinpoint toxicity drivers, enabling targeted pollutant reduction to reduce risk to marine communities. Since the make up of chemical mixtures in the environment constantly changes, this could additionally be used to extrapolate the obtained knowledge to ecosystems in other geographical areas if the identified toxicity drivers are present there, giving a quick potential risk indication. However, for a successful application, it is crucial to increase the amount of available ecotoxicological data, especially regarding marine organisms, and to further enhance this method.

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Appendix

Table 8: Criteria for the ecotoxicological data from US EPA regarding each of the three selected organism groups for the TU ranking of the chemicals present at site M3 and M4

Organism group	Site	Included common names	Excluded "sp_name"	Endpoints	Conc. sign	Time
Zooplankton	M3	• "Amphipod" • "Brine Shrimp" • "Cladocera" • "Copepod Order" • "Fairy Shrimp" • "Hydra" • "Invertebrates" • "Ostracod/Seed Shrimp Class" • "Rotifer" • "Rotifer Phylum" • "San Francisco Brine Shrimp" • "Scud" • "Scud, Amphipod" • "Water Flea"	• "Invertebrates"	• EC50 • LC50 • IC50	• =	• <48 h
Microalgae	M3	• "Algae" • "Blue-Green Algae" • "Blue-green Algae" • "Brown Algae Division" • "Chrysophyte" • "Cryptomonad" • "Cyanobacteria" • "Diatom" • "Diatom Class" • "Diatom Division" • "Green Algae" • "Green algae" • "Green Algae Class" • "Monera Kingdom" • "Red Algae"	• Algae • Chilomonas paramecium • Monera • Phaeophyta • Plumaria elegans	• EC50 • LC50 • IC50	• =	• <48 h
Bacteria	M3	• "Blue-Green Algae" • "Blue-green Algae" • "Cyanobacteria" • "Monera Kingdom"	None	• EC50 • LC50 • IC50	• =	• <48 h

Zooplankton	M4	• "Amphipod" • "Brine Shrimp" • "Cladocera" • "Copepod Order" • "Fairy Shrimp" • "Hydra" • "Invertebrates" • "Ostracod/Seed Shrimp Class" • "Rotifer" • "Rotifer Phylum" • "San Francisco Brine Shrimp" • "Scud" • "Scud, Amphipod" • "Water Flea"	• "Invertebrates"	• EC50 • LC50 • IC50	• =	• <48 h
Microalgae	M4	• "Algae" • "Blue-Green Algae" • "Blue-green Algae" • "Cryptomonad" • "Cyanobacteria" • "Diatom" • "Diatom Class" • "Green Algae" • "Green algae" • "Green Algae Class" • "Haptophyte" • "Monera Kingdom" • "Red Algae"	• "Algae" • "Chilomonas paramecium" • "Monera" • "Plumaria elegans"	• EC50 • LC50 • IC50	• =	• <48 h
Bacteria	M4	• "Blue-Green Algae" • "Blue-green Algae" • "Cyanobacteria" • "Monera Kingdom"	None	• EC50 • LC50 • IC50	• =	• <48 h

Table 9: Overview of chemicals that were detected in the mixtures at site M3 and M4, including their CAS-number if available, the respective measured environmental concentration, whether ecotoxicological data was available on US EPA and if they were included in the TU ranking for each organism group.

Site	Organism group	Chemical Name	CAS-Nr.	MEC [µg/L]	US EPA	Included in TU ranking
M3	Zooplankton	1,3-Diphenylguanidine	102-06-7	7,079	No	No
sM3	Zooplankton	1,5-Naphthalenediamine	2243-62-1	7,3	Yes	No
M3	Zooplankton	10,11-Dihydro-10-hydroxycarbamazepine	29331-92-8	2,247186	No	No

