ECOTOXICOLOGICAL ASSESSMENT USING SIX PHARMACEUTICALS COMMONLY FOUND IN WASTEWATERS

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Paula Taipale & Sisko Ursin

Lahti University of Applied Sciences
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ABSTRACT

Pharmaceutical residues in wastewaters are relatively new concern in environment protection. The usage of pharmaceuticals has increased a lot and their impact on the environment is unknown. Low levels of pharmaceuticals have been detected in many countries in sewage treatment plant effluents, surface waters, seawaters, groundwater and some drinking waters. Pharmaceutically-active compounds until recently have not been studied however even small residues of pharmaceuticals can have an effect on micro-organisms.

The main focus of this thesis is the ecotoxicological evaluation of selected pharmaceuticals using three different organisms (*Artemia salina*, *Daphnia magna* and *Pseudomonas fluorescens)*. The effect of six different pharmaceuticals were studied during a two months period. The test organisms were exposed to different concentrations of pharmaceuticals and observed for up to 48 hours. The selected pharmaceuticals were atenolol, diclofenac sodium salt, erythromycin hydrate, lidocaine, sodium diatrizoate hydrate and sulfamethoxazole. These were chosen from the key pharmaceuticals list of the on-going EU Pills project.

According to the results of this study, the most lethal pharmaceuticals were diclofenac sodium salt and lidocaine. With diclofenac sodium salt all three organisms reacted at all tested concentrations (100,1; 200,2 and 300,3 mg/l). For lidocaine only higher concentrations (200,2 and 300,3 mg/l) had an significant impact on the organisms. *Daphnia magna* was the most sensitive organism compared to other used and it was affected by all the pharmaceuticals.

Keywords: ecotoxicology, pharmaceuticals, Pills

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TIIVISTELMÄ

Lääkeainejäämät vesistöissä ovat suhteellisen uusi huolenaihe ympäristösuojelussa. Lääkkeiden käyttö on lisääntynyt, ja niiden vaikutuksia ympäristöön ei tiedetä. Monissa maissa on havaittu pieniä määriä lääkeainejäämiä jätevedenpuhdistamoilla, pintavesissä, merivedessä, pohjavesissä sekä juomavedessä. Lääkeaineita ja niiden vaikutuksia ei ole tutkittu riittävästi, sillä jopa pienillä lääkeainemäärillä voi olla vaikutus mikro-organismeihin.

Tämän opinnäytetyön tarkoituksena oli toteuttaa ekotoksikologisia kokeita valituilla lääkeaineilla käyttäen kolmea eri organismia (*Artemia salina*, *Daphnia magna* ja *Pseudomonas fluorescens*). Kokeissa käytettiin kuutta lääkeainetta ja koejärjestelyt kestivät kahden kuukauden ajan. Tutkitut organismit altistettiin erilaisille lääkeainepitoisuuksille ja niitä tarkkailtiin 48 tunnin ajan. Tutkimuksessa käytetyt lääkeaineet olivat atenolol, diclofenac sodium salt, erythromycin hydrate, lidocaine, sodium diatrizoate hydrate ja sulfamethoxazole. Nämä valittiin Pills – projektissa määritettävien lääkeaineiden listalta.

Saatujen tulosten perusteella diclofenac sodium salt ja lidocaine todettiin tappavimmiksi. Kaikki kolme organismia reagoivat diclofenac sodium salt:n tutkittuihin pitoisuuksiin (100,1; 200,2 ja 300,3 mg/l). Ainoastaan lidocainen suuremmilla pitoisuuksilla (200,2 ja 300,3 mg/l) oli merkittävä vaikutus organismeihin. *Daphnia magna* osoittautui kaikkein herkimmäksi verrattuna muihin kokeissa mukana olleisiin organismeihin, ja se reagoi kaikkiin lääkeiaineisiin.

Avainsanat: ekotoksikologia, lääkeaineet, Pills

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DICTIONARY / SANASTO

AAS = Atomic absorption spectrometer / Atomiabsorptiospektrometri

BOD = Biological oxygen demand / Biologinen hapenkulutus

COD = Chemical oxygen demand / Kemiallinen hapenkulutus

Pills = Phamaceutical Input and Elimination from Local Sources

SPE = Solid phase extraction

HPLC = High-performance liquid chromatography

1 INTRODUCTION

Pharmaceutical residues in wastewaters are a relatively new concern in environment protection. The pharmaceuticals effects not only the flora and fauna in water but also the entire food web and could eventually effect people. More than 100 personal care products and pharmaceuticals can be found in water after wastewater treatment and some of these can be harmful even in small concentrations (Fernández, Gonzáles-Doncel, Pro, Carbonell & Tarazona 2009; Kronberg 2010). Pharmaceutically-active compounds in water have not been studied or their environmental fate understood. Limited number of antibiotics are the exception because there are some studies about their fate in nature. (Bendz, Paxéus, Ginn & Loge 2005.)

Pills is an EU funded project which takes place in six countries in Europe. Germany, The Netherlands, Luxembourgh, Switzerland, The United Kingdom and France are involved. The project started at September 2007 and it runs until December 2012. The project is not unique studying pharmaceuticals in wastewaters but Pills is the only project where science and operators work closely together and where possible treatment techniques are tested on full-scale treatment plants which are operating under real conditions. All in all the main aim of the project is to study and learn about pharmaceuticals in wastewaters and increase knowledge among people. (Pills 2011.)

This report describes our contribution to Pills project during our practical training period in Glasgow in Spring 2011. During the period we did sampling in the field and basic analysis in the laboratory for the collected samples. From that part we have included results from the chemical characterisation of the wastewater since this consisted a significant part of our routine practical work. These results are presented in appendices 1 and 2 but not discussed. Our own final thesis project consisted of ecotoxicological test using two eucaryotic (*Artemia salina, Daphnia magna*) and one procaryotic organism (*Pseudomonas fluorescens*) and six pharmaceuticals in different concentrations. The chosen pharmacuticals are commonly found from waste waters and have been chosen to be monitoried during

the Pills -project (APPENDIX 3). In this report we present and discuss the results of our tests.

2 PILLS – EU PROJECT

The usage of pharmaceuticals has increased a lot and their impact to the environment is unknown (Fent, Weston & Caminada 2005). There is no standard means of treating wastewater with pharmaceuticals so that chemical compounds do not end up in the environment (Ferraria, Paxéusb, Lo Giudicec, Pollioc & Garric 2003). Low levels of pharmaceuticals have been detected in many countries in sewage treatment plant effluents, surface waters, seawaters, groundwater and some drinking waters (Fent, Weston & Caminada 2005).

Nowadays testing methods allows us to determine many pharmaceutical residues in very low concentrations, for example a thousandth of a gram can be measured. It is important to be aware of pharmaceutical residues even in small amounts since in bigger amounts they could be causing a problem. However, even small residues of pharmaceuticals can have effect to micro-organisms. (Pills 2010.)

There is generally two methods for evaluating pharmaceuticals, firstly by using sensitive chemical detection equipment such as HPLC, this is both expensive and relies on obtaining purified standards to act as controls. The second method utilizes the response of indicator organisms to the overall effect of the material(s) in solution. This approach has the drawback that a range or organisms may be need to fully quantify the effect. As there is no one optimum method both will be used during the Pills project. (Hunter 2011b.)

2.1 Pills objectives

The partners in the project have different tasks and work packages. Work package one is characterization of the pharmaceutically burdened wastewater. This work package is lead by The United Kingdom (Glasgow Caledonian University). (Pills 2010.)

The amount of pharmaceuticals in wastewater is researched during this work package of the project, especially at so called hot spots where pharmaceuticals are

heavily used but their use is closely monitored. These places are for example at hospitals and care homes. Fortunately there is a lot of co-operation between the different hospitals and research groups. (Pills 2011.)

The first steps where pharmaceuticals enter into the wastewater is the manufacturing processa and human consumption. At that stage the pharmaceuticals may be used but not all of them are absorbed by the body and they pass into the wastestream by natural processes. After that pharmaceuticals are eventually reach the wastewater treatment plant. These plants are designed to remove biodegradable substances and nutrients, however the pharmaceuticals can pass through them to surface waters. Pharmaceuticals are used also in veterinary medicine. In these cases pharmaceutical residues pass into surface water through the liquid manure. (Pills 2010.)

The Pills -project concentrates on pharmaceutical residues which come from human use. These are found especially from wastewater that comes from hospitals and care homes (PICTURE 1). One target is to research different ways to treat pharmaceutical residues in wastewaters and if it is worth while to treat them in situ at hospitals and care homes. Additionally the awareness about pharmaceuticals and environment is expanded. (Pills 2010.)

PICTURE 1. The Pills focus (Pills 2011).

3 SAMPLING

Samples were collected every week on Monday's from Glasgow and on Wednesday's from Melrose. Samples were collected in large jars which could hold about three litres of liquid when they were full. Reaching the sampling locations occurred by rented car and for safety reasons there always had to be at least two people to collect the samples. (Helwig 2011.)

3.1 Sampling places

There were six different sites in Scotland where the samples were taken. Three of them were in Glasgow and three in Melrose which is located about 120 kilometers from Glasgow. Both in Glasgow and Melrose, there was a hospital, a care home and a sewage water works involved. Samples were taken every week from hospitals and care homes and once a month from the wastewater treatment plants. (Helwig 2011.)

3.1.1 Glasgow sampling sites

Drumchapel Hospital is located in the west side of the city about nine kilometers from the center. There are 120 beds and the hospital provides stroke, general and ortho-geriatric rehabilitation services for older patients, including a day hospital. The three most commonly used pharmaceuticals in this hospital are paracetamol (painkiller), amoxicillin (mainly used to treat bacterial infections) and flucloxacillin (antibiotic). (Drumchapel 2009; Drumchapel Hospital 2011; Paracetamol 2011; Amoxicillin 2011; Flucloxacillin 2011.)

Western Infirmary is also located in the west side of the city about three kilometers from the center. The hospital provides most of the acute emergency and receiving functions serving this side of the city - accident and emergency, intensive care, orthopaedic trauma, emergency surgery, acute medicine and acute stroke. In addition, the hospital provides elective gastrointestinal, breast and cardiothoracic surgery. Medical specialties include cardiology, general medicine and renal medicine. (Western Infirmary 2011.)

Shieldhall wastewater treatment work is located in the west side of the city about 11 kilometers from the center and it is owned by Scottish Water. The sewage works originally opened in 1910 and were rebuilt in 1980. It is one of three such facilities in Glasgow, along with Dalmarnock and Daldowie. Shieldhall has a maximum treatment capacity of 574 000 population equivalents and it serves about 400 000 people. Shieldhall wastewater treatment work is considered a large one by Scottish standard. (Shieldhall 2011; Sampling places 2011.)

3.1.2 Melrose sampling sites

The Borders General Hospital is located in the west side of the town about one and half kilometers from the center. There are 300 beds and 63 767 m^3 of water was used in October 2009. This sampling site takes combined effluent from the entire hospital complex including the Main Hospital, Melburn Lodge, Huntlyburn House, Creche and all other services. The three most commonly used pharmaceuticals in

this hospital are paracetamol, amoxicillin and ibuprofen (a non-steroidal anti-inflammatory drug). (BGH 2009; Melburn 2011; Sampling places 2011; Ibuprofen 2011.) At the Borders General Hospital there is also a laundry which takes care of the hospitals washing. Thus wastewaters pharmaceutical compounds are diluted by, for example, water from the laundry and that is why samples are also taken from one of the geriatric unit as well.

Melburn Lodge is residential geriatric unit and it is located in the grounds of the Borders General Hospital. There are 16 beds which usage was approximately 86 % in October 2009. The water usage was 857 m^3 in October 2009 but the sampling station samples combined effluent from another care facility called Huntlyburn House which is a residential psychiatric unit. There are 26 beds which usage was 80 % and the usage of water was 2 430 m^3 in October 2009. The three most commonly used pharmaceuticals in Melburn Lodge are paracetamol, metformin (an anti-diabetic drug) and cephalexin (cephalosporin antibiotic). (Melburn Lodge 2009; Melburn 2011; Sampling places 2011; Cefalexin 2011; Metformin 2011.)

Galashiels wastewater treatment work is located to the west of the town about six and half kilometers from the center and it is owned by Scottish Water. Galashiels has a capacity of 25 000 population equivalents and it serves about 14 197 people. Galashiels wastewater treatment work is a medium size unit by Scottish standard. (Sampling places 2011.)

3.2 Samplers

There were two different kinds of static samplers used at the four locations and one mobile sampler that was used at the wastewater treatment works. The sampler that was used in places where it could be connected to mains current was the S320H (PICTURE 2). In these locations, samples were collected into glass jars held within the sampler. Jars were placed in rack and collecting pipe rotates automatically when sampler fills the jars. The sampler was connected to a flow meter (Q-Eye) that gave a signal to sampler to take a sample when certain amount

of water had passed through the meter. Then the sampler started to take a sample through a sampling pipe and it is collected into the jars. (Helwig 2011.)

PICTURE 2. Sampler S320H.

After one week, the jars were collected from the sampler. The rack was pulled out of the sampler and then lids were placed onto the jars. Then the full jars were put into coolboxes and delivered to the laboratory. Fresh jars were installed onto the sampler for the following weeks sample. (Helwig 2011.)

In General Borders Hospital the sampler S320H worked in a different fashion. Instead of flow meter there was a sensor that gave a signal to the sampler when a certain water level was reached in the sampling well. Then the sampler started to collect the sample and water level dropped. When the water level was reached again the sampler took another sample. (Helwig 2011.)

When there was no possibility to connect the sampler in mains current a P2 sampler (PICTURE 3) was used. The P2 was also connected to Q-Eye flow meter

but the flow meter was working with batteries. The P2 collected samples into a metal bucket that was placed within cooling elements inside the sampler. This sampler collected samples in a same manner as the S320H but the metal bucket only held a maximum of 3 000 ml in a week. That was why the flow meter was adjusted to take samples of approximately 100 ml every four hours so that bucket did not overflow. After a one week, the sample was poured out of the bucket into a glass jar and delivered to the laboratory. (Helwig 2011.) Sampler P2 and collecting the sample from it is shown in picture 3.

PICTURE 3. Sampler P2.

The P2 was also used as a mobile sampler when gathering samples from wastewater treatment works. About once a month a mobile sampler was taken to Galashiels and Shieldhall wastewater treatment works. When placing the sampler the pipe was lowered down a sampling well and then the sampler collected the samples during a one week in a similar fashion to that described previously. After that sample was collected to a glass jar and the whole sampler was taken to the laboratory. (Helwig 2011.)

3.3 Sampling problems

The problems with sampling could be roughly divided to two types: human mistakes and equipment errors. Almost all the problems were related to the samplers. Also blockages in the sampling drains were quite common problems. Common human mistakes with the samplers were forgetting to start the sampler

again after taking the samples out, not connecting all the needed cables or just simply not changing the batteries. There was unique equipment failure at Drumchapel Hospital with the flow meter and the sampler. The flow meter was sending a signal to the sampler but the sampler did not get the signal for some reason and this happened several times. (Helwig 2011.)

4 SAMPLE TREATMENT

Attempts were made to treat the samples on the same or the next day that they had been collected. The fast handling was important for obtaining the best results. For example COD and BOD values change during a long storing. After the samples were transported to the laboratory they were kept in a fridge. (Pelda 2011.)

Different amounts of water were taken from the sample during the filtration process for various analyses. Briefly total solids analyse was made using aluminium tray which had been weighted. A chlorine tablet was added to the 200 ml of the water and left for an hour before putting it into an oven at 104°C for 48 hours then a second oven at 450°C for 24 hours. The BOD sample (160 ml) was poured into an amber BOD bottle where was also a magnetic stirrer and a little rubber basket which contained lithium hydroxide. Then the BOD bottle was installed into a machine (Biotrak) that measured the variation of pressure in the bottle over 5 days. Suspended solids analyse sample (50 ml) was filtrated through a clean 0,45 µm filter after the filter had been weighted. Then the filter was put to oven for 48 hours and weighted after 24 and 48 hours. (Pelda 2011.)

All the results including total and suspended solids, wet chemistry and BOD are shown in appendix 1.

4.1 Filtration – pretreatment of water samples

If there were two or more bottles from the same location they were combined into one. At this stage of the unfiltered water was taken for determining the total solids weight of the sample. Then the rest of the sample was filtrated through 100 µm

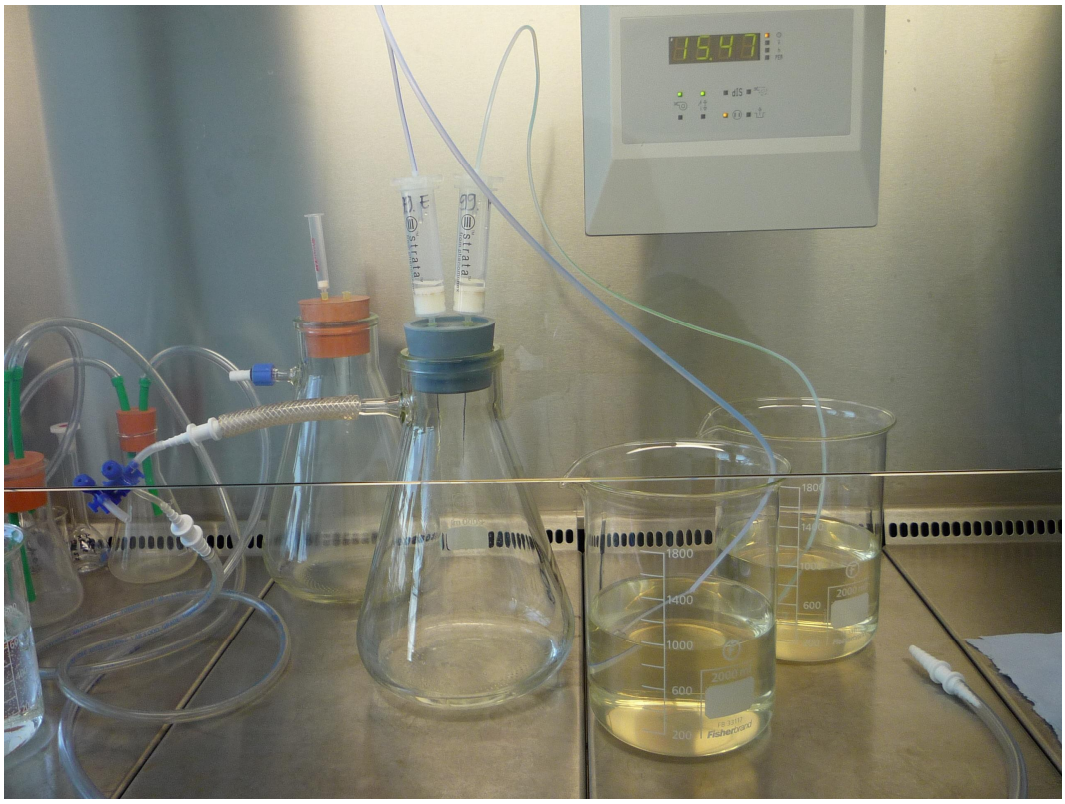
filter. At this point some of the water (as detailed above) was taken for BOD determination, suspended solids weight analysis and for the wet chemistry. (Pelda 2011.)

