CZECH UNIVERSITY OF LIFE SCIENCES
FACULTY OF ENVIRONMENTAL SCIENCES
DEPARTMENT OF APPLIED ECOLOGY

ASSESSMENT OF THE VALIDITY OF THE LUMINESCENCE TOXICITY TESTING

BACHELOR THESIS

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Faculty of Environmental Sciences

BACHELOR THESIS ASSIGNMENT

Maria Dolgaya

Environmental Engineering

Thesis title

Assessment of the validity of the luminescence toxicity testing

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Objectives of thesis

The overall aim of the bachelor thesis is to evaluate the influence of the source of Allivibrio fischeri (formerly Vibrio fischeri) on the validity of the luminescence toxicity testing. The theoretical part of the work will focus on the current state in the field of measuring acute toxicity in the aquatic environment, available standardized test protocols and related environmental legislation. Furthermore, selected environmental pollutants will be discussed with regard to their acute toxicity to the aquatic environment. The practical part of the work will be performed with the standardized luminescent bacteria on the selected water pollutants.

Methodology

The bachelor work is experimental. Methodologically, it will be formed as the comprehensive literature review followed by the practical part carried out in laboratory conditions in compliance with relevant the ISO 11348-3 standard.

The proposed extent of the thesis

50-60 pages incl. appendixes

Keywords

Allivibrio fischeri, light emission, ecotoxicity, water, pollutant, verification

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Recommended information sources

Abbas et al., 2018: Vibrio fischeri bioluminescence inhibition assay for ecotoxicity assessment: A review. Sci Total Environ. 626: 1295–1309. doi: 10.1016/j.scitotenv.2018.01.066.

AMIARD-TRIQUET, C. – AMIARD, J C. – MOUNEYRAC, C. *Aquatic ecotoxicology : advancing tools for dealing with emerging risks.* Amsterdam: Elsevier, 2015. ISBN 978-0-12-800949-9.

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Farré et al., 2008: Assessment of the acute toxicity of triclosan and methyl triclosan in wastewater based on the bioluminescence inhibition of Vibrio fischeri. Anal Bioanal Chem. 90(8):1999–2007. doi: 10.1007/s00216-007-1779-9.

ISO 11348-1:2007-ed.2.0/Amd1:2018: Water quality – Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) — Part 3: Method using freeze-dried bacteria. Geneva: International Organization for Standardization, 24 p.

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Bachelor thesis author's statement

I hereby declare that I have independently elaborated the bachelor/final thesis with the topic of: Assessment of the validity of the luminescence toxicity testing, and that I have cited all of the information sources that I used in the thesis as listed at the end of the thesis in the list of used information sources.

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Abstract

This bachelor thesis focuses on evaluation the source of *Aliivibrio fischeri* (formerly *Vibrio fischeri*) on the validity of luminescent bacteria testing. The theoretical part of the work deals with the current state in the field of acute ecotoxicity measurements in the aquatic environments, relative released legislations, and available standardized test protocols. The practical part is performed on the selected freshwater pollutants using the standardized marine luminescent bacteria *A. fischeri*.

According to the results obtained from the experimental part performance carried out in laboratory conditions in compliance with the relevant ISO 11348-3:2007 standard, the most stable, precise, and cost-effective testing bacteria sources are LUMIStox© (Germany) and Biolight (Belgium). The difference between the results obtained from testing on different luminescent bacteria suppliers shows that the source of bacteria has a slight effect on the final results depending on the required conditions and the procedure required by the suppliers. This fact should be taken into consideration during test performance.

Key words

Luminescence, light emission, *Aliivibrio fischeri*, acute toxicity, water, pollutant, validity

Table of contents

List of abbreviations

EC_x — Effective concentration

LC_x — Lethal concentration

CLP — Classification, Labelling and Packaging regulation

DCF — Diclofenac

ECHA — European Chemicals Agency

ERA — Environmental Risk Assessment

FDA — Food and Drug Administration

ISO — International Organisation for Standardization

LOEC — Lowest observed effect concentration

NOEC — No observed effect concentration

NSAIDs — Non-steroidal anti-inflammatory drugs

OECD — Organisation for Economic Cooperation and Development

PCBs — Polychlorinated biphenyls

PPCP's — Pharmaceuticals and personal care products

REACH — Registration, Evaluation, Authorisation and Restriction of Chemicals

SDGs — Sustainable Development Goals

TCS — Triclosan

U.S. EPA — United States Environmental Protection Agency

WFD — Waste Framework Directive

WWTP — Wastewater Treatment Plant

1. Introduction

The era of consumption, global overpopulation, and other anthropogenic problems are a huge challenge for the environment nowadays. Due to extensive soil and land degradation, high levels of chemical and waste pollution, deforestation, and species extinction humanity is forced to take care of nature to be able to secure its future and provide the next generations with suitable living conditions.

One of the most serious problems which has been taking place all around the world is chemical pollution caused by different anthropogenic activities. As the chemical industry has recently advanced, there are more and more synthetic materials that are used for various consumer product production and then released into the environment as toxic wastes (Asthana, 2014). Such chemicals not only can serve as unhealthy substances for the human body but also cause harmful effects on the living organisms and the environment exposed to them after a release of waste. Therefore, the topic of environmental pollution requires additional attention and contribution from different perspectives.

Ecotoxicology is an environmental science that plays a crucial role in the process of ecological problems' solutions and the elimination of their negative consequences. It carries out comprehensive research on separate chemical substances and evaluates their toxicity and negative effects that can appear in the presence of other chemicals. Because of the vast number of pollutants, today it is still quite challenging to evaluate the ones that pose the most serious risk to human health and improve efficient treatment techniques. Some of them are present in the environment below the detection limits available by modern technologies, and their toxic effects are not detected and established yet (Kyle et al., 2010). Therefore, it is necessary to improve the existing methodologies and techniques to provide more sophisticated research and experiments and obtain valuable results. In addition to it, it is crucial to analyse different sources of testing organisms and evaluate their influence on the results of ecotoxicological experiments.

2. Objectives of the thesis

The overall aim of the bachelor thesis was to evaluate the influence of the source of *Aliivibrio fischeri* (formerly *Vibrio fischeri*) on the validity of the luminescence toxicity testing.

The theoretical part of the work was focused on the current state in the field of measuring acute toxicity in the aquatic environment, available standardized test protocols, and related environmental legislation. Also, selected environmental pollutants were discussed with regard to their acute toxicity to the aquatic environment.

The practical part of the work was performed with three standardized luminescent bacteria on the selected water pollutants. The main purpose of the experimental part of this thesis was to compare and evaluate results obtained from tests performed, on the commonly applied chemical substances found in personal care products, using luminescent bacteria provided by different suppliers.

3. Literary research

3.1 Ecotoxicology

Ecotoxicology is a relatively new science that combines various disciplines such as chemistry, biology, ecology, and others. It was derived from toxicology in the 1970s as a response to the beginning of the development of environmental sciences at that time. The increased interest in this discipline was caused by many social and technological factors. Rapid population growth, intensive technological and scientific development, and the beginning of the era of consumerism directly and circumstantially led to many ecological problems including atmosphere, hydrosphere, and biosphere chemical pollution. Those problems had to and still need to be solved with the help of ecotoxicology which plays a key role in this process.

Nowadays, the environmental sciences are gaining the scientific interest of researchers and public awareness worldwide. It first received a lot of public attention with the help of the book *'Silent Spring'* written by the American biologist Rachel Carson. The author dedicated her book to ecological problems and highlighted the implementation of pesticides such as DDT (*dichlorodiphenyltrichloroethane*), arguably the most well-known insecticide in the world. It has harmed wildlife, could have detrimental impacts on human health, and clearly demonstrated the principal effects of environmental degradation brought on by synthetic chemicals (Turusov et al., 2002). The discussion about the pesticide was held by many researchers in different countries, and although the usage of them was banned, the problem of chemical pollution in general and its negative impact on the environment remains a big challenge.

Agricultural activities that include the implementation of pesticides are just one of the contributors to global environmental pollution. There is also a huge number of aquacultural works such as breeding and harvesting aquatic organisms which are known for a drastic amount of the overall released organic waste. Aquaculture is a fast-developing industry due to the high demand for the growing food industry, especially concerning seafood and fish products. This sector of the animal culture industry has been developing faster than any other, and such rapid growth has been causing increased environmental pollution accordingly (Gang et al., 2005). The main chemical pollutants found in aquaculture wastewater are nitrogenous compounds such as ammonia, which is the most common waste produced by aquatic animals. In addition to it, the existing technologies on wastewater are out-of-date and cannot manage to treat waste properly which aggravates the situation with the aquaculture environment (Cao et al., 2007).

Continuing the topic of anthropogenic pollution, for the last decades it has been causing a lot of different environmental problems. There is a huge negative effect on wildlife and human health in general, which is caused by many reasons such as

physical plastic waste left by humans, industrial production waste released into the environment, and discharge of bioavailable chemicals which are highly toxic for organisms. Once a chemical is exposed to a waterbody, it can accumulate in an organism's body and after some time can even affect the overall food chain of the ecosystem. One such example is lead (Pb), which contaminates the environment, enters food chains, and causes toxic effects on soil and human health as Pb is known to be highly bioavailable. It is one of the most toxic metals, and its ingestion via a food chain is a hazard for plants and humans (Kumar et al., 2020). Also, another particular concern of Pb contamination is the influence on children's neural system development and the decrease of their intelligence quotient due to their hand-to-month activities (Shannon, 1998). This is one of the reasons why Pb and other heavy metals should remain a matter of concern for scientific research along with other chemicals and pollutants exposed to the environment in enormous concentrations.