M3	Zooplankton	1H-Benzotriazole	95-14-7	15,061717	Yes	Yes
M3	Zooplankton	2-(2-Pyridyl)ethanol	103-74-2	32,8	Yes	Yes
M3	Zooplankton	2-(Diethylamino)-6-methylpyrimidin-4-one	42487-72-9	2	No	No
M3	Zooplankton	2,4-Dinitrophenol	51-28-5	305,446184	Yes	Yes
M3	Zooplankton	2-Benzothiazolesulfonic acid	941-57-1	6,1	No	No
M3	Zooplankton	2-Hydroxycarbazole	86-79-3	3,3	No	No
M3	Zooplankton	2-Hydroxyquinoline	59-31-4	8,170833	Yes	No
M3	Zooplankton	2-Isopropyl-6-methyl-pyrimidin-4-ol	2814-20-2	2,040607	Yes	No
M3	Zooplankton	3-Cyclohexyl-1,1-dimethylurea	31468-12-9	226,9	No	No
M3	Zooplankton	4'-Aminoacetanilide	122-80-5	137,419528	No	No
M3	Zooplankton	4-Aminodiphenylamine	101-54-2	1,7	Yes	Yes
M3	Zooplankton	5-Methyl-1H-benzotriazole	136-85-6	13,695891	Yes	Yes
M3	Zooplankton	6,2-fluorotelomer sulfonic acid	27619-97-2	124,897539	Yes	No
M3	Zooplankton	Acetaminophen	103-90-2	45,573748	Yes	Yes
M3	Zooplankton	Adiponitrile	111-69-3	205,8	Yes	Yes
M3	Zooplankton	Amcinonide	51022-69-6	3,194846	No	No
M3	Zooplankton	Amphetamine	300-62-9	11,1	No	No
M3	Zooplankton	Atenolol	29122-68-7	2,492178	Yes	Yes
M3	Zooplankton	Atorvastatin	134523-00-5	12,552489	Yes	No
M3	Zooplankton	Azelaic acid	123-99-9	40,3	No	No
M3	Zooplankton	Benzoylecgonine	519-09-5	0,7	No	No
M3	Zooplankton	Candesartan	139481-59-7	4,786029	No	No
M3	Zooplankton	Carbamazepine	298-46-4	2,527522	Yes	Yes
M3	Zooplankton	Chenodeoxycholic acid	474-25-9	0,2	Yes	No
M3	Zooplankton	Chloridazon	1698-60-8	3,463612	Yes	No
M3	Zooplankton	Cholic acid	81-25-4	0,9	No	No
M3	Zooplankton	Climbazole	38083-17-9	0,835068	No	No
M3	Zooplankton	Cotinine	486-56-6	113,970114	No	No
M3	Zooplankton	Cyclamate	100-88-9	105,890383	No	No
M3	Zooplankton	Cyclohexylphenylketone	712-50-5	120,4	No	No
M3	Zooplankton	DEET carboxylic acid	72236-23-8	2,7	No	No
M3	Zooplankton	Dicyclohexylurea	2387-23-7	62,9	No	No
M3	Zooplankton	Dimethachlor CGA369873	NOCAS	16,465127	NA	No
M3	Zooplankton	Dimethenamid ESA	205939-58-8	0,870814	No	No
M3	Zooplankton	Ephedrine	299-42-3	21,6	No	No
M3	Zooplankton	Eprosartan acid	NOCAS	0,8	NA	No
M3	Zooplankton	epsilon-Caprolactam	105-60-2	216,8	Yes	Yes
M3	Zooplankton	Equilin	474-86-2	10,8	No	No

M3	Zooplankton	Flunisolide	3385-03-3	11,41289	No	No
M3	Zooplankton	Fluorometholone	426-13-1	14,2425	No	No
M3	Zooplankton	Formetanate	22259-30-9	18,6	Yes	Yes
M3	Zooplankton	Gabapentin-Lactam	64744-50-9	4,154403	No	No
M3	Zooplankton	Labetalol	36894-69-6	1,311685	No	No
M3	Zooplankton	Lamotrigine	84057-84-1	4,236749	No	No
M3	Zooplankton	Losartan Carboxylic acid	124750-92-1	3,8	No	No
M3	Zooplankton	Metalaxyl CGA108906	104390-56-9	4,2	No	No
M3	Zooplankton	Metazachlor BH479-12	NOCAS	73,893499	NA	No
M3	Zooplankton	Metazachlor ESA	172960-62-2	13,720956	No	No
M3	Zooplankton	Metazachlor OA	1231244-60-2	17,629777	No	No
M3	Zooplankton	Methamphetamine	537-46-2	0,2	No	No
M3	Zooplankton	Metolachlor_ESA	171118-09-5	5,38678	Yes	No
M3	Zooplankton	Mometasone fuorate	83919-23-7	12,527324	No	No
M3	Zooplankton	m-Xylene-4-sulfonic acid	88-61-9	15,966182	No	No
M3	Zooplankton	Nicosulfuron	111991-09-4	0,038645	Yes	No
M3	Zooplankton	Norfloxacin	70458-96-7	30,219175	Yes	Yes
M3	Zooplankton	Palmitoylethanolamide	544-31-0	22,1	No	No
M3	Zooplankton	Phenylethylmalonamide	7206-76-0	17,155085	No	No
M3	Zooplankton	Phloretin	60-82-2	5,4	Yes	No
M3	Zooplankton	Propranolol	525-66-6	1,066297	Yes	Yes
M3	Zooplankton	Prosulfocarb	52888-80-9	3,675312	No	No
M3	Zooplankton	Quinmerac	90717-03-6	2,516006	No	No
M3	Zooplankton	Raloxifene	84449-90-1	17,408061	No	No
M3	Zooplankton	Spinosyn A	131929-60-7	2,563476	Yes	Yes
M3	Zooplankton	Sucralose	56038-13-2	254,241841	No	No
M3	Zooplankton	TDCPP	13674-87-8	11,376391	No	No
M3	Zooplankton	Telmisartan	144701-48-4	0,067529	No	No
M3	Zooplankton	Tetrabutylammonium	10549-76-5	0,8	No	No
M3	Zooplankton	Tetraethyleneglycol monobutyl ether	23783-42-8	8,1	No	No
M3	Zooplankton	Tetraglyme	143-24-8	9,93268	No	No
M3	Zooplankton	Theobromine	83-67-0	10,2	Yes	No