After this the water was centrifuged if needed and filtered through a 1,6 µm filter and again through a 0,7 µm filter. Usually these ran through quite fast unless the sample was very thick. The next step was to filter the water through a 0,45 µm filter. At this point water was taken for element analysis using the AAS and for total organic carbon determination. (Pelda 2011.) The whole filtration equipment is shown in picture 4.

PICTURE 4. Filtration equipment.

The pH of the filtered water was adjusted to pH 2 using 0,5 M HCl. Once the pH had settled to two, water was divided into two beakers each containing one litre. At this last stage pharmaceuticals were extracted from the water using two SPE syringes (PICTURE 5). One SPE syringe was used to quantify the different

pharmaceuticals by HCLC and the other used for ecotoxicological tests. (Pelda 2011.)



PICTURE 5. SPE syringes.

4.2 Wet chemistry

Wet chemistry tests were made with Palintest® Tubetests®. Three replicates were done for every sample to exclude casual errors. Photometer 7100 was used for reading all Tubetests® analyses results. It reads the results from tubes and automatically calculates the results in concentration units. Digital Reactor Block (DRB200) by HACH called was used to digest the samples which demanded digesting during the test. It was used to process COD, Total Phosphorous and Total Nitrogen sample. (Hach 2011; Effluent and Wastewater Testing 2011; Photometer 7100 2011.)

One of the biggest potential errors when making the Tubetests® was using wrong procedure for wrong analyse. Another possibility was to use too much or too small amount of sample in certain analyse and this could have an effect to the results. For example if twice as much sample as needed was used the results are bigger than the reality. Wrong reagents could also be used but this was very unlikely when different Tubetests® were kept in their own boxes.

4.2.1 Aluminium

10 ml of sample was added to test tubes by using a pipettor. First one Aluminium No 1 tablet was added to each tube and they had to be crushed and mixed well into the water. Then one Aluminium No 2 tablet was added and also crushed and mixed gently to dissolve. At this point vigorous mixing had to be avoided because the contents could foam over the tubes. After the tablets had been added and dissolved the tubes stood for five minutes to allow full colour development. For reading the results deionized water was used as blank. (Aluminium.)

4.2.2 Ammonia

1,0 ml of sample was added to test tubes by using a pipettor. The caps were replaced and samples inverted three times to mix. 0,5 ml of Ammonia (Nessler) Reagent was added and once again the caps were replaced and samples inverted several times to mix. After this the tubes stood for one minute to allow full colour development. For reading the results an unused Ammonia tube or deionized water was used as blank. (Ammonia/50N (Nessler) 2007.)

4.2.3 COD

The tubetests heater was turned on before preparing the samples. The control was setted up for 150 °C and 120 minutes. Sample tube was shaken vigorously to suspend all sediment from the tube. 2,0 ml of sample was added to test tubes by using a pipettor. The caps were replaced and tubes inverted gently to mix contents and at this point the tubes became very hot. Blank tube was prepared the same way

as the sample tubes but the only difference was that 2,0 ml of deionized water was used instead of sample. After these preparations the tubes were taken to the heater. 120 minutes later the tubes were taken out from the heater. Tubes were inverted gently a couple of times to mix and then allowed to cool to room temperature before reading the results. (Chemical oxygen demand – COD/2000 2008.)

4.2.4 Nitrate

1,0 ml of sample was added to test tubes by using a pipettor. The sample was added slowly without disturbing the contents of the tube and it was forbidden to shake the tube at that point to ensure the best result. One level scoop of Nitrate Powder using Size 1 dosing scoop was added. The caps were replaced and tubes inverted gently five or six times to mix contents and at this point the tubes became very hot. After this the tubes stood for five minutes to allow full colour development. For reading the results an unused Nitrate tube was used as the blank. (Nitrate/30N 2003.)

4.2.5 Total nitrogen

The tubetests heater was turned on before preparing the samples. The heater was setted up for 105 °C and 30 minutes. Three level scoops of Total Nitrogen Reagent No 1 using Size 1 dosing scoop was added to the tubes. 5,0 ml of sample was added to test tubes by using a pipettor. The caps were replaced and tubes shaked vigorously for 30 seconds to mix contents. After these preparations the tubes were taken to the heater and 30 minutes later the tubes were taken out. The tubes were allowed to cool to room temperature before adding one level scoop of Total Nitrogen Reagent No 2 using Size 4 dosing scoop. The caps were once again replaced and tubes shaked for 15 seconds to mix contents and then stood for three minutes. (Total nitrogen/30 2003.)

1,0 ml of digested sample from Total Nitrogen tube was transferred by using a pipettor to a Nitrate tube. The sample was added slowly without disturbing the contents of the tube and it was forbidden to shake the tube at this point to ensure

the best result. One level scoop of Nitrate Powder using Size 1 dosing scoop was added. The caps were replaced and tubes inverted gently ten times to mix contents and at this point the tubes became very hot. After this the tubes stood for five minutes to allow full colour development. For reading the results an unused Nitrate tube was used as a blank. (Total nitrogen/30 2003.)

4.2.6 Phosphate

2,0 ml of sample was added to test tubes by using a pipettor. First one Phos No 1 tablet was added to each tube and they had to be crushed and mixed completely to dissolve. Then one Phos No 2 tablet was added and also crushed and mixed to dissolve. The caps were replaced and tubes inverted gently several times to mix contents. After this the tubes stood for ten minutes to allow full colour development. For reading the results an unused Phosphate tube or deionized water was used as blank. (Phosphate/12P 2003.)

4.2.7 Total phosphorus

The tubetests heater was turned on before preparing the samples. The heater was setted up for 105 °C and 60 minutes. 2,0 ml of sample was added to test tubes by using a pipettor and then two Digest Ox tablets were added to each tube, crushed and mixed well to dissolve. The caps were replaced and tubes inverted gently to mix contents. After these preparations the tubes were taken to the heater and 60 minutes later the tubes were taken out. Tubes were allowed to cool to room temperature before adding 2,0 ml of PhosNeut Solution by using a pipettor. After that one Phos No 1 tablet was added to each tube, crushed and mixed completely with the water. Then one Phos No 2 tablet was added and also crushed and mixed to dissolve. The caps were replaced and tubes inverted gently several times to mix contents. After this the tubes stood for ten minutes to allow full colour development. For reading the results an unused Total Phosphorus tube or deionized water was used as blank. (Total phosphorus/12 2003.)

4.3 Cadmium, lead and zinc

Besides the Palintest® Tubetests® we did other analyses for the samples to determine the concentration for some elements and total organic carbon. Elements we were looking for were cadmium, lead and zinc. These have been observed in drinking water and that was why they were measured before the possible purification. Lead is used in old piping and it can damage nervous system when present in large amounts. Cadmium and zinc are used in paints and metal industry.

Before cadmium, lead and zinc could be determined the sample had to be filtered through a 0,22 µm filter. Then it could be fed into a AAS (PICTURE 6) which breaks up the compounds in a sample to atoms using heat. After that light is passed thorough the sample and the device detects how much light the sample absorbs. (Atomiabsorptiospektrometri 2011.)

PICTURE 6. Atomic absorption spectrometer.

There is a different lamp for different metals. Each element absorbs at certain wavelength and when the lamp is changed in the AAS various metals can be measured. AAS does not work for halogens or nonmetals and samples must be in liquid form before fed to AAS. (Atomiabsorptiospektrometri 2011.)

All the results from analyzing cadmium, lead and zinc are shown in appendix 2.

5 ECOTOXICOLOGICAL TESTS

Ecotoxicological data is available for less than 1 % of pharmaceuticals in the open peer-reviewed literature and ecotoxicological databases and only a small number of new pharmaceuticals have been subjected to a complete risk assessment, including a battery of appropriate ecotoxicological tests in the EU. (Sanderson, Brain, Johnson, Wilson & Solomon 2004.)

5.1 Pharmaceuticals used in the tests

Atenolol is used to treat abnormally rapid heart rhythms. These kinds of drugs are called beta-adrenergic agent because they block sympathetic nervous system to make the heart to beat more rapidly. It also lowers blood pressure by reducing the force of contraction of heart muscle. Atenolol also eases chest pain and can be used to treat heart attack. Side effects are rare but there can be for example insomnia, nausea, diarrhea, abdominal cramps and fever. (Atenolol 2011.)

Bezafibrate it used to treat high cholesterol levels along with a diet and an exercise program. It can have serious side effects when combined with other drugs such as muscle injury. Other side effect of using this drug is stomach upset, stomach pain, gas or nausea. These may occur in first days after starting to use the drug. Another side effects are itchy skin, redness, headache and dizziness. (Bezafibrate 2011.)

Diclofenac sodium salt is non-steroidal anti-inflammatory drug which are used to treat pain. These drugs have an effect that lowers the action of enzymes and as a result inflammation, pain and fever are reduced. Other non-steroidal anti-inflammatory drugs are for example ibuprofen and naproxen. Common side effects for using diclofenac are ulcerations, abdominal burning, pain, cramping, nausea, gastritis, serious gastrointestinal bleeding and liver toxicity. (Diclofenac sodium salt 2011.)

Erythromycin hydrate is a macrolide antibiotic that is used to treat for example upper / lower respiratory tract infections, skin infections, acute pelvic inflammatory disease and erythrasma. These infections are caused by bacteria. Erythromycin hydrate does not effect on human cells but it prevents bacterial cells to multiply and grow. Side effects such as nausea, vomiting, loss of appetite, diarrhea and abdominal pain are usually dose-related which means that they are more common with higher dose. (Erythromycin 2011.)

Ibuprofen is non-steroidal anti-inflammatory drug and it is used to treat pain, fever and inflammation. It works in same fashion as diclofenac sodium salt and it can inhibit the blood pressure drugs effect. The most common side effects for this drug are rash, ringing in the ears, headaches, dizziness, abdominal pain, nausea, diarrhea, constipation and heartburn. (Ibuprofen 2011.)

Lidocaine is anesthetic drug which is usually taken as an injection. All the vital signs such as cardiovascular and respiratory should be carefully and constantly monitored after anesthetic injection. (Drugs.com 2011.) Lidocaine is used for relieve itching, burning and pain from skin inflammations. It is also injected as a dental anesthetic. (Lidocaine 2011.)

Sodium diatrizoate hydrate is an X-ray contrast that is taken by mouth usually 15 to 30 min before test. It helps to view patients throat, stomach and intestines more clearly. There may occur nausea, vomiting, diarrhea, stomach cramps, rash, itching or heartburns as side effects. (Sodium diatrizoate hydrate 2011.)

Sulfamethoxazole is almost outdated anti-bacterial drug. It is mostly used in combination with other drugs such as trimethoprim. Earlier it was useful antibiotic but bacteria have developed resistance to its effects. Sulfamethoxazole suppress the formation of dihydrofolic acid that is vital for bacteria. It can cause dizziness, headache, lethargy, diarrhea, norexia, nausea, vomiting and rash as side effects. (Sulfamethoxazole 2011.)

For the ecotoxicological analyses we prepared pharmaceutical stock solutions which contained approximately 0,1 g of the pharmaceutical dissolved to 10 ml of 99,8 % methanol. The accurate amount and concentration of each pharmaceutical is shown at table 1. These stocks were easy to dilute to water or saltwater and use for testing the toxicity of each pharmaceutical.

TABLE 1. The amount of pharmaceuticals in the stocks (made 28.2.2011).

Pharmaceutical	**g**	**g/l**
Atenolol	0.1001	10,01
Bezafibrate	0.1003	10,03
Diclofenac sodium salt	0.1001	10,01
Erythromycin hydrate	0.1015	10,15
Ibuprofen	0.1004	10,04
Lidocaine	0.1001	10,01
Sodium diatrizoate hydrate	0.1010	10,10
Sulfamethoxazole	0.1009	10,09

To test the toxicity of pharmaceuticals we used *Artemia salina*, *Daphnia magna* and *Pseudomonas fluorescens*. We made three dilutions of the pharmaceutical stocks into deionised water so that the test solution contained approximately 100, 200 or 300 mg/l pharmaceutical. Unfortunately, at this point we had to discard bezafibrate and ibuprofen because they precipitated out of solution after dilution.

We needed to make new stock solutions of the pharmaceuticals for the bacteria tests. The accurate amount and concentration of each pharmaceutical is shown at table 2.

TABLE 2. The amount of pharmaceuticals in the stocks (made 15.4.2011).

Pharmaceutical	**g**	**g/l**
Atenolol	0,1003	10,03
Diclofenac sodium salt	0,1010	10,10
Erythromycin hydrate	0,1006	10,06
Lidocaine	0,1002	10,02
Sodium diatrizoate hydrate	0,1004	10,04
Sulfamethoxazole	0,1005	10,05

5.2 *Artemia salina*

Artemia salina or brine shrimp belongs to phylum of Arthropoda, subphylum of Crustacea, in family of Artemiidae and genus of Artemia. They live in different places in the world in saltwater lakes but not in oceans. Their biological life-cycle is about one year and their mature length is approximately one centimeter. (Brine shrimp 2011.)

Artemia salina eggs are known from their cryptobiosis which means that they can survive through very rough conditions and still maintain their viability. For example *Artemia salina* eggs can survive in dry desert for even ten years and when they are put to seawater they hatch and start a new life-cycle. (Fossweb 2009.)

Artemia salina reaches maturity in three to six weeks. Female *Artemia salina* generate live offspring initially, after that it lays eggs following mating with males. Young *Artemia salina* develops fast in beneficial conditions. (Fossweb 2009.)

Artemia salina is mainly used as fish food. Both eggs and live shrimps are good and nutritious food for tank fish. *Artemia salina* is also used in biological and ecotoxicological studies because they are easy to grow and maintain. (Fossweb 2009.)

5.2.1 Growing the cultures

20 g of sea salt was weighted and added to 500 ml of deionized water (Sea salts 2011) and mixed together in a conical flask. The liquid was stirred as long as needed to get all the salt to dissolved. After that 2,0 ml of *Artemia salina* eggs (Waterlife Research Ind. Ltd.) were added to the sea salt water. The inoculated conical flask was placed close to a light source and air was bubbled through the solution via an air tube. The eggs hatched in two to three days and were ready to be used in tests.

After hatching some of the *Artemia salina* were grown to adulthood by using the same sea salt water broth as mentioned before. At this time 10 g of sea salt was weighted and added to 250 ml of deionized water, to that 10 ml of the hatched *Artemia salina* were added to the water (Sea salts 2011).

We tried three different feeding methods in an attempt to grow the *Artemia salina* to adulthood as you can see from picture 7. In first flask we fed the *Artemia salina* daily with 0,5 ml of yeast solution. It was made by weighting 0,1 g Allison Dried Active Yeast to 1 l of deionized water. This was not successful because almost all the *Artemia salina* died during the first day and after two days all of them had died. In second flask we fed the *Artemia salina* daily with five drops (about 0,5 ml) of brine shrimp food (NT Laboratories LTD UK). This was not good either because after three days all the *Artemia salina* died. In third flask we fed the *Artemia salina* daily with 0,5 ml of vegetable mixture (consisting of 80 g peas, 20 g carrots, 300 ml water blended to a puree) and this turned out to be the best way of feeding them. (Hunter 2011c). The *Artemia salina* seemed active and healthy and was growing great.

PICTURE 7. Different growing methods for *Artemia salina*.

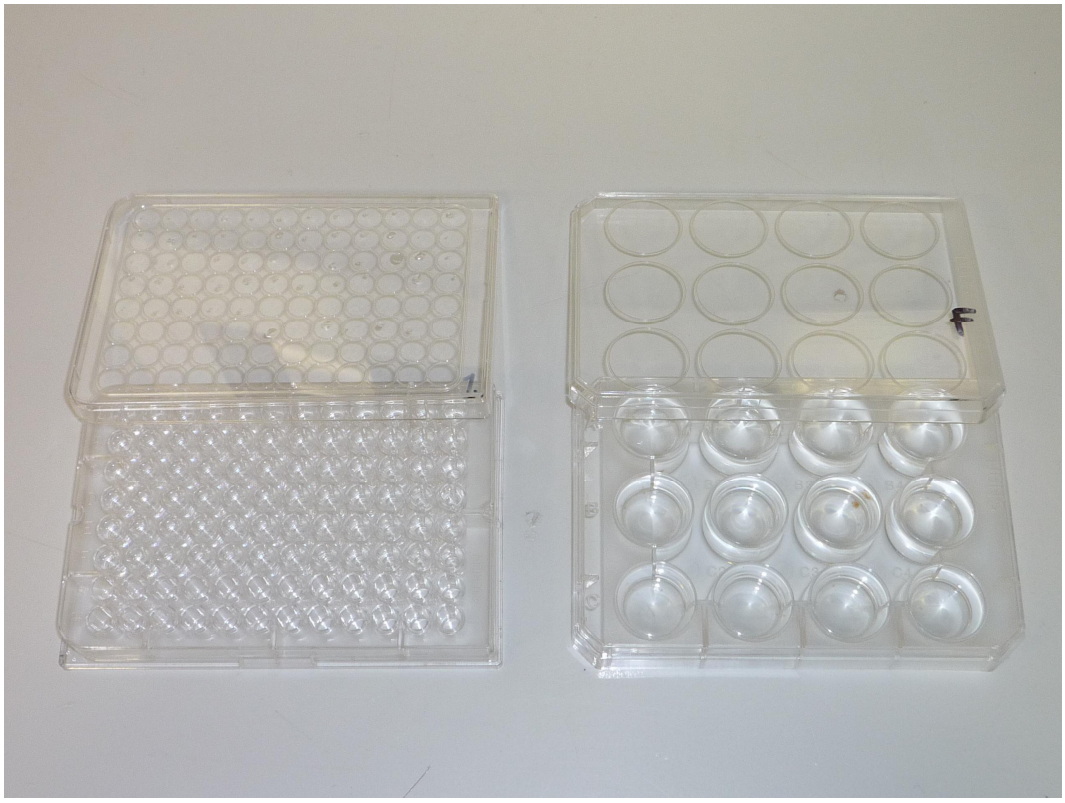
For testing we used juvenile and adult *Artemia salina*. Juvenile *Artemia salina* were taken straight from the saltwater broth where they had hatched. We had problems repeating our attempts to grow the *Artemia salina* to adulthood after the first test when they grew fine with the vegetable mixture. However, when we added aeration and only fed them with 0,5 ml vegetable mixture every other day we succeeded. When using this modification adult *Artemia salina* were smaller and lighter colored than before.

