UNIVERSITAT D'ALACANT
Facultat de Ciències
Facultad de Ciencias

FACULTY OF SCIENCES

DEGREE IN BIOLOGY

FINAL PROJECT

ACADEMIC YEAR [2017 -2018]

TITLE:

THE EFFECT OF COMMON PHARMACEUTICAL COMPOUNDS (IBUPROFEN & PARACETAMOL) ON *GAMMARUS PULEX*, A FRESHWATER INVERTEBRATE

AUTHOR:

CARLOS MATAIX RIGAL

Abstract

Pollution in rivers is a problem on the agenda in many countries, specifically byproducts derived from the pharmaceutical industry are detected globally in surface waters; Unfortunately the ecological impacts of these biologically active and ubiquitous chemicals are largely unknown; For this purpose, techniques such as biomonitoring through the use of benthic macroinvertebrates are used in many cases to evaluate water quality; The truth is that most methods use measurements at the community level, and the use of responses from unique species has been limited despite its potential benefits. In this work, toxicity tests were developed for a single species, in which the symptomatology was evaluated by exposure to two different and common drugs (Paracetamol and Ibuprofen) on the freshwater amphipod *Gammarus pulex*. The trial was implemented in a non-contaminated reference site to quantify the effects at different concentrations in terms of doses of the relevant drugs and to elucidate changes in behaviour and mortality rates.

The ability of the assay to detect the sensitivity of this organism was evaluated by using different concentrations of the compound diluted in the medium, with controlled replicates; It is also observed the ecological relevance that the presence of this type of derivative active principles would have, once they are exposed in the environment due to their accidental release from the industry itself; Gammarus pulex is therefore an important detritivore in stream communities, there was a correlation between exposure to paracetamol which had severe effects with concentrations greater than 16 mg/L, after 24 hours. And a strong negative correlation with severe effects after long exposure from ibuprofen even at low doses; Ibuprofen did not significantly affect any of the measured parameters of Gammarus spp. The *G. pulex* toxicity test is a short-term sublethal biomonitor of water quality that is indicative of responses at the community and ecosystem levels that occur over longer periods of time; It is robust, receptive and relevant.

Keywords: "*Gammarus pulex", "*Toxicity test", "Drugs", "Behaviour", "Mortality"

Resumen

La contaminación en los ríos es un problema a la orden del día en muchos países, concretamente subproductos derivados de la industria farmacéutica son detectados globalmente en aguas superficiales, desafortunadamente los impactos ecológicos de estos productos químicos biológicamente activos y ubicuos, son en gran parte desconocidos; Para ello, técnicas como la biomonitoración mediante el uso de macroinvertebrados bentónicos son utilizadas en muchos casos para evaluar la calidad del agua; Lo cierto es que la mayoría de los métodos usan mediciones a nivel comunitario, y el uso de respuestas a nivel específico ha sido limitado a pesar de sus beneficios potenciales. En este trabajo se desarrollaron test de toxicidad para una sola especie, en los que se evaluó la sintomatología por exposición a dos fármacos distintos y comunes (Paracetamol e Ibuprofeno) sobre el anfípodo de agua dulce *Gammarus pulex*. El ensayo se implementó en un sitio de referencia no contaminado para cuantificar los efectos a distintas concentraciones en cuanto a dosis de los fármacos pertinentes y para también dilucidar cambios en el comportamiento.

La capacidad del ensayo para detectar la sensibilidad de este organismo se evaluó pues empleando distintas concentraciones del compuesto diluido en el medio, con réplicas controladas; Se observa también la relevancia ecológica que tendría la presencia de este tipo de principios activos derivados, una vez quedan expuestos en el medio por su liberación accidental desde la propia industria; *Gammarus pulex* es pues un importante detritívoro en las comunidades de arroyos, existió correlación entre la exposición al paracetamol, el cual tenía efectos severos con concentraciones mayores a 16 mg/L al cabo de 24 horas. Y una fuerte toxicidad tras una larga exposición por parte del ibuprofeno incluso a bajas dosis. El test de toxicidad para *G. pulex* concluye pues, como un biomonitor subletal a corto plazo de la calidad del agua, que es indicativo de las respuestas a nivel de la comunidad y del ecosistema que se producen durante períodos de tiempo más largos; Es robusto, receptivo y relevante.

Palabras clave: "*Gammarus pulex", "*Toxicity test", "Fármacos", "Comportamiento", "Mortalidad"

Index

1. Introduction, Topic Background and Objectives

Species of aquatic amphipods grouped in the set of freshwater macroinvertebrates (i.e. *Gammarus sp*) play an increasingly important role in the field of freshwater ecology, often dominating the benthos in areas of well oxygenated near-shore and shallow waters (Barton and Hynes 1976; Clemens 1950; Winnell and Jude 1987); Moreover, amphipods are a major, but potentially vulnerable, component of the food webs of rivers; So consequently the presence of certain pollutants could set them in a precarious state due to prolonged exposure to low concentrations of anthropogenic chemicals that may lead to sublethal effects, including changes in behaviour, added to their relative sensitivity to toxicants, the suggestion put amphipods in the spotlight of being one of the most vulnerable groups of organisms to chemical pollution (De Lange HJ, 2018).

Taking into consideration previous assessments from the past 10 years based on biological indicators as sentinel species, about 60% are based on macroinvertebrate analysis (De Pauw & Hawkes, 1993); Therefore, interest has been shown in the application of biological water-quality monitoring techniques using macroinvertebrates, which tend to be advantageous, cost-effective and simple in use.

Since 1999, an attempt has been made towards the elaboration of a biological method, in relation with European Union requirements, for assessing the biological quality of running water (Czerniawska-Kusza, 2005) as ideas and suggested implementations are put into the context of the European Water Framework Directive guidelines (WFD; 2000/06/EC) in a more holistic approach, as it is aimed at maintaining of the integrity of the ecosystem characteristics. There is, however, a large group of chemicals that have not been studied or regulated well, namely pharmaceuticals as their harmful effects in the environment were ignored (e.g. Daughton and Ternes, 1999).

Gammarus was the focal species for this research study as it is commonly found across most rivers and lakes in Scotland and as they are considered “keystone species”, since they sustain the ecological integrity (structure and productivity) of their ecosystems; Furthermore, amphipods are usually very sensitive to toxic substances and often used in toxicology studies. (Anderson 1982; Green *et al.* 1985; Williams *et al.* 1985).

1.1.Objectives

The objectives of the present study were to assess *Gammarus sp*. toxicity tests to investigate the effects of paracetamol and ibuprofen on physiological and behavioural responses, and to evaluate mechanisms of action of these drugs. To achieve these goals, different levels of pharmaceuticals were used to assess its effects on survival and activity responses.

1.2.Taxa

The crustacean sub-order *Gammaridea* comprises over 4500 species, that is, approximately 85% of the order Amphipoda (Bousfield, 1973); *Gammaridea* are widespread throughout a range of marine, freshwater and terrestrial habitats (Bousfield, 1973; Lincoln, 1979; Lincoln & Boxshall, 1989). The amphipod genus with the highest number of epigean freshwater species is *Gammarus*, which embraces over 100 freshwater species distributed widely throughout the northern hemisphere (Karaman & Pinkster, 1977).

The most representative species are found in freshwater aquatic ecosystems in Scotland, include *Gammarus pulex* and *G. zaddachi* both inhabitants of high-energy environments, such as high courses of rivers and estuaries;

They show the classic amphipod profile: flattened body from side to side, with seven pairs of thoracic walking legs, and six pairs of abdominal limbs; Gammarids grow to about 5 to 30 mm (0.2 to 1.2 inches).

Figure 1: Gammarus pulex, pre-copula pair, male on the top (Cart, 2014).

Target species occur primarily in freshwater, even though, being quite tolerant to salinity and temperature variations; They are locally abundant among aquatic plant growth, since they are omnivorous, ingesting algae, rests of plants, mud and faecal pellets, playing an important role in detritus processing in streams and is an important prey species for fish (Andersen *et al*., 1993).

Gammarus species can localize their food (De Lange *et al*., 2005) and detect predators through chemical cues from fish and injured conspecifics; The most sensitive primary target are the gills of those crustaceans (Wood, 2011), multifunctional organs crucial for respiration, osmotic and ionic regulation, ammonia excretion and acid-base balance (Henry *et al*., 2012); Also changes in behaviour such as hiding in response to detected predators are crucial to optimize survival chances (e.g. Williams and Moore, 1985; Wudkevich *et al*., 1997; Wisenden *et al*., 1999, 2001; Baumgartner *et al*., 2002; Åbjörnsson *et al*., 2004).