M3	Zooplankton	Theophyllin	58-55-9	27,689562	Yes	Yes
M3	Zooplankton	Ursolic acid	77-52-1	30,6	No	No
M3	Algae	1,3-Diphenylguanidine	102-06-7	7,079	No	No
M3	Algae	1,5-Naphthalenediamine	2243-62-1	7,3	Yes	No
M3	Algae	10,11-Dihydro-10-hydroxycarbamazepine	29331-92-8	2,247186	No	No
M3	Algae	1H-Benzotriazole	95-14-7	15,061717	Yes	No
M3	Algae	2-(2-Pyridyl)ethanol	103-74-2	32,8	Yes	No
M3	Algae	2-(Diethylamino)-6-methylpyrimidin-4-one	42487-72-9	2	No	No
M3	Algae	2,4-Dinitrophenol	51-28-5	305,446184	Yes	Yes
M3	Algae	2-Benzothiazolesulfonic acid	941-57-1	6,1	No	No
M3	Algae	2-Hydroxycarbazole	86-79-3	3,3	No	No
M3	Algae	2-Hydroxyquinoline	59-31-4	8,170833	Yes	Yes
M3	Algae	2-Isopropyl-6-methyl-pyrimidin-4-ol	2814-20-2	2,040607	Yes	No
M3	Algae	3-Cyclohexyl-1,1-dimethylurea	31468-12-9	226,9	No	No
M3	Algae	4'-Aminoacetanilide	122-80-5	137,419528	No	No
M3	Algae	4-Aminodiphenylamine	101-54-2	1,7	Yes	No
M3	Algae	5-Methyl-1H-benzotriazole	136-85-6	13,695891	Yes	No
M3	Algae	6,2-fluorotelomer sulfonic acid	27619-97-2	124,897539	Yes	No
M3	Algae	Acetaminophen	103-90-2	45,573748	Yes	Yes
M3	Algae	Adiponitrile	111-69-3	205,8	Yes	No
M3	Algae	Amcinonide	51022-69-6	3,194846	No	No
M3	Algae	Amphetamine	300-62-9	11,1	No	No
M3	Algae	Atenolol	29122-68-7	2,492178	Yes	No
M3	Algae	Atorvastatin	134523-00-5	12,552489	Yes	No
M3	Algae	Azelaic acid	123-99-9	40,3	No	No
M3	Algae	Benzoylecgonine	519-09-5	0,7	No	No
M3	Algae	Candesartan	139481-59-7	4,786029	No	No
M3	Algae	Carbamazepine	298-46-4	2,527522	Yes	Yes
M3	Algae	Chenodeoxycholic acid	474-25-9	0,2	Yes	No
M3	Algae	Chloridazon	1698-60-8	3,463612	Yes	Yes
M3	Algae	Cholic acid	81-25-4	0,9	No	No
M3	Algae	Climbazole	38083-17-9	0,835068	No	No
M3	Algae	Cotinine	486-56-6	113,970114	No	No
M3	Algae	Cyclamate	100-88-9	105,890383	No	No
M3	Algae	Cyclohexylphenylketone	712-50-5	120,4	No	No
M3	Algae	DEET carboxylic acid	72236-23-8	2,7	No	No
M3	Algae	Dicyclohexylurea	2387-23-7	62,9	No	No
M3	Algae	Dimethachlor CGA369873	NOCAS	16,465127	NA	No

M3	Algae	Dimethenamid ESA	205939-58-8	0,870814	No	No
M3	Algae	Ephedrine	299-42-3	21,6	No	No
M3	Algae	Eprosartan acid	NOCAS	0,8	NA	No
M3	Algae	epsilon-Caprolactam	105-60-2	216,8	Yes	Yes
M3	Algae	Equilin	474-86-2	10,8	No	No
M3	Algae	Flunisolide	3385-03-3	11,41289	No	No
M3	Algae	Fluorometholone	426-13-1	14,2425	No	No
M3	Algae	Formetanate	22259-30-9	18,6	Yes	No
M3	Algae	Gabapentin-Lactam	64744-50-9	4,154403	No	No
M3	Algae	Labetalol	36894-69-6	1,311685	No	No
M3	Algae	Lamotrigine	84057-84-1	4,236749	No	No
M3	Algae	Losartan Carboxylic acid	124750-92-1	3,8	No	No
M3	Algae	Metalaxyl CGA108906	104390-56-9	4,2	No	No
M3	Algae	Metazachlor BH479-12	NOCAS	73,893499	NA	No
M3	Algae	Metazachlor ESA	172960-62-2	13,720956	No	No
M3	Algae	Metazachlor OA	1231244-60-2	17,629777	No	No
M3	Algae	Methamphetamine	537-46-2	0,2	No	No
M3	Algae	Metolachlor_ESA	171118-09-5	5,38678	Yes	No
M3	Algae	Mometasone fuorate	83919-23-7	12,527324	No	No
M3	Algae	m-Xylene-4-sulfonic acid	88-61-9	15,966182	No	No
M3	Algae	Nicosulfuron	111991-09-4	0,038645	Yes	Yes
M3	Algae	Norfloxacin	70458-96-7	30,219175	Yes	Yes
M3	Algae	Palmitoylethanolamide	544-31-0	22,1	No	No
M3	Algae	Phenylethylmalonamide	7206-76-0	17,155085	No	No
M3	Algae	Phloretin	60-82-2	5,4	Yes	Yes
M3	Algae	Propranolol	525-66-6	1,066297	Yes	Yes
M3	Algae	Prosulfocarb	52888-80-9	3,675312	No	No
M3	Algae	Quinmerac	90717-03-6	2,516006	No	No
M3	Algae	Raloxifene	84449-90-1	17,408061	No	No
M3	Algae	Spinosyn A	131929-60-7	2,563476	Yes	Yes
M3	Algae	Sucralose	56038-13-2	254,241841	No	No
M3	Algae	TDCPP	13674-87-8	11,376391	No	No
M3	Algae	Telmisartan	144701-48-4	0,067529	No	No