5.2.2 Tests

Due to the nature of the pharmaceuticals, we needed to use methanol to dissolve the pharmaceuticals. That was why we had to carry out some tests to evaluate the toxicity of methanol to *Artemia salina*. We made dilutions of standard 99,8 % methanol to 5, 10, 15, 20 and 30 %. In addition we made further dilutions to 2, 4, 6, 8 and 10 % based on our observation with the first set of dilutions. The

methanol tests were made using the juvenile and adult *Artemia salina* because we wanted to see if there was a different reaction between them.

The juvenile *Artemia salina* were put into 96 well microtitre plates which could contain 0,3 ml of liquid. We put 0,1 ml *Artemia salina* in sea water and 0,1 ml methanol dilution. Adult *Artemia salina* were put into 12 well multiplates which could contain 5 ml of liquid. Here we put 2 ml *Artemia salina* In sea water and 2 ml of the methanol dilution. In both plates the concentration of studied liquid was a half from dilution. We used 12 replicates for the juvenile and the adult *Artemia salina.* The two different plates are shown in picture 8.



PICTURE 8. Plates (at left the 96 well microtitre plate and at right the 12 well multiplate).

After the *Artemia salina* and methanol dilutions were added to the plates, we left the plates under room conditions where there was enough light for them. After two

hours we observed the plates and counted the dead individuals. Death was defined as a shrimp that did not move even after gentle tapping of the plate. After counting we put the plates back into the light. We repeated this after 4, 6, 24 and 48 hours from plating.

We used this same protocol to evaluate the toxicity of the six pharmaceuticals.

5.2.3 Results from juvenile and adult *Artemia salina*

We gathered the results from ecotoxicological tests and put them to tables. Then we used tables to draw figures and all the figures are shown below. For easier comparison all figures are in same scale and in every pharmaceutical figure there are results from 2, 4 and 6 % methanol tests.

Result of the methanol evaluation with juvenile *Artemia salina* in 2,5; 5; 7,5; 10 and 15 % concentrations is shown in figure 1. Differences between the different concentrations began to show between 2 and 24 hours.

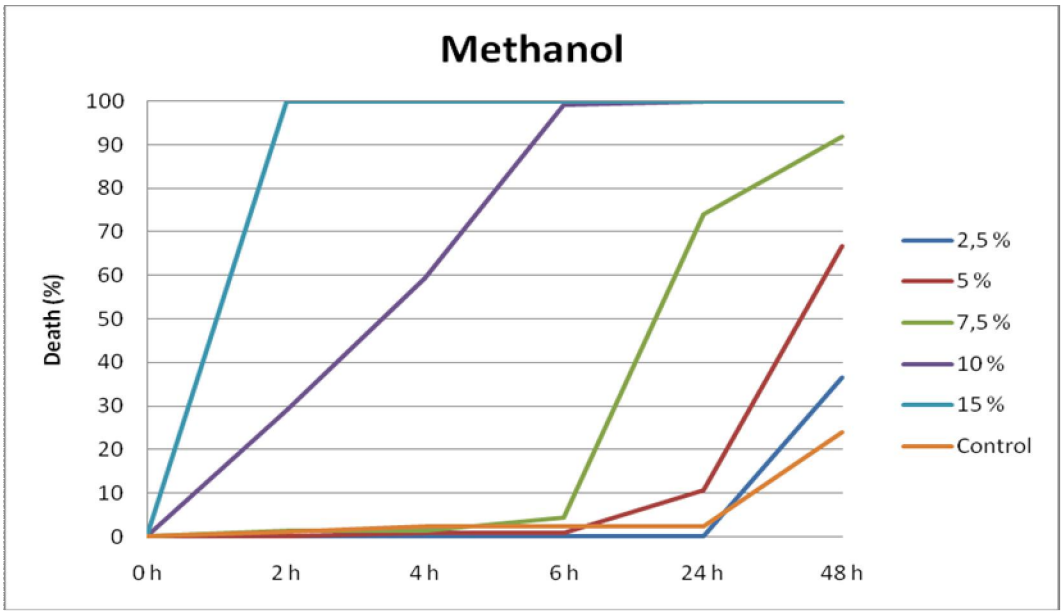


FIGURE 1. Result of the methanol test with juvenile *Artemia salina* in 2,5; 5; 7,5; 10 and 15 % concentrations.

Result of the methanol assessment with juvenile *Artemia salina* in 1, 2, 3, 4 and 5 % concentrations is shown in figure 2. Differences between the different concentrations began to show this time only after 24 hours.

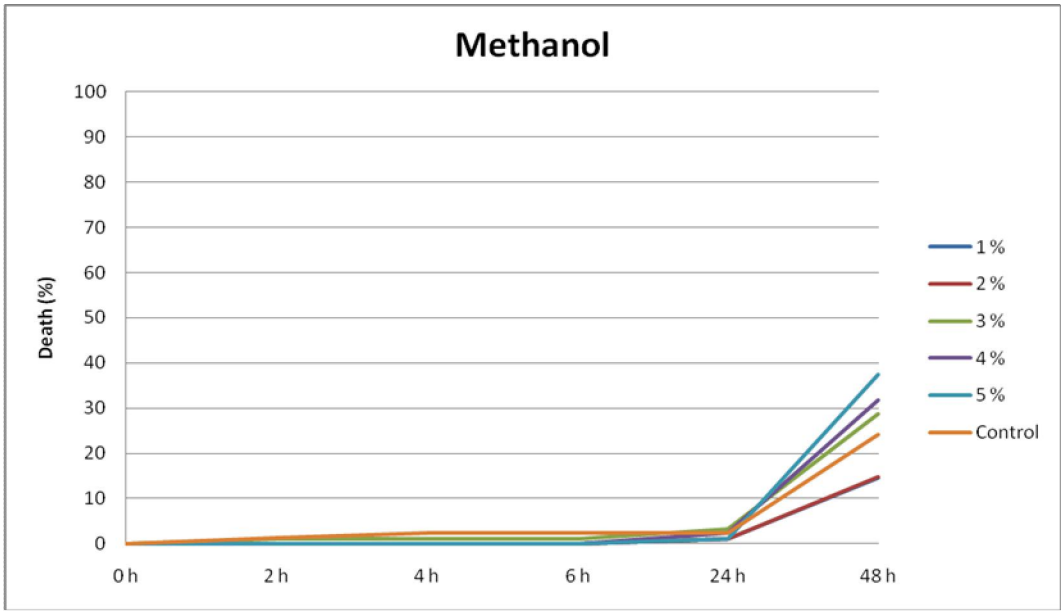


FIGURE 2. Result of the methanol test with juvenile *Artemia salina* in 1, 2, 3, 4 and 5 % concentrations.

Result of the atenolol test with juvenile *Artemia salina* is shown in figure 3. Differences between the concentrations began to show after 24 hours.

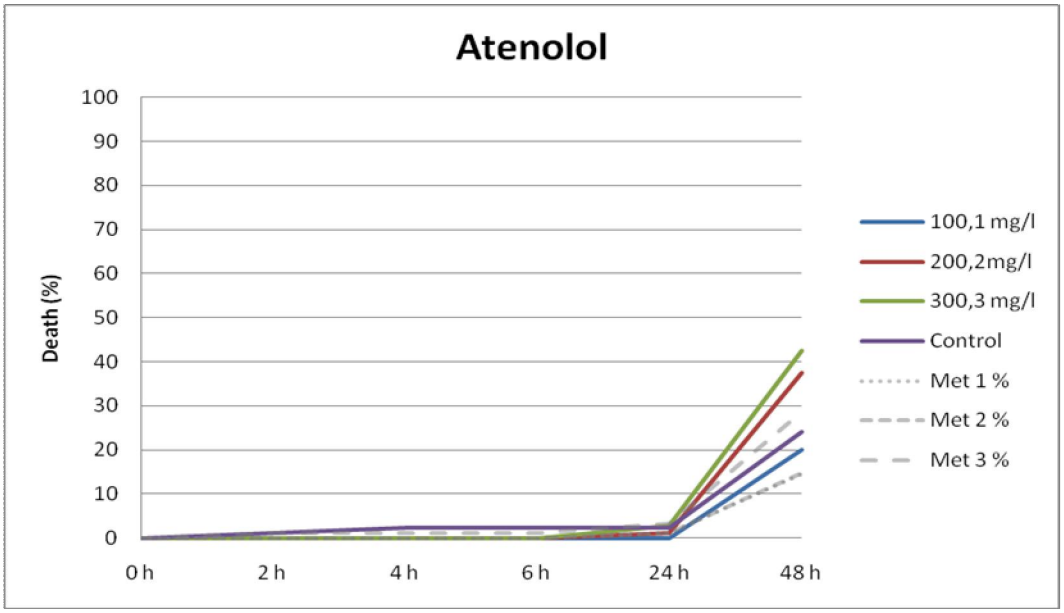


FIGURE 3. Result of the atenolol test with juvenile *Artemia salina*.

Result of the diclofenac sodium salt test with juvenile *Artemia salina* is shown in figure 4. Differences between the concentrations began to show after 6 hours.

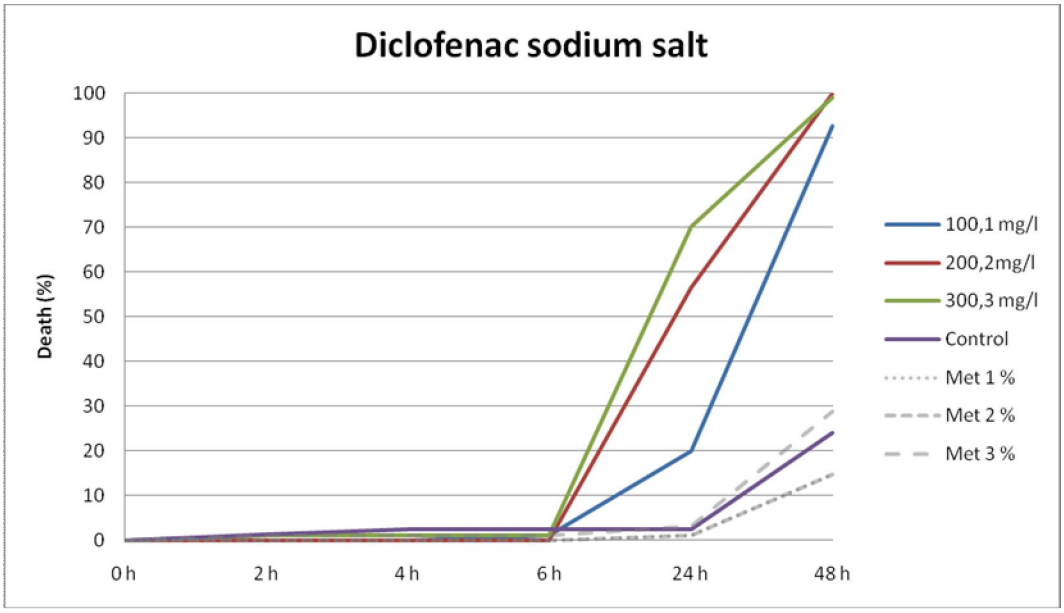


FIGURE 4. Result of the diclofenac sodium salt test with juvenile *Artemia salina*.

Result of the erythromycin hydrate test with juvenile *Artemia salina* is shown in figure 5. Differences between the concentrations began to show after 24 hours.

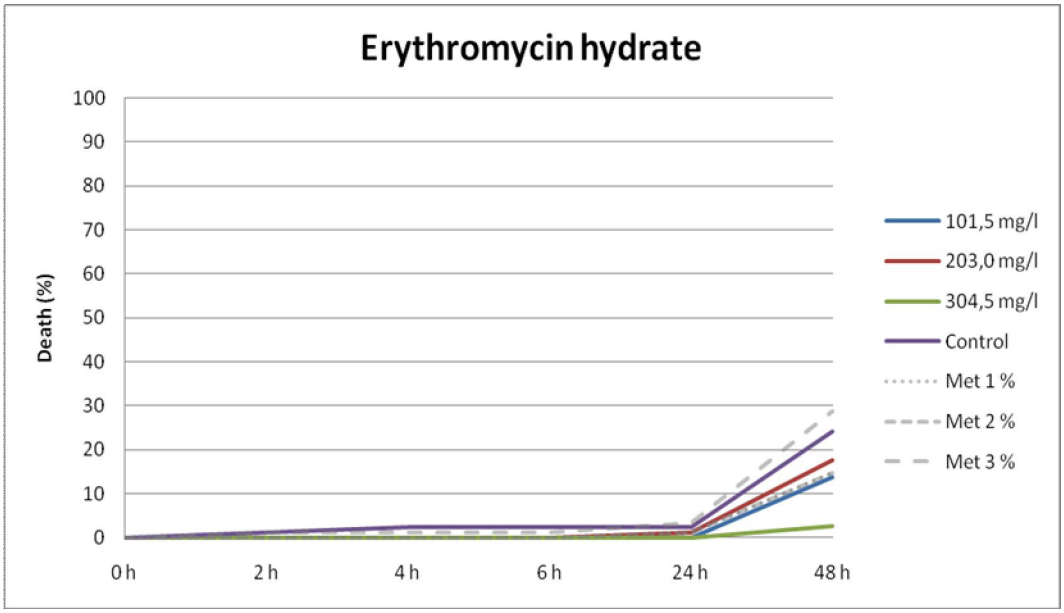


FIGURE 5. Result of the erythromycin hydrate test with juvenile *Artemia salina*.

Result of the lidocaine test with juvenile *Artemia salina* is shown in figure 6. Differences between the concentrations began to show after 6 hours.

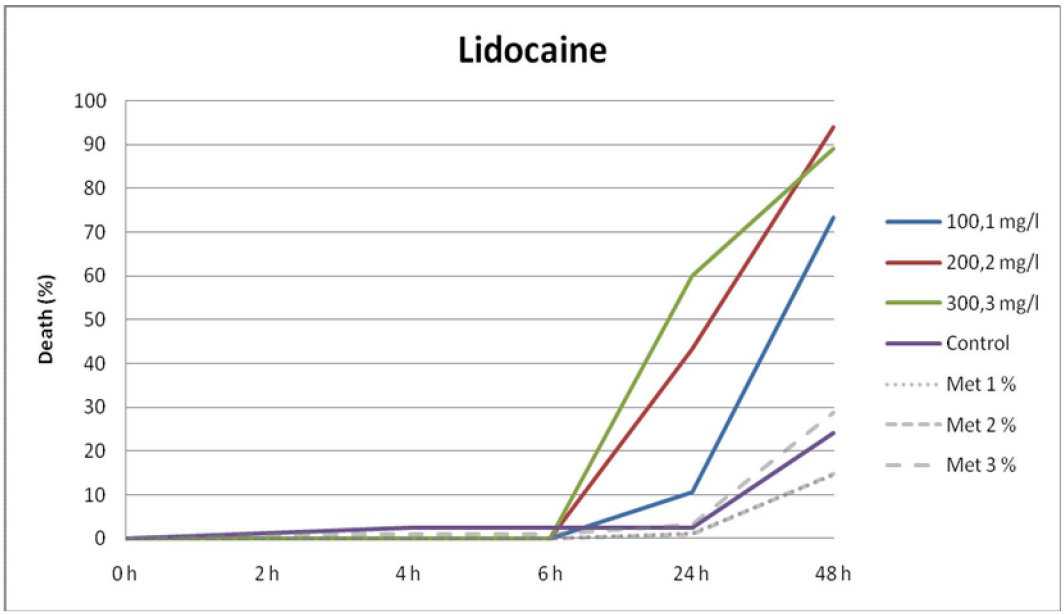


FIGURE 6. Result of the lidocaine test with juvenile *Artemia salina*.

Result of the sodium diatrizoate hydrate test with juvenile *Artemia salina* is shown in figure 7. Differences between the concentrations began to show after 24 hours.

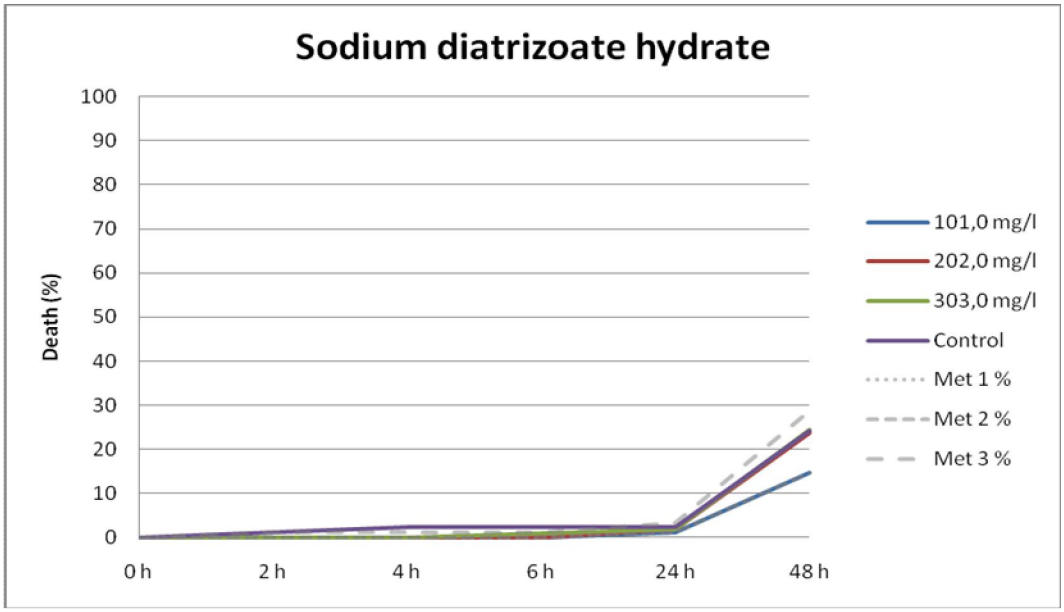


FIGURE 7. Result of the sodium diatrizoate hydrate test with juvenile *Artemia salina*.

