Effect of individual toxicants and their mixtures to the marine bacterium *Vibrio fischeri*
Abstract

Due to the prevalent use of different compounds in industry, agriculture, health care and other activities, the release of these compounds into the aquatic environment have been increasing. That is why marine coastal organisms are exposed to many different types of compounds. Different single compounds in the environment will interact with each other in some way and mixture of compounds will be made. Regarding the risk assessment of chemicals, the risk of mixture of compounds might have been underestimated since most studies have been focused on effects of single exposures. Bacteria are generally regarded as insensitive compared to algae, Daphnia and fish, except for chemicals which are used as antibiotics, however they are responding rapidly (within minutes) if luminescence is used as response. *Vibrio fischeri* was selected in this study as a suitable model organism to evaluate the effects of single substance and mixture exposure scenarios. A group of compounds with similar and dissimilar mode of action (MoA), including copper chloride (Cu), triclosan, linear alkylbenzene sulfonate (LAS), silver nitrate (Ag+), ciprofloxacin, zinc pyrithione (ZnPT), and zinc oxide, were selected. The endpoint of this study was growth inhibition by measuring optical density of bacterial suspension. All substances demonstrated toxicities to the bacteria. Results of the mixture toxicity with all seven compounds, mostly followed the pattern of IA predicted curve, consequently IA is shown more toxicity than CA which is unusual. Mixture toxicity of copper, zinc and zinc pyrithione indicated less toxicity than expected. Generally ZnPT is known to be highly toxic to aquatic organisms, but in combination with Zn and Cu, the whole mixture observed less toxicity. The binary mixture of Cu and ZnPT presented a little more toxicity than expected, because growth inhibition occurred at lower concentration than predicted. In addition, mixture toxicity of tested compounds was predicted by using two classic concepts namely Concentration Addition (CA) and Independent Action (IA).
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1. Introduction

1.1. General Introduction

Every year, many new compounds are registering into the chemical database system and sooner or later, they will be used in new different products in industry, health care, agriculture or other activities. Consequently all these compounds will end up in the environment; most of these compounds will be transferred directly to the aquatic environment through Sewage Treatment Plants (STPs), sludge and streams. Chemicals in the environment have different mechanisms; they act differently on animals and plants, they can slow down or grow worse. They act as selection pressure that harm some species while favor others. The prevalent use of different compounds, modify environment’s ecology. With increasing delivery of chemical into the coastal waters, both people and the environment get affect because pollutants can produce long and short-term environmental impacts (Laws Edward A. 2000). For predicting toxicity of compounds, samples of contaminated area will be selected and transferred to laboratory to be compared with samples of cleaner areas (Douglas et al., 2005).

Eco-toxicological studies have mostly focused on exposure and effect of single compounds while in a polluted environment such as a coastal environment; organisms are exposed to many different chemicals. Single-substance risk evaluation is the most common regulatory approach for management of chemical compounds (Zwart et al. 2005). In addition there are different arguments for making mixture risk assessment; the nature of chemicals in the mixture, the variability of exposure routes and the range of sensitivities of the organism’s receptor (Zwart et al. 2005). During the last 15 years, predictive mixture toxicology has improved a lot and multi component mixtures with clear results are now possible (Backhaus et al. 2009). But still it is not clear how effects on particular species translate into ecological effects and it is because of low understanding of uptake and elimination of a compound in a body (Kooijman et al. 1996). Marine coastal organisms are exposed to many different types of compounds that some of them might be toxic. It is therefore not surprising that even limited survey finds complex mixtures of chemicals in the coastal environment (Kortenkamp et al. 2009).
1.2. *Vibrio fischeri*: A well-established model system for eco-toxicological research

In order to avoid time consuming aquatic species tests for chemicals and commercial products, researchers have found an encouraging alternative which is a marine bacterium called *Vibrio fischeri*. It was formally known as *Photobacterium phosphoreum* (Kaiser. 1998). This bacterium is a heterotrophic gram-negative, rod shaped bacterium in marine environments which has bioluminescent properties. It is a key research organism to understand relationship between bacteria and animal (Zhou et al. 2006). Bioluminescent bacteria will be used to detect toxic chemicals in a standardized assay in eco-toxicology (Zhou et al. 2006).

The features that make *Vibrio fischeri* a suitable model species include short reproduction cycle and colonizing light-producing organ on fishes and squid and help it develop (DOE. 2010). This bacterium is known to be used in toxicity studies of aquatic environments and help researchers to testing water samples (Maiden S. 2004). For the acute toxicities of most standardized aquatic bioassays, shorter than 96hrs (except some fish tests) is the usual period of test, while the bacteria test can be performed in a matter of minutes. Moreover, *Vibrio fischeri* bacteria can be kept frozen and take a few minutes to reactivation. It also can be available in the laboratory on demand without the need for keeping fishes or other mammals. So in compared to other assays with more complex organisms, it is obviously less money and time consuming. On the other hand it should be noted that in some cases, the biochemical mechanisms of which compounds handle a toxic or bioluminescence reducing effect on the bacterium is not well understood (Kaiser. 1998).