To sum it up, the problem of environmental pollution is very complex, so it requires a lot of attention and investigation. After all, people must be able to preserve the habitat for species and provide appropriate measures to protect human health. To achieve it, ecotoxicology assesses the main sources of chemical pollutants of different types and studies their impacts on the environment and organisms on different biological levels of organization. Ecotoxicological research includes the theoretical, experimental, and statistical parts, which involve many people and mostly require a lot of time. Ecological studies are usually conducted on molecular, cell, or organism levels, experiments are designed with the usage of living organisms and a chosen chemical. After a series of repetitive experiments, the overall impact on other higher biological levels can be predicted by using various statistical and analytical tools which have been improved during the last decades.

Ecotoxicology is being developed and studied all around the world. There are common environmental groups of tested toxicants such as PCBs (polychlorinated biphenyls), pesticides, heavy metals, and others which have already been described in detail and create a base for future studies and predictions. These groups of chemicals which are the main concern of ecotoxicology as well as species used for toxicology testing will be covered in detail in the following chapters.

3.2 Ecotoxicity testing

Testing on the organisms is a crucial part of ecotoxicological research as it lets scientists assess the toxic effect of chemicals on organisms, populations, and the environment in general.

Ecotoxicity tests are performed under specific conditions in accordance with standardized rules and legislation. They define all major steps of the procedure, necessary laboratory equipment, the tested species, and other important characteristics

and settings of the experiments. All the above-mentioned factors are described in the following chapters in more detail.

3.2.1 Types of tests

There is a wide range of methods of ecotoxicity testing that help to evaluate the level of danger that a particulate matter can cause. The choice of a test depends on what toxicity effect the project is going to explore, what type of chemical will be used, and what equipment and laboratory conditions are available.

Ecotoxicological tests can be divided according to several crucial factors:

- exposure time (acute and chronic toxicity tests);
- observed effect (mortality, reproduction, behavioral changes);
- effective response (lethal, sublethal) (Kapanen and Itavaara, 2001).

However, apart from the mentioned categories, there are three main types of testing on organisms that are widely used all around the world:

- Acute toxicity tests (mortality tests), which assess the toxicity of chemicals within a short period (24 hrs). Such tests can provide a half maximal effective concentration value (EC_{50}) which corresponds to a concentration of a chemical expected to cause 50% of a certain effect, and lethal concentration value (LC_{50}) which is a concentration that causes the death of 50% of the tested population. Acute toxicity tests are crucial to identify the target organ of toxicity, obtain data on the adverse effects of a chemical and provide safety measures for testing substances (Arome and Chinedu, 2013);

- Chronic toxicity tests, which are conducted with long-term exposure to a chemical, referring to various sublethal effects. During this type of test, the values of no observed effect concentration (NOEC) and the lowest observed effect concentration LOEC can be calculated. This type of test involves bigger groups of tested organisms, and they are designed to identify the affected organs and encompass the entire life cycle or several life stages of the organisms.

- Avoidance tests (behavioral tests) which are rapid assays of the bioavailability of contaminants in soil. The response of the tested system can be defined as its action or reaction to changes in the environment, and such behavioral response is caused by the integration of metabolic and physiological processes in the tested system. This type of test has a significant value because the factor of avoidance indicates a decrease in populations which is caused by various stressors such as chemical contamination (Gainer et al., 2019).

3.2.2 Test organisms

There is a list of defined species on which the ecotoxicity tests can be performed. They can be terrestrial or aquatic organisms which mainly depends on the goals of the research that required testing. All the mentioned procedures are controlled by official documentation and standardized guidelines, which will be partially covered in the following chapters.

As it was mentioned before, tested biota is divided into two groups of organisms:

- terrestrial (plant seeds such as *Sorghum saccharatum*, *Lepidium sativum*, *Sinapis alba*)
- aquatic (various types of organisms such as *Artemia franciscana, Daphnia magna, Phaeodactylum tricornutum, Lemna minor*)

Terrestrial species are often used for ecotoxicology tests connected to the analysis of chemical pollutants affecting the land, soil, and different terrestrial ecosystems in general. The group of aquatic biota can be represented by either freshwater or marine species depending on the type of studied system. According to the species, the biota is provided by suppliers in the form of immobilized organisms such as cysts, ephippia, or turions, but not as living cultures as such. This factor makes it possible to transport and store the organisms for up to half a year and use them for testing at the time when it is necessary (MicroBioTest, ©2023).

The choice of an organism for ecotoxicological studies depends on many factors. It should fulfill a range of criteria such as easy sampling, fast achievement of sufficient population size, visibility, measurability, and high sensitivity to chemicals and other environmental changes. For example, the genus *Artemia* (brine shrimp) is introduced by six species which can be used for ecotoxicological testing and characterized by common features such as adaptability to wide ranges of salinity (5–250 g/L) and temperatures (6–35 °C). They also easily adapt to adverse environmental conditions and various nutrition sources, have a short life cycle and give large offspring production which makes this genus reliable, feasible, and cost-effective for some acute and short-term ecotoxicology research tests (Nunes et al., 2006).

On the other hand, one of the most common species, which is quite often chosen for chronic ecotoxicity tests such as sediment toxicity testing, is the benthic oligochaete, *Lumbriculus variegatus*. This species occupies water and sediment compartments and experience exposure to tested contaminants through overlaying and pore sediment water and particles of the sediment. Such assays provide more relevant experimental results and data which can be further used to assess the toxicity of a chemical (Little et al., 2021).

The ethical factor is also met while choosing the living organisms for the experiment's performance. Recently, ethical considerations have got more attention and pressure to decrease the use of invertebrates and fish for ecotoxicity testing. As a result, the National Centre for the Replacement, Refinement and Reduction of Animals in Research (*NC3Rs*) was established. The main goal of this organization is to minimize the use of animal species in research by integrating other methods and creating reliable alternatives. In 2008, NC3Rs initiated a program dedicated to ecotoxicology research which has been integrating authorities, research institutions, and industry to reduce animal testing in this field (Burden et al., 2015). One of the achieved results of the programme includes testing on fish cell cultures and embryos which today replaces experiments on fish larvae or adult species. Although fish cells are less sensitive to chemicals, it does not require such strict conditions as other animal cell cultures. The environment for those cells does not have to be sterile, and there is a wide range of temperatures and salinity that can be set for its storing and tests conduction, therefore there are also some practical advantages of using them instead of common alternatives such as mammal cells cultures (Breitholtz et al., 2006).

Among other goals, which NC3Rs is focusing on, there is an intention to harmonize the global data and a shift toward the complete elimination of vertebrate testing in environmental risk assessment (Burden et al., 2015). Now, considering all the above-mentioned factors, the most common species for ecotoxicological testing are related to bacteria, yeast, algae, protozoa, and invertebrates (populations obtained from conserved dormant stages such as eggs, embryos, or cysts) (Viegas, 2021).

In addition to it, there is Directive 86/609/EEC, which deals with the protection of animals used for testing and experiments on the level of the European Union. It incorporates the principle of the 3Rs, develops legislation and necessary documents for the protection of animals, which are still used in scientific research, and aims to fully replace them with other methods.

Although the topic of animal testing remains controversial meeting many social movements against it, there are no existing alternatives for efficient ecotoxicological analysis for now. However, there is a specific range of organisms that are allowed to be tested and strict and organized instructions on laboratory conditions for each species. These protocols must be followed during the conduction of ecotoxicological tests, and they have to meet all the described criteria.

3.2.3 Guidelines and methodologies

As it was mentioned before, a lot of documentation has been developed for accurate test procedures in laboratory conditions. For instance, the OECD (*Organization for Economic Co-operation and Development*) has been coordinating policies for many international problems including environmental ones. A series of guidelines for testing

chemicals on living organisms were promoted by OECD at the end of the 20th century, which are now being used by different organizations to determine the safety of chemicals. For instance, Test Guideline OECD 471:2020 refers to Bacterial Reverse Mutation Test and provides the manual on how to proceed it with *Salmonella typhimurium* and *Escherichia coli*. It describes the principle of testing, preparation of bacteria and medium, test substance preparation, the procedure itself, and interpretation of the results.

Although there are many guidelines for testing available for purchase, ecotoxicology research addresses the necessity of developing new ones, and more protocols are being released quite often. For instance, in 2004 the OECD initiated the development of a new testing guideline focusing on fish embryo toxicity assessment. The draft version of the project included testing on embryos of zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*), and Japanese medaka (*Oryzias latipes*). As a result, a new document OECD 236:2013 on Fish Embryo Acute Toxicity (FET) test was issued for testing chemicals on embryonic stages of fish instead of adult species which were common before, and it was an important step forward to limit the use of animals for testing (Busquet et al., 2014).