Result of the sulfamethoxazole hydrate test with juvenile *Artemia salina* is shown in figure 8. Differences between the concentrations began to show after 24 hours.

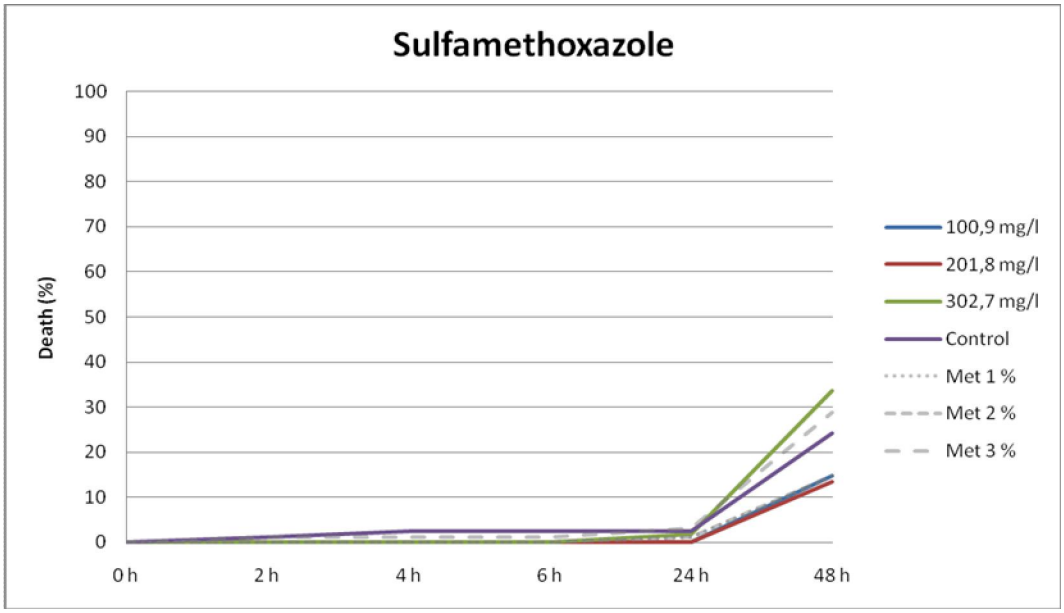


FIGURE 8. Result of the sulfamethoxazole hydrate test with juvenile *Artemia salina*.

Result of the methanol test with adult *Artemia salina* in 2,5; 5; 7,5; 10 and 15 % concentrations is shown in figure 9. Differences between the different concentrations began to show after 2 hours.

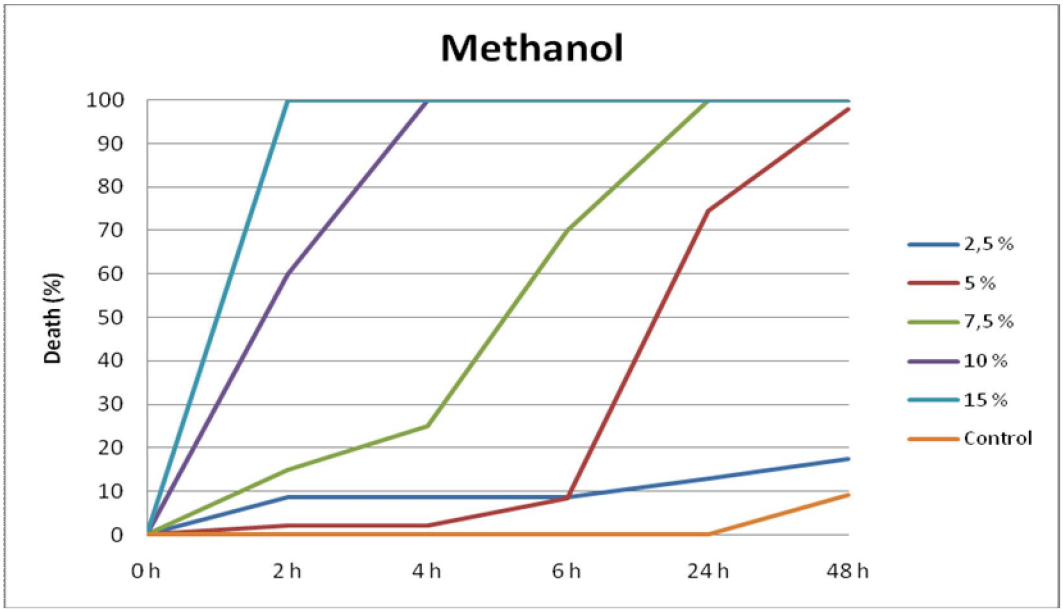


FIGURE 9. Result of the methanol test with adult *Artemia salina* in 2,5; 5; 7,5; 10 and 15 % concentrations.

Result of the methanol test with adult *Artemia salina* in 1, 2, 3, 4 and 5 % concentrations is shown in figure 10. Differences between the different concentrations began to show between 6 and 24 hours.

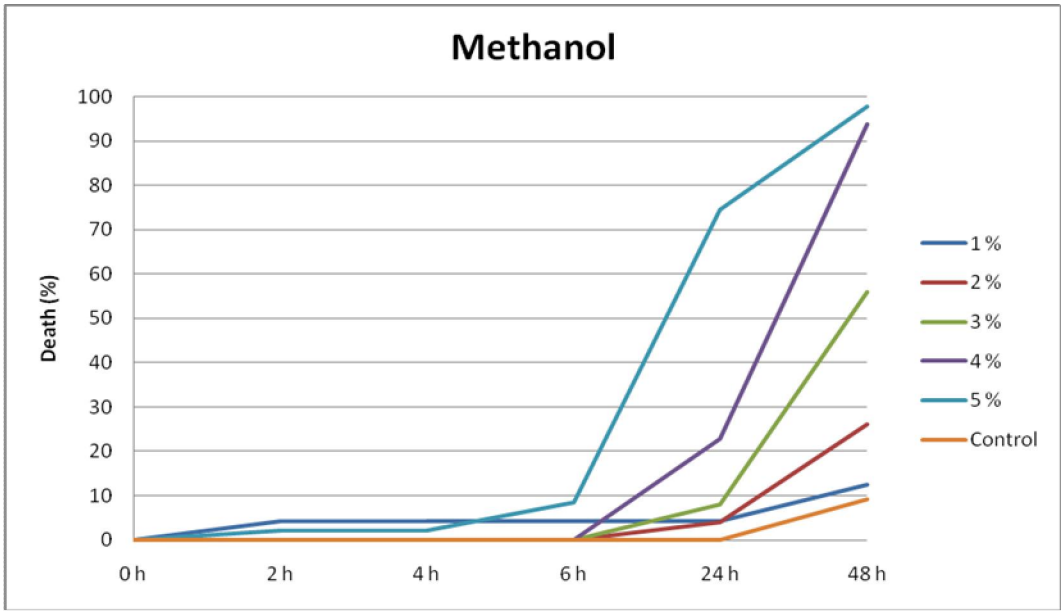


FIGURE 10. Result of the methanol test with adult *Artemia salina* in 1, 2, 3, 4 and 5 % concentrations.

Result of the atenolol test with adult *Artemia salina* is shown in figure 11. Differences between the concentrations began to show after 6 hours.

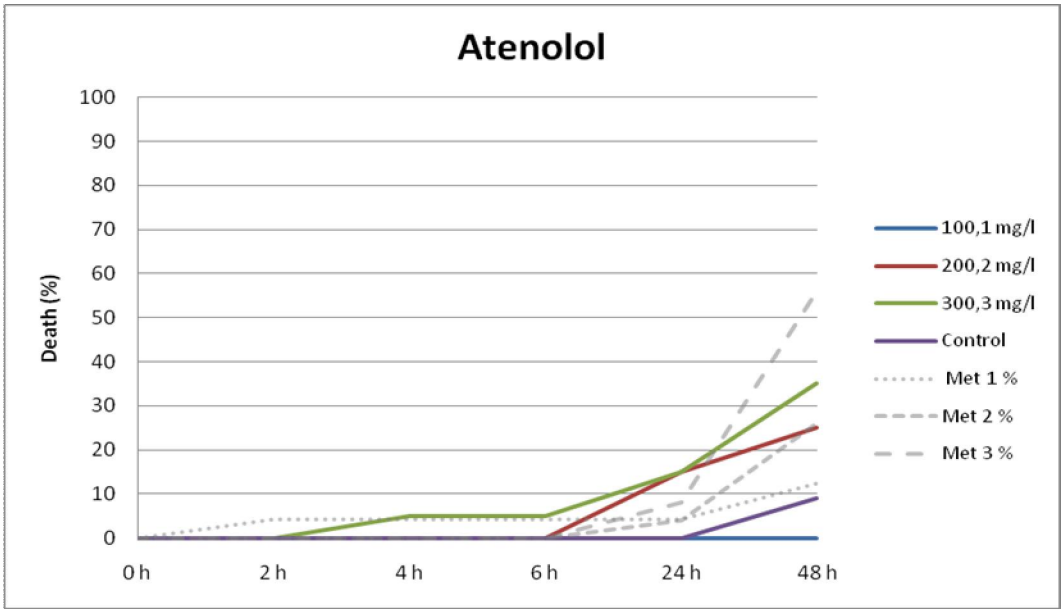


FIGURE 11. Result of the atenolol test with adult *Artemia salina*.

Result of the diclofenac sodium salt test with adult *Artemia salina* is shown in figure 12. Differences between the concentrations began to show after 6 hours.

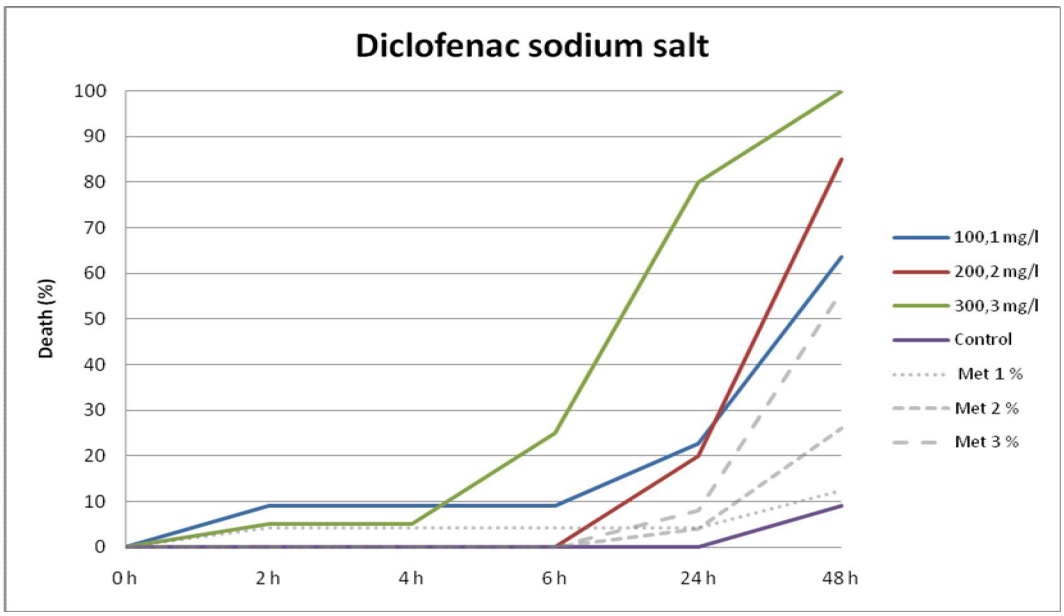


FIGURE 12. Result of the diclofenac sodium salt test with adult *Artemia salina*.

Result of the erythromycin hydrate test with adult *Artemia salina* is shown in figure 13. Differences between the concentrations began to show after 6 hours.

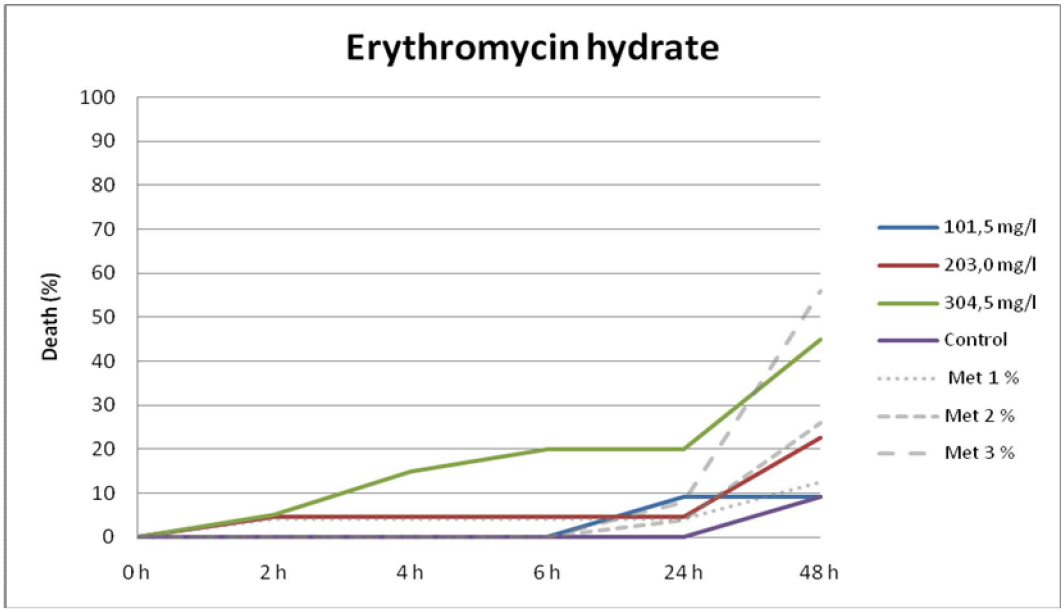


FIGURE 13. Result of the erythromycin hydrate test with adult *Artemia salina*.

Result of the lidocaine test with adult *Artemia salina* is shown in figure 14. Differences between the concentrations began to show after 4 hours.

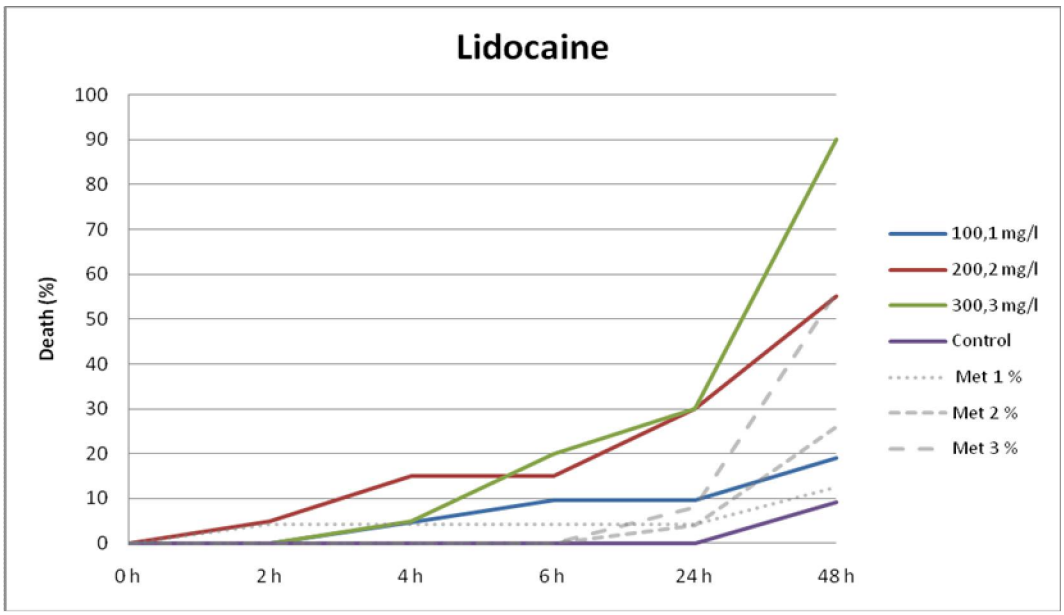


FIGURE 14. Result of the lidocaine test with adult *Artemia salina*.

Result of the sodium diatrizoate hydrate test with adult *Artemia salina* is shown in figure 15. Differences between the concentrations began to show after 4 hours.

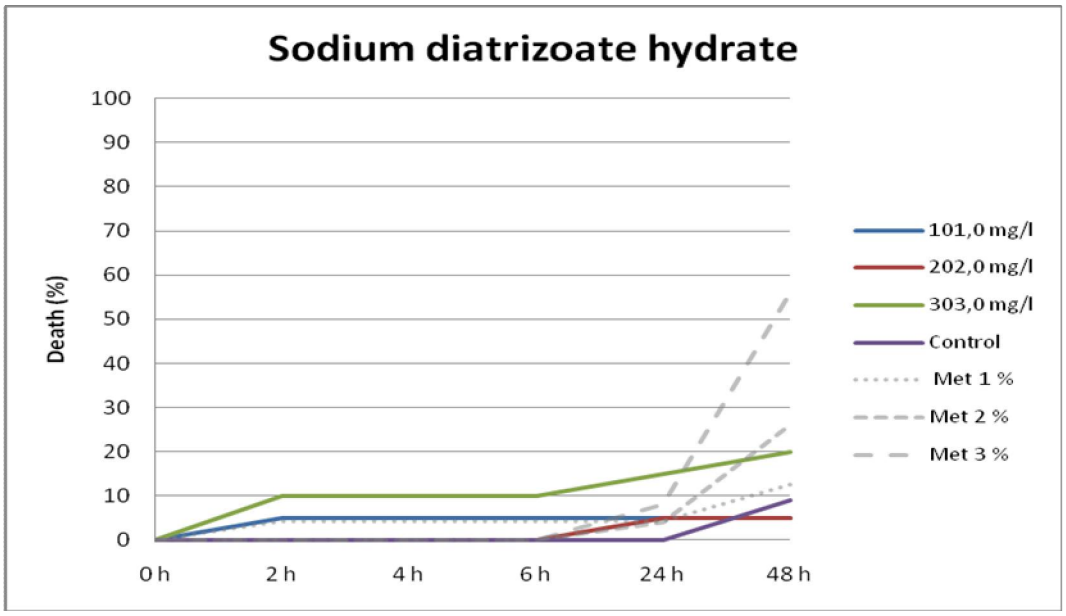


FIGURE 15. Result of the sodium diatrizoate hydrate test with adult *Artemia salina*.