Therefore, crustaceans exposed to water-borne pollutants, environmental stressors, and pathological agents usually exhibit disruptions of osmoregulation, including alterations in the structure of the branchial and excretory organs. Due to this reasons *G. pulex* has been incorporated in a variety of toxicity tests, including feeding activity (Taylor *et al*., 1993), precopula separation (Pascoe *et al*., 1994), scope for growth (Maltby *et al*., 1990), in situ tests (Crane and Maltby, 1991), and behaviour (e.g. Gerhardt *et al*., 1994).

The increasing awareness as far as emergent pollutants are concerned has resulted in decreasing concentrations of the emissions of at least those toxics forming part of the "conventional" set (la Farré et al., 2008); Consequently *Gammarus* has been used in diverse tests of exposure to chemicals such as dissolved metals (e.g. cadmium and copper) or more complex substances like pesticides and studies related with oil and derivatives, which are common constituents in coastal pollution especially in dredged and harbour sediments (Sanz-Lázaro C, 2018).

It has been in recent studies when, due to continuous discharge of pharmaceuticals and personal care products may result in a chronic exposure of aquatic organisms to these substances and their metabolites; That despite of not result in lethal toxicity (D, 2018), its prolonged exposure to low concentrations of anthropogenic chemicals may lead to sublethal effects, including changes in behaviour.

1.3.Pharmaceuticals

The final destination after use, of drugs belonging to pharmaceutical industries is scarcely known, and already low concentrations of these bioactive compounds may have significant effects on aquatic organisms (Halling-Sørensen *et al*., 1998); It is acknowledged that removal efficiencies of the sewage treatment plans (STP) vary widely both from one group of substances to another, and from one individual active ingredient to another within the same group (Schrap *et al*., 2003); So part of these pharmaceuticals and their metabolites that are excreted via faeces and urine end up in the aquatic environment since they can pass through the STPs

The problem is therefore generated due to those pharmaceuticals consumed by humans and animals that can be either completely, partially or not metabolized at all, which generates either intact molecules or metabolites (Kümmerer, 2010). This represents a real danger, especially that these discharges into the environment are continuous.

Just in the first broad investigation of drugs and their residues in water, 80% of 32 selected drugs were detectable in at least one sewage treatment plant effluent, and 20 different drugs and four corresponding metabolites were detected in rivers and streams (Ternes, 1998).

On the other hand, disposal of pharmaceuticals may enter the environment via another pathway. Bound and Voulvoulis (2005) carried out a survey in which they interviewed the members of 400 households in the UK to investigate the household disposal of unused and expired pharmaceuticals. They found that about half of the respondents did not finish their drugs and among those 63.2% discarded their unfinished drugs in household waste, 21.8% brought them back to a pharmacist, and 11.5% discarded them into the sink or toilet.

In this experiment two common and widely known drugs were used; Ibuprofen (non-steroidal anti-inflammatory, antipyretic and analgesic); And paracetamol (acetamidophenol an analgesic–antipyretic).

2. Materials and methods

2.1. ***Gammarus*** **collection**

"Site" and dates

Specimens were collected between the months of February and May of 2018 in the River of Dee, Aberdeenshire, Scotland. The collection was performed in one affluent allocated close to Peterculter, a suburb found in the southern-western part of the city of Aberdeen (Lat: 57.10063 | Long: -2.202725).

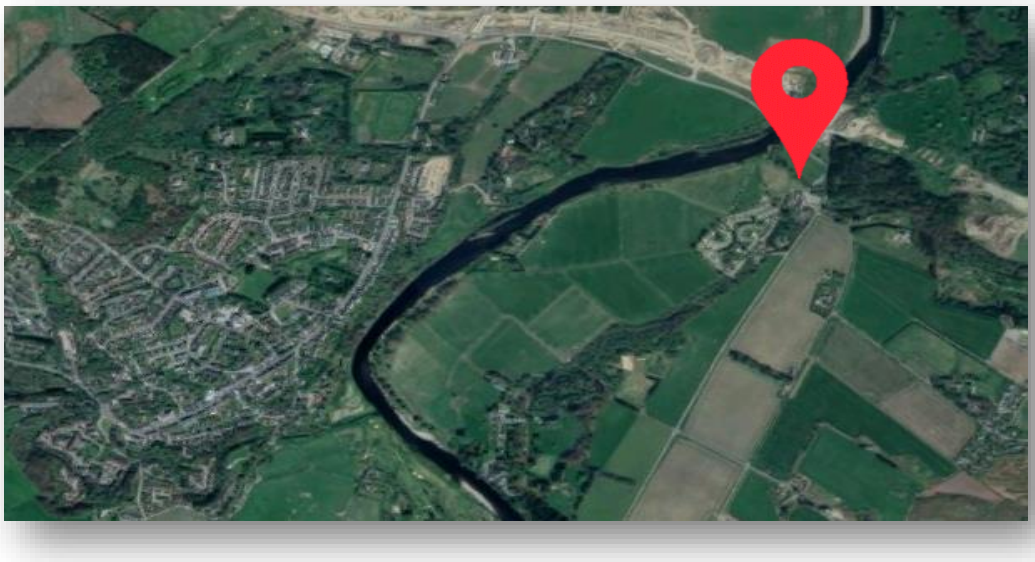


Figure 2: Sampling location: Affluent of the Dee River close to the main meander of Peterculture suburb.

Five weeks of lab work were carried out, the samples were taken with a week in advance, so the first visit to Dee River took place the second week of February, and the next ones were just labours to restock the population of the aquariums; Therefore, the second and fourth week of March were the moments when, and the last one took place in May.

The sampling-sorting performance was planned using a whole day, that week before the toxicity test, just to ensure the organisms to adapt to captivity into the tanks, feed them properly, and normalize the conditions in the media.

Gammarids capture protocol

These crustaceans were first sorted from river samples mostly composed by litter fall and the proper substrate, where many other kinds of invertebrates were found as well.

First day of sampling, I counted with some extra help, students forming part of the Master (MSc) "Environmental and Ecological Sciences" were taking samples too, so it got easier to stablish the first colony of individuals that would become separated in two tanks. *Gammarus* searching was then carried out using nets scratching on the river bed and under stones in areas where shallow waters separated from the riffles were found. The strategy was first removing some stones using the wellies and taking advantage of the water flow current putting the net downstream, so all the matter and organisms in suspension were flowing towards it and trapped in there.

*Figure 3: **Left**: Affluent of river Dee were the catchment was done, low tide; **Right**: Net used for sampling.*

A noteworthy fact during the course of the catches was the influence of the tides on all rivers invertebrates; Depending on the rain regime, amount of sediments or water level of the river, in the case of the chosen amphipods, they were changing the place of establishment; Thus if the water came all brownish charged with more than usual sediments in suspension (more nutrients) and higher tides, the target crustacean preferred then to be attached to the riparian vegetation that was submerged into the river, showing higher amounts of individuals (Jenkins, 1985), so the collection task was even easier to carry out; Conversely clear waters and lowers levels of tides, set them under the rocks at midpoints of the river, just stuck on the bottom of it.

2.2. ***Gammarus*** **sorting**

Once the samples were brought from the river to the lab, they first had to be carried in two kinds of buckets:

1) Water from the river that was used to refill the water level of the thanks and for rushing the tank cycle-time the first moment of the setup.

2) Mixture of substrate, literfall, water and organic matter where all organisms were included.

Water buckets could be easily added to the tanks, but in case of the other ones containing organic matter, the procedure was much more laborious; Since the samples arrived at the laboratory at midday the delay could not be too big for the separation of the crustaceans; The new artificial conditions of steady state in which water was found placed the set now in frail conditions; Because, despite of not having constant flow of water and even a lower temperature due to the conditions of the lab, organisms were still doing their proper metabolism rates; So the runoff of oxygen was performed just in a few hours due to respiration from even cellular level (e.g. Bacteria, Protozoa…); So organisms normally adapted to high concentrations of oxygen, own of the upper course of the rivers, were the first ones suffering the consequences.