Another example of documentation used all around the world is provided by ISO (*International Organization for Standardization*). This organization has been developing and publishing international standards not only for environmental management but also for health and safety, energy management, food safety, and other subjects. Concerning ecotoxicology, ISO actively provides guidelines for testing on various types of organisms including both terrestrial and freshwater ones. ISO has been developing new test guidelines and changing the existing ones. For example, it has implemented some changes regarding the international harmonization of terrestrial plants toxicity tests. As a result, the standardized protocols in the section of soil quality control on measuring the inhibition of root growth (ISO 11269-1:2012) and the emergence and early growth of higher plants (ISO 11269-2:2012) were updated and complemented with the screening methods tested on single species of *Lactuca sativa L*., so ISO 17126:2005 was developed (Tarazona et al., 2013).

There are also other international organizations that deal with environmental regulations, legislation, and standards. Examples of those are:

- ECHA (*European Chemical Agency*) that implements European Union legislation to protect human health and the environment by various regulations and directives;
- U.S. EPA (*U.S. Environmental Protection Agency*) that has been implementing protection programs in the fields of water quality, chemical manufacture and usage, and others.

All above-mentioned organizations play a crucial role in ecotoxicological research and provide all necessary documentation and guidelines which can be used for as safe and precise testing as it is possible. However, technological and scientific development makes existing legislations adapt to new changes, therefore updated versions of documents as well as completely new guidelines are released by each organization periodically.

3.2.4 Reference substances

Besides strict standards and guidelines that help to control the process of testing, there are also several standardized solutions that are commonly used during the experiment. Because it is quite challenging to maintain indicators of the state of health of the organisms under laboratory conditions, it is necessary to conduct an experiment on a standardized solution before testing the chemicals of interest (Krejčí and Palíková, 2006). These standardized solutions are also called reference substances, and they are used for ecotoxicity research to be able to evaluate the results of a newly tested chemical or a developed methodology for ecotoxicological testing.

Some guidelines recommend using specific reference substances depending on the type of organisms for testing. For example, the OECD 201:2006 protocol for freshwater algae and cyanobacteria growth inhibition test recommends conducting ecotoxicity tests on 3,5-dichlorphenol and potassium dichromate and specifies that the tests on such reference solutions should be done at least twice a year. The OECD 202:2004 guideline on acute immobilization tests on Daphnia magna does not specify the list of recommended substances but highlights that the tests on the reference chemicals must be done preferably every month and at least twice a year.

The ISO standards also mention preferable substances that can be used as a reference. For instance, in the standardized protocol EN ISO 11348-3:2007 developed for testing on *Aliivibrio fischeri* 3,5-dichlorphenol ($C_6H_4Cl_2O$, CAS 591-35-5), potassium dichromate ($K_2Cr_2O_7$, CAS 7778-50-9) and zinc sulphate heptahydrate ($ZnSO_4.7H_2O$ CAS 7446-20-0) are mentioned in the list of chemicals for testing.

Different research studies are performed using different reference substances. However, typical examples of such solutions which are commonly used for ecotoxicity testing on living organisms are 3,4-dichloroaniline ($Cl_2C_6H_3(NH_2)$, CAS 95-76-1), 3,5-dichlorophenol, chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$, CAS 56-75-7), potassium dichromate, zinc sulphate heptahydrate, and many others. For instance, 3,5-dichlorophenol was used in the study whose main objective was the development and optimization of ecotoxicological testing methods on *Navicula libonensis*, and its sensitivity to the chemical was checked and compared to the results published in the

literature (Vidal et al., 2014). Similarly, another study used all the above-mentioned reference compounds for developing an automatic system to conduct tests on the luminescent bacteria *Aliivibrio fischeri* (Menz et al., 2013).

These are only a couple of examples in which reference substances were used. Practically, the above-mentioned ones are the most widely used in ecotoxicological research, which play a crucial role in the validity control of ecotoxicity experiments.

3.3 Bioluminescent tests

One of the most common types of ecotoxicity tests is based on the process of bioluminescence. This type has got a lot of attention due to modern and sensitive equipment which allows to read and compare the level of natural light emissions.

Organisms that are often chosen for bioluminescent tests on water samples are luminescent bacteria. Compared to other species, the bacteria do not require much space and effort to take care of, and it is also financially efficient. The bacteria are also highly sensitive to the chemicals, and it is easy to assess the toxicity because of the luminescence which is its unique feature (Figure 1). The process and mechanisms of bioluminescence will be covered in detail in the following chapters.

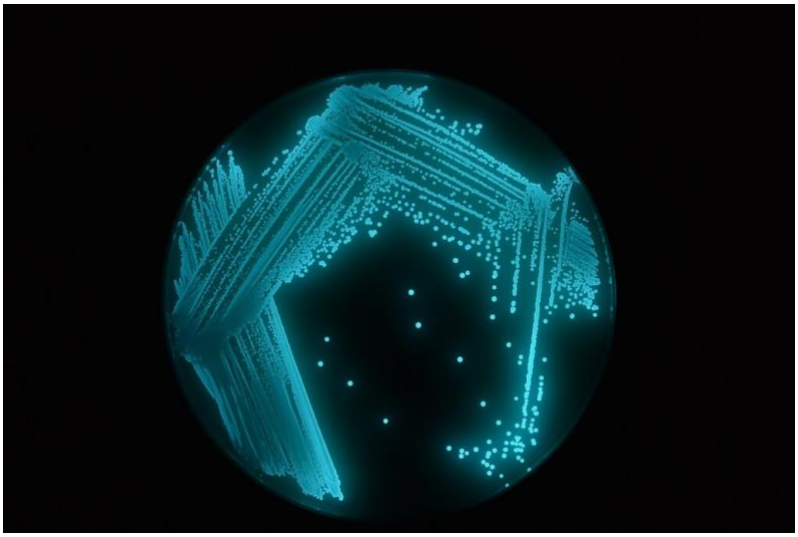

Figure 1. Bioluminescent plate (MicroBioTests, ©2023)

In addition to it, in comparison with other living organisms which are widely used for testing, luminescent bacteria allow to conduct acute toxicity experiments significantly

faster and require a short time (minutes) to determine the toxicity of water samples (Garcia et al., 2012), that is another advantage of testing on it. This type of bacteria emits light during metabolic chemical reactions, and this factor can be easily assessed and measured in a short time using specific equipment in the laboratory. The higher the rate of luminescence inhibition is, the more toxic the tested chemical is because it has ecotoxicological effects on the metabolism of bacteria. The stimulation of luminescence can be also recorded from the sample with a low concentration of some chemicals as they can stimulate metabolism. Therefore, the level of luminescence which is recorded during the experiment lets directly analyze the toxicity effects of chemicals.

One of the most frequently applied conventional standard methods for toxicity testing on luminescent bacteria is ISO 11348:2007. It refers to water quality determination and assesses the toxicity of water samples on the bacteria. This method is applicable for many environmental samples for acute toxicity tests due to the short exposure time of a chemical. On the other hand, due to this characteristic, it is impossible to use luminescent bacteria and the standardized method for some long-term experiments such as testing of chronic effects and others. This standard is described in detail in the chapter below.

3.3.1 Aliivibrio fischeri

Aliivibrio fischeri (formerly *Vibrio fischeri*) is a gram-negative luminescent bacterium that got its name after the German microbiologist Bernhard Fischer, who contributed to the bacteria classification in the beginning of the 20th century. Back in 2007, this species was reclassified from the genus *Vibrio* to the newly designed *Aliivibrio* (Urbanczyk et al., 2007). Despite this fact, a new name is not commonly used by researchists yet, and it is still possible to face the old version of the name in recent scientific papers and articles.

A. fischeri is normally found in the marine environment. It can be freely floating in the water as well as commonly create symbiotic relationships with other marine organisms, that is why it also plays an important role in ecology in marine ecosystems. One example of such symbiosis can be found with the Hawaiian bobtail squid. The bacteria are ecologically crucial for this species as the bioluminescence provides the squid with counter-illumination camouflage and lets it not cast a shadow on the ocean floor at night. In this way, this nocturnal animal uses bacterial bioluminescence in an antipredation strategy (Jones et al., 2004). In this symbiosis, the bacteria are found in a light organ which is a ventrally located tissue on the body of the squid (Fig. 2).