1.3. Compounds of interest

Due to the wide discharge of chemical contaminants into coastal ecosystems from different sources like STPs, industry and agricultural nonpoint source runoff, high toxicity to aquatic organisms has been reported (Scott et al. 2012). Developed areas, including marinas, collect pollutants of all types; potential impact of transformation products in marine environment is an understudied topic (The coastal environment and pollution impacts. April 2001). The types of pollutants which have been tested in the present study are common potential pollutants to marine environment that harms organisms in different ways. In addition, to be able to assess toxicity of pollutions from coastal waters, a basic knowledge of pollutants and their impacts on the environment is needed (Tab 1.3.)
Table 1.3. Compounds of interest with their information from ChemSpider database

<table>
<thead>
<tr>
<th>Substance</th>
<th>IUPAC name</th>
<th>CAS no.</th>
<th>Molecular weight</th>
<th>Log Kow</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper chloride</td>
<td>Copper(II) chloride</td>
<td>7447-39-4</td>
<td>134.45</td>
<td>−0.17</td>
<td>Protein denaturation, producing cell damage and leakage by binding to functional groups of protein molecules</td>
</tr>
<tr>
<td>Triclosan</td>
<td>5-chloro-2-(2,4-dichlorophenoxy)phenol</td>
<td>3380-34-5</td>
<td>289.54</td>
<td>4.76</td>
<td>Kills bacteria through inhibiting bacteria fatty acid synthesis by inhibiting the enzyme Enonyl-acyl carrier protein reductase (ENR)</td>
</tr>
<tr>
<td>Linear Alkylbenzene Sulfonate</td>
<td>Dodecylbenzene sulfonic acid, sodium salt</td>
<td>25155–30-0</td>
<td>348.48</td>
<td>3.32</td>
<td>Affects cell functions such as growth, viability, and NH4+ oxidation activity</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>Nitric acid silver (1+) salt</td>
<td>7761-88-8</td>
<td>169.87</td>
<td>1.68</td>
<td>Changes in the bacterial cell membranes which might be the cause of cell death</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-quinoline-3-carboxylic acid</td>
<td>85721–33-1</td>
<td>331.346</td>
<td>0.28</td>
<td>Inhibition of DNA gyrase</td>
</tr>
<tr>
<td>Zinc Pyrithione</td>
<td>2-pyridinethiol-1-oxide, zinc salt</td>
<td>13463–41-7</td>
<td>317.7</td>
<td>&lt;2.8 &gt;4.8</td>
<td>Inhibition of uptake of several unrelated substrates</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>Oxozinc</td>
<td>7440-66-6</td>
<td>81,408</td>
<td>1.2</td>
<td>Inhibition and inactivation of cell growth</td>
</tr>
</tbody>
</table>

Table 1.4. Reported concentrations of selected compounds in five marinas and harbors at the Swedish west-coast from a sampling campaign in 2012 (M/L)

<table>
<thead>
<tr>
<th>Substances</th>
<th>Instöränna</th>
<th>Fiskebäckskil</th>
<th>Port of Gothenburg</th>
<th>Lerkil</th>
<th>Stenungsund</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper sulfate</td>
<td>8.18E-09</td>
<td>1.71E-08</td>
<td>8.18E-09</td>
<td>6.25E-09</td>
<td>3.64E-08</td>
</tr>
<tr>
<td>Triclosan</td>
<td>5.53E-09</td>
<td>1.49E-08</td>
<td>1.07E-08</td>
<td>1.55E-08</td>
<td>6.22E-09</td>
</tr>
<tr>
<td>LAS</td>
<td>n.d,</td>
<td>n.d,</td>
<td>n.d,</td>
<td>n.d,</td>
<td>n.d,</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>4.89E-11</td>
<td>5.00E-11</td>
<td>4.00E-11</td>
<td>4.24E-11</td>
<td>5.77E-11</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>4.53E-10</td>
<td>5.58E-10</td>
<td>1.88E-09</td>
<td>4.53E-10</td>
<td>4.53E-10</td>
</tr>
<tr>
<td>ZnPT</td>
<td>n.d,</td>
<td>n.d,</td>
<td>n.d,</td>
<td>n.d,</td>
<td>n.d,</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>4.91E-12</td>
<td>4.91E-12</td>
<td>4.91E-12</td>
<td>4.91E-12</td>
<td>4.91E-12</td>
</tr>
</tbody>
</table>
Metals

Combination of several factors leads to environmental pollution and when water pollution is the subject, some important changes such as pH, temperature, and oxygen concentration are included (Fulladosa et al. 2004). In case of metal pollution, presence of heavy metals in solid and liquid wastes is an important issue. Generally, physicochemical parameters like pH, hardness, interactive effects and presence of natural organic matter play distinguished role in solubility, bioavailability and toxicity of heavy metals. For example, pH affects the solubility, speciation and transportation of metals from solid to liquid phase (Tsiridis et al. 2005). In recent 15 years, toxicity of metals has received increasing attention and it is because of their wide spread release from different sources into the environment. High toxicity of metals to aquatic organisms has been reported (Utgikar et al. 2004). In this present study, three essential metals: copper sulfate and zinc oxide & silver nitrate are used.