Taking random parts of the submerged leaf litter from the buckets, pieces of the set were placed then on a big white tray with a certain amount of water as all the swimming macroinvertebrates were able to swim away from all solid bodies, mostly composed by broken plant parts and little stones;

Between all the organisms present in the samples, those of the *Amphipoda* order were the most abundant (the target species), but also their relative abundance during sampling in comparison with other species was influenced by the state in which the river was found, as it could be the season of the year, etc; They were followed by the *Plecoptera* order (stoneflies), which were in more abounds than amphipods when the river came down with low levels of tide and its waters were not turbid at all; Other orders found for example were: *Ephemeroptera, Megaloptera*, and *Trichoptera* among others, all corresponding to their first life stages (aquatic phases); On the other hand other further clades, such as earthworms (belonging to the *Annelida* Phylum) and other organisms with its life cycle closely linked to the river bed.

In this way the *Gammarus* sorting after their identification in the samples could be just separated using a couple of plastic spoons, especially for the largest size individuals, enclosing the passage helped by the small level of water that the tray allowed, but the most effective way was to improvise a plastic pipette with a diameter enough to "suck" the ones of medium and small size, this process was much faster and easier.

After several rounds extracting the solid parts, when finishing each one´s searching and sorting, the rest of organic matter and substrate, and the rest of the organisms of course, were discarded; So, the target species was directly added to the tanks.

2.3. **Setups**

- Aquarium setup

To maintain a stable population of *Gammarus sp.* individuals, two aquariums of 50 liters each (50x30x34 cm) were prepared to house enough organisms for at least two or three rounds for the experiment before re-stocking them.

Both aquariums were arranged with internal filters and aerators, for maintaining a constant flow of water and oxygen; The first filling was made with a major part of river water and another one of tap water that was previously treated for a few hours with a chlorine removal aquarium chemical.

No substrate was used in the aquaria, since the main idea was to acclimatize the animals and keep them only for a short period of time, and the presence of it would have made difficult the task of capturing them within the tanks afterwards; On the other hand they were provided with plastic plants, as artificial refuges, since these organisms prefer to be attached to solid structures, places also where they usually feed on; So just using an aquarium net, taking the whole plastic plant was the way how a lot of individuals became trapped, thus suffering less stress by manipulation.

The maintenance of the tanks was easy-going, the aquariums received a cycle of natural light and the populations of *Gammarus* were fed with pellets for fish, once every 4 or 5 days; Finally, the refilling of the tanks was done every two weeks.

Figure 4: Aquariums display; 50 liter capacity each

- Experiment setup

For the assembly of the experiment itself, it was performed in laboratory conditions (NIST standard conditions: 20ºC and 1 atm) and under average photoperiods of ≥ 10 h of light/day; So plastic cups with a capacity of half a liter each were used as the containers of the groups of 10 *Gammarus* that would be tested in each one; All concentrations used had a total number of three replicates, starting from a base concentration of zero, called control (C), and increasing the dose according to the volume of solution containing the pills (dissolved with mili Q water) that was added into the cups.

The concentrations that were used of paracetamol the first two weeks out of four during testing, were lower than the last two, since the lethal dose of this drug was overestimated and the second week only served as a double check adding an extra concentration [8 mg/L], but the lethal doses of this chemical still was not found; So starting from the maximum concentration of that second week, a new range was then established; That latter concentration was in that way being doubled till reaching eight times the initial one, becoming the new highest dose [64 mg/L]. In case of Ibuprofen, the experimental time lasted only a week, because this drug showed more severe effects in terms of its reaction but it would serve to establish a comparative.

Week	Concentrations of Paracetamol [mg/L]						
1st, 2nd	C	0,2	0,5	1	2	4	8
3rd, 4th	C	8	16	32	64		

Week	Concentrations of Ibuprofen [mg/L]				
5th	C	0,5	4	8	16

The purpose of this experiment was to test both the percentage of mortality at different concentrations and the study the behavioural patterns that the causing effects of the drugs have on these organisms.

The way to carry out these two analyses was the following:

- **Mortality**: The first count was then the number of casualties, for establishing a chiefly percentage of mortality; That is why, in each replica, a group of ten individuals (chosen by the same characteristics in terms of size and body shape), were selected to work in a more reliable conditions, and already ten-based built so the value was easily known; In this way phenomena such as cannibalism could be avoided, since the specimens that were to be tested would not have food contribution until the end of the experiment itself, which could last up to a total of 96 hours (a maximum of 5 days for taking the measurements).

- **Behaviour**: For this last purpose, two perpendicular lines were drawn using a marker on the base of the plastic cups in a cross-like shape; In this way, the "behavioural" fact of these crustaceans would be evaluated in relation with their swimming periods, and thus to see how active they were when subjected to certain doses of the specific tested drugs, counting the times random animals were crossing the lines during an established period of time that lasted roughly 2 minutes, but picking on just one individual from each cup; To sum up, the tests were conducted by placings the cups, before any measurement, randomly on the table, and leaving the relevant time slots occurred between each data collection; In this way the experiment tried to be the more stochastic as possible.

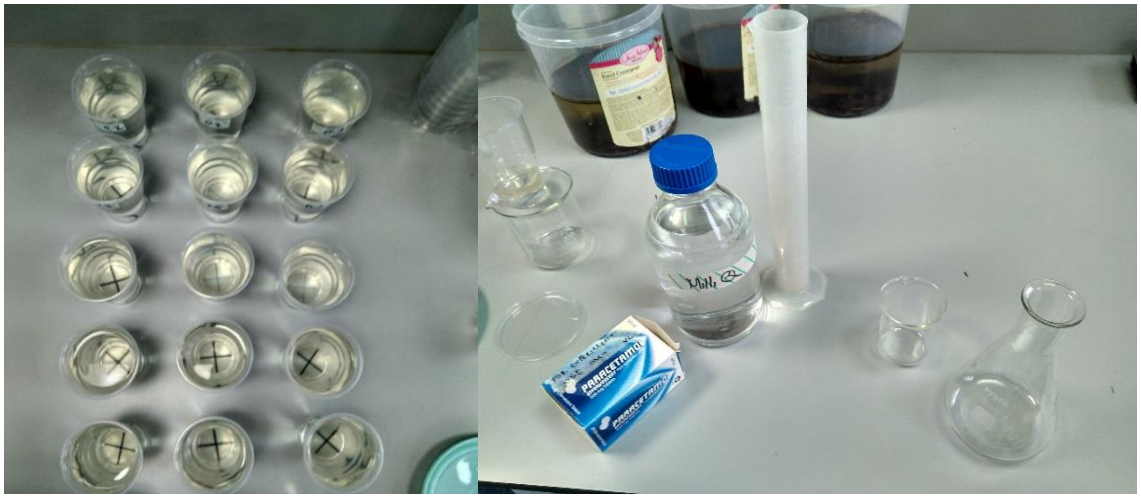


*Figure 5: **Left**: Setup of the three replicas for every single concentration; **Right**: Material used for the dilution preparation.*

2.4. **Statistical analysis**

The weekly collected data was included into tables of Excel, bounded by the time of the measurement and the concentration used; Grouping then the measures related with mortality, expressed in number of casualties in each plastic cup; And as the activity of the individuals or behavioural test (where only a random individual was chosen for each replica), that was expressed as number of times crossing any of the lines that formed the cross.

Once the data was arranged in the tables, it proceeded to establish possible correlations; IBM SPSS Statistics version 24 program was the statistical tool used for this purpose, as in it bivariate correlations, probit analyses tests (regressions) and toxicity bar charts comparisons were performed.

First, based on the Spearman's Rank correlation test (measure based on association or interdependence between two continuous random variables), relationships were established by arranging the drug concentration and later the time, both as independent variables (x); In this way it could be observed the evolution of: 1st Behaviour and 2nd Mortality, acting dependent variables, included in the y axis.

Therefore the groups where the independent variable (x) was the concentration, the absent factor would be in this case the time, and it would be necessary to choose by hand one group of data; This particular case was grouping at 24h for behaviour, and 48h for the activity test. The same happened then with the independent variable acting as time; The concentration in this case would be the picked one after the screening of the total plotted graphs.