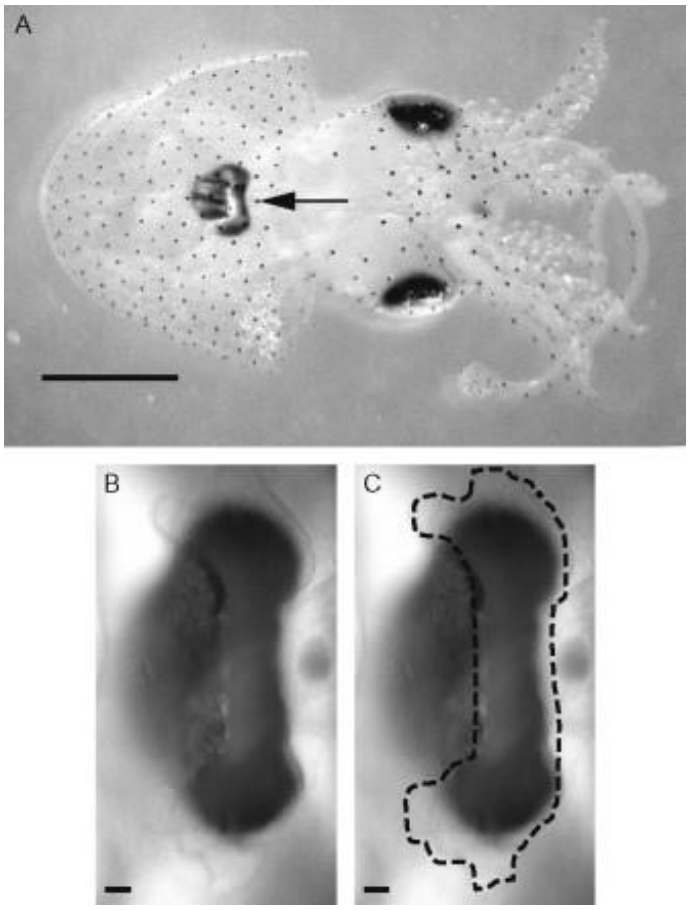

Figure 2. The light organ that houses Allivibrio fischeri consists of transparent tissue located on the ventral side of the animal (Dunn, 2012)
(A) Light microscopy image of the ventral side of a juvenile squid. The arrow points to the location of the light organ. Scale bar represents ~ 1 mm. (B) Higher magnification of the light organ region. Scale bar represents ~ 10 mm. (C) Same image as (B), highlighting the location of the transparent light organ

A. fischeri is mainly known for its ability to light production, and due to this feature this bacterium has been used a lot in research that has consequently led to important scientific discoveries. The ability to register changes in light emission activity which are invisible with the naked eye, cost-effectiveness, and other mentioned factors, which are common for testing on luminescent bacteria, make this species very suitable for many ecotoxicological experiments.

To sum it up, the special feature of bioluminescence made *A. fischeri* both ecologically and scientifically crucial, as light emission provides a range of various functions and uses.

3.3.2 Mechanisms of bioluminescence

The biochemical and genetic mechanisms of bacterial light emissions have been studied and fully understood (Meighen, 1993). The regulation of bioluminescence is led by the transcription of the *'lux'* operon which is included in the species genome. The *'lux'* operon is a bacterial luciferin-luciferase system that consists of five genes responsible for light emission and two genes regulating the operon. It controls light production through catalytic reactions with an enzyme called luciferase. The overall process can be described by the following formula ([eq.1]):

$$FMNH_2 + O_2 + R\text{-}CHO \rightarrow FMN + R\text{-}COOH + H_2O + light \qquad [eq.1]$$

Where:

$FMNH_2$ is a flavin mononucleotide called luciferin;

R-CHO is some long-chain aldehyde;

R-COOH is an acid corresponding to an aldehyde;

FMN is flavin mononucleotide.

The light is emitted during the slow decomposition of the complex called luciferase-hydroxyflavin, which is an intermediate substance of the mentioned reaction (Abbas et al., 2018).

The level of emissions is different, and it changes during the day that helps bacteria to camouflage and be less visible if it is necessary. The process of bioluminescence is controlled by the circadian rhythms of *A. fischeri*, so the light emission is more active during the daytime and more dimmed at night (Bulich, 1982).

There is also another intercellular signaling mechanism, which plays a key role in the bioluminescence regulation of *A. fischeri*, and it is called quorum sensing (Dunlap, 1999). This phenomenon is based on bacterial population density and related to two genes (luxI and luxR). LuxI is involved in the synthesis of an autoinducer molecule (acylated homoserine lactone) which accumulates in the bacterial cells up to the critical concentration for lux operon activation. Due to this, there is a necessary bacterial cell density which lets the lux operon transcription and following bioluminescence emission. Free-living bacteria which can be found in seawater have low population density and do not produce much light. Once the bacteria multiply exponentially in a photophore of marine animals, the transcription of the lux operon starts, and this process leads to luminescence (Nunes-Halldorson and Duran, 2003).

3.3.3 ISO 11348:2007 standard

Standardized instructions on how to proceed with a specific type of experiment play a crucial role in modern science and let scientists all around the world get sufficient results and be able to compare them to each other. ISO Standards (*International Organization for Standards*) was founded in 1947 and since then it has been developing standardized protocols for different industries. For now, there can be found over 19.500 international standards which are applied to all Sustainable Development Goals (SDGs). They include 17 topics with smaller and detailed goals within each of them. The topics which are connected to science and more related to the topic of this work are Clean water and sanitation, Industry, innovation and infrastructure, and Life on land.

ISO standards are approved by professionals and include guidance and step-by-step instructions on specific procedures. The standard ISO 11348:2007 describes a method for determining the inhibition of the luminescence emitted by the marine bacterium *A. fischeri*. These methods can be used for ecotoxicological tests of waste waters, leachates, fresh water, special single substances diluted in water, and others. Three methods differ by the type of bacteria activation. The standards for using freshly prepared bacteria are described in ISO 11348-1:2007, in the second part (ISO 11348-2:2007) the instructions on how to proceed with the tests with liquid-dried bacteria are given, and in the final part (ISO 1348-3:2007) the guidance explains the procedure with freeze-dried bacteria.

The choice of bacteria can affect the overall results of the experiments, and it depends on the ecotoxicological test, testing substances, and the laboratory and its available equipment. If the procedures given in the ISO standards are followed, the precise and validated results of conducted experiments are guaranteed. In the following chapters, the third part with procedures on freeze-dried bacteria will be described more in detail as this method was chosen for the experimental part of this work.

3.3.4 Freeze-dried bacteria

The principle of the test described in ISO 11348-3:2007 is based on the inhibition of luminescence emissions of the bacteria influenced by a toxic effect of a chemical substance. The test samples or diluted test samples should be verified by a specific volume of the bacterial suspension. According to the standardized protocols, the activity of light emission shall be measured 15 and 30 min after the beginning of the exposure.

The method described in the standards can be implemented for testing on:

- waste waters;
- leachates;

- freshwater and seawater samples;
- single chemical substances diluted in water;
- and others.

The above-mentioned standards provide all necessary information including some abiotic factors which can significantly affect the results of the tests, the amount of oxygen, pH, conductivity, and other parameters of the tested sample which should be measured and adjusted if needed. It also gives information on reagents and materials, such as diluent for bacteria which has a specific composition, solutions for adjusting pH, and reference substances that can be used before testing on chemicals of interest.

The bacteria which are used for this type of test are freeze-dried and they should be properly stored in the freezer at a specific range of temperatures, according to ISO 11348-3:2007. However, it does not describe the methods and time for bacteria activation, because this is always provided by the bacteria supplier and can considerably vary. Some bacteria require room temperature activation, while other types must be always stored in the refrigerator at a specific range of lower temperatures. Activation often includes two steps – during the first step the reconstitution agent should be added, and during the second step the diluent is used. The solutions often include NaCl as the main component as it is essential for *A. fischeri*, because this species is marine, and it requires a specific amount of salts dissolved in water for being able to maintain its life.

The standards also highlight what type of apparatus is necessary for the test conduction and defines some important details on it. Another important part of ISO 11348-3:2007 is a description of samples' preparation, preparations of stock and test suspensions, and the test procedure itself. It includes specific steps on bioluminescence measurements before and after tested chemical exposure by using an available luminescence instrument. The values given by this equipment provide information on how less or more intensive emissions were observed compared to the ones measured before exposure. The toxic chemicals are supposed to suppress the light emission; however, the small concentrations can also lead to more intensive light emission compared to the samples before exposure.

After all data on the gain or loss of luminescence is obtained, the values can be assessed and calculated. These steps can be found in the standards in the chapters dedicated to the evaluation and determination of values. One of the most common values, which is necessary to find out, is the half maximal effective concentration (EC_{50}). This number corresponds to a concentration of a toxicant, which leads to the specific effect in 50 % of the population, which was used for testing. In ecotoxicity testing, the expected effect is the death of the organisms exposed to a toxicant, which refers to the toxic effect of the substance.

EC_{50} can be found by concentration-effect relationship analysis using various tools and mathematical models and equations. According to the standards, the first type of evaluation of this relationship can be done with a linear regression technique. For this, the gamma value should be evaluated for each concentration ([eq.2]) (ISO 11348-3:2007):

$$\Gamma_t = [\overline{H_t}/(100 - \overline{H_t})] \quad \text{[eq.2]}$$

Where:

Γ_t is the gamma value of the sample after a specific amount of time;

$\overline{H_t}$ is the mean of the inhibitory effect of the test sample.

After the gamma value is found, it can be used for further calculation, according to the following equation ([eq.3]) (ISO11348-3:2007):

$$\lg c_t = b \lg \Gamma_t + \lg a \quad \text{[eq.3]}$$

Where:

c_t is the percentage of water in the sample (%);

b is the slope of a described line;

$\lg a$ is the intercept of the described line.

EC_{50} value can be calculated with the corresponding confidence limits, according to the statistical models:

$$c_t = EC_{50,t} \text{ at } \Gamma_t = 1{,}00.$$

Another type of evaluation to find EC_{50} is using the non-linear analysis. They are conducted by various software programs and different functions based on a normal distribution. The values can be obtained by statistical tools or graphically using a double logarithmic system.