Result of the sulfamethoxazole test with adult *Artemia salina* is shown in figure 16. Differences between the concentrations began to show after 4 hours.

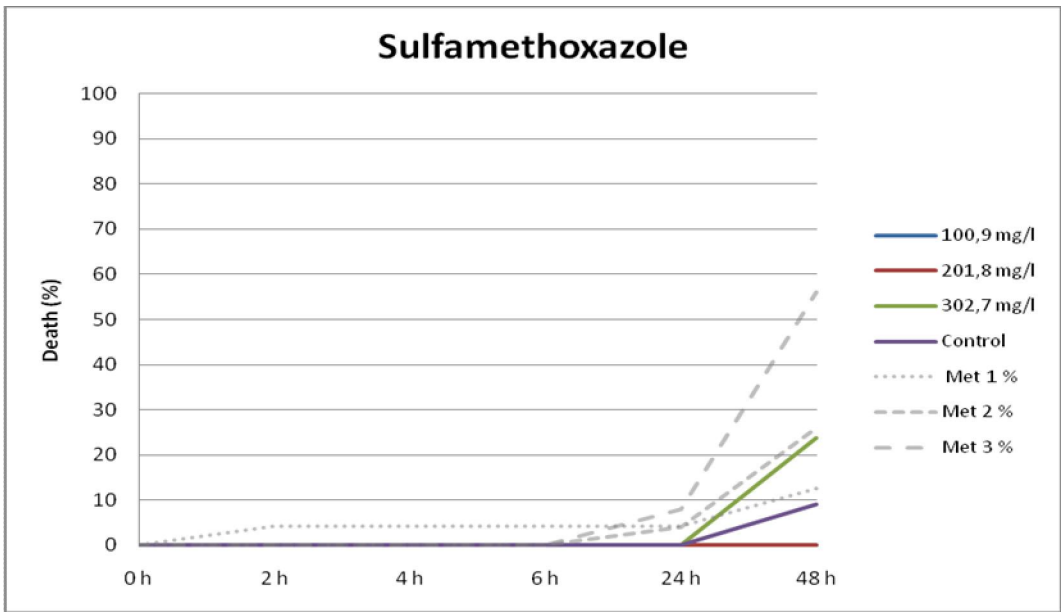


FIGURE 16. Result of the sulfamethoxazole test with adult *Artemia salina*.

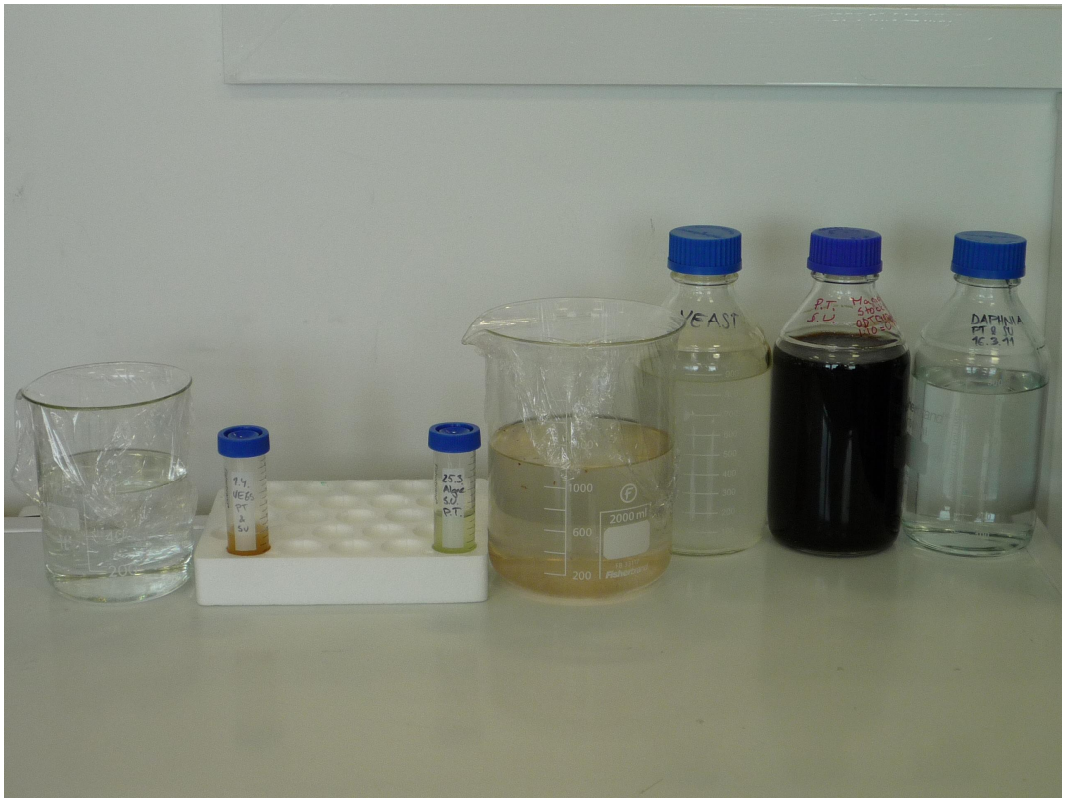
5.3 *Daphnia magna*

Daphnia magna is used widely for fish food because it is relatively easy to culture and it is more commonly used in ecotoxicity tests than *Artemia salina*. It belongs to Crestacea subphylum, its family is Daphniidae. Originally they occurred in northern and western North America. At 25 °C *Daphnia magna* lives approximately two months and reproduces quickly. (Daphnia magna 2011.)

Daphnia magna is easy to use in laboratory and therefore it is usually used for standard testing. *Daphnia magna* has been used to test for example sediment, water and environmental contaminants. It is also important for aquatic food webs and reacts to different stimulation. (Yegane, Parlak, Arslan & Boyacioğlu 2008.) *Daphnia magna* is widely used in pharmaceutical toxicity testing.

5.3.1 Growing the cultures

The *Daphnia magna* was cultured in 2 l beakers containing 1 000 ml of deionized water, 200 ml of stock salt solution, 4 ml of nutrient supplement (Marinune, The Glenside Group Limited), 0,5 ml of algae and 0,5 ml of yeast (Allison Dried Active Yeast) (PICTURE 9.). Right concentration of nutrient supplement was inspected by measuring the optical density with 400 nm to give result of 0,800 and with algae the wavelength was 490 nm. To each beaker 15 adult *Daphnia magna* were added and the liquid was replaced weekly. (Heckmann & Connon 2007.)



PICTURE 9. Different growing methods for *Daphnia magna*.

The stock salt solution was made as follows: firstly 291,9 mg of calcium chloride was weighted and dissolved in 500 ml of deionized water that was constantly stirred. The liquid was stirred as long as it took to get the salt fully dissolved. After that 82,2 mg of magnesium sulphate, 64,80 mg of sodium hydrogen carbonate, 5,8 mg of potassium carbonate and 0,002 mg of sodium selenite were weighted and dissolved together in 500 ml of deionized water that was also stirred. The liquid was stirred until all the salts dissolved. At this point the two separate liquids were combined and left stirring at least 12 hours to ensure proper mixing of the salts. The stock salt solution can be used for up to one month and after that it has to be disposed. (Heckmann & Connon 2007.)

We wanted to develop a method for the long term culture of *Daphnia magna* so we compared the standard method of fedding them daily with1,0 ml of nutritional supplement, 1,0 ml of algae and 0,5 ml of yeast with only giving them 0,5 ml of vegetable mixture daily. Thus the growth media was also a bit different from the

other (PICTURE 9.). We changed the water at normal way but we did not put there nutritional supplement, algae or yeast. We observed if there were any differences in *Daphnia magna* activity and health when feeding and changing the water in the two different ways. We did not observe a difference but obviously there were less work when feeding daphnids with just vegetable mixture.

At last we decided to grow our cultures by feeding the *Daphnia magna* daily with 1,0 ml of algae and 1,0 ml of vegetable mixture to ensure the best results. The water was replaced weekly at same manner as mentioned before. We believe this method was optimal to our use.

5.3.2 Tests

As previously discussed, we needed to use methanol to dissolve the pharmaceuticals into an aqueous solution. That was why we had to evaluate the toxicity of methanol to *Daphnia magna*. We made dilutions of 99,8 % methanol to 2, 4, 6, 8 and 10 %.

Tests were carried out in 12 well multiplates which contained 5 ml of liquid per well. Into which we put 2 ml *Daphnia magna* in growth liquid and 2 ml of the test solution. Then after two hours we observed the plates and counted the dead individuals. Death was defined as a *Daphnia magna* that did not move even after gentle tapping of the plate. We repeated this observation after 4, 6, 24 and 48 hours from plating.

5.3.3 Results

We gathered the results from ecotoxicological tests and put them to tables. Then we used tables to draw figures and all the figures are shown below. For easier comparison all figures are in same scale and in every pharmaceutical figure there are results from 2, 4 and 6 % methanol tests.

Result of the methanol test with 1, 2, 3, 4 and 5 % concentrations is shown in figure 17. Differences between the concentrations began to show between 2 and 24 hours.

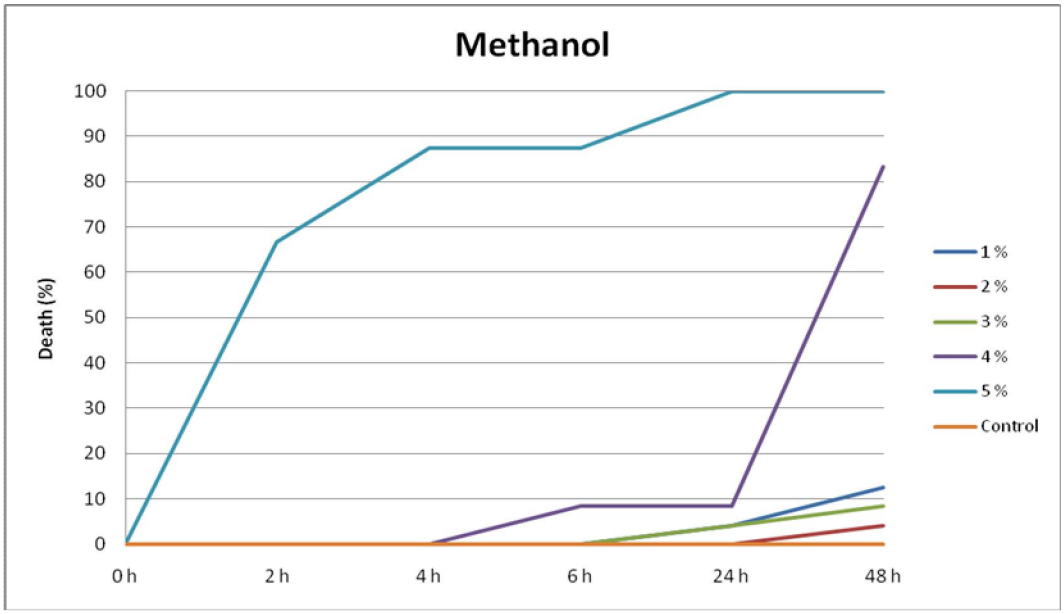


FIGURE 17. Result of methanol test in 1, 2, 3, 4 and 5 % concentrations.

Result of the atenolol test is shown in figure 18. Differences between the concentrations began to show between 6 and 24 hours.

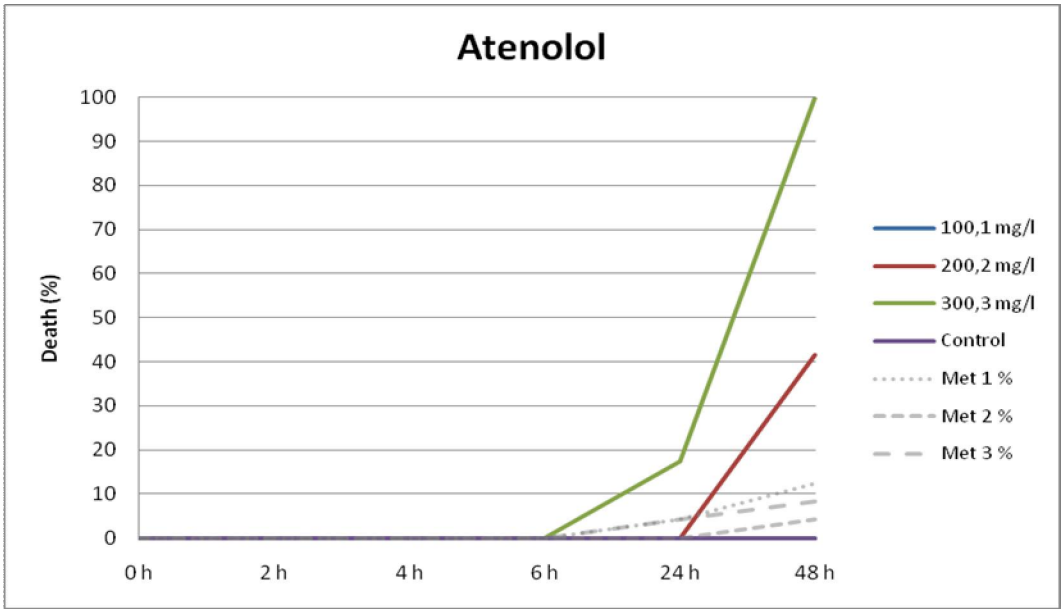


FIGURE 18. Result of the atenolol test.

Result of the diclofenac sodium salt test is shown in figure 19. Differences between the concentrations began to show after 2 hours.

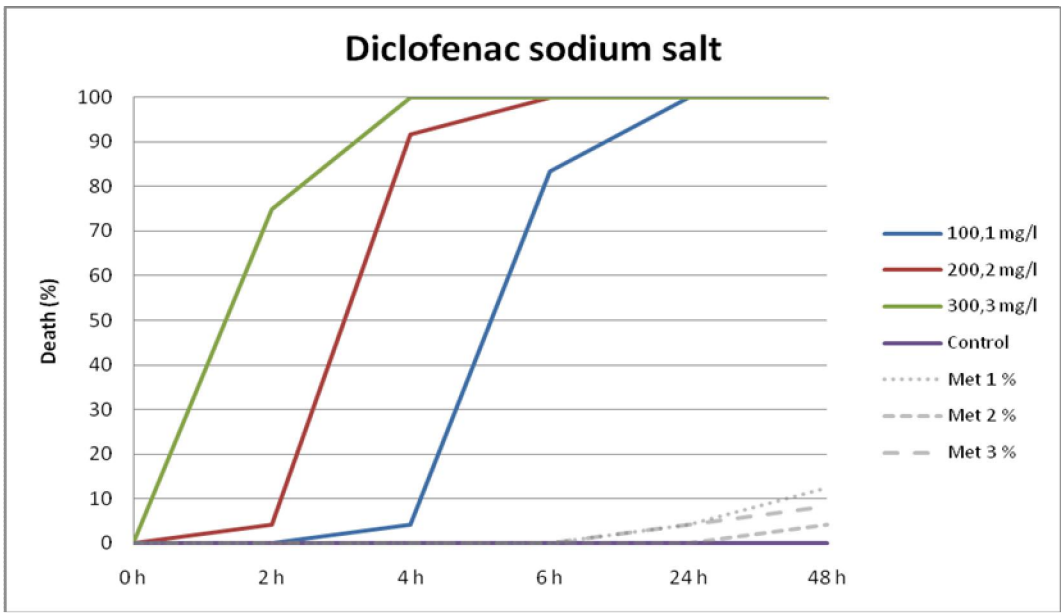


FIGURE 19. Result of the diclofenac sodium salt test.

Result of the erythromycin hydrate test is shown in figure 20. Differences between the concentrations began to show between 4 and 24 hours.

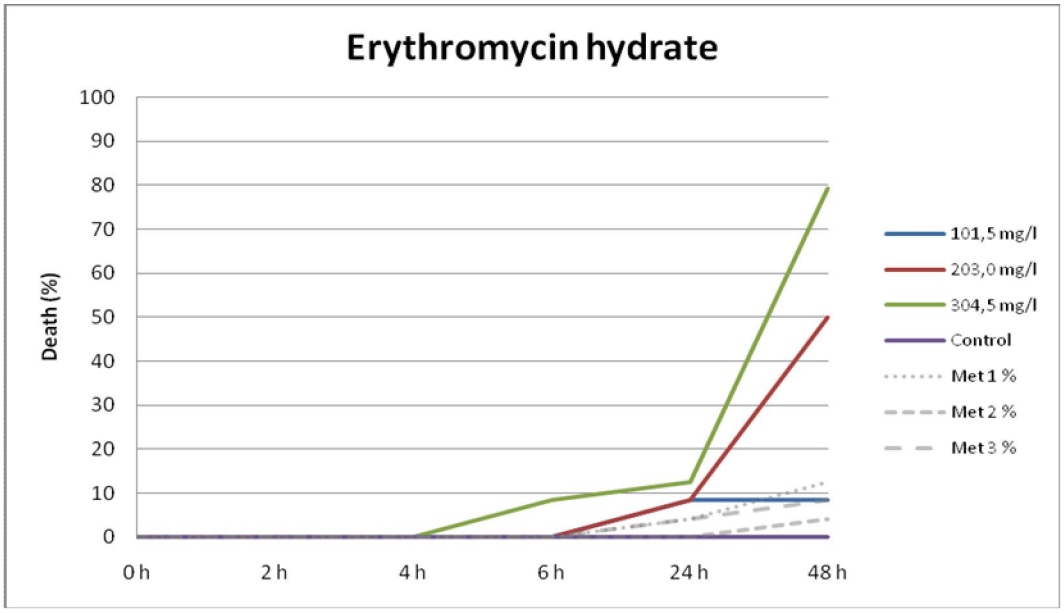


FIGURE 20. Result of the erythromycin hydrate test.

Result of the lidocaine test is shown in figure 21. Differences between the concentrations began to show between 6 and 24 hours.