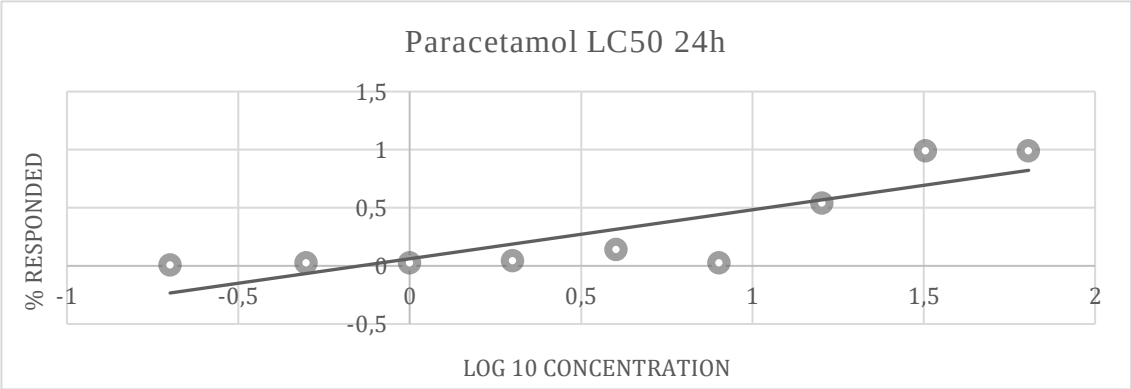
For establishing a better valuation, note that only those displays that showed conclusive data and some interesting facts to be mentioned were plotted in this work.

The closest comparative analysis regarding the two drugs was performed using t a bar chart with the two independent samples, so grouping the same values of concentrations for ibuprofen and paracetamol they were arranged as tandem bars, measured at a certain time in relation to the observed mortality, in a way of checking the toxicity of the drugs.

To sum up, the LC50 was already obtained for some periods of measurement, this calculation is well known in environmental Sciences as it is used for toxicological studies; It is defined as the dose required to kill 50% of exposed organisms; Thus, providing a full range of values, or in other words, broad enough, it can be known which is the concentration of the studied element that manages to eliminate half of the population studied.

3. Results

3.1. LC50 (Paracetamol)



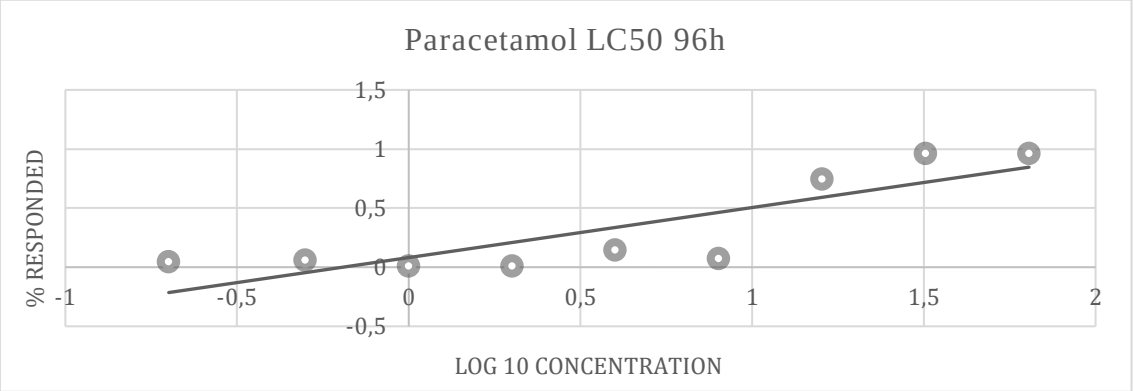
Graph 1: LC50 for paracetamol at 24 h; Value between 13,4 and 18,1 mg/L

Conc [mg/L]	Conc log10	Mortality %
8	0,9	0,025
16	1,2	0,54
32	1,5	0,99

***Table 1**: Estimation PROBIT after 24 h paracetamol exposure,*

LC50= 15,5 mg/L;

Lethal dose at some point between 16 and 32 mg/L



Graph 2:: LC50 for paracetamol at 96 h; Value between 10,9 and 14,9 mg/L

Conc [mg/L]	Conc log10	Mortality %
8	0,9	0,069
16	1,2	0,74
32	1,5	0,95

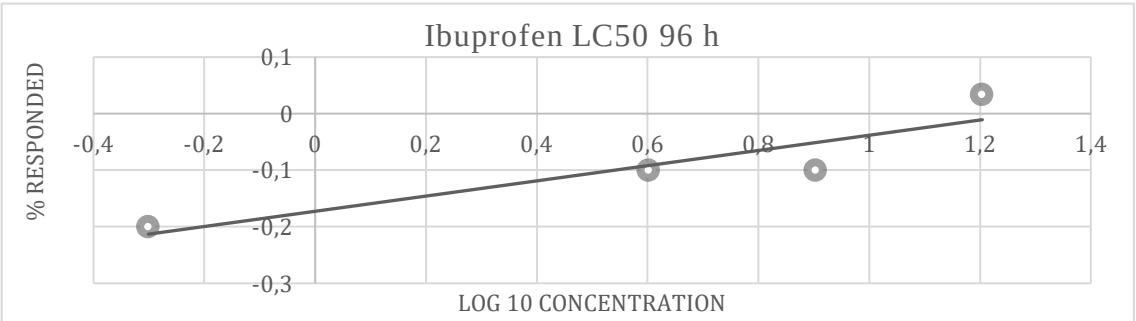
***Table 2**: Estimation PROBIT after 96 h paracetamol exposure,*

LC50= 12,83 mg/L;

Lethal dose at some point between 16 and 32 mg/L

3.1. LC50 (Ibuprofen)

It was supposed to perform the Ibuprofen LC50 at 24h but inaccurate results were obtained, same happened using data from 48 hours of exposure; Most likely because it was required to perform at least one more week of work in the lab with this drug, and higher concentrations for finding first the lethal dose, so not too much causalities in the first stages were observed according with the chosen concentrations in just one week of experiment.



Graph 3: LC50 for Ibuprofen at 96 h;

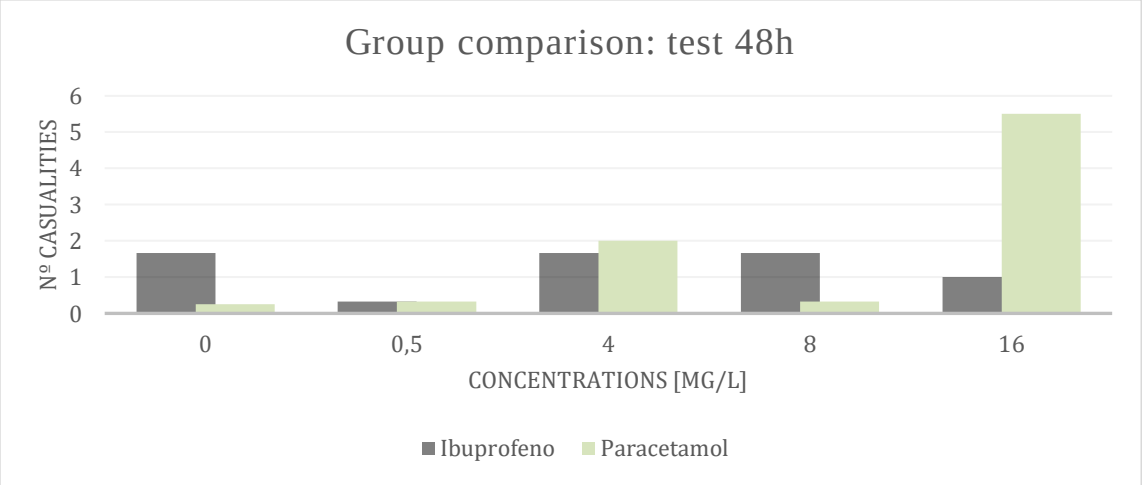
Conc [mg/L]	Conc log10	Mortality %
8	0,9	-0,1
16	1,2	0,033

***Table 4**: Estimation PROBIT after 96 h Ibuprofen exposure,*

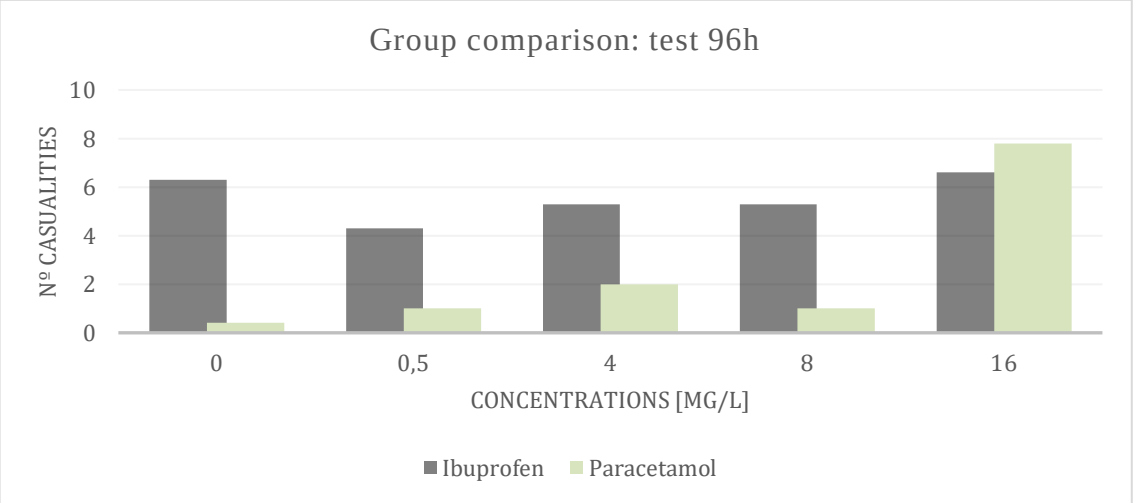
LC50= 1,956 mg/L;

Lethal dose not found; Bigger than 16 mg/L

3.2.Bar chart plotting



Bar chart 1: Testing toxicity after two days of exposure.