3.3.5 Colour correction

The visible colour of the samples can also provide some information on changes in luminescence. The procedure of colour-correction method described in ISO 11348-3:2007 can be used during ecotoxicity experiment if there is a visible colour in the range of red and brown at the EC_{20} and it should be done if the sample concentration is close to the known EC_{50}. For this method, the colour-correlation tube is needed and the whole procedure should be conducted at a specific range of temperatures, according to the protocols (ibid.).

As an example of such a testing method, the effect of brown food addictive on toxicity measurement can be described. Quite often Caramel is used in the food industry to give various types of food and drinks brown colour. This addictive is not toxic, and this feature makes it appropriate for using it as a reference during ecological testing. In one of the studies, Caramel Colour Number 106 (E 150) was used to evaluate the changes in light output of *A. fischeri* bacteria suspension with and without exposure to $ZnSO_4$, and the range of brownish colours, as well as the level of bioluminescence, helped to evaluate it (Lappalainen et al., 2001).

3.4 Freshwater pollutants

Water pollution remains one of the main global environmental problems. Chemicals that are exposed to fresh and saltwater cause behavioural changes in the organisms and lead to various toxicological effects. Moreover, due to inappropriate wastewater treatment, there is a huge negative impact of the chemicals on human health starting from minor poisoning and finishing with irreversible health changes and death. Considering all these factors, the topic of water pollution requires a lot of attention and further research.

Freshwater pollutants have been detected in the environment at trace concentrations for many years. The highest concentrations have been found for the following groups of chemicals:

- industrials – PFOA (*perfluorooctanoic acid*), PFOS (*perfluorooctane sulphonic acid*), and DEHP (*di(2-ethylhexyl) phthalate*);
- pesticides – diazinon, methoxychlor, and dieldrin;
- PPCPs (*Pharmaceuticals and Personal Care Products*) – Ethinyl Estradiol (EE2), carbamazepine, *17β-estradiol* (βE2), N,N-diethyl-meta-toluamide (DEET), triclosan (TCS), diclofenac (DCS), acetaminophen etc.

These pollutants are extremely dangerous to the environment due to their occurrence in the highest concentrations. They still need to be examined more and new strategies and directions should be issued to protect wildlife and human health (Kyle et al., 2010).

The toxic effects of some groups of pollutants remain unclear. Carbon-based nanomaterials (CNM) have been found in aquatic systems in increased amounts recently. Although CNM concentrations observed in the environment do not cause negative effects on the organisms, some ecotoxicological effects occurred at its high concentrations and strongly depended on the type of organisms. In addition to it, the synergetic effect between CNM and other micro-pollutant interactions was also observed, and it related to the characteristics and chemical properties of each type of a substance (Freixa et al., 2018). Therefore, there is a need to conduct complex studies on different chemicals to be able to predict their effects on the biotic systems in the long term.

However, a lot of actions have been already taken to improve the situation. Nowadays, studies on environmental protection are focused on preserving or restoring the ecological status of specific environmental factors. As a result, chemical compounds have been registered and authorized for use in ecotoxicity and environmental impact research, which have to be conducted in the accordance with 2004/35/CE Directive. In addition to it, all member states of the European Water Framework Directive (WFD) must monitor and maintain the appropriate ecological status of different water bodies such as lakes, rivers, and others (Drzymała and Kalka, 2020).

3.4.1 Pharmaceuticals and Personal Care Products

As it was mentioned before, one of the main groups of freshwater pollutants is PPCP's (*Pharmaceuticals and Personal Care Products*). They include various types of chemicals and lots of them are released into the environment in drastic amounts and concentrations with urine and feces (Daughton, 2001), that is why concentrations of PPCP's found in wastewaters are enormous.

This group of pollutants is very diverse and includes various chemicals such as different analgesics, antibiotics and antimicrobials, synthetic hormones, and many other diverse groups (Esplugas et al., 2007) that make the problem of pollution a way more complicated. As an example, it is known that during the time microorganisms perform resistance to antibiotics and there is a need to adapt the drugs more to have some benefit from it that leads to further pollution with chemicals of more complicated structure and toxic effects.

PPCP's can easily accumulate in the aquatic organisms' tissues and cause chronic low-level effects. An example of this can be fluoxetine and paroxetine which are commonly prescribed antidepressants. Significantly high concentrations of those two chemicals were found in fish muscle tissue in Hamilton Harbour, Canada. Other PPCP's were also found accumulated in fish liver tissue and plasma (Chen et al., 2015). Some chemical substances are also known to have a high level of biomagnification, which

means that its concentrations are greater following each step of the food chain. This phenomenon leads to considerable ecological problems.

At the same time, another popular topic for scientific discussion is the usage of synthetic hormones that becomes more and more common in different developed countries. After release into the environment, these substances and their metabolites cause behavioural changes in water organisms and can affect their reproduction system and normal population development. For instance, in the regions of some wastewater flows, male fishes are noticed to produce vitellogenin which is a hormone usually produced by females, and show other types of feminization. This is a result of high levels of oestrogen and its substances contained in water which are used in birth control pills (Kidd et al., 2007).

All these and many other factors make the topic of PPCP's in the environment a topic of intensive scientific research and public concern.

3.4.2 Triclosan

Triclosan (TCS) is an antibacterial and antifungal agent, and it is considered to be one of the most common PPCP's pollutants. Due to its high safety rating, it has been used in a wide range of personal care products, such as deodorant soaps, underarm deodorants, shower gels, and health care personnel handwashes (Bhargava et al., 1996).

Triclosan is also called by its short form TCS; the full name of the substance is 2,4,4′-trichloro-2′-hydroxydiphenyl, CAS 3380-34-5. It is a chlorinated organic compound, and its functional groups correspond to both ethers and phenols (Figure 3).

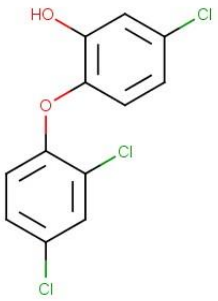

Figure 3. Molecular structure of Triclosan (ECHA, ©2023)

The antibacterial is quite soluble in water (10 mg/L, t = 20 °C) and it is considered a lipophilic substance with a high level of bioaccumulation. In addition to it, its by-products, for example, methyl triclosan and other chlorinated phenols have higher resistance to degradation, and they are more toxic compared to the parent compound (Dann et al., 2011).

Triclosan first appeared in the end of the 20th century, and it was originally used as a component for healthcare products and toothpaste. Triclosan was prohibited from being used in soap products in September 2016, because of a risk evaluation done by the U.S. Food and Drug Administration (FDA). However, it is still present at trace concentrations in other products such as surgical soaps, toothpaste, and sanitizers, therefore humans quite often get in contact with the chemical. Human skin and oral mucosa are both easily permeable to triclosan, so it is also present in different human tissues (Weatherly et al., 2017).

Also, due to its chemical structure, TCS is quite similar to specific oestrogens which can potentially affect the sex ratios of fish and the length of its fins (Ishibashi et al., 2004). In addition to it, in recent years the chemical has been detected in many aquatic organisms, and further effects of triclosan on organisms have to be assessed. All these and many other factors are the consequence of huge chemical releases and inappropriate wastewater treatment.

Most PPCP's, which are used as antiseptics, are freely released into sewage drains, and treated by wastewater treatment plants (WWTP). Although there are various wastewater treatment methods and techniques which allow to remove many types of chemicals, municipal sewage treatment plants are not designed to eliminate personal care products from water. Triclosan is one of those that remain in the water even after treatment procedures - the studies show that it is found in wastewater treatment plants influents in the range of concentrations between 52 and 21,900 ng/L (Bedoux et al., 2012).

Some research on triclosan and its ecotoxicity has been conducted on different living organisms. As in most scientific studies the long-term exposure to triclosan was assessed, and the toxic effect of the chemical was observed on *Daphnia magna* and other species. However, some studies were also performed on the luminescent bacteria *A. fischeri* and some toxicity parameters of triclosan and its main metabolite methyl triclosan were found and compared to other chemicals (Table 1). From the table is it visible that TCS is more toxic than the other chemicals used in the research due to their lower 50 % effective concentrations, a higher number of toxicity units and quite low values for the lowest observed effect concentrations. To obtain the results a wide range of concentrations was used, and the bioluminescence inhibition was measured (Farré et al., 2008).

Table 1. Toxicity parameters obtained using A. fischeri, expressed as 50 % bioluminescence inhibition (EC50; μg/mL*), toxicity units (TU), and lowest observed effect concentration (LOEC; μg/mL) for standard substances (Farré et al., 2008)*

Compound	EC_{50} (μg/mL)	TU	LOEC (μg/mL)
Triclosan	0.28	357	0.10
Methyl triclosan	0.21	476	0.075
NP	0.36	278	0.12
OP	0.30	333	0.12
NP_2EO	2.04	49	0.90
$NP_{9-5}EO$	2.70	37	1.25
OP_1EC	2.38	42	1.00
NP_1EC	2.64	38	1.22
NP_2EC	3.05	33	1.00
LAS	144.00	0.7	55.00
CDEA	5.46	18	6

**NP – nonylphenol; OP – organophosphate; NP_2EO – nonylphenol diethoxylate; $NP_{9-5}EO$ – polyethoxylated nonylphenols; OP_1EC – 4-tert-octylphenoxyacetic acid; NP_1EC – nonylphenoxy acetic acid; NP_2EC – nonylphenoxyethoxy acetic acid; LAS – linear alk ylbenzene sulfonate; CDEA – coconut diethanol amide*

Unfortunately, a single-effect assessment is not enough to properly evaluate the harmful consequences of triclosan exposure to the environment. E.g., the synergetic effect of triclosan and methyl triclosan in the mixture with other chemical compounds was shown. Higher bacterial bioluminescence inhibition was observed in the mixture of triclosan and linear alkylbenzene sulfonates (LAS), which means that TCS can cause a more toxic effect in the combination with some other pollutants (Farré et al., 2008).