M3	Algae	Tetrabutylammonium	10549-76-5	0,8	No	No
M3	Algae	Tetraethyleneglycol monobutyl ether	23783-42-8	8,1	No	No
M3	Algae	Tetraglyme	143-24-8	9,93268	No	No
M3	Algae	Theobromine	83-67-0	10,2	Yes	No
M3	Algae	Theophyllin	58-55-9	27,689562	Yes	No
M3	Algae	Ursolic acid	77-52-1	30,6	No	No
M3	Bacteria	1,3-Diphenylguanidine	102-06-7	7,079	No	No
M3	Bacteria	1,5-Naphthalenediamine	2243-62-1	7,3	Yes	No
M3	Bacteria	10,11-Dihydro-10-hydroxycarbamazepine	29331-92-8	2,247186	No	No
M3	Bacteria	1H-Benzotriazole	95-14-7	15,061717	Yes	No
M3	Bacteria	2-(2-Pyridyl)ethanol	103-74-2	32,8	Yes	No
M3	Bacteria	2-(Diethylamino)-6-methylpyrimidin-4-one	42487-72-9	2	No	No
M3	Bacteria	2,4-Dinitrophenol	51-28-5	305,446184	Yes	No
M3	Bacteria	2-Benzothiazolesulfonic acid	941-57-1	6,1	No	No
M3	Bacteria	2-Hydroxycarbazole	86-79-3	3,3	No	No
M3	Bacteria	2-Hydroxyquinoline	59-31-4	8,170833	Yes	No
M3	Bacteria	2-Isopropyl-6-methyl-pyrimidin-4-ol	2814-20-2	2,040607	Yes	No
M3	Bacteria	3-Cyclohexyl-1,1-dimethylurea	31468-12-9	226,9	No	No
M3	Bacteria	4'-Aminoacetanilide	122-80-5	137,419528	No	No
M3	Bacteria	4-Aminodiphenylamine	101-54-2	1,7	Yes	No
M3	Bacteria	5-Methyl-1H-benzotriazole	136-85-6	13,695891	Yes	No
M3	Bacteria	6,2-fluorotelomer sulfonic acid	27619-97-2	124,897539	Yes	No
M3	Bacteria	Acetaminophen	103-90-2	45,573748	Yes	No
M3	Bacteria	Adiponitrile	111-69-3	205,8	Yes	No
M3	Bacteria	Amcinonide	51022-69-6	3,194846	No	No
M3	Bacteria	Amphetamine	300-62-9	11,1	No	No
M3	Bacteria	Atenolol	29122-68-7	2,492178	Yes	No
M3	Bacteria	Atorvastatin	134523-00-5	12,552489	Yes	No
M3	Bacteria	Azelaic acid	123-99-9	40,3	No	No
M3	Bacteria	Benzoylecgonine	519-09-5	0,7	No	No
M3	Bacteria	Candesartan	139481-59-7	4,786029	No	No
M3	Bacteria	Carbamazepine	298-46-4	2,527522	Yes	No
M3	Bacteria	Chenodeoxycholic acid	474-25-9	0,2	Yes	No
M3	Bacteria	Chloridazon	1698-60-8	3,463612	Yes	Yes
M3	Bacteria	Cholic acid	81-25-4	0,9	No	No
M3	Bacteria	Climbazole	38083-17-9	0,835068	No	No
M3	Bacteria	Cotinine	486-56-6	113,970114	No	No
M3	Bacteria	Cyclamate	100-88-9	105,890383	No	No