- **Copper chloride**

Copper chloride is an essential mineral with high solubility in water that is toxic to algae, bacteria and fungi. Copper sulfate was first registered in United States in 1956 and has been used since the 1700s, registration of copper sulfate completed by Environmental Protection Agency (EPA) in 2009. Copper is available in the food, water and environment. It is used in both agriculture and non-agricultural settings. The copper ions cause protein denaturation, producing cell damage and leakage by binding to functional groups of protein molecules (National Pesticide Information Center, 2012).

- **Zinc oxide**

Zinc has shown significant toxicity to bacteria; inhibition and inactivation of cell growth has been reported as antibacterial effect of zinc (Xie et al. 2011).

- **Silver Nitrate**

There are different kinds of heavy metals in the environment which are toxic, but silver ion is one of the most toxic forms of them. The source of silver in the environment is mostly from industrial wastes. It has been estimated at approximately 2,500 tones, which 150 tones will end up into the sludge of wastewater treatment plants and 80 tones is released into surface waters. Generally, bioaccumulation of silver in soil is rather low. In natural waters, toxicity of dissolved silver ion is less than in laboratory tests. It could be because of more opportunities for possible covalent, complexing or colloidal binding silver with reactants (Ratte H.T. 1998).
**Zinc Pyrithione**

ZnPT is widely used in personal-care products such as anti-dandruff shampoos (Zhou et al. 2006). In coastal areas it is really interesting since it is used in antifouling paints (Dinning et al. 1998). It has been reported that this compounds is membrane active and this is indicated by the inhibition of uptake of several unrelated substrates in both bacteria and fungi (Dinning et al. 1998). In addition, reduction in intracellular ATP levels is a direct membrane effect of a biocide (Dinning et al. 1998). Detected concentration of ZnPT in the aquatic environment is between 1.9 – 32 µg/L (Woldegiorgis et al. 2007).

**Triclosan**

Triclosan is a synthetic, broad-spectrum antimicrobial agent that has been used in a wide variety of household and personal care products. It is a stable lipophilic compound (with log$K_{ow}$ =4.8) which can be bio-accumulated and effects on non-target species. This compound has been found in the environment, food, plasma and human breast milk (Farre et al. 2008). Triclosan has been also detected in Sewage treatment plants (STPs) because treatment plants are not designed to remove personal care products and pharmaceuticals. The adverse effect of triclosan is on aquatic organisms such as bacteria and algal communities and toxicity of triclosan was found higher for bacteria than algae (Farre et al. 2008). It acts as a biocide to kill bacteria through inhibiting bacteria fatty acid synthesis by inhibiting the enzyme Enyol reeducates (ENR). Finally, studies have shown that triclosan of surface waters can degrade in the presence of sun light and it leads to harmful products in the environment (Farre et al. 2008).

**Linear Alkylbenzene Sulfonate**

Linear Alkylbenze Sulfonate (LAS) is a surfactant compound that has been used in many industrial applications. Industrial surfactants are mostly produced by using of complex mixtures of many individual surface active components. Surfactants are organic compounds containing both water soluble and water-insoluble components.

They have been used as consumer and industrial cleaning compounds such as detergents and cleansing agents (Ma et al. 2006). They act as an active surface agent to reduce surface tension of liquids, so they can bind to particles that are suspended in the liquid. They have been used in household products such as shampoo, toothpaste and soaps (Environmental Risk Assessment, HERA Report. 2013). The seasonal average variations of LAS concentration in the aquatic environment during summer and spring periods had the highest concentration at surface (0.13±0.04 mg/L) and bottom (0.12±0.10 mg/L) (Okbah et al. 2013).
Ciprofloxacin

Currently, several antibiotics as a subgroup of pharmaceuticals are being used in medicine to treat human and animal infectious disease. Pharmaceuticals will be transferred into the environment through sewage treatment plants (STPs). Consequently, they will be found in the sludge and aquatic environment. Ciprofloxacin is a second generation of fluoroquinolone (FQ) antibiotic (Lindberg et al. 2007). Result of previous studies on FQ, indicated that approximately 70% of the amount of this substance is available in sludge (Lindberg et al. 2007). The widespread release of antibiotics in the water, have adverse effects on microorganisms in the aquatic environment (Näslund et al. 2008). Ciprofloxacin is an example of extensively used, broad-spectrum antibiotics and this made it important in an environmental risk assessment perspective (Näslund et al. 2008).

1.4. Mixture Toxicity Study

One of the most critical and emergent issue in human and environmental toxicology is the risk assessment of complex chemicals mixtures. Both humans and the environment are exposed to these mixtures via concurrent or consecutive release through multifarious routes, modulated by both biotic and abiotic factors (Groten et al., 2001); however at present there are quite meager approaches for prospective assessment of these multiple and sequential exposures (Kortenkamp et al., 2009).