3.4.3 Diclofenac

Diclofenac (DCF) is included in a group of non-steroidal anti-inflammatory drugs (NSAIDs) and it is well-known under the name of the brand Voltaren. It is mostly available for purchase without a prescription in the form of oral pills or an ointment to be applied to the skin (Lonappan et al., 2016). It is also possible to apply the pharmaceutical rectally or as an injection. Diclofenac was first patented in the middle and became widely prescribed and used all around the world in the end of the 20th century.

The chemical is not only found in the human health industry but also applied to livestock in different countries, it is commonly used to control some bacterial diseases of farm animals. Due to the direct exposure to the environment after being used, DCF caused some ecological disasters such as the Indian vulture crisis, which lead to the extinction of 95% species' population in the end of the 20th and in the beginning of the 21st century (Cuthbert et al., 2014). The reason for this was that vultures feed on the dead stock to which diclofenac was applied. Nowadays, the use of the chemical in agricultural work is forbidden.

The International Union of Pure and Applied Chemistry (IUPAC) name of diclofenac is 2-[2-(2,6-dichloroanilino) phenyl] acetic acid, CAS 15307-86-5, and it is relatively highly lipophilic (Figure 4). In the cells it acts by inhibiting the cyclooxygenase enzyme which is responsible for prostanoids synthesis, and it leads to pain elimination (Kyle et al., 2010). Similar to other nonsteroidal anti-inflammatory drugs, it is associated with gastrointestinal and cardiovascular adverse effects, which are very dose-dependent (Altman et al., 2015).

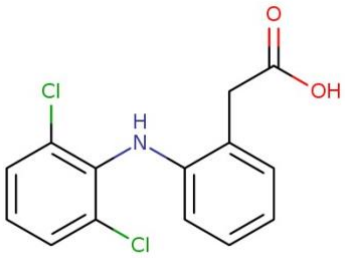

Figure 4 Molecular structure of Diclofenac (ECHA, ©2023)

As it was mentioned before, the main source of PPCP's (including diclofenac) in the environment is residential wastewater. However, pharmaceuticals that are used in veterinary and stock farming can be directly exposed to natural environments, for example, to pastures or manures (Foucquier et al., 2015). This is the reason why chemicals such as diclofenac should be studied more to give a clearer picture of the level of toxicity on different organisms as it directly interacts with them in nature. Moreover, the question of synergism also takes place in the overall toxic effect because chemicals may interact with each other and show different bioactivity that causes various environmental problems.

According to the studies, diclofenac was classified as a chemical with low or average toxicity on different organisms. However, in the mixture with sulfamethoxazole

(SMX), which is a broad-spectrum antibiotic against various types of bacteria and microorganisms, the experiments showed different results. The mixture of the two chemicals was considered as average or highly toxic (Table 2).

Table 2. Comparison of toxicity of DCF, SMX and their mixture (Drzymała and Kalka, 2020)

Test organism		**DCF toxicity classification**	**SMX**	**Mixture**
A. fischeri		average toxicity	low toxicity	average toxicity
D. magna	24 h	low toxicity	low toxicity	average toxicity
	48 h	average toxicity	low toxicity	average toxicity
L. minor		low toxicity	low toxicity	high toxicity
Summary		average toxicity	low toxicity	high toxicity

*The result for the most sensitive test organism is taken as a summary.

A huge number of complex pollutants and compounds can be found in the environment today, and it is a challenge to assess their toxicity as the content and chemicals ratio of such wastewater is constantly changing. Micropollutants must be observed for a long-time exposure, and the complex effects must be assessed. Despite all these facts, it is extremely important to continue research on this topic and improve prediction and evaluation tools which can be also useful during environmental studies (Drzymała and Kalka, 2020).

3.5 Related environmental legislation

Environmental regulations are a crucial part of contemporary law, especially in the countries which are members of the European Union. Most of the environmental documentation deals with the green and circular economy, endangered species protection, air and water quality control, and other environmental factors which are necessary to track to ensure safe and good quality of life. They are also a result of the cooperation of government agencies, the public, stakeholders, and risk assessors, who equally contribute to the process and look at it from different perspectives.

Among several categories of legislative documentation belong:

- policies;
- legislations;

- and regulations.

Policies are documents that represent a plan or a program within a specific topic. Environmental policies deal with the prevention and mitigation of the negative effects of some anthropogenic activity on the environment. Policies as a type do not have any legal implications, however, they lead to the development of new laws and related limits.

Legislations are laws or sets of laws defined and implemented for a particular level of government. In the field of the environment, legislations refer to the regulation of different types of pollution emissions, limiting or prohibition of human activities that are harmful to nature.

Regulations are rules within a law that are crucial for its realization, and they can be changed without modifying the law itself. This type of documentation can specify the limits of emissions, requirements for toxic waste, and others (Welbourn et al., 2022).

3.5.1 PPCP's legislation

Pharmaceuticals and personal care products are extremely diverse, and they include cosmetics, veterinary medicines, agriculture, and health care items. Some research has calculated that an approximate number of PPCP's in the world reached six million different chemicals (Eckstein, 2012). Due to this great number which is constantly increasing over time, and a lot of complications connected to PPCPs assessment, there are no strict and exact regulations on it so far.

Most of the chemicals of this group are said to be resistant to current wastewater treatment techniques, which leads to its exposure to natural water bodies such as lakes, rivers, groundwater, and others. The existing legislation is not adopted for the treatment of PPCP's specifically, but there are certain chemical substances that are under some legislation. For example, in 2013 a list of chemicals for monitoring all around the European Union was established. It controls substances in the field of water policy in accordance with Directive 2008/105/EC of the European Parliament and of the Council and includes pharmaceuticals such as diclofenac, various hormones, antibiotics erythromycin, clarithromycin, and other substances included in the group of insecticides and herbicides (Hrkal et al., 2018). This directive is a part of environmental quality standards (EQS), it specifies pollutants mentioned in Article 16 of Directive 2000/60/EC and aims to achieve good surface water conditions in terms of its chemical composition (ECOLEX, ©2023).

In addition to it, there are several regulations released under the European Chemical Agency (ECHA), which include the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) and the Classification, Labelling and Packaging (CLP) regulations, and others that contribute to the protection of human health and the

environment. REACH applies to various chemicals and provides with procedures on the properties and hazardous effects of chemicals. For instance, according to REACH, diclofenac can cause damage to organs during prolonged exposure, it is also toxic for aquatic organisms, and it can be harmful to unborn children or damage fertility.

CLP deals with the classification and labelling of chemical substances and ensures a high level of protection of human health and the environment. According to the 1272/2008/EC Regulation under CLP, pharmaceutical substances are supposed to be treated under the procedures as long as they are not the substances in their finished state intended for a final user. For example, the regulation defines triclosan as a substance that is very toxic for aquatic organisms with long-term effects, which also can cause eye and skin irritation (ECHA, ©2023).

There are certain numbers and concentrations set for the specific use of triclosan and diclofenac in products and goods. According to 358/2014/EU Commission Regulation, the maximum concentration of triclosan which is allowed for toothpaste, soaps, and cosmetics production is 0.3 % and 0.2 % for mouthwashes. Diclofenac was mentioned in the 582/2009/EC Commission Regulation which provided maximum residue limits of veterinary use in foodstuffs (10 μg/Kg in the kidney and 5 μg/Kg in the liver of bovine). These numbers are not enough for organized chemical treatment and regulations, and further laws are required.

In addition to it, there are some regulations under ECHA such as PIC (Prior Informed Consent Regulation), which deals with storage, usage, and disposal measures of hazardous substances but do not include pharmaceutical and veterinary products. Therefore, the topic of PPCP's regulation must be developed, and the lists of substances with details on their usage and properties have to be expanded.

3.5.2 Waste legislation

Waste is another crucial topic of concern within the field of ecotoxicology. A large number of chemicals from different categories occur in the environment as a result of various anthropogenic activities. It was estimated that around 70 thousand substances are produced by the chemical industry nowadays (Asthana, 2014). Many of them are released into the environment and cause harmful effects on living organisms and human health. That is why the political awareness and contribution to the stated problem have increased considerably and developed several legislations on proper waste management (Rogowska et al., 2020).

One of the most important directives of the European Union is the 2008/98/EC Waste Framework Directive (WFD). WDF defines waste as: *'any substance or object which the holder discards or intends or is required to discard'*. The principles of this regulation are in the accordance with the concepts of the circular economy and waste

hierarchy which considers waste prevention as the priority and landfill waste collection as the last option during the process of waste management. The whole hierarchy of waste management activities provided by Directive 2008/98/EC is the following:

1. waste prevention;
2. re-use of materials;
3. recycling and recovery;
4. waste disposal.