M3	Bacteria	Cyclohexylphenylketone	712-50-5	120,4	No	No
M3	Bacteria	DEET carboxylic acid	72236-23-8	2,7	No	No
M3	Bacteria	Dicyclohexylurea	2387-23-7	62,9	No	No
M3	Bacteria	Dimethachlor CGA369873	NOCAS	16,465127	NA	No
M3	Bacteria	Dimethenamid ESA	205939-58-8	0,870814	No	No
M3	Bacteria	Ephedrine	299-42-3	21,6	No	No
M3	Bacteria	Eprosartan acid	NOCAS	0,8	NA	No
M3	Bacteria	epsilon-Caprolactam	105-60-2	216,8	Yes	No
M3	Bacteria	Equilin	474-86-2	10,8	No	No
M3	Bacteria	Flunisolide	3385-03-3	11,41289	No	No
M3	Bacteria	Fluorometholone	426-13-1	14,2425	No	No
M3	Bacteria	Formetanate	22259-30-9	18,6	Yes	No
M3	Bacteria	Gabapentin-Lactam	64744-50-9	4,154403	No	No
M3	Bacteria	Labetalol	36894-69-6	1,311685	No	No
M3	Bacteria	Lamotrigine	84057-84-1	4,236749	No	No
M3	Bacteria	Losartan Carboxylic acid	124750-92-1	3,8	No	No
M3	Bacteria	Metalaxyl CGA108906	104390-56-9	4,2	No	No
M3	Bacteria	Metazachlor BH479-12	NOCAS	73,893499	NA	No
M3	Bacteria	Metazachlor ESA	172960-62-2	13,720956	No	No
M3	Bacteria	Metazachlor OA	1231244-60-2	17,629777	No	No
M3	Bacteria	Methamphetamine	537-46-2	0,2	No	No
M3	Bacteria	Metolachlor_ESA	171118-09-5	5,38678	Yes	No
M3	Bacteria	Mometasone fuorate	83919-23-7	12,527324	No	No
M3	Bacteria	m-Xylene-4-sulfonic acid	88-61-9	15,966182	No	No
M3	Bacteria	Nicosulfuron	111991-09-4	0,038645	Yes	No
M3	Bacteria	Norfloxacin	70458-96-7	30,219175	Yes	Yes
M3	Bacteria	Palmitoylethanolamide	544-31-0	22,1	No	No
M3	Bacteria	Phenylethylmalonamide	7206-76-0	17,155085	No	No
M3	Bacteria	Phloretin	60-82-2	5,4	Yes	No
M3	Bacteria	Propranolol	525-66-6	1,066297	Yes	No
M3	Bacteria	Prosulfocarb	52888-80-9	3,675312	No	No
M3	Bacteria	Quinmerac	90717-03-6	2,516006	No	No
M3	Bacteria	Raloxifene	84449-90-1	17,408061	No	No
M3	Bacteria	Spinosyn A	131929-60-7	2,563476	Yes	No

M3	Bacteria	Sucralose	56038-13-2	254,241841	No	No
M3	Bacteria	TDCPP	13674-87-8	11,376391	No	No
M3	Bacteria	Telmisartan	144701-48-4	0,067529	No	No
M3	Bacteria	Tetrabutylammonium	10549-76-5	0,8	No	No
M3	Bacteria	Tetraethyleneglycol monobutyl ether	23783-42-8	8,1	No	No
M3	Bacteria	Tetraglyme	143-24-8	9,93268	No	No
M3	Bacteria	Theobromine	83-67-0	10,2	Yes	No
M3	Bacteria	Theophyllin	58-55-9	27,689562	Yes	No
M3	Bacteria	Ursolic acid	77-52-1	30,6	No	No
M4	Zooplankton	1,3-Diphenylguanidine	102-06-7	4,165	No	No
M4	Zooplankton	1,3-Diphenylurea	102-07-8	0,8	No	No
M4	Zooplankton	1,5-Naphthalenediamine	2243-62-1	3,9	Yes	No
M4	Zooplankton	10,11-Dihydro-10-hydroxycarbamazepine	29331-92-8	1,731286	No	No
M4	Zooplankton	1H-Benzotriazole	95-14-7	10,543649	Yes	Yes
M4	Zooplankton	2-(2-Pyridyl)ethanol	103-74-2	23,3	Yes	Yes
M4	Zooplankton	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	6846-50-0	5,7	No	No
M4	Zooplankton	2,4-Dinitrophenol	51-28-5	112,282984	Yes	Yes
M4	Zooplankton	2-Benzothiazolesulfonic acid	941-57-1	4,1	No	No
M4	Zooplankton	2-Hydroxycarbazole	86-79-3	2,5	No	No
M4	Zooplankton	2-Hydroxyquinoline	59-31-4	9,974531	Yes	No
M4	Zooplankton	3-Cyclohexyl-1,1-dimethylurea	31468-12-9	191,6	No	No
M4	Zooplankton	4,4'-Methylenedianiline	101-77-9	2	No	No
M4	Zooplankton	4'-Aminoacetanilide	122-80-5	103,194587	No	No
M4	Zooplankton	4-Aminodiphenylamine	101-54-2	1,9	Yes	Yes
M4	Zooplankton	5-Methyl-1H-benzotriazole	136-85-6	11,560275	Yes	Yes
M4	Zooplankton	Acetaminophen	103-90-2	27,223345	Yes	Yes
M4	Zooplankton	Adiponitrile	111-69-3	123,5	Yes	Yes
M4	Zooplankton	Amphetamine	300-62-9	9,2	No	No
M4	Zooplankton	Azelaic acid	123-99-9	34,9	No	No
M4	Zooplankton	Benzoylecgonine	519-09-5	0,7	No	No
M4	Zooplankton	Budesonide	51333-22-3	0,072017	No	No
M4	Zooplankton	Carbamazepine	298-46-4	2,480491	Yes	Yes
M4	Zooplankton	Carbetamide	16118-49-3	0,942835	Yes	No
M4	Zooplankton	Chenodeoxycholic acid	474-25-9	0,3	Yes	No
M4	Zooplankton	Chloridazon	1698-60-8	2,396792	Yes	No
M4	Zooplankton	Cholic acid	81-25-4	1	No	No
M4	Zooplankton	Climbazole	38083-17-9	0,742616	No	No
M4	Zooplankton	Cotinine	486-56-6	83,052369	No	No