Aquatic organisms are exposed to mixture of toxic compounds, mixture toxicity and their additive effects is mostly depends on their mode of action (MoA). If compounds have same MoA, they would have more additive effects, while if the compounds are different in their mode of action, they would act independently (IA concept) (Schei et al. 2009).

CA concept: Concentration Addition

In aquatic toxicology, for the assessment of combination effect, concentration addition has been mostly used. CA applies to chemicals with the same mode of action and assumes that all the components present in a mixture act on the same biological/biochemical target site. This concept actually says that every single substance can contribute to the whole toxicity; it means substances in the given mixture can be replaced by another or changed while the whole toxicity does not change as far as toxic unit (TU) of the mixture remain the same. Toxic unit is a measure of toxicity in an effluent as determined by the acute toxicity units or chronic toxicity units. Higher TUs indicate greater toxicity. Concentration addition in the simple cases occurs if a chemical acts as a dilution of another (Backhaus et al., 2000).
1.4.1. The mathematical model used to describe this concept is:

\[ \text{EC}_{\text{mix}}(x) = \left[ \sum_{i=1}^{n} \frac{p_i}{	ext{EC}_{\text{xi}}} \right] - 1 \]

\( \text{EC}_{\text{xi}} \) stands for concentration of the compound that causes x% effect in single exposure and \( p_i \) denote for relative fraction of a compound in the mixture. \( \text{EC}_{\text{mix}}(x) \) is the concentration of the total mixture that provoke x% effect.

**IA concept: Independent Action**

This concept is based on the idea that all components have a dissimilar mode of action. In contrast to CA, IA states that low-dose compounds do not influence the overall toxicity of a mixture if present below their individual threshold. According to this concept the combined effects of the exposure to a mixture of dissimilarly acting chemicals can be calculated by the individual mixture’s components effects \( E(C_i) \) by using the statistical concept of independent random effects (Backhaus et al., 2010); which simply means that the concept is based on the known effects of mixture components.

1.4.2. The mathematical model used to describe this concept is:

\[ E(C_{\text{mix}}) = 1 - \prod_{i=1}^{n} [1 - E(C_i)] \]

In this concept \( E(C_i) \) denotes the effect of the compound \( i \) at concentration \( C_i \) in single exposure. \( E(C_{\text{mix}}) \) is the concentration of the total mixture that provoke x% effect.

1.5. **Aim of this study**

- The general aim of this research was to establish the *Vibrio fisheri* test and to test a range of compounds regularly found at the Swedish west-coast and study realistic mixtures of these. Is there any effect of these exposures to *Vibrio fisheri* and what can that tell us about the contamination levels – are those risky or not?

- Are the effects of those dissimilarly acting compounds predictable according to IA or CA?
2. Materials and Methods

In order to start experiments, some preparations need to be done. The test organism (*Vibrio fischeri*) is in form of liquid-dried bacteria and needs to be reactivated. Reactivated bacteria should be inoculated in growth medium to make bacterial stock culture. Finally, toxicant solutions should be made specifically for each test substance. All these steps are explained further below.

2.1. Preparation of the nutrient solution

All seven compounds were purchased from Sigma Aldrich (Table 2.3).

According to the given test protocol from university, nutrient solution for the experiments containing the following compounds in 1 liter autoclaved Milli-Q water: \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \) (0.2 g/L), \( (\text{NH}_4)_2\text{HPO}_4 \) (0.5 g/L), Glycerin (3 ml/L), Peptone (5 g/L), Yeast Extract (0.5 g/L), \( \text{NaCl} \) (30 g/L), \( \text{H}_2\text{NaO}_4\text{P}_2\text{O}_7 \) (4.045 g/L), and \( \text{HK}_2\text{PO}_4 \) (2.79 g/L).

Growth medium has been prepared 10 times stronger, so all these compounds have been measured double in 2 liters of MQ water. In order to reach the wanted amount of substances in the media to run the assay, nutrient solution should be diluted with autoclaved MQ water, in a ratio of 1:9. The pH value was checked at the beginning of the experiment and was adjusted to 7.0 ± 0.2.

2.2. Preparation of bacterial stock culture

For preparing the stock culture, liquid-dried luminescent bacteria which is delivered by DRLANGE Company, were reactivated with a specific sterile solution which comes with the package. According to the instruction from the manufacturer, freeze reactivation solution was warmed up until to be melted, which it takes approximately 5 minutes. The luminescent bacteria were kept at room temperature for 2 minutes before reactivation, and then 0.5 ml of reactivation solution will be added to the bacteria. After 15 minutes, the rest of reactivation solution was mixed with the suspended luminescent bacteria. After reactivation, the whole bacteria suspension was added to 200ml of growth medium in 250ml E-flask and placed in a hood at 21±1°C in darkness. Re-inoculation of the bacteria should be done every day, at the same time.
In order to have fresh exponentially multiplying population, 200µL of the previous bacterial stock was re-inoculated daily with the freshly prepared nutrient solution, stirring on stir bar in a hood at 21±1°C in darkness.