WFD was developed under the ECHA agency to develop effective measures for waste management and improve the usage of resources, which is also a crucial idea of the circular economy.

Hazardous waste has been observed more due to its high toxicological effects on human health and the environment. The WFD provides with detailed information on how to monitor and control such substances and defines several categories into which they can be divided. It also includes methods for the evaluation of hazardous wastes, limit values and criteria for it, and waste sampling and methodology which should be used for laboratory testing and during ecotoxicological studies. This and other necessary information can be found in the SCIP (the database for information on Substances of Concern In Products) established under WFD.

Another important document that was released in the field of chemical legislation is the European List of Waste (LoW). This list includes hazardous materials and substances by a decision-making process on the evaluation of chemicals from the European Waste Catalogue, in which both hazardous and non-hazardous wastes can be found. The process of assessment includes the identification of hazardous ingredients present in the waste chemical and its properties and based on this information the final statement is made (Maraboutis et al., 2016).

Although a lot of steps have been made, it is still a challenge to protect the environment from waste and maintain its good conditions. Waste compounds are still having a harmful impact on surface waters due to the fact that a narrow spectrum of chemicals is described in the present legislation (Rogowska et al., 2020).

3.5.3 Water legislation

Water management and wastewater treatment are other topics of ecotoxicology concern. There are wastes exposed to aquatic environments which have to be analyzed by ecotoxicology testing and categorized for specific treatment measures afterward. Non-polluted water is required everywhere and by every structure, that is why it has to be protected by specific organizations, and strict rules and limits have to be clarified.

Water Framework Directive 2000/60/EC (WFD) was first issued in October 2000, establishing the framework for the topic of water policy. The directive ensures natural ecosystem protection from the key issues present in the environment, including chemical pollution of water, wastewater treatment, and drinking water management. It also focuses on the decision-making process and mentions the political structures which need to be involved in it as well as the clear timeline and deadlines which should be followed for the implementation of each step. One of the main goals of the Directive is to reach a *'good status'* for surface waters, groundwaters, and coastal and transitional waters to be maintained both from chemical and ecological perspectives. According to Annex V of WFD, good chemical status can be achieved by following all the requirements specified for parameters such as the content of oxygen, pH, conductivity, nitrate, and ammonium content (Greim and Snyder 2018).

One part of the directive focuses on strategies against water pollution. According to the Article 16 of Directive 2000/60/EC following main ideas shall be obeyed:

- specific measures against water pollution should be adopted by state organizations regarding individual pollutants or groups of them at significant risk to aquatic environments;
- the priority hazardous substances should be identified;
- recommendation from the Scientific Committee on Toxicity, Ecotoxicity and the Environment, the European Environmental Agency, and other organizations dealing with the environment should be taken into account;
- the controls for discharges and emissions reduction should be taken for priority substances;
- and others.

The Environmental Quality Standards Directive 2008/105/EC (EQSD) is another law document, which plays role in surface water regulation and management and considers pollutants as components of ecological status. This directive provides a list of priority substances aimed to tackle eco-toxicological effects, bioaccumulation, and health impacts and establishes environmental quality standards for surface waters for these 33 priority chemicals (Greim and Snyder, 2018). The document includes pesticides, personal care products, *'forever chemicals'*, plastics, several pharmaceuticals (painkillers and antibiotics), and others.

As it was mentioned above, the released directives and other types of documentation are regularly updated depending on the knowledge and scientific development. For example, some pharmaceuticals are expected to be reconsidered as priority hazardous substances by challenging current regulatory approaches due to the stereochemical effects of chiral chemicals. Therefore, it is stated that current documentation such as ERA (*Environmental Risk Assessment*) can lead to under or overestimation of the

toxicity of some chemicals. Consequently, it can cause some toxic effects on living organisms and the environment where the chemicals are exposed in higher concentrations (Andrés-Costa et al., 2017).

4. Methodology

The methodology, which was used for conducting the experimental part of this thesis, was in the accordance with the ČSN EN ISO 11348-3:2007 standard – Water quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) – Part 3: Method using freeze-dried bacteria, as amended in 2018. This method is applicable for:

- wastewater;
- aqueous extracts and leachates;
- surface or ground fresh water;
- and others, such as seawater or single substances, diluted in water.

The experimental part also complied with the good laboratory practice (GLP) and the ČSN 01 8003:2017 standard – Safety code for working in chemical laboratories, as amended in 2021.

The objective of this thesis was to evaluate the influence of a source of *A. fischeri* on the validity of the luminescence toxicity testing by comparing three different freeze-dried bacteria suppliers (Microtox® – Modern Water, U.S.A.; Biolight – Microbiotest, Belgium, and LUMIStox – Hach Lange, Germany). The comparison was performed considering the efficiency of achieved test results, the price of materials, and related shipping costs.

4.1 Materials and equipment

4.1.1 Chemicals

- Potassium dichromate, $K_2Cr_2O_7$, CAS: 7778-50-9 (Sigma Aldrich, Czech Republic)
- Triclosan (TCS) $C_{12}H_7Cl_3O_2$, CAS: 3380-34-5 (Sigma Aldrich, Czech Republic)
- Diclofenac (DCF) $C_{14}H_{11}Cl_2NO_2$, CAS: 15307-86-5 (Sigma Aldrich, Czech Republic)
- Sodium chloride, NaCl, CAS: 7647-14-5 (Sigma Aldrich, Czech Republic)
- Microtox® Diluent solution, 2% NaCl (Modern Water, U.S.A.)
- Microtox® Salinity Adjustment Solution, 22% NaCl (Modern Water, U.S.A.)
- Biolight Recon Solution, BIO2001, 22% NaCl (Microbiotest, Belgium)
- Biolight Diluent Solution, BIO2002, 2% NaCl (Microbiotest, Belgium)

- Hach® Reconstitution Solution, LCX 047, 22% NaCl (Hach Lange, Germany)
- Hach® Diluent Solution, LCX 048, 2% NaCl (Hach Lange, Germany)

4.1.2 Test organisms

For the experiment conduction, the vials with freeze-dried luminescent bacteria *Aliivibrio fischeri* (formerly *Vibrio fischeri*) were ordered from three different suppliers based in different countries:

- SOLO Microtox® Reagent, Acute Toxicity Test, vials (Modern Water, U.S.A.)
- Biolight, Single Test Reagent, BIO2007, vials (Microbiotest, Belgium)
- LUMIStox, Hach® LCK 491, bottles (Hach Lange, Germany)

All supplied vials and bottles with bacteria delivered by all suppliers were preserved in the freezer at -18°C.

4.1.3 Equipment

Most equipment used was made of laboratory glass. It was properly washed, rinsed with ultrapure water, and dried at 45°C before conducting the experimental part. The following glassware was used:

- Beakers 25, 50 and 100 mL (P-Lab, Czech Republic)
- Volumetric flasks 15, 25 and 50 mL, (P-Lab, Czech Republic)
- Volumetric flasks stoppers (P-Lab, Czech Republic)
- Glass cuvettes (Modern Water, U.S.A.)
- Cuvette rack (Modern Water, U.S.A.)
- Pasteur pipettes
- Automatic pipettes WITOPET and Acura®, 100-1000 µL; 0.5-5 mL (Witeg, Germany; SOCOREX, Switzerland)
- Pipette tips (100-1000 µL; 0.5-5 mL)
- MS Excel program (Microsoft, U.S.A.)

4.1.4 Apparatus

- InoLab® pH meter 7130 with the SenTix 81 probe (WTW CZ, Czech Republic)
- MultiLine® Multi 3620 IDS meter with conduction TetraCon 925 and oxygen FDO 925 probes (WTW, Czech Republic)
- Analytical balances ABJ 220-4JN (KERN & SOHN, Czech Republic)
- Freezer F 6248 W (Gorenje, Slovenia)
- Refrigerator NORDline UR 600 S (TEFCOLD CZ, Czech Republic)
- Hot air dryer ED 115 (BINDER, Germany)
- Ultrapure water device PURELAB flex 1 (ELGA LabWater, United Kingdom)
- Microtox® FX bioluminescence analyser (Modern Water, U.S.A.)

4.2 Methodology

4.2.1 Calibration of bacteria concentrations

As the main objective of this thesis can be only achieved by comparing three different luminescent bacteria suppliers performing the same ecotoxicity tests, the concentrations of bacteria in tested samples should be the same to be able to obtain reliable, precise, and comparable results.

According to the SOLO Microtox® (Modern Water, U.S.A.) and Biolight (Microbiotest, Belgium) suppliers, the initial number of bacteria was around 100 million cells per vial, the amount of LUMIStox (Hach Lange, Germany) bacteria per vial was significantly higher, as the bottle was purchased. Therefore, it was necessary to test different dilution rates and choose the best options for each supplier considering the fact that their performance shall be comparable.

Before the experiment on the tested chemicals was conducted, several tests on the calibration of bacteria concentrations were performed. For this test, the Microtox® supplier was chosen to find the most sufficient concentration which shows reliable results compared to the Microtox® 81.9% Basic test (Microtox® 2023).