M4	Zooplankton	Cyclohexylphenylketone	712-50-5	114,5	No	No
M4	Zooplankton	DEET carboxylic acid	72236-23-8	2,2	No	No
M4	Zooplankton	Dicyclohexylurea	2387-23-7	17,9	No	No
M4	Zooplankton	Dimethachlor CGA369873	NOCAS	12,451334	No	No
M4	Zooplankton	Dimethenamid ESA	205939-58-8	0,895335	No	No
M4	Zooplankton	Enalapril	75847-73-3	2,169639	No	No
M4	Zooplankton	Ephedrine	299-42-3	19,9	No	No
M4	Zooplankton	Eprosartan acid	NOCAS	0,8	No	No
M4	Zooplankton	epsilon-Caprolactam	105-60-2	117	Yes	Yes
M4	Zooplankton	Equilin	474-86-2	8,6	No	No
M4	Zooplankton	Flunisolide	3385-03-3	0,043028	No	No
M4	Zooplankton	Formetanate	22259-30-9	12,8	Yes	Yes
M4	Zooplankton	Gabapentin-Lactam	64744-50-9	3,612414	No	No
M4	Zooplankton	Labetalol	36894-69-6	0,876499	No	No
M4	Zooplankton	Lamotrigine	84057-84-1	4,073097	No	No
M4	Zooplankton	Losartan Carboxylic acid	124750-92-1	1,3	No	No
M4	Zooplankton	Metalaxyl CGA108906	104390-56-9	3,1	No	No
M4	Zooplankton	Metazachlor BH479-12	NOCAS	108,599505	No	No
M4	Zooplankton	Metazachlor ESA	172960-62-2	9,701094	No	No
M4	Zooplankton	Metazachlor OA	1231244-60-2	11,900537	No	No
M4	Zooplankton	Metolachlor_ESA	171118-09-5	4,487213	Yes	No
M4	Zooplankton	Metolachlor-OA	152019-73-3	4,577012	Yes	No
M4	Zooplankton	m-Xylene-4-sulfonic acid	88-61-9	9,794387	No	No
M4	Zooplankton	Nicosulfuron	111991-09-4	0,039304	Yes	No
M4	Zooplankton	Norfloxacin	70458-96-7	27,442044	Yes	Yes
M4	Zooplankton	Palmitoylethanolamide	544-31-0	14,1	No	No
M4	Zooplankton	Phenothiazine	92-84-2	1,9	No	No
M4	Zooplankton	Phenylethylmalonamide	7206-76-0	9,977054	No	No
M4	Zooplankton	Phloretin	60-82-2	6,4	Yes	No
M4	Zooplankton	Prosulfocarb	52888-80-9	4,099272	No	No
M4	Zooplankton	Quinmerac	90717-03-6	2,112783	No	No
M4	Zooplankton	Raloxifene	84449-90-1	11,55843	No	No
M4	Zooplankton	Sidenafil	139755-83-2	0,5	No	No

M4	Zooplankton	Spinosyn A	131929-60-7	5,335221	Yes	Yes
M4	Zooplankton	Sucralose	56038-13-2	163,288356	No	No
M4	Zooplankton	Telmisartan	144701-48-4	0,066441	No	No
M4	Zooplankton	Tetrabutylammonium	10549-76-5	0,8	No	No
M4	Zooplankton	Tetraglyme	143-24-8	7,360509	No	No
M4	Zooplankton	Ursolic acid	77-52-1	25,4	No	No
M4	Algae	1,3-Diphenylguanidine	102-06-7	4,165	No	No
M4	Algae	1,3-Diphenylurea	102-07-8	0,8	No	No
M4	Algae	1,5-Naphthalenediamine	2243-62-1	3,9	Yes	No
M4	Algae	10,11-Dihydro-10-hydroxycarbamazepine	29331-92-8	1,731286	No	No
M4	Algae	1H-Benzotriazole	95-14-7	10,543649	Yes	No
M4	Algae	2-(2-Pyridyl)ethanol	103-74-2	23,3	Yes	No
M4	Algae	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	6846-50-0	5,7	No	No
M4	Algae	2,4-Dinitrophenol	51-28-5	112,282984	Yes	Yes
M4	Algae	2-Benzothiazolesulfonic acid	941-57-1	4,1	No	No
M4	Algae	2-Hydroxycarbazole	86-79-3	2,5	No	No
M4	Algae	2-Hydroxyquinoline	59-31-4	9,974531	Yes	Yes
M4	Algae	3-Cyclohexyl-1,1-dimethylurea	31468-12-9	191,6	No	No
M4	Algae	4,4'-Methylenedianiline	101-77-9	2	No	No
M4	Algae	4'-Aminoacetanilide	122-80-5	103,194587	No	No
M4	Algae	4-Aminodiphenylamine	101-54-2	1,9	Yes	No
M4	Algae	5-Methyl-1H-benzotriazole	136-85-6	11,560275	Yes	No
M4	Algae	Acetaminophen	103-90-2	27,223345	Yes	Yes
M4	Algae	Adiponitrile	111-69-3	123,5	Yes	No
M4	Algae	Amphetamine	300-62-9	9,2	No	No
M4	Algae	Azelaic acid	123-99-9	34,9	No	No
M4	Algae	Benzoylecgonine	519-09-5	0,7	No	No
M4	Algae	Budesonide	51333-22-3	0,072017	No	No
M4	Algae	Carbamazepine	298-46-4	2,480491	Yes	Yes
M4	Algae	Carbetamide	16118-49-3	0,942835	Yes	No
M4	Algae	Chenodeoxycholic acid	474-25-9	0,3	Yes	No
M4	Algae	Chloridazon	1698-60-8	2,396792	Yes	Yes
M4	Algae	Cholic acid	81-25-4	1	No	No
M4	Algae	Climbazole	38083-17-9	0,742616	No	No
M4	Algae	Cotinine	486-56-6	83,052369	No	No
M4	Algae	Cyclohexylphenylketone	712-50-5	114,5	No	No
M4	Algae	DEET carboxylic acid	72236-23-8	2,2	No	No
M4	Algae	Dicyclohexylurea	2387-23-7	17,9	No	No