To be able to start an experiment, the OD of the bacterial suspension should reach the value of 0.2 and this happened approximately 20 hours after the first inoculation. Sometimes the OD’s value was less than 0.2. In order to have fresh bacterial stock for the tests, 200µL of the previous bacterial stock should be re-inoculated daily with the new prepared nutrient solution. The pH of the solution was adjusted to 7.0 ± 0.2, during stirring it placed in a hood at 21±1°C in darkness. For pH adjustment, 700 µL of 5M NaOH was used.

2.3. Preparation of toxicant solutions

Table 2.3. The tested substances and their solvents.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper chloride</td>
<td>NaCl 3%</td>
</tr>
<tr>
<td>Triclosan</td>
<td>Methanol</td>
</tr>
<tr>
<td>LAS</td>
<td>MQ Water</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>MQ Water</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Methanol</td>
</tr>
<tr>
<td>ZnPT</td>
<td>DMSO</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>NaCl 3%</td>
</tr>
</tbody>
</table>

For preparing the test solution for copper chloride and zinc, specific amount of toxicant were dissolved in NaCl (3%) before starting the experiment.

Zinc pyrithione, has been solved in DMSO just because it is not soluble in any other usable solvent, DMSO concentration of 0.1% was used in all treatments. The DMSO with the same concentration was used in controls as well. DMSO is light sensitive, so all the flasks and vials used in this test, were covered by aluminum foil to avoid the light and stored at -20°C. To run this experiment, two dilution series have been made; first in DMSO and finally, dilution series prepared in NaCl (3%).
For water soluble substances such as Linear Alkylbenzene Sulfonate (LAS) and silver nitrate, stock solution has been prepared in MQ water. In order to reach the wanted amount of NaCl (3%) in the experiment, growth medium for these tests has been prepared with double amount of NaCl.

Stock solution of triclosan and ciprofloxacin were prepared in methanol and stored at -20°C. For preparing test solution for each independent experiment, specific amount of the stored toxicant has been dissolved in methanol, and then left it in the hood for the methanol to be evaporated. After evaporating, NaCl (3%) was added to the toxicant and placed on the shaker in darkness overnight.

### 2.4. Toxicity tests

In this study, independent single substances experiments for 7 selected compounds and also three different mixture toxicity tests have been tested. Three mixture experiments, where (1) a mixture of all seven compounds, (2) a mixture of copper, zinc & zinc pyrithione, and (3) a mixture of copper and zinc pyrithione were tested.

According to the standard protocol for tests with *Vibrio fischeri* (ISO11348-2), toxicant solutions were made in 3% NaCl. For all the experiments, 3 replicates of each concentration and 6 controls have been tested. To run the assay, 175 µL of the bacteria stock has been distributed to all the vessels. For the treatments, 175 µL of the prepared dilution series will be added into the correct vessels as well. In the controls, 175 µL of NaCl (3%) have been added. The tests were run for 8 hours on the shaker at 350 rpm in a hood at 21±1°C in darkness. Always, the OD at time 0 and after 8 hours has been measured.

To start mixture toxicity test, first of all toxicant solution for each compound was made. Then specific volume of each solution transferred into one flask. The mixture flask placed on the shaker during the night. The day after, dilution series for the mixture has been made in NaCl (3%).

In order to find concentration range of the mixture, parameters $a$ and $b$ are calculated by using NonLinear Regression (Nlreg) software. Then parameter $p$ (fraction) for each compound is calculated by using this formula:

$$P = \frac{EC50 \text{ of each compound}}{\text{Sum of EC50 of all the compounds}}$$
After mentioned steps, CA and IA for the mixture have been calculated with use of their mathematical formula which is explained earlier and then CA and IA curves predicted. The test concentration range is decided to be on the base of EC50 of the mixture. From the EC50 for the mixture, concentration of each compound was calculated.

For this experiment, 3 replicates for each treatment + 6 controls + 6 controls with DMSO have been tested. The reason to have controls with DMSO is that zinc pyrithione has been solved in DMSO, so DMSO in the controls should be tested as well.

The conditions like temperature, darkness, shaker and test long for the experiment were exactly the same as in the single species tests.

2.5. Endpoint

The endpoint of this study was bacterial growth rate by recording turbidity of the bacterial suspension. For evaluating the growth inhibition rate, Optical Density (OD) of the unexposed controls was compared with the exposed samples. When the Optical Density indicates higher value, it means higher biomass. Naturally, lower OD means lower biomass or higher growth inhibition rate. For Optical Density measurement, 96-well plates was used and they were placed in the spectrometer at $\lambda = 700$ nM wave length.

2.6. Data treatment

2.6.1. The growth inhibition rate calculation

In order to calculate the inhibition rates of the exposed treatments, OD values of the samples have been used in following equation:

$$\text{Relative inhibition of growth (\%) } = \frac{Bc - Bn}{Bc - B0} \times 100$$

Where $Bc$ stands for average of the ODs of the controls at the end of the test, $Bn$ is average of the ODs of each treatments and $B0$ is the initial OD of the controls at time 0.