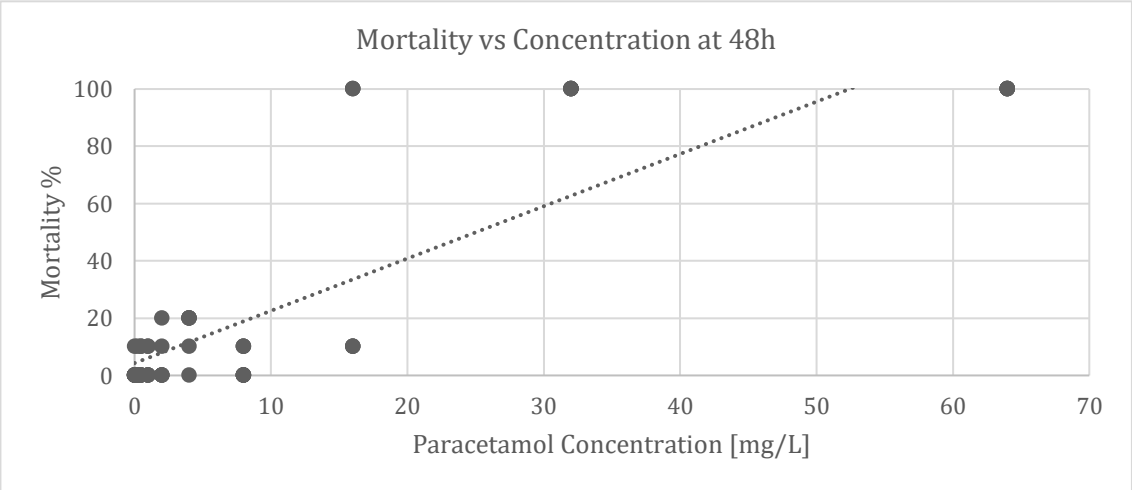
Bar chart 2: Causalities found after 96h for both drugs according different concentrations.

3.3.Spearman´s Rank Correlation Test

Value of Rho: between 0 and 1 for positive correlations

Concentrations:

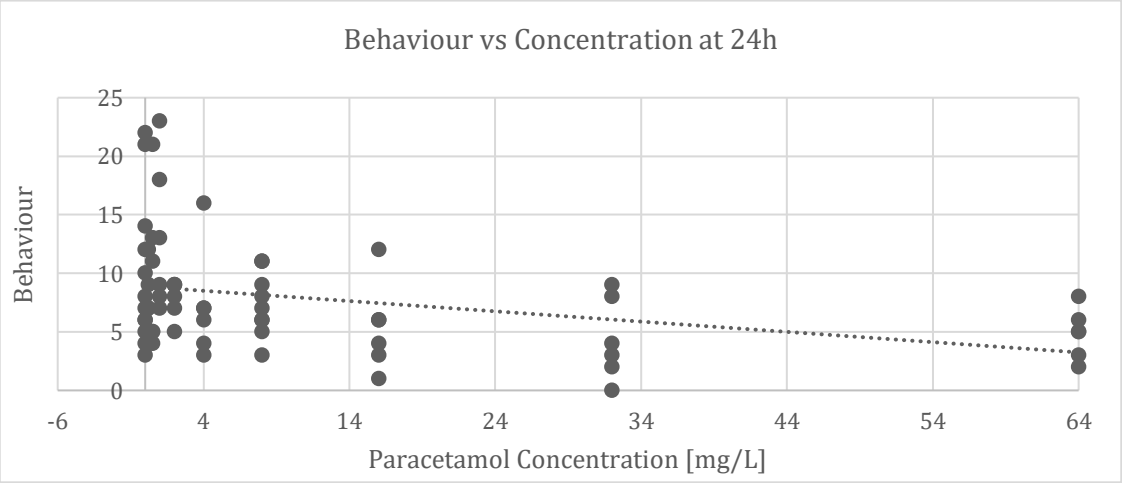
- H0: There is no correlation between the concentration of Paracetamol and the mortality rates in *Gammarus sp.*
- H1: There exists correlation between the concentration of Paracetamol and the mortality rates in *Gammarus sp*.



Graph 4: Mortality percentage according different concentrations two days later of exposure

Rho of Spearman: 0,732;

Sig (2-tailed) p-value: 0 < 0,05 H_1 is accepted, also strong positive correlation



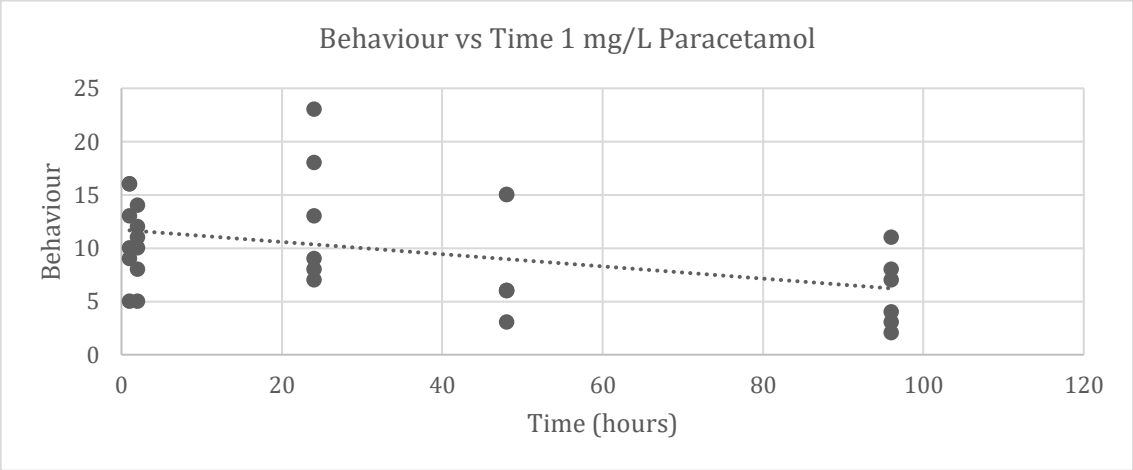
Graph 5: Graph 1: Activity related with different concentrations 24 hours later of exposure

Rho of Spearman: -0,365, negative correlation;

Sig (2-tailed) p-value: 0,002 < 0,05; H_0 is rejected

Time:

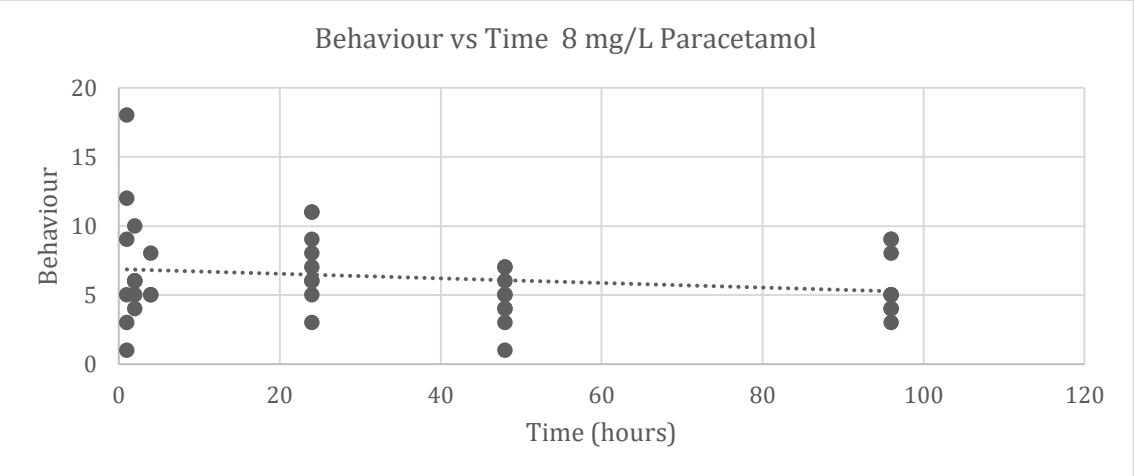
- H0: There is no correlation between the time exposure of Paracetamol and the mortality rates in *Gammarus sp.*
- H1: There exists correlation between the time exposure of Paracetamol and the mortality rates in *Gammarus sp*.