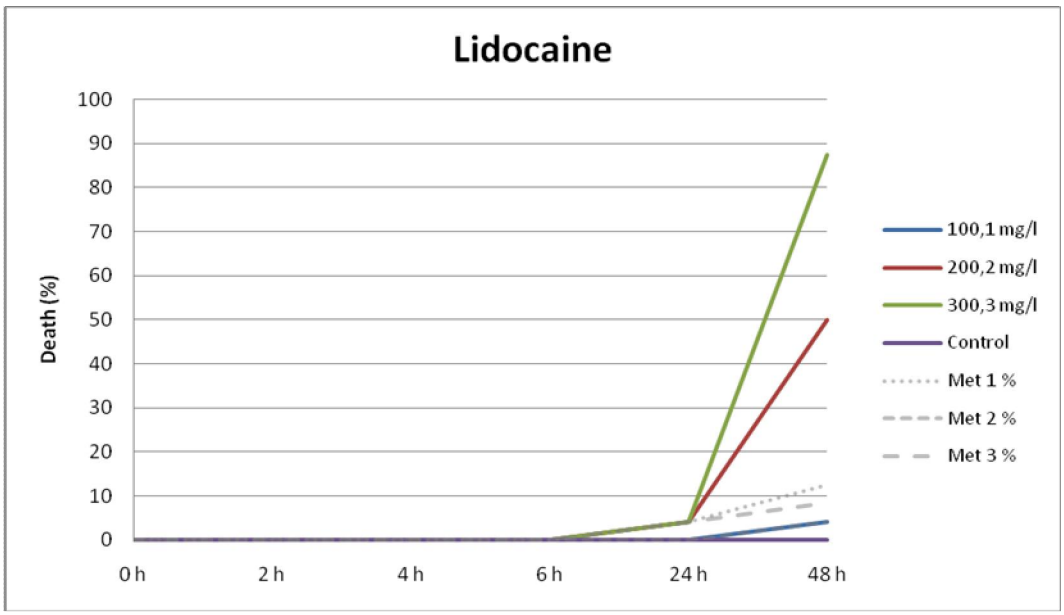


FIGURE 21. Result of the lidocaine test.

Result of the sodium diatrizoate hydrate test is shown in figure 22. Differences between the concentrations began to show between 6 and 24 hours.

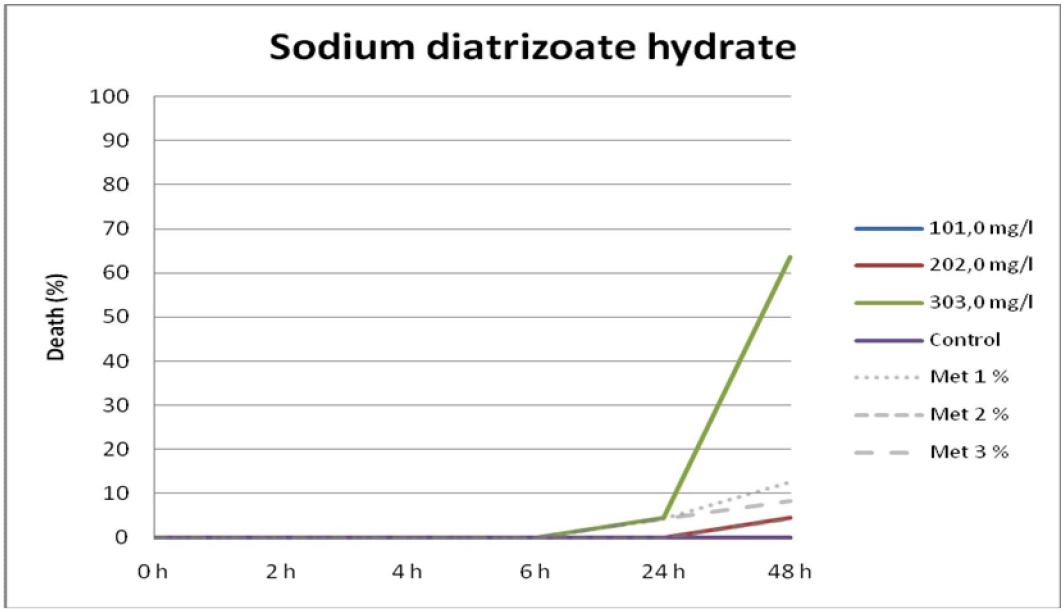


FIGURE 22. Result of the sodium diatrizoate hydrate test.

Result of the sulfamethoxazole test is shown in figure 23. Differences between the concentrations began to show after 6 hours.

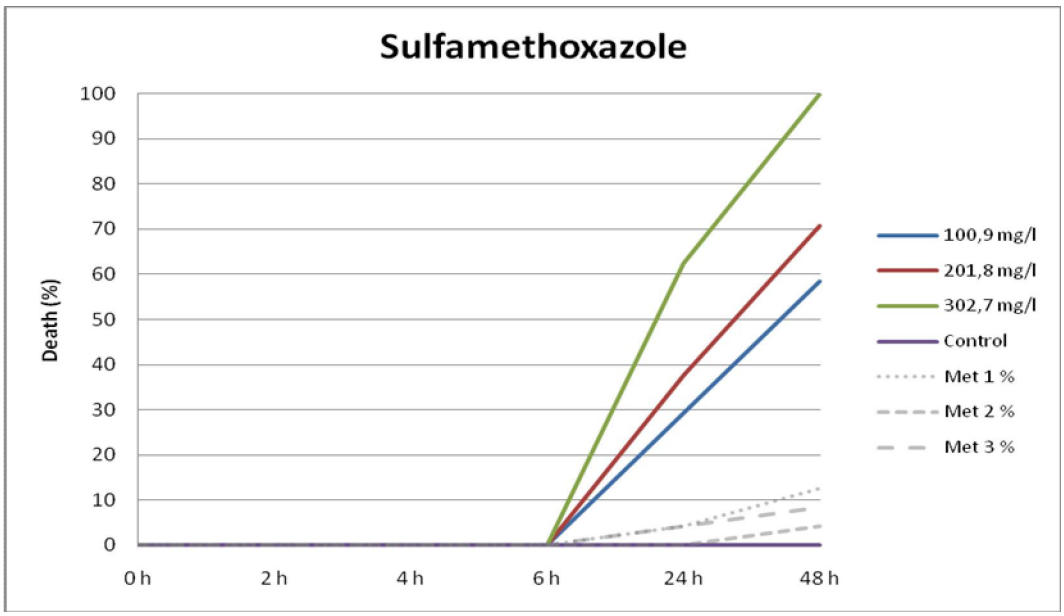


FIGURE 23. Result of the sulfamethoxazole test.

5.4 *Pseudomonas fluorescens*

The bacteria we used belongs to *Pseudomonas* genera and is very common everywhere. It can be found in soil, water and vegetation but also in healthy person's skin, throat and stool. They are Gram-negative and measuring 0,5 to 0,8 µm by 1,5 to 3,0 µm. This bacillus is also aerobic. It can be cultured in general purpose media and that is why it is easy to use and grow. (Baron 1996.) It belongs to Proteobacteria phylum, its family is Pseudomonadaceae (Pseudomonas flurescens 2011).

We used *Pseudomonas fluorescens* to test pharmaceuticals' toxicity to bacteria. The main idea was similar to the other ecotoxicological test which was to expose the selected organism to pharmaceuticals and observe them. The bacteria we used were obtained from the German Collection of Micro-organisms and Cell Cultures as a freeze-dried culture (Hunter 2011a).

5.4.1 Growing the cultures

Firstly we had to re-activate the bacterial culture, the outer vial was opened by heating the tip above flame and crushing the tip by gently tapping with forceps. Then few drops of Nutrient Broth (Oxoid) were dropped into the vial and the freeze-dried culture was left to hydrate for 30 minutes. After that the bacterial culture was moved to a sterile glass test tube which contained approximately 10 ml of nutrient broth. The test tube was incubated at 27 °C for 24 hours.

The next day the bacteria were transferred to agar slopes for long therm storage. Some of the bacteria broth was removed using a flame-sterilized wire loop and then the loop was drawn across the face of the slope. Using the loop the bacteria were spreaded by moving the loop from right to left across the slope. A total of three slopes were made and taken back to the incubator.

After a few days of incubation, the bacteria had grown on the slopes. These slopes were used in all subsequent tests as a source of inoculum. Fresh nutrient broth (10

ml) was transferred to sterile test tubes and then one colony of bacteria was transferred to each test tube using a sterile loop. These test tubes were taken to the incubator and slopes were put to the fridge.

5.4.2 Tests

After 24 hours of incubation, 1 ml of bacteria culture was transferred to individual wells on a 12 well multiplate. Then 1 ml of the test solution was added to each well. We used two controls, the first where we only added sterile deionised water to the bacteria. The second was a positive control where we added 1 ml of nutrient broth. We tested methanol in 2, 4, 5, 6, 8, 10, 15, 20 and 30 % dilutions and all six pharmaceuticals at three different concentrations. We did not use replicates at any concentration of methanol or pharmaceuticals.

After adding the bacteria and the test solution into the wells, the plates were incubated for 24 hours. After that we added 20 µl of CellTiter 96® which is broken down by living cells into a coloured liquid. We incubated the bacteria with CellTiter 96® for 30 minutes and after that we read the results with a spectrometer. For reading the results we used two wavelengths: 490 and 600 nm.

5.4.3 Results

We gathered the results from ecotoxicological tests and put them to tables. Then we used tables to draw figures and all the figures are shown below. For easier comparison all figures are in same scale. We used the deionised water control as the blank and thus this is the nil value in figures.

Result of the methanol test with 2,5; 5; 7,5; 10 and 15 % concentrations is shown in figure 24.

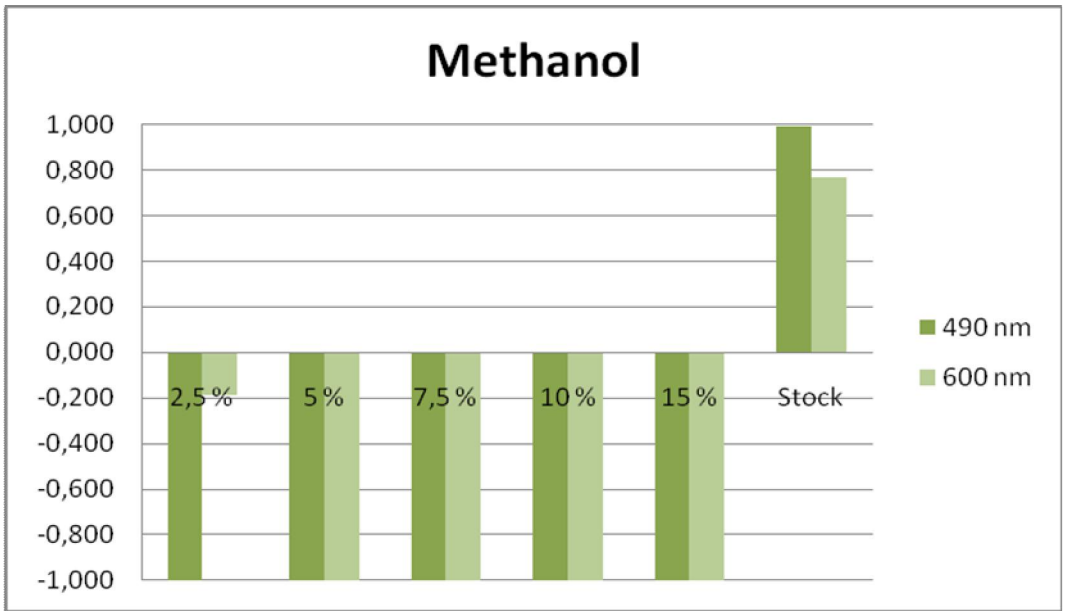


FIGURE 24. Result of the methanol test with 2,5; 5; 7,5; 10 and 15 % concentrations.

Result of the methanol test with 1, 2, 3, 4 and 5 % concentrations is shown in figure 25.

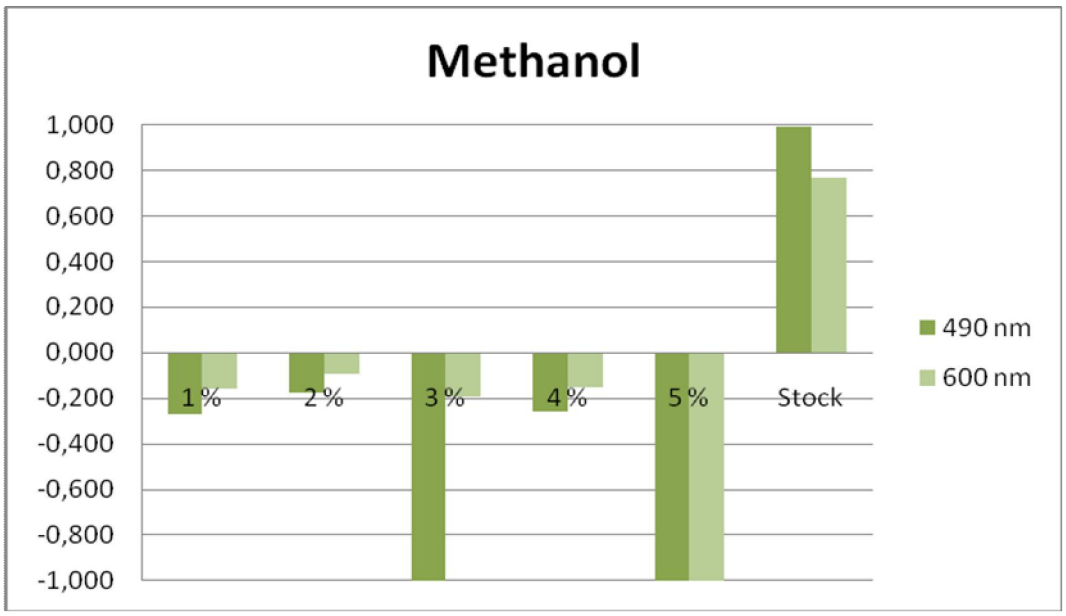


FIGURE 25. Result of the methanol test with 1, 2, 3, 4 and 5 % concentrations.

Result of the atenolol test is shown in figure 26. At all concentrations the bacterial growth was slightly inhibited.

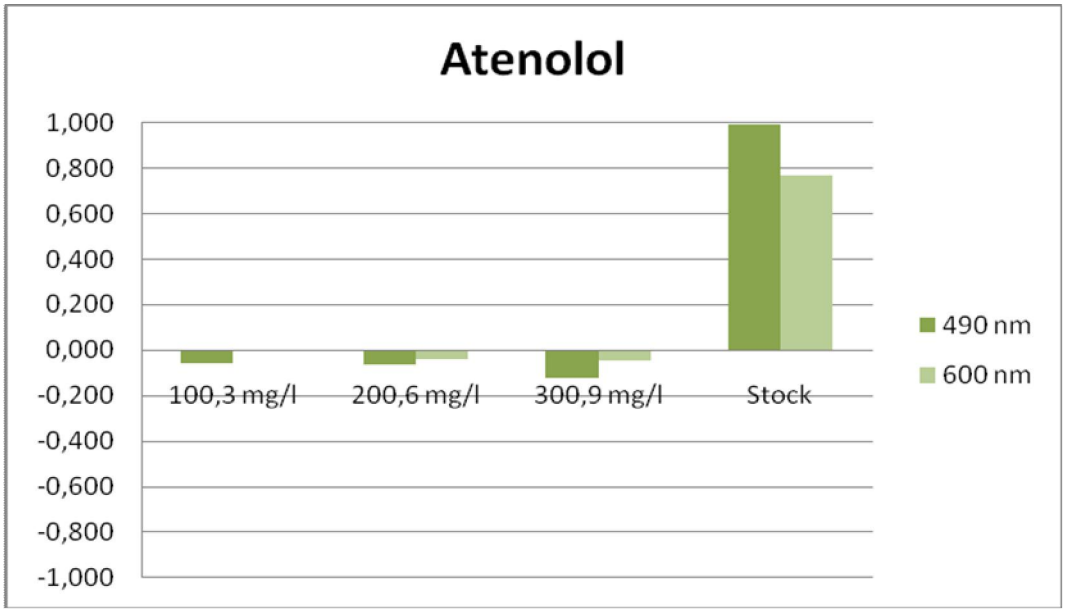


FIGURE 26. Result of the atenolol test.

Result of the diclofenac sodium salt test is shown in figure 27. Again, at all concentrations the bacterial growth was slightly inhibited.

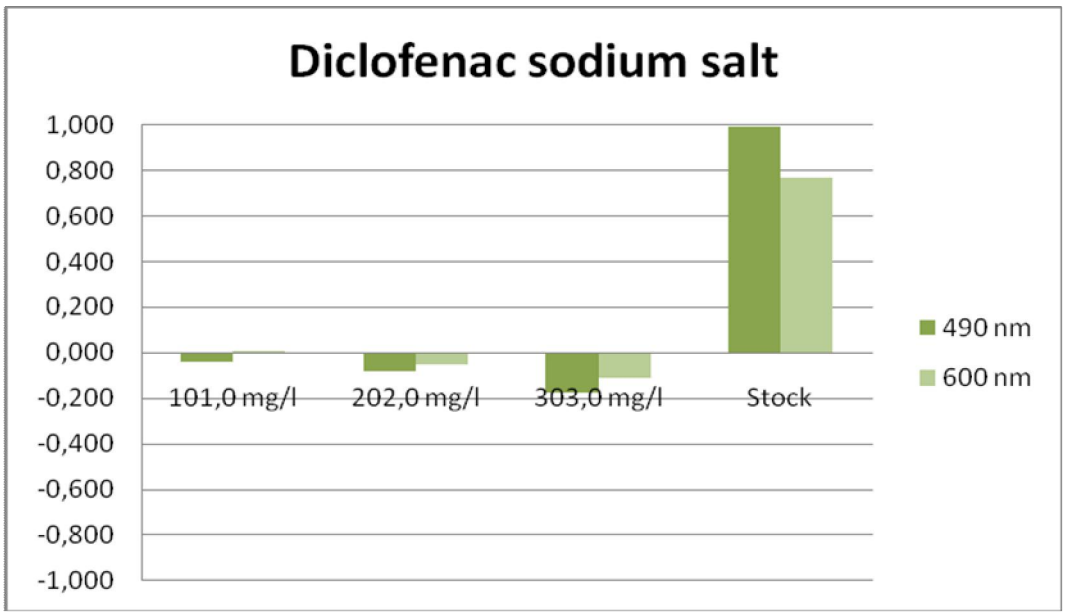


FIGURE 27. Result of the diclofenac sodium salt test.