Inhibition rates calculating should be done first and after that, the calculated values will be used to plot the inhibition rates versus tested concentration ranges for each test.
2.6.2. EC\_x values calculation

To determine the toxicity of a substance, several parameters such as EC1 and EC50 values rather can be used. Since the concentration-response curves are not symmetric in EC\_x values, it is needed to have a fitted curve. For fitting the curve, there are different models such as Probit, Weibull and logistic, but the Weibull model is the most widely used model (Backhaus 2008). Weibull equation is as follow:

\[ E(\text{conc}) = 1 - \exp(-\exp(a + b \times \log_{10}(\text{conc}))) \]

Where E(conc) is the effect from a certain concentration.

To calculate \( a \) and \( b \) parameters, software named Nonlinear Regression and curve fitting (available to download from webpage: http://www.nlreg.com/) have been used for the inhibition values. Then with using these parameters, the fitted curves were plotted in the excel sheets.

To prepare CA predictive curve, calculated EC\_x values and fraction of each substance were used. Then EC\_x mix was calculated which provided required data to plot the CA predictive curve.

To prepare IA predictive curve, molar amount of each compound in the mixture were calculated by using the predicted concentrations in CA and the fraction of each substance. Then, single substance effect which are needed for IA model, were calculated for each substance with using \( a \) and \( b \) values. Estimated mixture effects were then calculated, thus the IA predictive curve was plotted with using the estimated mixture effects versus mixture effect concentration.
3. Results and Discussion

Data on EC5, EC10, EC50 & EC95 values from Weibull fit model for each substance, also their parameters a & b are presented in Table 3.1.

Table 3.1. Parameters a & b and estimated toxicity of the tested substances to *Vibrio fischeri*

<table>
<thead>
<tr>
<th>Substances</th>
<th>EC5</th>
<th>EC10</th>
<th>EC50</th>
<th>EC95</th>
<th>Parameter a</th>
<th>Parameter b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper chloride</td>
<td>3.02E-04</td>
<td>1.37E-04</td>
<td>1.01E-04</td>
<td>5.60E-4</td>
<td>18.87</td>
<td>5.46</td>
</tr>
<tr>
<td>Triclosan</td>
<td>1.31E-05</td>
<td>9.71E-06</td>
<td>8.64E-06</td>
<td>1.60E-05</td>
<td>68.69</td>
<td>14.17</td>
</tr>
<tr>
<td>LAS</td>
<td>6.85E-05</td>
<td>8.30E-06</td>
<td>3.65E-06</td>
<td>2.56E-04</td>
<td>7.84</td>
<td>2.01</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>1.51E-07</td>
<td>5.87E-09</td>
<td>1.70E-09</td>
<td>1.87E-06</td>
<td>8.76</td>
<td>1.34</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1.07E-07</td>
<td>1.80E-10</td>
<td>1.56E-11</td>
<td>1.53E-05</td>
<td>4.37</td>
<td>0.68</td>
</tr>
<tr>
<td>ZnPT</td>
<td>1.02E-07</td>
<td>9.42E-09</td>
<td>3.78E-09</td>
<td>6.54E-07</td>
<td>12.34</td>
<td>1.82</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>6.75E-06</td>
<td>3.22E-11</td>
<td>2.98E-13</td>
<td>9.20E-02</td>
<td>1.46</td>
<td>0.35</td>
</tr>
</tbody>
</table>

3.1. Effect of single substances on *Vibrio fischeri*

The tested toxicants inhibited the growth of the *Vibrio fischeri*.

Different concentration ranges of the compound have been tested on the bacterium to predict a curve between 0 – 100% inhibition. Results are presented as typical concentration-response curve. As an example, different experiments of Triclosan will be shown in Figures to show how good or bad the test system performed.
Figure 3.1.1. Growth inhibition of Triclosan on Vibrio fischeri at 2013

Figure 3.1.2. Growth inhibition of Triclosan on Vibrio fischeri at 2013
The inhibition of triclosan on *Vibrio fischeri* from three independent experiments is shown in Figure 3.1.4.

Figure 3.1.3. Growth inhibition of Triclosan on *Vibrio fischeri* at 2013

Figure 3.1.4. Inhibition of triclosan on *Vibrio fischeri* from three independent experiments
The concentration-response curves of the 6 other tested substances are presented as below:

![Figure 3.1.5. Growth inhibition of Zinc pyrithione on *Vibrio fischeri*](image)

![Figure 3.1.6. Growth inhibition of Cipofloxacin on *Vibrio fischeri*](image)
Figure 3.1.7. Growth inhibition of Zn$^{2+}$ on *Vibrio fischeri*

![Graph showing growth inhibition of Zn$^{2+}$ on Vibrio fischeri](image)

Figure 3.1.8. Growth inhibition of Cu$^{2+}$ on *Vibrio fischeri*

![Graph showing growth inhibition of Cu$^{2+}$ on Vibrio fischeri](image)
Figure 3.1.9. Growth inhibition of LAS on *Vibrio fischeri*

Figure 3.1.10. Growth inhibition of Silver on *Vibrio fischeri*
Standard deviation of the controls in these experiments was usually less than 20%, but in some cases like ciprofloxacin and zinc, the controls variation was more than 20%.