Graph 6: Activity along time according low concentration.

Negative correlation; Rho of Spearman= -0,42;

Sig (2-tailed) = 0,021< 0,05; So H_0 is rejected;

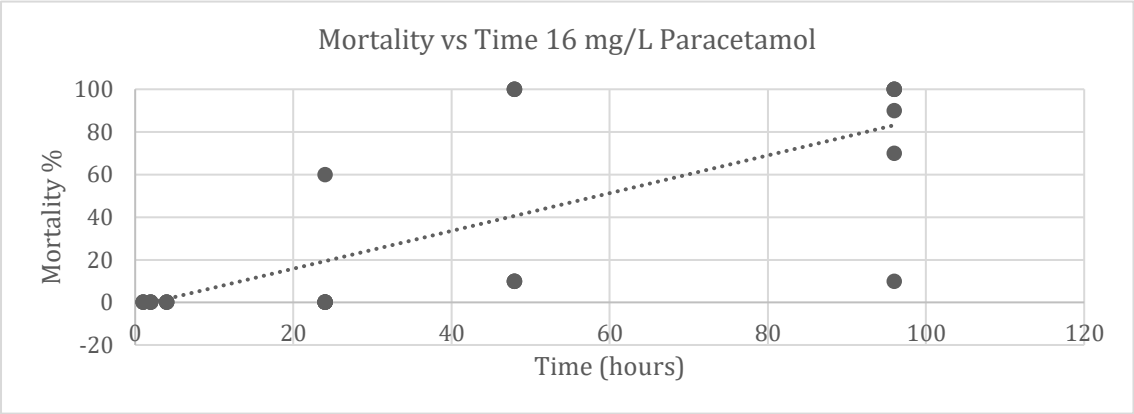


Graph 7: Activity along time at medium concentration.

Light negative slope; Rho of Spearman= -0,206;

Sig (2-tailed) = 0,089> 0,05; So H_0 is accepted; No correlated factors

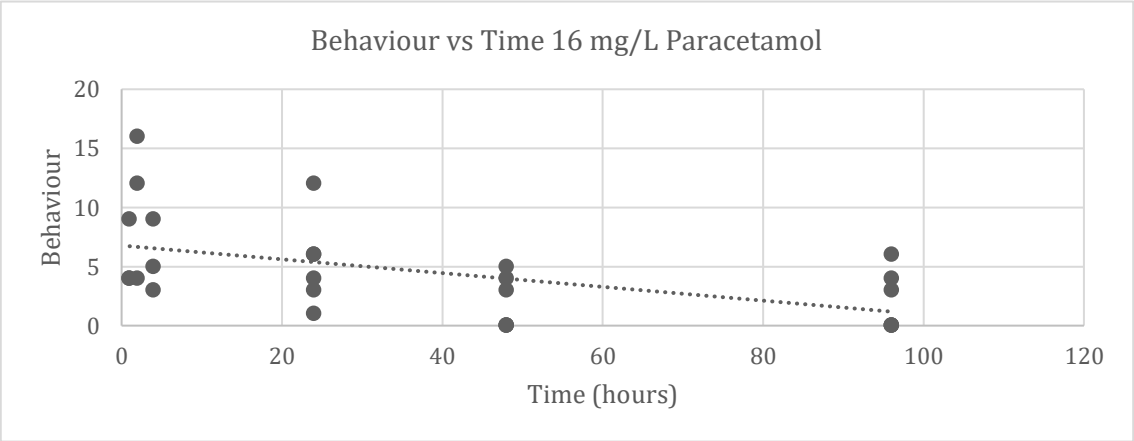
Lethal doses from 16 mg/L of Paracetamol; The borderline of total mortality



Graph 8: Critical concentration; Mortality vs time analysis

Strong positive; Rho of Spearman= 0,836;

Sig (2-tailed) = 0,000 < 0,05; So H_0 is rejected; Both factors are correlated.



Graph 9: : Critical concentration; Activity vs time analysis

Negative correlation; Rho of Spearman= -0,547;

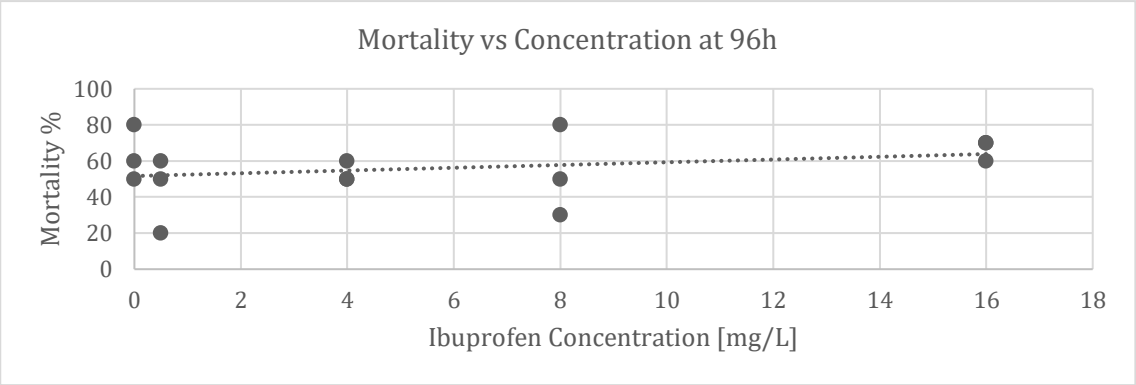
Sig (2-tailed) = 0,003 < 0,05; So H_0 is rejected; Both factors are correlated.

Ibuprofen test; Contrasting data

Concentrations:

- H0: There is no correlation between the concentrations of Ibuprofen and the mortality or activity rates in *Gammarus sp*.
- H1: There exists correlation between the concentrations of Ibuprofen and the mortality or activity rates in *Gammarus sp*.

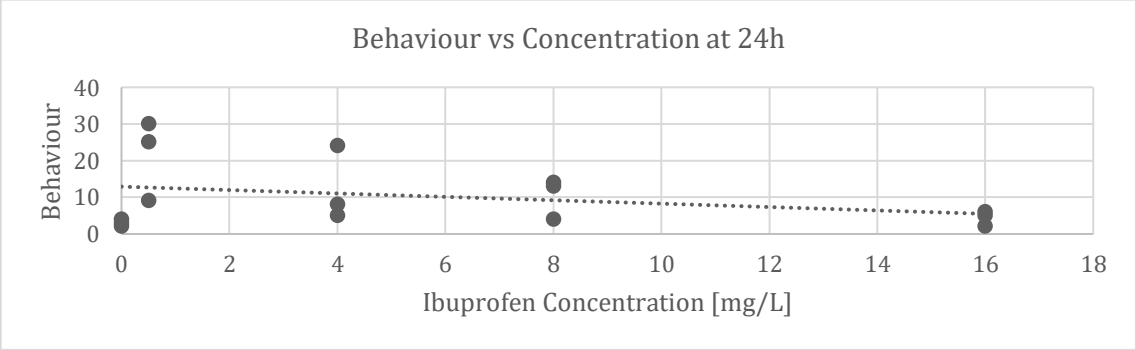
Mortality according concentration graph only was plotted as it observable at the latest exposure time (96h)



Graph 10: Plot according the evolution along the whole time tested (96h) for every sample; Most representative.

Light positive; Rho of Spearman= 0,191;

Sig (2-tailed) = 0,495> 0,05; The factors are not correlated, H_0 is accepted.