Result of the erythromycin hydrate test is shown in figure 28. As expected erythromycin completely inhibited the growth of *Pseudomonas fluorescens*.

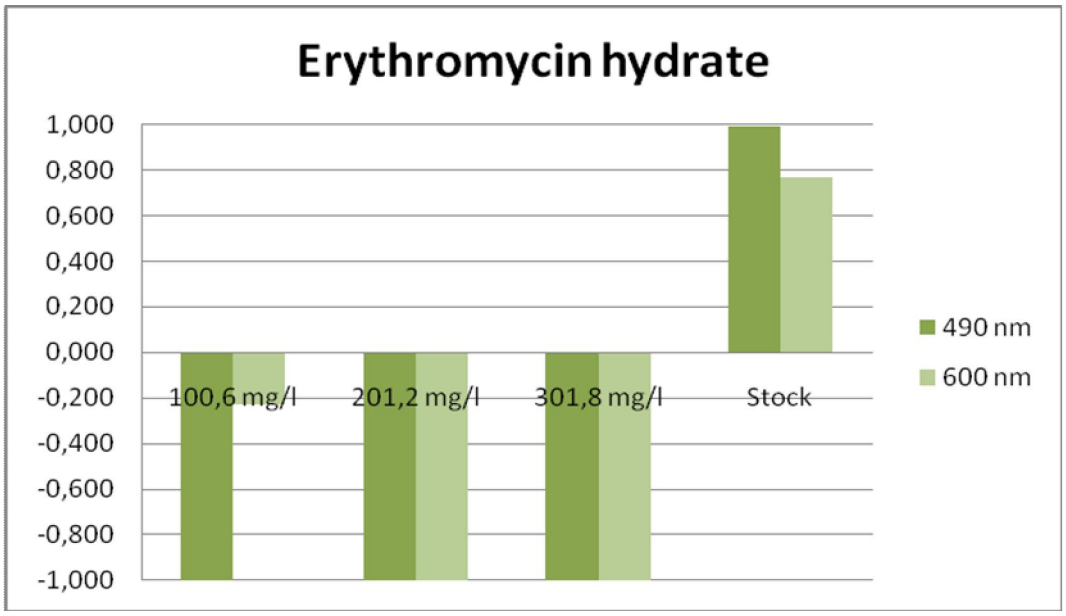


FIGURE 28. Result of the erythromycin hydrate test.

Result of the lidocaine test is shown in figure 29. Only at 300,6mg/l was the bacterial culture inhibited. At lower concentrations it appears that lidocaine provides a nutrient source for the bacteria.

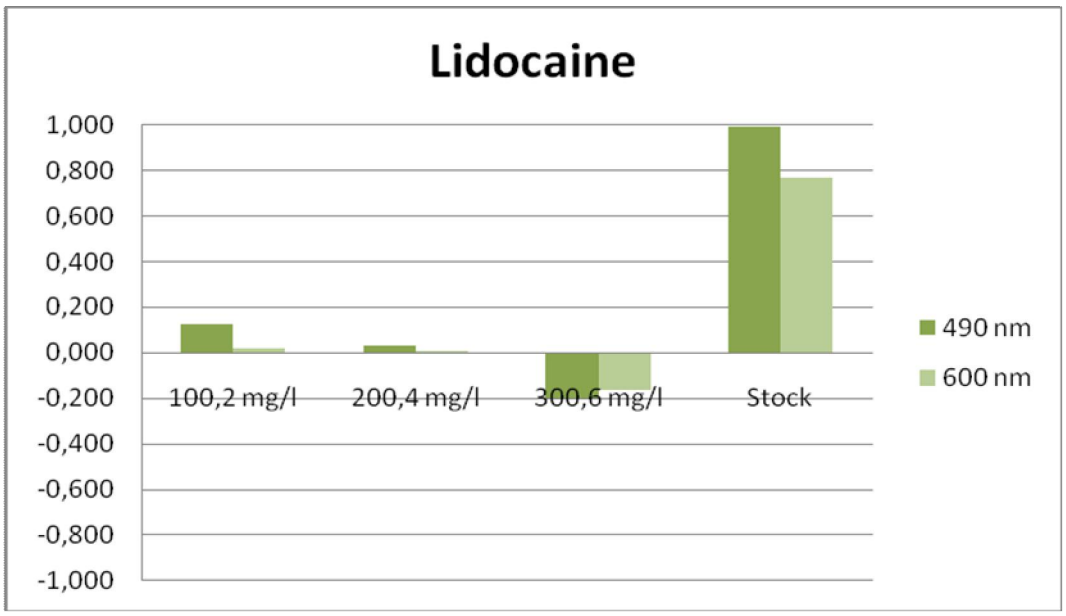


FIGURE 29. Result of the lidocaine test.

Result of the sodium diatrizoate hydrate test is shown in figure 30. As with atenolol and diclofenac growth was slightly inhibited. We believe that the finding at 100.4mg/l was an experimental error.

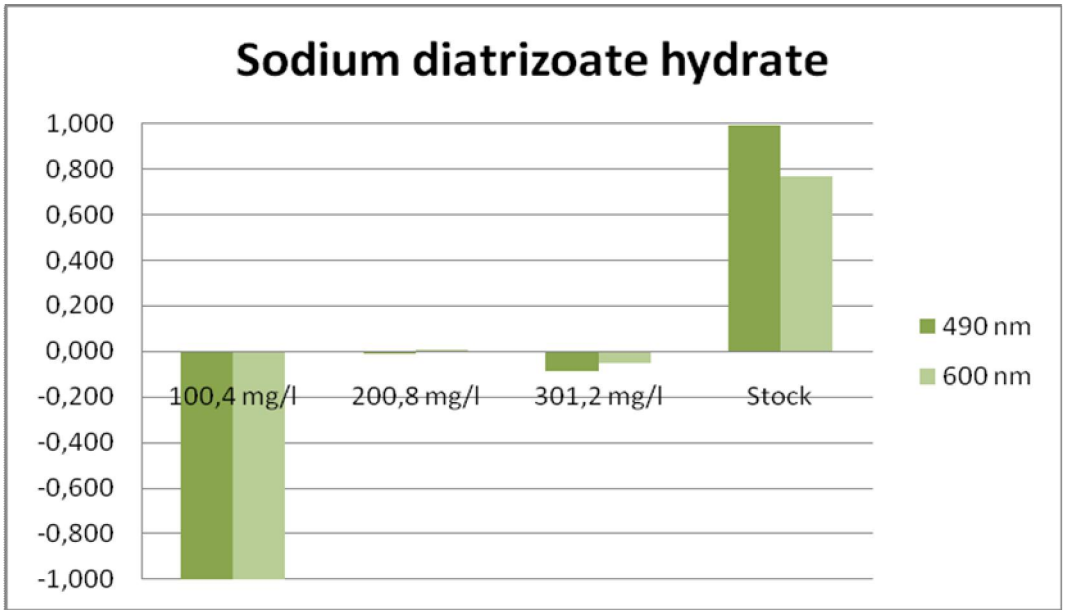


FIGURE 30. Result of the sodium diatrizoate hydrate test.

Result of the sulfamethoxazole test is shown in figure 31. As noted with lidocaine, the lowest concentration appears to stimulate growth while the other two higher concentrations inhibit the bacteria.

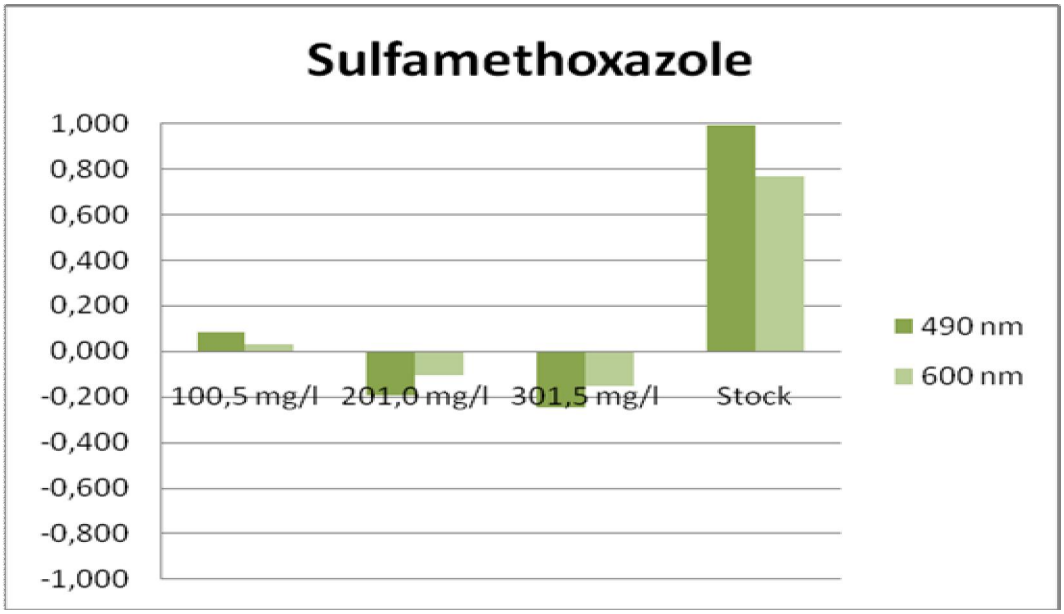


FIGURE 31. Result of the sulfamethoxazole test.

6 DISCUSSION

The conclusion we can draw from all of our results is that most of the studied pharmaceuticals do not seem to have a noticeable impact to organisms. In fact some of the organisms seem to utilize pharmaceuticals as a food source but we can not be sure about these effects without further investigations. The most toxic pharmaceuticals appears to be diclofenac sodium salt and lidocaine. They had a negative effect to all organisms studied. This is worrying because diclofenac sodium salt is widely used as a painkiller and lidocaine as anesthetic drug in hospitals.

We are satisfied with our results and we believe they are giving a true indication of the organisms response to selected pharmaceuticals.

6.1 Eukaryotic organisms

In our tests (TABLE 3) the lowest concentration of atenolol (100,1 mg/l) did not have a significant impact onthe organisms testedcompared to the control and 1 % methanol. Indeed in some cases they survived better, for example there were no dead during the test period using *Daphnia magna* and adult *Artemia salina*. Why this occurred we are not sure. At concentrations of 200,2 mg/l and 300,3 mg/l a greater number of juvenile *Artemia salina* and *Daphnia magna* died compared to the control, 2 or 3 % methanol. The impact of atenolol started to show between 24 and 48 hours. This suggests that atenolol has an impact to juvenile *Artemia salina* and *Daphnia magna* at higger concentrations.

TABLE 3. Results from the atenolol tests.

		Juvenile *Artemia salina*			Adult *Artemia salina*			*Daphnia magna*		
		Death %			Death %			Death %		
	h	C	Met	Ate	C	Met	Ate	C	Met	Ate
100,1 mg/l	2	1	0	0	0	4	0	0	0	0
	4	2	0	0	0	4	0	0	0	0
	6	2	0	0	0	4	0	0	0	0
	24	2	1	0	0	4	0	0	4	0
	48	24	15	20	9	13	0	0	13	0
200,2 mg/l	2	1	0	0	0	0	0	0	0	0
	4	2	0	0	0	0	0	0	0	0
	6	2	0	0	0	0	0	0	0	0
	24	2	1	1	0	4	15	0	0	0
	48	24	15	38	9	26	25	0	4	42
300,3 mg/l	2	1	1	0	0	0	0	0	0	0
	4	2	1	0	0	0	5	0	0	0
	6	2	1	0	0	0	5	0	0	0
	24	2	3	3	0	8	15	0	4	17
	48	24	29	42	9	56	35	0	8	100

In our tests (TABLE 4) 100,1 mg/l diclofenac sodium salt had a significant impact on the organisms compared to the the control and 1 % methanol, as judged by the percentage dead. The most sensitive was *Daphnia magna* which reacted almost immediately. In concentrations 200,2 mg/l and 300,3 mg/l all organisms had a larger death percentage compared to the control, 2 and 3 % methanol. The impact of diclofenac sodium salt started to show in *Artemia salina* between 6 and 48 hours, however with *Daphnia magna* the effect was almost immediate. The results suggest that diclofenac sodium salt has a great impact on the organisms.

TABLE 4. Results from the diclofenac sodium salt tests.

		Juvenile *Artemia salina*			Adult *Artemia salina*			*Daphnia magna*		
		Death %			Death %			Death %		
	h	C	Met	Dic	C	Met	Dic	C	Met	Dic
100,1 mg/l	2	1	0	0	0	4	9	0	0	0
	4	2	0	0	0	4	9	0	0	4
	6	2	0	1	0	4	9	0	0	83
	24	2	1	20	0	4	23	0	4	100
	48	24	15	93	9	13	64	0	13	100
200,2 mg/l	2	1	0	0	0	0	0	0	0	4
	4	2	0	0	0	0	0	0	0	92
	6	2	0	0	0	0	0	0	0	100
	24	2	1	56	0	4	20	0	0	100
	48	24	15	100	9	26	85	0	4	100
300,3 mg/l	2	1	1	1	0	0	5	0	0	75
	4	2	1	1	0	0	5	0	0	100
	6	2	1	1	0	0	25	0	0	100
	24	2	3	70	0	8	80	0	4	100
	48	24	29	99	9	56	100	0	8	100

In our tests (TABLE 5) the lowest concentration of erythromycin hydrate (101,5 mg/l) did not have significant impact to organisms compared to the control and 1 % methanol. At a concentration of 203,0 mg/l more *Daphnia magna* died compared to control and 2 % methanol after 24 hours. At the higest concentration (304,5 mg/l) adult *Artemia salina* and *Daphnia magna* had larger death percentages compared to the control and 3 % methanol. However, juvenile *Artemia salina* had a smaller death percentage compared to control or 3 % methanol. The results suggest that erythromycin hydrate impacts on the adult *Artemia salina* and *Daphnia magna* in bigger concentrations.

TABLE 5. Results from the erythromycin hydrate tests.

		Juvenile *Artemia salina*			Adult *Artemia salina*			*Daphnia magna*		
		Death %			Death %			Death %		
	h	C	Met	Ery	C	Met	Ery	C	Met	Ery
101,5 mg/l	2	1	0	0	0	4	0	0	0	0
	4	2	0	0	0	4	0	0	0	0
	6	2	0	0	0	4	0	0	0	0
	24	2	1	0	0	4	9	0	4	8
	48	24	15	14	9	13	9	0	13	8
203,0 mg/l	2	1	0	0	0	0	5	0	0	0
	4	2	0	0	0	0	5	0	0	0
	6	2	0	0	0	0	5	0	0	0
	24	2	1	1	0	4	5	0	0	8
	48	24	15	18	9	26	23	0	4	50
304,5 mg/l	2	1	1	0	0	0	5	0	0	0
	4	2	1	0	0	0	15	0	0	0
	6	2	1	0	0	0	20	0	0	8
	24	2	3	0	0	8	20	0	4	13
	48	24	29	3	9	56	45	0	8	79

In our tests (TABLE 6) 100,1 mg/l lidocaine had a significant impact on *Artemia salina* compared to the control and 1 % methanol. In concentrations of 200,2 mg/l and 300,3 mg/l all organisms had a greater death percentage compared to control, 2 and 3 % methanol. The impact of lidocaine started to show in the juvenile *Artemia salina* and *Daphnia magna* after 24 hours. Adult *Artemia salina* reactions could be seen after only 4 hours. The results suggest that lidocaine has a great impact on the organisms and especially the adult *Artemia salina*.

TABLE 6. Results from the lidocaine tests.

		Juvenile *Artemia salina*			Adult *Artemia salina*			*Daphnia magna*		
		Death %			Death %			Death %		
	h	C	Met	Lid	C	Met	Lid	C	Met	Lid
100,1 mg/l	2	1	0	0	0	4	0	0	0	0
	4	2	0	0	0	4	5	0	0	0
	6	2	0	0	0	4	10	0	0	0
	24	2	1	10	0	4	10	0	4	0
	48	24	15	73	9	13	19	0	13	4
200,2 mg/l	2	1	0	0	0	0	5	0	0	0
	4	2	0	0	0	0	15	0	0	0
	6	2	0	0	0	0	15	0	0	0
	24	2	1	43	0	4	30	0	0	4
	48	24	15	94	9	26	55	0	4	50
300,3 mg/l	2	1	1	0	0	0	0	0	0	0
	4	2	1	0	0	0	5	0	0	0
	6	2	1	0	0	0	20	0	0	0
	24	2	3	60	0	8	30	0	4	4
	48	24	29	89	9	56	90	0	8	88

In our tests (TABLE 7) 101,0 mg/l and 202,0 mg/l concentrations of sodium diatrizoate hydrate had no significant impact on the organisms compared to the control, 1 or 2 % methanol. In fact adult *Artemia salina* and *Daphnia magna* survived better. At a concentration of 303,0 mg/l adult *Artemia salina* and *Daphnia magna* had a larger percentage dead compared to control and 3 % methanol. The impact of sodium diatrizoate hydrate started to show in adult *Artemia salina* from the beginning of the test and in *Daphnia magna* after 48 hours. The results suggest that sodium diatrizoate hydrate has impact to adult *Artemia salina* and *Daphnia magna* in higher concentrations.

TABLE 7. Results from the sodium diatrizoate hydrate tests.

		Juvenile *Artemia salina*			Adult *Artemia salina*			*Daphnia magna*		
		Death %			Death %			Death %		
	h	C	Met	Sod	C	Met	Sod	C	Met	Sod
101,0 mg/l	2	1	0	0	0	4	5	0	0	0
	4	2	0	0	0	4	5	0	0	0
	6	2	0	0	0	4	5	0	0	0
	24	2	1	1	0	4	5	0	4	0
	48	24	15	15	9	13	5	0	13	0
202,0 mg/l	2	1	0	0	0	0	0	0	0	0
	4	2	0	0	0	0	0	0	0	0
	6	2	0	0	0	0	0	0	0	0
	24	2	1	2	0	4	5	0	0	0
	48	24	15	24	9	26	5	0	4	5
303,0 mg/l	2	1	1	0	0	0	10	0	0	0
	4	2	1	0	0	0	10	0	0	0
	6	2	1	1	0	0	10	0	0	0
	24	2	3	2	0	8	15	0	4	5
	48	24	29	25	9	56	20	0	8	64

In our tests (TABLE 8) all concentrations sulfamethoxazole had no significant impact to *Artemia salina* compared to the control, 1, 2 and 3 % methanol. However *Daphnia magna* was more sensitive showing a greater percentage dead after 24 hours in every concentration. The results suggest that sulfamethoxazole impacts on *Daphnia magna* in all concentrations.