In order to compare the toxicity in single substance exposure, the concentration-response curves are shown in the same figure (Fig 3.1.11).

![Figure 3.1.11. Growth inhibition of Vibrio fischeri caused by the 7 tested substances](image)

The toxicity of the individual substance is mode of action dependent. As a comparison in term of toxicity of different tested substances presented in Fig3.1.11 the concentration-response curves were different in steepness. Due to antibacterial characteristics of silver, zinc pyrithione and ciprofloxacin, they have shown inhibition effect on growth on *Vibrio fischeri* at a very low concentration (approximately 1E-10 mol/L), because they are made to kill bacteria. LAS, triclosan and copper all showed toxicity at high concentrations. As it presented in the figure above zinc has a very shallow curve, and the reason might be that zinc is an essential mineral so bacteria might benefit at low. But at higher concentrations zinc would have an inhibiting effect on growth on bacteria.
3.2. Effects of mixture toxicity on *Vibrio fischeri*

In this study, three different mixture scenarios have been tested on *Vibrio fischeri* to see effects of combination of different compounds in the coastal environment.

3.2.1. Mixture of all compounds

For the first mixture scenario, all tested compounds have been mixed together on the base of their EC50s. The predicted CA and IA curves are indicated in figure 3.2.1.1.

![Figure 3.2.1.1. Predicted mixture toxicity of CA and IA curves](image-url)
In order to compare the mixture toxicity tests with the predicted CA and IA curves, and to see if the results are reasonable and they follow the same pattern, all the data are presented in figure 3.2.1.2.

According to the data presented in Fig3.2.1.2 the results of these three mixtures experiments with all seven compounds, 1) CA and IA provide two predictions that are quite close to each other 2) The experimental results are between both predictions in the range of approximately 30% effect to 60% effect. Below 30% both predictions and the data overlap, above 60% the toxicity data are to the right of both predictions (i.e. the experimental toxicity is less than predicted. The reason might be presence of different toxicants from different groups with different mechanisms of actions in the mixture. Generally in the multi component mixtures, toxicity of compounds might change when they mix with the other substances, because they would interact in some way, so they could show more toxic effect or less. Bioavailability of different compounds could be another reason.
3.2.2. Mixture of Copper, Zinc & Zinc pyrithione

The second mixture scenario is the mixture of three compounds (Cu, Zn & ZnPT). The predicted CA and IA curves and the curves for these three tested compounds are shown in figure 3.2.2.1.

![Figure 3.2.2.1. Comparison of single substance tests of Cu, Zn & ZnPT and predicted CA and IA curves](image1)

In order to compare the mixture toxicity tests with the predicted CA and IA curves, all the data are presented in figure 3.2.2.2.

![Figure 3.2.2.2. Predicted CA and IA curves and observations for 2 mixture experiments](image2)
The results of the mixtures of copper, zinc oxide and zinc pyrithione showed in Fig. 3.2.2 suggested less toxicity than expected. The actual growth inhibition of the mixture on *Vibrio fischeri* occurred approximately at concentration 0.0001mol/L, while suspected concentration of inhibition was at 1E-16 mol/L. This result was actually opposite of the previous studies (Vivien et al. 2008), where binary mixture of ZnPT and Cu were shown to be more toxic than CA, while in the present study the observed toxicity of the mixture of Cu, Zn & ZnPT to *Vibrio fischeri* did not indicate synergistic effects. Generally, ZnPt is well known to be highly toxic to aquatic organisms, but, when combined with Zn and Cu, the whole mixture was less toxic than suspected. The reason might be presence of Zn in the mixture in the present study or it might just be different for the *Vibrio fischeri* assay.

### 3.2.3. Mixture of Copper and Zinc pyrithione

The last mixture scenario is from combination of copper and zinc pyrithione. The predicted CA and IA curves and the curves for these two tested compounds are shown in figure 3.2.3.1.

![Figure 3.2.3.1. Comparison of single substance tests of Cu & ZnPT and predicted CA and IA curves](image-url)
In order to compare the mixture toxicity test with the predicted CA and IA curves, the data are presented in figure 3.2.3.2.

![Figure 3.2.3.2. Predicted CA and IA curves and observations for the Cu & ZnPT mixture.](image)

Figure 3.2.3.2. Predicted CA and IA curves and observations for the Cu & ZnPT mixture.