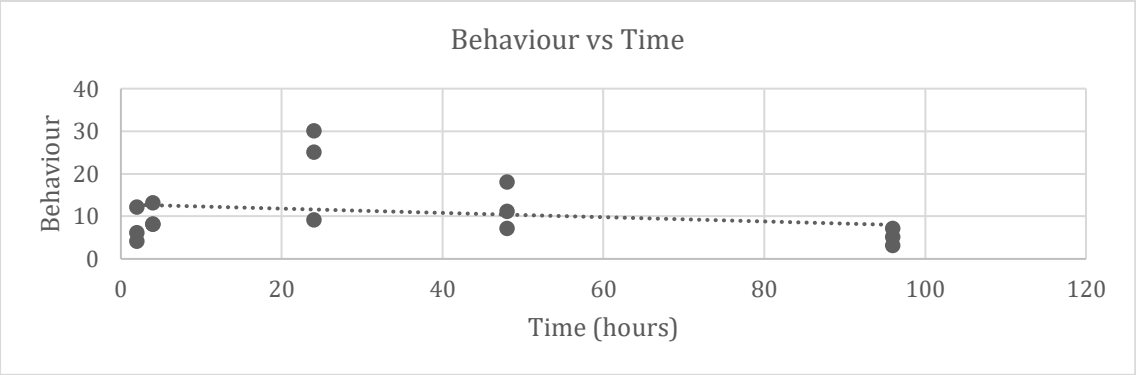
Graph 11: Activity evolution due to the different concentrations was represented within a day of exposure

Rho of Spearman= 0,027; Scarcely positive, almost 0.

Sig (2-tailed) = 0,923> 0,05; Clear null hypothesis, misleading data.

Time:

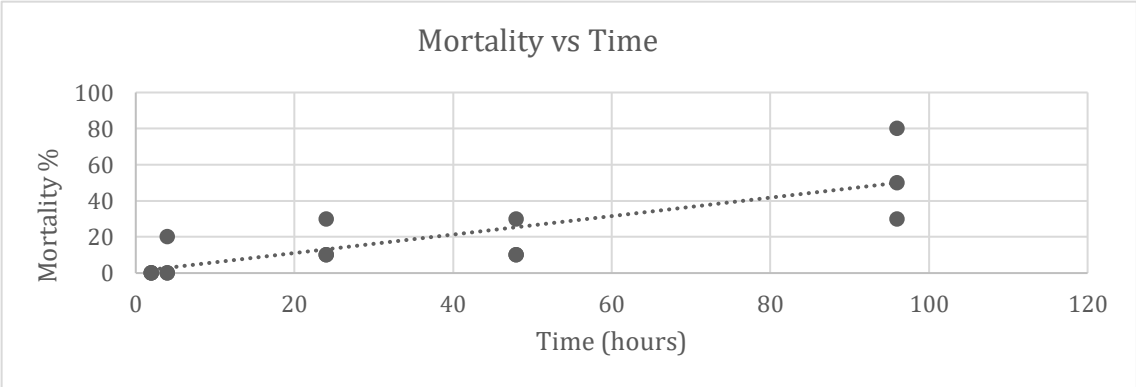
- H0: There is no correlation between the time exposure of Ibuprofen and the mortality or activity rates in *Gammarus sp*.
- H1: There exists correlation between the time exposure of Ibuprofen and the mortality or activity rates in *Gammarus sp*.



Graph 12: Activity along time with low concentration of the drug; 0,5 mg/L

Barely negative slope; Rho of Spearman= -0,148.

Sig (2-tailed) = 0,6 > 0,05; Alternative hypothesis rejected, so H_0 is accepted.



Graph 13: Mortality according different measures in time with medium concentration of ibuprofen; 8 mg/L

Rho of Spearman= 0,844; Strong positive

Sig (2-tailed) = 0,00< 0,05; H_0 is in this case rejected; So alternative hypothesis is accepted; There exist a positive correlation.

4. Discussion

The aim of this study was to evaluate the effects of paracetamol and ibuprofen in *Gammarus sp.* developing toxicity tests using it as a biomonitor of water quality; Two aspects of the assay were investigated: Death percentage (number of causalities), and Behavioural test (based on activity).

For the LC50 analysis, paracetamol required greater quantities dissolved in the water to achieve fifty percent of losses compared to ibuprofen, demonstrating that ibuprofen has apparently more damaging effects in the short and medium term. As well, for a longer exposure time for this first mentioned drug (*graph 2*) it was required a lower concentration in comparison with a lesser period of exposure; This is attributed to the cumulative effects of the byproducts of the drug in the organism (Levy, 1998).

A strong positive correlation was observed between *G. pulex* mortality rate measured in situ over time and the increase in the concentration (*graphs 4 & 8*); It came closely related then with a decrease in the activity level of the analysed specimens, showing decreasing slopes (*graphs. 5 & 9*) exposing negative correlations. *Graph 7* was showing no correlation; This plot concerns medium-high concentration of paracetamol [8 mg/L], this is because there were probably other factors affecting that correlation, which made the relationship between these two parameters somewhat doubtful, not as in the case of low and high concentrations (*graph 6 & 9*) respectively.

Moreover, as the analysis for paracetamol was way more extensive due to the greater availability of time, causes of high toxicity could be observed, *bar charts 1 & 2* showed an increasing number of causalities as concentration was growing, also became reflected in a sharp increase of the mortality test from concentrations of [16 mg/L]; Here then (*graphs 8 & 9*) the lethal doses was found when it crossed the barrier of 48 hours of exposure, reaching the total number of casualties in the cups.

On the other hand, no significant effects of ibuprofen were observed, but physiological data of ibuprofen was interesting to be shown; *Graph 11* was showing a rare pick according first doses in terms of activity, what made things interesting for plotting later in the time-related graph (*graph 12*) for that low concentration [0,5 mg/L].

After comparing the different results obtained with all concentrations used for each compound, it is clear that the low concentrations of ibuprofen are the origin of an higher level of activity due to a respiratory metabolism stimulation, that resulted in the incensement of locomotion in *Gammarus sp*. (De Lange *et al*. 2006); In fact, this seems

to be the induction of a hormetic response specific to this measured parameter, that triggers a remarkable change in behaviour due to low doses in this case, of ibuprofen; Whereas all the rest concentrations of ibuprofen used un this study yielded similar results as in the controls both for mortality and behaviour.

That´s why in this work was noted two types of responses in behaviour for the two tested drugs. The final observation is that organism's exposure to low concentrations of Ibuprofen [0,5-2 mg/L] developed a certain increase in activity level; Not matching with the general tendency showed with those exposed to paracetamol having a decrease in the activity as time went by or the dose was increased.

To sum up the ecological view is that habitat provides a refuge from predation, and the ability to recognise the scent of a predator, and thereby avoid direct interactions, is essential for the survival of an organism. To identify and locate food, habitats and predators, crustaceans rely on a combination of sophisticated chemosensory systems (Derby & Sorensen 2008). If these systems are disturbed by xenobiotics, the consequences could be severe, especially for Baltic Sea Gammarus spp., since their habitat, the stands of Fucus and its epibiota, are also their main food source.

5. Conclusion

In conclusion, lab toxicity tests on *G. pulex* have been performed using it as a short-term biomonitor for possible future uses in the evaluation of water quality; Which it is as well an indicative of community health and testable for checking ecosystem-level responses occurring over longer time periods. It is robust, responsive, and relevant.

Locomotor activity is one of the most pronouncedly affected parameter. This behavioural response is highly relevant as an ecological marker reflecting potential effects at the population level; The review of the toxicological effects of Ibuprofen on *Gammarus sp*, shows that at lower concentrations, Ibuprofen doesn't affect gammarids mortality but increases activity.

The application of this tool may grant a significant contribution to the assessment, interpretation and understanding of the impact of contaminants in freshwater ecosystem. Further studies are needed to elucidate the mechanisms involved at the cellular (i.e. structure of osmoregulators organ: gill epithelium, green gland) and molecular levels (e.g. Na+/K+-ATPase activity in green gland), to assess the relevance of these markers with other pollutants and also to determine their natural variability in different gammarid populations (in situ).

Therefore, research is required with a larger number of samples, and should be focused on the use of a wide range of concentrations with temporal evaluations.

Conclusión

En conclusión, las pruebas de toxicidad para *G. pulex* se han realizado basando a esta especie como un biomonitor a corto plazo para futuros posibles usos en la evaluación de la calidad del agua; El cual es también un indicativo de la salud de la comunidad y factible para verificar las respuestas del ecosistema que ocurren durante períodos de tiempo más largos. Es robusto, receptivo y relevante.

La actividad locomotora es pues, uno de los parámetros más afectados. Esta respuesta conductual es muy relevante como un marcador ecológico que refleja los efectos potenciales a nivel de la población; La revisión de los efectos toxicológicos del ibuprofeno en *Gammarus sp*. muestra que, a concentraciones más bajas, el ibuprofeno no afecta la mortalidad de los gamáridos, pero si aumenta su actividad.