M4	Algae	Dimethachlor CGA369873	NOCAS	12,451334	No	No
M4	Algae	Dimethenamid ESA	205939-58-8	0,895335	No	No
M4	Algae	Enalapril	75847-73-3	2,169639	No	No
M4	Algae	Ephedrine	299-42-3	19,9	No	No
M4	Algae	Eprosartan acid	NOCAS	0,8	No	No
M4	Algae	epsilon-Caprolactam	105-60-2	117	Yes	Yes
M4	Algae	Equilin	474-86-2	8,6	No	No
M4	Algae	Flunisolide	3385-03-3	0,043028	No	No
M4	Algae	Formetanate	22259-30-9	12,8	Yes	No
M4	Algae	Gabapentin-Lactam	64744-50-9	3,612414	No	No
M4	Algae	Labetalol	36894-69-6	0,876499	No	No
M4	Algae	Lamotrigine	84057-84-1	4,073097	No	No
M4	Algae	Losartan Carboxylic acid	124750-92-1	1,3	No	No
M4	Algae	Metalaxyl CGA108906	104390-56-9	3,1	No	No
M4	Algae	Metazachlor BH479-12	NOCAS	108,599505	No	No
M4	Algae	Metazachlor ESA	172960-62-2	9,701094	No	No
M4	Algae	Metazachlor OA	1231244-60-2	11,900537	No	No
M4	Algae	Metolachlor_ESA	171118-09-5	4,487213	Yes	No
M4	Algae	Metolachlor-OA	152019-73-3	4,577012	Yes	No
M4	Algae	m-Xylene-4-sulfonic acid	88-61-9	9,794387	No	No
M4	Algae	Nicosulfuron	111991-09-4	0,039304	Yes	Yes
M4	Algae	Norfloxacin	70458-96-7	27,442044	Yes	Yes
M4	Algae	Palmitoylethanolamide	544-31-0	14,1	No	No
M4	Algae	Phenothiazine	92-84-2	1,9	No	No
M4	Algae	Phenylethylmalonamide	7206-76-0	9,977054	No	No
M4	Algae	Phloretin	60-82-2	6,4	Yes	Yes
M4	Algae	Prosulfocarb	52888-80-9	4,099272	No	No
M4	Algae	Quinmerac	90717-03-6	2,112783	No	No
M4	Algae	Raloxifene	84449-90-1	11,55843	No	No
M4	Algae	Sidenafil	139755-83-2	0,5	No	No
M4	Algae	Spinosyn A	131929-60-7	5,335221	Yes	Yes
M4	Algae	Sucralose	56038-13-2	163,288356	No	No