The presented results of the binary mixture of copper and zinc pyrithione (Fig 3.2.3.2) indicate that inhibition effect occurred at a bit lower concentration than expected which means more toxicity than expected in the range of 30% inhibition or less. Generally, ZnPT and Cu have shown synergistic effects on *Vibrio fischeri* but ZnPT alone was found to be much more toxic than Cu alone. The binary mixture of ZnPT and Cu showed a very strong synergistic effect on *Vibrio fischeri* and the current result support that ZnPT is highly toxic to marine organisms. In the previous study, which was about synergistic effects of ZnPT and Cu to the three marine species (diatom *Thalassiosira pseudonana*, polychaete larvae *Hydroides elegans* and amphiod *Elasmopus eapax*), a combination of ZnPT and Cu showed a strong synergistic effect as well (Vivien et al. 2008). Vivien et al. also mentioned that all Cu added in this binary mixture was transchelated with ZnPT to form Cu-complexed organic compounds (presumably CuPT), so that all Cu was only found in the organic phase. As Vivien et al. (2008) demonstrated in their research, the synergistic effects of this binary mixture are partially attributable to the formation of CuPT, since CuPT has been shown to be more toxic than ZnPT to marine organisms. In contrast to the observations in marine animals, CuPT is less toxic to the algae *C. gracilis* and *S. costatum*, and the reason could be that Cu is as essential element for their growth.
3.3. Conclusions

According to the aim of this study, two questions have been posed. To answer to the first question (Is there any effect of these exposures to *Vibrio fisheri* and what can that tell us about the contamination levels - are those risky or not?) some calculations and comparisons on the basis of ration of EC10s have been done (Table 3.3.1).

Table 3.3.1. Ratio of EC10 of the compounds to the detected concentration.

<table>
<thead>
<tr>
<th>Substances</th>
<th>Instöränna</th>
<th>Fiskebäckskil</th>
<th>Port of Gothenburg</th>
<th>Lerkil</th>
<th>Stenungsund</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper sulfate</td>
<td>16685</td>
<td>7980</td>
<td>16685</td>
<td>21849</td>
<td>3746</td>
</tr>
<tr>
<td>Triclosan</td>
<td>1757</td>
<td>654</td>
<td>907</td>
<td>625</td>
<td>1562</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>120</td>
<td>117</td>
<td>147</td>
<td>138</td>
<td>102</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.40</td>
<td>0.32</td>
<td>0.10</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
</tr>
</tbody>
</table>

To calculate the ratio of the EC10 to the detected concentration, the value of EC10 of each compound has been divided by the real concentration in the environment. For the two compounds (LAS & ZnPT) that they did not detect in those specific areas, comparison of the ratio of EC10 has been done generally for the concentration in the aquatic environment. The ratio of EC10 for LAS in the aquatic environment is between 22 and 72 and for ZnPT is between 0.09 and 1.57. The real concentration of most of the tested compounds are much lower than concentration that effects starts, so there should be no adverse effect on *Vibrio fisheri*, except for ciprofloxacin for which a 19% effect is expected to take place at the reported concentration.

Regarding the second posed question (Are the effects of those dissimilarly acting compounds predictable according to IA or CA?), the EC50 that is predicted by CA and IA is compared with the EC50 that is actually observed (Table 3.3.2).

Table 3.3.2. Comparison between predicted EC50 and observed EC50.

<table>
<thead>
<tr>
<th>Substances</th>
<th>Predicted EC50</th>
<th>Observed EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper sulfate</td>
<td>1.00E-05</td>
<td>3.02E-04</td>
</tr>
<tr>
<td>Triclosan</td>
<td>1.00E-05</td>
<td>1.31E-05</td>
</tr>
<tr>
<td>LAS</td>
<td>1.00E-05</td>
<td>6.85E-05</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>1.00E-05</td>
<td>1.51E-07</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1.00E-05</td>
<td>1.07E-07</td>
</tr>
<tr>
<td>ZnPT</td>
<td>1.00E-05</td>
<td>1.02E-07</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>1.00E-05</td>
<td>6.75E-06</td>
</tr>
</tbody>
</table>
As a comparison in term of toxicity of mixture of all 7 compounds and predicted CA and IA curve, all the curves are shown in one figure (Fig3.3.3). In this figure, the curve of the mixture of all 7 compounds was in the middle of CA and IA curves.

In order to compare toxicity of mixture of Cu, Zn & ZnPT and predicted CA and IA curve, all the curves are presented in figure 3.3.4. As shown in the figure, a mixture of these compounds indicated less toxicity to *Vibrio fischeri* than both the CA and IA predictions. This result is unexpected in view of the literature data, so this is something for future work.
In order to compare toxicity of binary mixture of Cu & ZnPT and predicted CA and IA curve, all the curves are presented in figure 3.3.5. As it shows in the figure, the binary mixture curve is in the middle CA and IA curves.

![Figure 3.3.5. Comparison of single substances tests of 7 compounds and mixture curves](image)

The *Vibrio fischeri* toxicity test is a powerful assay to evaluate the effect of chemicals in single and mixed exposure, which can be prolonged to follow the effects. The comparisons indicate that CA and IA can both be used for predicting the mixture toxicity of the compounds and it does not really matter which model we choose.
Acknowledgements

I take this opportunity to express my profound gratitude and deep regards to my supervisor Professor Thomas Backhaus and co-supervisor Dr. Åsa Arrhenius for their cordial support, valuable information and guidance throughout the course of this thesis.
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