TABLE 8. Results from the sulfamethoxazole tests.

		Juvenile *Artemia salina*			Adult *Artemia salina*			*Daphnia magna*		
		Death %			Death %			Death %		
	h	C	Met	Sul	C	Met	Sul	C	Met	Sul
100,9 mg/l	2	1	0	0	0	4	0	0	0	0
	4	2	0	0	0	4	0	0	0	0
	6	2	0	0	0	4	0	0	0	0
	24	2	1	0	0	4	0	0	4	29
	48	24	15	15	9	13	0	0	13	58
201,8 mg/l	2	1	0	0	0	0	0	0	0	0
	4	2	0	0	0	0	0	0	0	0
	6	2	0	0	0	0	0	0	0	0
	24	2	1	0	0	4	0	0	0	38
	48	24	15	14	9	26	0	0	4	71
302,7 mg/l	2	1	1	0	0	0	0	0	0	0
	4	2	1	0	0	0	0	0	0	0
	6	2	1	0	0	0	0	0	0	0
	24	2	3	2	0	8	0	0	4	63
	48	24	29	34	9	56	24	0	8	100

6.2 Prokaryotic organism

In our tests (TABLE 9) all the pharmaceuticals except lidocaine and sulfamethoxazole had a negative effect on bacterial growth. Lidocaine and sulfamethoxazole in low concentrations actually increased the growth. These results suggest that these pharmaceuticals are harmful to bacteria growth.

TABLE 9. Results from the *Pseudomonas fluorescens* tests.

Pseudomonas fluorescens					
Atenolol			Diclofenac sodium salt		
	490 nm	600 nm		490 nm	600 nm
100,3 mg/l	-0,06	-0,01	101,0 mg/l	-0,040	0,010
200,6 mg/l	-0,06	-0,04	202,0 mg/l	-0,081	-0,049
300,9 mg/l	-0,12	-0,05	303,0 mg/l	-0,175	-0,108
Erythromycin hydrate			Lidocaine		
	490 nm	600 nm		490 nm	600 nm
100,6 mg/l	-1,000	-0,229	100,2 mg/l	0,123	0,022
201,2 mg/l	-1,000	-1,000	200,4 mg/l	0,031	0,006
301,8 mg/l	-1,000	-1,000	300,6 mg/l	-0,201	-0,164
Sodium diatrizoate hydrate			Sulfamethoxazole		
	490 nm	600 nm		490 nm	600 nm
100,4 mg/l	-1,000	-1,000	100,5 mg/l	0,085	0,033
200,8 mg/l	-0,008	0,009	201,0 mg/l	-0,195	-0,104
301,2 mg/l	-0,086	-0,049	301,5 mg/l	-0,249	-0,149

6.3 Potential errors

While we conducted the Eukayotes testing using sufficient replicates, the bacterial test was only done using one tube. We had only a limited time to carry out all the ecotoxicological tests and that made it impossible to repeat the tests for more reliable results. In addition, identifying when an organism was dead introduced some error into the analysis. We believe that alternative methods, to visual assessment, should be considered when judging if an organism is dead or alive.There is always a change for human error when doing tests where you need to be accurate and careful.

We had difficulties to read the bacteria results with spectrophotometer because there was a problem with alignment of the machine. In addition the coloured product of the breakdown of CellTiter® settled with time in the curvettes and thus the use of microtitre plates and a suitable reader is suggested as an approach to be considered. The main purpose of these tests was to develope the methodology for future bacteria testing with pharmaceuticals. We managed to do that but there are still some things to figure out, for example the incubating time with CellTiter® and right wavelength to read the results.

7 CONCLUSIONS

Ecotoxicological tests were successful and we managed to get results from every pharmaceutical studied. The results from this study suggest that pharmaceuticals have an acute impact to organisms certainly at high concentrations. We managed to establish methods for long term culturing of *Daphnia magna* and *Artemia salina*.

There are many things to study when talking about pharmaceuticals in wastewater. Some studies of the subject have been made but the knowledge about pharmaceuticals' impact to environment is poor. All the tests we made should be made in bigger scale.

We did not have time to test with pharmaceutical mixtures. There have been a few studies that suggest that many pharmaceuticals which are not harmful in single compound can be more toxic in mixture with other pharmaceuticals (Fent, Weston & Caminada 2005). This would be interesting field to study because of the lack of information.

There are a lot of acute tests made when studying the effects of pharmaceuticals. However chronic toxicity testing is minor compared to acute toxicity tests. The lifecycle of many organisms are longer than few days so the chronic effects do not show up in short time exposure. (Fent, Weston & Caminada 2005). However, as mentioned previously, we established methods for long term culture of *Daphnia*

magna and *Artemia salina* and these methods could be utilised during the evaluation of the chronic effects of pharmaceticals on test organisms.

The main purpose of bacteria tests was to develope the methodology for future tests. We managed to do so and we are hoping that maybe someone working in Pills will continue our work. It would be interesting to test the effect of pharmaceuticals to bacteria in longer time period.

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APPENDICES

APPENDIX 1 The results of wet and filter weight, wet chemistry and BOD

APPENDIX 2 The results of cadmium, lead and zinc

APPENDIX 3 The list of pharmaceuticals from Pills

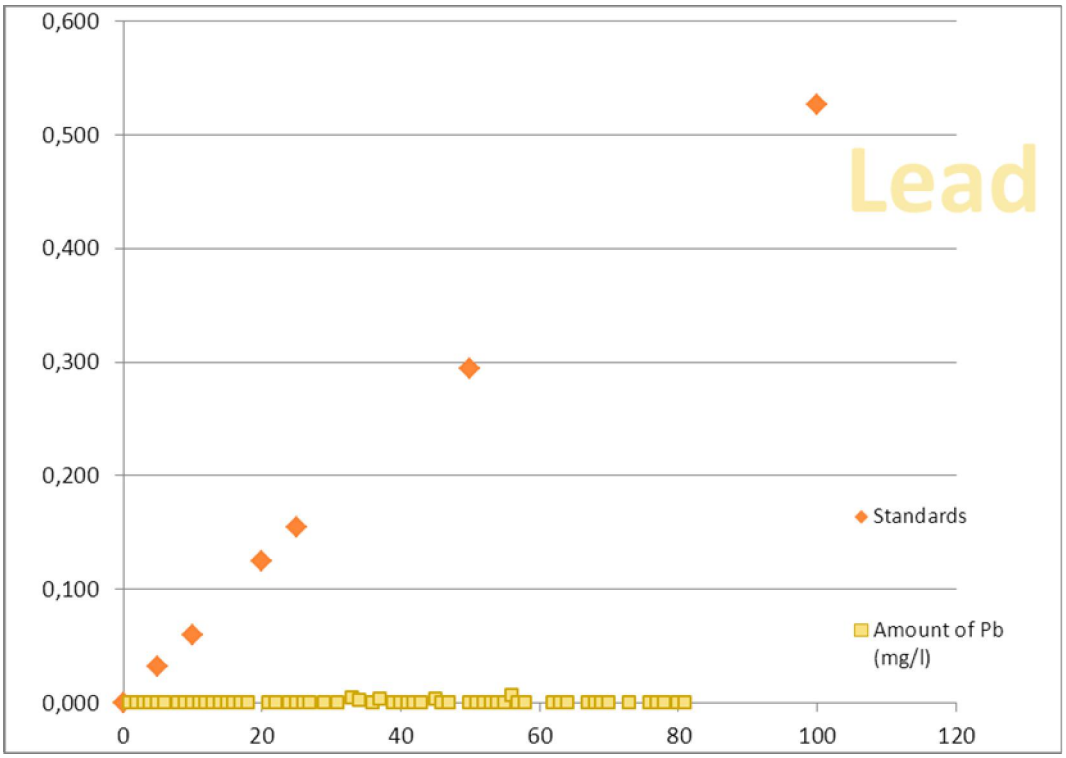
APPENDIX 1 The results of wet and filter weight, wet chemistry and BOD

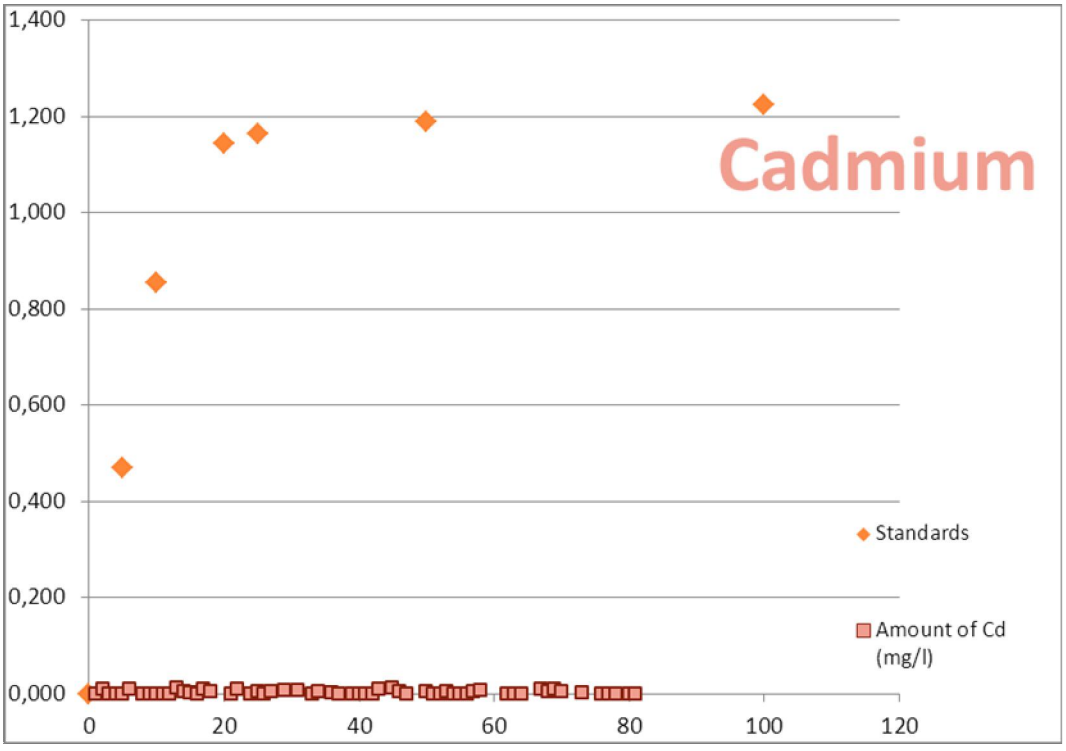
Place	Season	Total solids g/l	Volatile solids g/l	Suspend ed solids	BOD (5)	COD	Total nitrogen	Nitrate nitrogen	Ammoni acal	Total phosphorus	Orthoph os.	Nitrate	Ammonia	Orthoph osphate	Aluminium
Western Infirmary	Autumn	1,19	0,82	0,07	385,29	615,71	8,80	0,08	12,10	5,00	3,67	0,00	12,10	13,09	0,12
	Winter	2,11	1,84	0,06	315,43	720,00	27,82	0,92	31,61	5,65	4,01	4,15	31,61	12,25	0,14
Drumchapel	Autumn	2,49	2,04	0,36	354,33	681,67	10,73	0,71	23,91	10,61	7,68	3,23	23,91	22,37	0,06
	Winter	1,50	1,66	0,20	284,00	794,44	17,68	1,34	32,97	8,94	6,50	5,83	32,97	20,06	0,29
Shieldhall	Winter	2,78	2,63	0,14	153,00	494,44	20,28	0,88	21,16	9,27	8,78	4,04	21,16	27,04	0,23
BGH	Autumn	1,59	0,90	10,46	286,00	562,14	13,23	0,60	19,39	8,00	6,10	3,38	19,39	18,58	0,07
	Winter	1,48	1,09	3,95	284,73	611,52	22,11	0,62	22,92	10,06	6,91	2,54	22,92	21,29	0,07
	Spring	1,50	1,38	0,05	272,40	740,00	18,34	0,55	26,23	11,29	9,15	2,41	26,23	28,03	0,12
Melburn Lodge	Winter	1,19	0,96	0,03	113,00	315,56	9,03	0,71	15,06	2,68	1,92	2,77	15,06	5,88	0,05
	Spring			0,05	303,00	1298,33	34,50	0,83	23,90	4,31	6,81	3,72	23,90	20,81	0,28

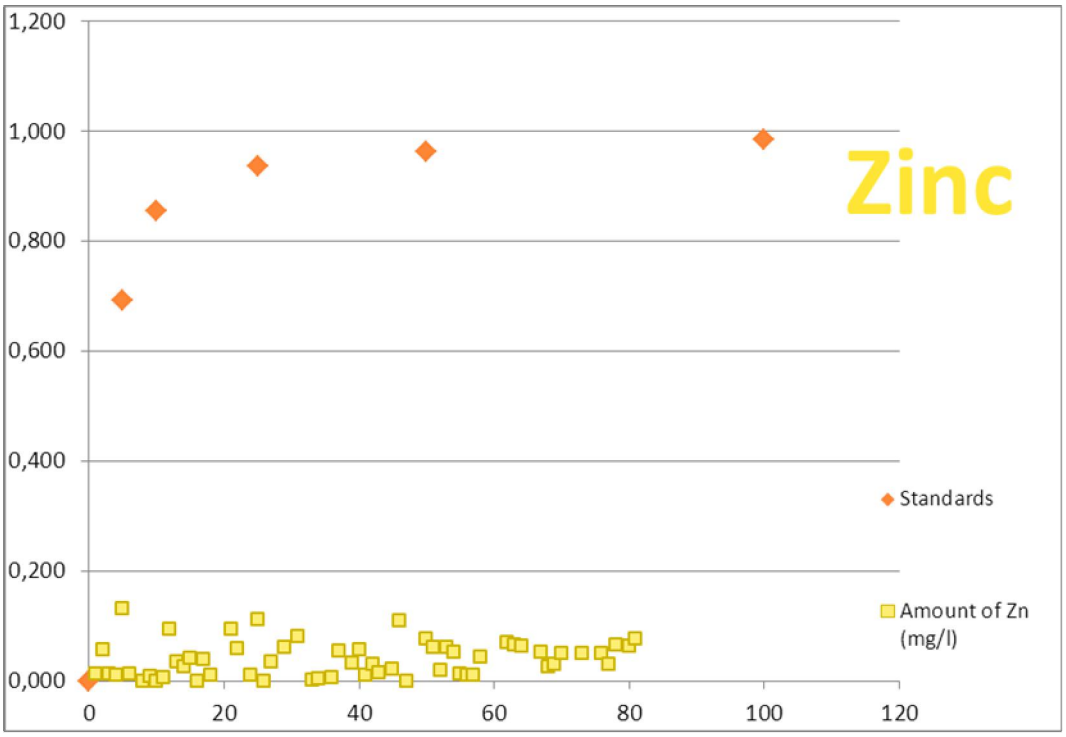
APPENDIX 2 The results of cadmium, lead and zinc

Glasgow			
Drumchapel Hospital			
Date	mg/l		
	Pb	Cd	Zn
8./12.10.2010	0,000	0,000	0,000
26.10.2010	0,004	0,000	0,002
2.11.2010	0,003	0,000	0,054
15.11.2010	0,000	0,010	0,015
22.11.2010	0,000	0,005	0,110
29.11.2010	0,000	0,005	0,076
14.3.2011	0,000	0,000	0,030
21.3.2011	0,000	0,000	0,077
Western Infirmary			
Date	mg/l		
	Pb	Cd	Zn
21.9.2010	0,000	0,000	0,093
29.9.2010	0,000	0,010	0,059
12.10.2010	0,000	0,004	0,112
2.11.2010	0,000	0,000	0,033
8.11.2010	0,000	0,000	0,010
7.2.2011	0,000	0,000	0,065
14.3.2011	0,000	0,000	0,065
Shieldhall WWTP			
Date	mg/l		
	Pb	Cd	Zn
20.12.2010	0,000	0,000	0,012

Melrose			
Borders General Hospital			
Date	mg/l		
	Pb	Cd	Zn
15.10.2010	0,000	0,007	0,061
20.10.2010	0,000	0,007	0,080
27.10.2010	0,000	0,002	0,006
3.11.2010	0,000	0,000	0,057
10.11.2010	0,000	0,000	0,031
17.11.2010	0,003	0,011	0,022
24.11.2010	0,000	0,000	0,000
15.12.2010	0,000	0,000	0,062
15.12.2010	0,000	0,000	0,020
15.12.2010	0,000	0,003	0,060
15.12.2010	0,000	0,000	0,052
12.1.2011	0,006	0,000	0,011
19.1.2011	0,000	0,004	0,011
26.1.2011	0,000	0,007	0,044
2.2.2011	0,000	0,000	0,069
9.2.2011	0,000	0,000	0,063
15.2.2011	0,000	0,010	0,052
23.2.2011	0,000	0,004	0,050
2.3.2011	0,000	0,001	0,051
9.3.2011	0,000	0,000	0,051
16.3.2011	0,000	0,000	0,064
Melburn Lodge			
Date	mg/l		
	Pb	Cd	Zn
27.10.2010	0,002	0,003	0,004
15.2.2011	0,000	0,005	0,026
23.2.2011	0,000	0,010	0,030


0,600
0,500
0,400
0,300
0,200
0,100
0,000
0
20
40
60
80
100
120
Lead
Standards
Amount of Pb (mg/l)


1,400
1,200
1,000
0,800
0,600
0,400
0,200
0,000
0
20
40
60
80
100
120
Cadmium
Standards
Amount of Cd (mg/l)


Zinc
1,200
1,000
0,800
0,600
0,400
0,200
0,000
0
20
40
60
80
100
120
Standards
Amount of Zn (mg/l)

APPENDIX 3 The list of pharmaceuticals from Pills

Type of pharmaceutical	Specific compound
Analgesics / Anti-inflammatories	Diclofenac Lidocaine Naproxen
Cytostatics	Cyclophosphamide Ifosfamide
Antibiotics	Amoxicillin Ciprofloxacin Clarithromycin Erythromycin Sulfamethoxazole Acetyl-sulfa methoxazole
X-Ray Contrast	Diatrizoate Iopamidol Iopromide
Anticonvulsants / tranquilisers	Carbamazepin
Lipid Regulators	Bezafibrate
Betablockers / Anti-hypertensives	Atenolol