La aplicación de esta herramienta puede otorgar una contribución significativa a la evaluación, interpretación y comprensión del impacto de los contaminantes en el ecosistema de agua dulce. Se necesitarían, sin embargo, más estudios para dilucidar los mecanismos implicados en el nivel celular (i.e. estructura del órgano osmorregulador: epitelio branquial, glándula verde) y niveles moleculares (e.g. actividad Na + / K + -ATPasa en la glándula verde), para evaluar la relevancia de estos marcadores con otros contaminantes y también para determinar su variabilidad natural en diferentes poblaciones de gamáridos (in situ).

Por lo tanto, se requeriría investigación con un mayor número de muestras, y debería enfocarse en el uso de una amplia gama de concentraciones con evaluaciones temporales.

6. Work Plan

	Month	Jan	Feb	Mar	Apr	Ma	Jun
Work Plan							
Revision and methodology							
Collection				X2			
Testing							
Data analysis							
Report writing							

Table 2: Chronogram representing the duration of each of the phases of the project performed

7. Acknowledgements

I specially thank Dr Vasilis Louca for helping me perform the experiment, his interest in the project and his practical help, including the installation of the laboratory display; Also, the projects coordinating staff (Department of Zoology, Aberdeen University) for supplying the freshwater lab to work in, and all the infrastructure for the experiment.

8. Bibliography

1. Åbjörnsson, K., Hansson, L.-A., Brönmark, C., 2004. Responses of prey from habitats with different predator regimes: local adaptation and heritability. Ecology 85, 1859–1866.
2. Andersen TH, Friberg N, Hansen HO, Iversen TM, Jacobsen D, Krojgaard L. 1993. The effects of introduction of brown trout (Salmo trutta L.) on Gammarus pulex L. drift density in two fishless Danish streams. Arch Hydrobiol 126:361–371.
3. Anderson RL (1982) Toxicity of fenvalerate and permethrin to several nontarget aquatic invertebrates. Environ Entomol 11:1251-1257
4. Barton DR, Hynes HBN (1976) The distribution of amphipoda and isopoda on the exposed shores of the Great Lakes. J Great Lakes Res 2:207-214
5. Baumgartner, D., Jungbluth, A.-D., Koch, U., Von Elert, E., 2002. Effects of ¨ infochemicals on microhabitat choice by the freshwater amphipod Gammarus roeseli. Arch. Hydrobiol. 155, 353–367.
6. Bousfield, E. L. (1973). Shallow-water Gammaridean Amphipoda of New England. Cornell University Press, Ithaca and London.
7. Czerniawska-Kusza, I. (2005). Limnologica - Ecology and Management of Inland Waters. pp.169-176.
8. D, M. (2018). Use of Gammarus pulex (L.) in safety evaluation tests: culture and selection of a sensitive life stage. - PubMed - NCBI. [online] Ncbi.nlm.nih.gov. Available at: https://www.ncbi.nlm.nih.gov/pubmed/3168873 [Accessed 1 Jun. 2018].
9. Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ. Health Persp. 107, 907–938.
10. De Lange HJ, e. (2018). Behavioural responses of Gammarus pulex (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. - PubMed - NCBI. [online] Ncbi.nlm.nih.gov. Available at: https://www.ncbi.nlm.nih.gov/pubmed/16624423 [Accessed 10 May 2018].
11. De Lange HJ, Noordoven W, Murk AJ, Lürling M, Peeters ETHM. Behavioural responses of Gammarus pulex (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. Aquat Toxicol. 2006; 78:209-216

12. De Lange, H.J., Lurling, M., Van den Borne, B., Peeters, E.T.H.M., 2005. ¨ Attraction of the amphipod Gammarus pulex to water-borne cues of food. Hydrobiologia 544, 19–25.
13. De Pauw, N. and Vanhooren, G. (n.d.). Method for biological quality assessment of watercourses in Belgium.
14. Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lutzhøft, H.C., Jørgensen, S.E., 1998. Occurrence, fate and effects of ¨ pharmaceutical substances in the environment—a review. Chemosphere 36, 357–393.
15. Henry, R.P, Lucu, C., Onken, H. & Weihrauch, D., 2012. Multiple Functions of the Crustacean Gill: Osmotic/ionic Regulation, Acid-Base Balance, Ammonia Excretion, and Bioaccumulation of Toxic Metals. Front. Physiol. 3:431. doi: 10.3389/fphys.2012.00431
16. Jenkins, D. (1985). The Biology and management of the River Dee. Banchory: [Verlag nicht ermittelbar].
17. Karaman, G. S. & Pinkster, S. (1977). Freshwater Gammarus species from Europe, North Africa and adjacent regions of Asia (Crustacea-Amphipoda). Part 1. Gammarus pulex-group and related species. Bijdragen tot de Dierkunde 47(1), 1–97
18. Kümmerer K. Pharmaceuticals in the Environment. Annu Rev Environ Resour. 2010; 35:57-75
19. la Farré, Marinel & Pérez, Sandra & Kantiani, Lina & Barcelo, Damia. (2008). Fate and Toxicity of Emerging Pollutants, Their Metabolites and Transformation Products in the Aquatic Environment. TrAC Trends in Analytical Chemistry. 27. 991-1007. 10.1016/j.trac.2008.09.010.
20. Levy, M. (1998). *Unwanted drug effects of antipyretic analgesics: epidemiological data*. [online] Springerlink. Available at: https://link.springer.com/chapter/10.1007/978-3-0348-6387-2_4 [Accessed 4 Jun. 2018].
21. Lincoln, R. J. & Bousfield, G. A. (1989). The Cambridge Illustrated Dictionary of Natural History. Cambridge University Press, Cambridge.
22. Lincoln, R. J. (1973). British Marine Amphipoda: Gammaridea. (British Museum (Natural History), London.

23. Maltby, L., Naylor, C., Calow, P., 1990. Effect of stress on a freshwater benthic detritivore: scope for growth in Gammarus pulex. Ecotoxicol. Environ. Saf. 19, 285–291.
24. Pascoe, D., Kedwards, T.J., Maund, S.J., Muthi, E., Taylor, E.J., 1994. Laboratory and field evaluation of a behavioural bioassay—the Gammarus pulex (L.) precopula separation (GaPPS) test. Water Res. 28, 369–372.
25. Sanz-Lázaro C, e. (2018). Toxicity Studies of Polynuclear Aromatic Hydrocarbons (PAHs) on European Amphipods. - PubMed - NCBI. [online] Ncbi.nlm.nih.gov. Available at: https://www.ncbi.nlm.nih.gov/pubmed/20020897 [Accessed 1 Jun. 2018].
26. Schrap, S.M., Rijs, G.B.J., Beek, M.A., Maaskant, J.F.N., Staeb, J., Stroomberg, G., Tiesnitsch, J., 2003. Humane en veterinaire geneesmiddelen in Nederlands oppervlaktewater en afvalwater. RIZA report 2003.023 (in Dutch).
27. Taylor, E.J., Jones, D.P.W., Maund, S.J., Pascoe, D., 1993. A new method for measuring the feeding activity of Gammarus pulex (L.). Chemosphere 26, 1375–1381.
28. Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.
29. Williams, D.D., Moore, K.A., 1985. The role of semiochemicals in benthic community relationships of the lotic amphipod Gammarus pseudolimnaeus: a laboratory analysis. Oikos 44, 280–286
30. Wisenden, B.D., Cline, A., Sparkes, T.C., 1999. Survival benefit to antipredator behavior in the amphipod Gammarus minus (Crustacea: Amphipoda) in response to injury-released chemical cues from conspecifics and heterospecifics. Ethology 105, 407–414.
31. Wisenden, B.D., Pohlman, S.G., Watkin, E.E., 2001. Avoidance of conspecific injury-released chemical cues by free-ranging Gammarus lacustris (Crustacea: Amphipoda). J. Chem. Ecol. 27, 1249–1258.
32. Wood, C.M., 2011. Silver. In: Wood, C.M., Farrell, A.P. & Brauner, C.J. (eds) Homeostasis and Toxicology of Non-Essential Metals. Fish Physiology Volume 31 B, Academic Press/Elsevier, New York, NY, USA, 1-65.
33. Wudkevich, K., Wisenden, B.D., Chivers, D.P., Smith, R.J.F., 1997. Reactions of Gammarus lacustris to chemical stimuli from natural predators and injured conspecifics. J. Chem. Ecol. 23, 1163–1173.