M4	Algae	Telmisartan	144701-48-4	0,066441	No	No
M4	Algae	Tetrabutylammonium	10549-76-5	0,8	No	No
M4	Algae	Tetraglyme	143-24-8	7,360509	No	No
M4	Algae	Ursolic acid	77-52-1	25,4	No	No
M4	Bacteria	1,3-Diphenylguanidine	102-06-7	4,165	No	No
M4	Bacteria	1,3-Diphenylurea	102-07-8	0,8	No	No
M4	Bacteria	1,5-Naphthalenediamine	2243-62-1	3,9	Yes	No
M4	Bacteria	10,11-Dihydro-10-hydroxycarbamazepine	29331-92-8	1,731286	No	No
M4	Bacteria	1H-Benzotriazole	95-14-7	10,543649	Yes	No
M4	Bacteria	2-(2-Pyridyl)ethanol	103-74-2	23,3	Yes	No
M4	Bacteria	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	6846-50-0	5,7	No	No
M4	Bacteria	2,4-Dinitrophenol	51-28-5	112,282984	Yes	No
M4	Bacteria	2-Benzothiazolesulfonic acid	941-57-1	4,1	No	No
M4	Bacteria	2-Hydroxycarbazole	86-79-3	2,5	No	No
M4	Bacteria	2-Hydroxyquinoline	59-31-4	9,974531	Yes	No
M4	Bacteria	3-Cyclohexyl-1,1-dimethylurea	31468-12-9	191,6	No	No
M4	Bacteria	4,4'-Methylenedianiline	101-77-9	2	No	No
M4	Bacteria	4'-Aminoacetanilide	122-80-5	103,194587	No	No
M4	Bacteria	4-Aminodiphenylamine	101-54-2	1,9	Yes	No
M4	Bacteria	5-Methyl-1H-benzotriazole	136-85-6	11,560275	Yes	No
M4	Bacteria	Acetaminophen	103-90-2	27,223345	Yes	No
M4	Bacteria	Adiponitrile	111-69-3	123,5	Yes	No
M4	Bacteria	Amphetamine	300-62-9	9,2	No	No
M4	Bacteria	Azelaic acid	123-99-9	34,9	No	No
M4	Bacteria	Benzoylecgonine	519-09-5	0,7	No	No
M4	Bacteria	Budesonide	51333-22-3	0,072017	No	No
M4	Bacteria	Carbamazepine	298-46-4	2,480491	Yes	No
M4	Bacteria	Carbetamide	16118-49-3	0,942835	Yes	No
M4	Bacteria	Chenodeoxycholic acid	474-25-9	0,3	Yes	No
M4	Bacteria	Chloridazon	1698-60-8	2,396792	Yes	Yes
M4	Bacteria	Cholic acid	81-25-4	1	No	No
M4	Bacteria	Climbazole	38083-17-9	0,742616	No	No
M4	Bacteria	Cotinine	486-56-6	83,052369	No	No
M4	Bacteria	Cyclohexylphenylketone	712-50-5	114,5	No	No
M4	Bacteria	DEET carboxylic acid	72236-23-8	2,2	No	No
M4	Bacteria	Dicyclohexylurea	2387-23-7	17,9	No	No
M4	Bacteria	Dimethachlor CGA369873	NOCAS	12,451334	No	No
M4	Bacteria	Dimethenamid ESA	205939-58-8	0,895335	No	No

M4	Bacteria	Enalapril	75847-73-3	2,169639	No	No
M4	Bacteria	Ephedrine	299-42-3	19,9	No	No
M4	Bacteria	Eprosartan acid	NOCAS	0,8	No	No
M4	Bacteria	epsilon-Caprolactam	105-60-2	117	Yes	No
M4	Bacteria	Equilin	474-86-2	8,6	No	No
M4	Bacteria	Flunisolide	3385-03-3	0,043028	No	No
M4	Bacteria	Formetanate	22259-30-9	12,8	Yes	No
M4	Bacteria	Gabapentin-Lactam	64744-50-9	3,612414	No	No
M4	Bacteria	Labetalol	36894-69-6	0,876499	No	No
M4	Bacteria	Lamotrigine	84057-84-1	4,073097	No	No
M4	Bacteria	Losartan Carboxylic acid	124750-92-1	1,3	No	No
M4	Bacteria	Metalaxyl CGA108906	104390-56-9	3,1	No	No
M4	Bacteria	Metazachlor BH479-12	NOCAS	108,599505	No	No
M4	Bacteria	Metazachlor ESA	172960-62-2	9,701094	No	No
M4	Bacteria	Metazachlor OA	1231244-60-2	11,900537	No	No
M4	Bacteria	Metolachlor_ESA	171118-09-5	4,487213	Yes	No
M4	Bacteria	Metolachlor-OA	152019-73-3	4,577012	Yes	No
M4	Bacteria	m-Xylene-4-sulfonic acid	88-61-9	9,794387	No	No
M4	Bacteria	Nicosulfuron	111991-09-4	0,039304	Yes	No
M4	Bacteria	Norfloxacin	70458-96-7	27,442044	Yes	Yes
M4	Bacteria	Palmitoylethanolamide	544-31-0	14,1	No	No
M4	Bacteria	Phenothiazine	92-84-2	1,9	No	No
M4	Bacteria	Phenylethylmalonamide	7206-76-0	9,977054	No	No
M4	Bacteria	Phloretin	60-82-2	6,4	Yes	No
M4	Bacteria	Prosulfocarb	52888-80-9	4,099272	No	No
M4	Bacteria	Quinmerac	90717-03-6	2,112783	No	No
M4	Bacteria	Raloxifene	84449-90-1	11,55843	No	No
M4	Bacteria	Sidenafil	139755-83-2	0,5	No	No
M4	Bacteria	Spinosyn A	131929-60-7	5,335221	Yes	No
M4	Bacteria	Sucralose	56038-13-2	163,288356	No	No
M4	Bacteria	Telmisartan	144701-48-4	0,066441	No	No
M4	Bacteria	Tetrabutylammonium	10549-76-5	0,8	No	No

M4	Bacteria	Tetraglyme	143-24-8	7,360509	No	No
M4	Bacteria	Ursolic acid	77-52-1	25,4	No	No