The overall procedure of preparing final samples with bacteria is described in the commercially available system Microtox® (Modern Water, U.S.A.). It includes the activation of bacteria with reconstitution and dilution solutions and proper dilution of the sample with the tested chemical. Before the experiment, some calculations were done to define possible final bacteria concentrations and compare test results afterward.

The chosen volumes of diluent added per vial with bacteria for its activation were 600 µL, 900 µL, and 1.5 ml. After 15-minute activation bacteria suspension was added to the cuvettes placed in a cuvette rack and mixed with the dilution solution (Table 3). Each dilution was checked separately and compared to the 81.9% Basic test which was used as a control reference. Samples with the final bacteria concentrations of 0.238 mL/mL (low), 0.159 mL/mL (medium), and 0.095 mL/mL (high) were tested.

Table 3. Tested concentration of luminescent bacteria per sample – SOLO Microtox® vials

Tested dilution - SOLO Microtox®	**size (type of pck)**	**dilution. solution (mL)**	**volume per sample (mL)**	**volume of diluent (mL)**	**bact. conc. in pck (mL/mL)**	**bact. conc. in sample (mL/mL)**
81.9% Basic test	vial	0.30	0.10	0.90	3.33	0.333
Dilution - high	vial	1.50	0.15	0.90	0.67	0.095
Dilution - medium	vial	0.90	0.15	0.90	1.11	0.159
Dilution - low	vial	0.60	0.15	0.90	1.67	0.238

The measurement on the Microtox FX device was done each 5 min during the first hour, then after 30 min up to 2 hrs to track the luminescence activity drop. All data were recorded in a separate sheet of the MS Excel program (Microsoft, U.S.A.) and necessary calculations were done using this programme tool. This process is described in detail in the following chapters.

After a series of experiments, it was found out that the most sufficient was the low dilution with a concentration of bacteria of 0.238 mL/mL, therefore it was used in the following experiments on tested chemicals. Also, the experiment was repeated at room-temperature and cooled diluent (6–8°C), and it showed that more sufficient results can be got using room-temperature materials.

For the other two suppliers, the same calculations were done and the similar concentrations of bacteria, as well as volumes of needed diluent per vial, were validated (Table 4).

Table 4. Tested concentration of luminescent bacteria per sample – Biolight vials and LUMINOStox bottle

Tested dilution - Biolight	size (type of pck)	reconst. solution (ml)	Dilution solution (mL)	volume per sample (mL)	volume of sample (mL)	bact. conc. in pck (mL/mL)	bact. conc. in sample (mL/mL)
Dilution - standard (high)	vial	0.10	1.00	0.10	0.90	0.91	0.091
Dilution - medium	vial	0.10	1.00	0.15	0.90	0.91	0.130
Dilution - low (as SOLO Microtox® standard)	vial	0.10	1.00	0.40	0.70	0.91	0.331
Tested dilution - LUMINOStox							
Dilution - standard (high)	bottle	1.00	50.00	0.50	0.50	0.20	0.098
Dilution - medium	bottle	1.00	15.00	0.20	0.80	0.63	0.125
Dilution - low (as SOLO Microtox® standard)	bottle	1.00	15.00	0.60	0.40	0.63	0.357

4.2.2 Preparation of potassium dichromate stock solution

After the most reliable concentration of SOLO Microtox® luminescent bacteria was found, a series of experiments were done on a reference solution of potassium dichromate ($K_2Cr_2O_7$) to check the methodology and confirm setting before the real experiment on tested chemicals (TCS and DCF).

For the experiment, the stock solution of potassium dichromate of a concentration of 25 mg/L was prepared. Using the pH meter, the conductivity and oxygen meter to the following abiotic parameters were measured and compared to standardized values from the ISO 11348-3:2007 guidelines:

- pH = 6.8
- NaCl-equivalents (salinity) = 25.5 g/L
- oxygen concentration (O_2) = 8 mg/L
- t = 25°C

Salinity (NaCl-equivalents) of the solution was originally lower, therefore some Salinity Adjustment Solution (22% NaCl) was added to it to reach higher values

required by the guidelines (20-50 mg/L). The rest of the parameters was in the required ranges (6.0-8.5 for pH; > 3 mg/L for oxygen concentration).

4.2.3 Preparation of dilution series

According to the ISO 11348-3:2007 standards, the EC_{50} value for $K_2Cr_2O_7$ for freeze-dried bacteria is 18.71 mg/L. Considering this value, the dilution series of concentrations was prepared using the pre-prepared stock solution. The final concentrations were: 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.19, 0.097, and 0.048 mg/L. As the control (blank sample) was used the dilution solution, 2% NaCl (Figure 1). The dilution series was prepared according to the mentioned Microtox® 81.9% Basic test manual.

4.2.4 Activation of bacteria

The SOLO Microtox® bacteria (Modern Water, U.S.A.) was activated prior to the test by adding the diluent solution (2% NaCl) and placing them into the fridge for 15 min.

For the Biolight BIO2007 bacteria (Microbiotest, Belgium), the procedure of its activation was different. First, 0.1 mL of the reconstitution solution (22% NaCl) was added to the cuvette at 5 °C. After 15 min, 1 mL of thermostated diluent solution was put in a reagent cuvette, and the mixture was placed in the thermostat at 15 °C for 15 min.

The Hach® LUMIStox, LCK 491 bacteria (Hach Lange, Germany) was activated by 1 mL of a reconstitution solution at refrigerator temperature and placed there for 15 min. After this step, 15 mL of a dilution solution was added and left for other 15 min. in the refrigerator.

All the above-mentioned procedures follow the commercially available instructions provided by each of the tested suppliers. The procedure of the Biolight and LUMIStox bacteria activations follows also the ČSN EN ISO 11348-3:2007 requirements.

4.2.5 Testing on potassium dichromate

For testing on potassium dichromate, the standard concentration (81.9% Basic test – slightly toxic, 0.333 mL/mL) and the chosen concentration of 0.238 mL/mL was used for the SOLO Microtox® supplier; three concentrations of 0.091, 0.13 and 0.331 mL/mL for the Biolight bacteria, and three concentrations of 0.098, 0.125 and 0.357

mL/mL for the Hach® LUMIStox. All measurements were repeated 3 times to validate data.

All obtained values were recorded into separate tables using MS Excel Sheets, the representative graphs were created to calculate the EC_{50} values for each experiment and compare them afterward. More detailed information on the procedure of EC_{50} values calculation and data analysis is available in the following chapters.

As a result of this experiment, the concentrations of bacteria which showed the most precise values compared to a standardized table concentration (18.71 mg/L), were chosen to perform the verification tests on TCS and DCF solutions:

- SOLO Microtox®: 0.238 mL/mL (low dilution)
- Biolight: 0.331 mL/mL (low dilution)
- Hach® LUMIStox: 0.125 mL/mL (medium dilution)

4.2.6 Preparation of stock solutions of diclofenac and triclosan

The stock solutions of DCF and TCS were prepared prior to the test. According to the available literature, EC_{50} of DCF was *A. fischeri* 11.45 mg/L for 30-min exposure time (Ferrari et al., 2003), and of TCS 0.28 μg/mL for 15-min exposure time (Farré et al., 2008). Therefore, the stock solutions were prepared at concentrations of 20 mg/L and 2 μg/L correspondingly.

Both stock solutions were stored in the refrigerator at the temperature of 6 °C without UV-light exposure. As the measurements were conducted the following day after the stock solutions were prepared, their concentration decreased. This drop was more considerable in the case of TCS as this chemical is unstable and fast degraded under UV-light exposure.

The final concentrations of the chemicals were measured using the HPLC-UV-VIS (High Performance Liquid Chromatography combined with the UV-Vis detector) UltiMate™ 3000 (Thermo Fisher Scientific©, U.S.A.) at the day of the performing the measurement were:

- TCS: 1.106 μg/mL
- DCF: 19.177 mg/L

4.2.7 Testing on diclofenac

The dilution series was prepared for DCF at the concentrations of 19.177, 9.589, and 4.794 mg/L. Each concentration was triplicated, therefore there were nine samples in total and one control to measure.

All bacteria were activated according to the above-mentioned procedures. The tests on each bacteria supplier were performed twice.

The bioluminescence of activated bacteria was measured first, and afterward DCF was added immediately. The timer on the instrument was set for 15 min of exposure. After 15 min, the bioluminescence measurements were performed, and the results were recorded. The same procedure was repeated and the results for 30 min exposure were recorded.

The test was first performed on the SOLO Microtox® bacteria, then on Biolight, and finally on the HACH® LUMIStox. Each supplier required a specific volume of the reconstitution solution and the diluent added for activation, as well as the volume of the bacteria added into each sample, and the volume of the sample used (Table 5).

Table 5. Dilution procedures used to get the experimentally established best-fit final bacteria concentrations

	size (type of pck)	**reconst. solution (mL)**	**dilution solution (mL)**	**volume per sample (mL)**	**volume of sample (mL)**	**bact. conc. in pck (mL/mL)**	**bact. conc. in sample (mL/mL)**
SOLO Microtox®	vial	0.60	0.00	0.15	0.90	1.67	0.238
Biolight	vial	0.10	1.00	0.40	0.70	0.91	0.331
Hach® LUMIStox	bottle	1.00	15.00	0.20	0.80	0.63	0.125

4.2.8 Testing on triclosan

The TCS concentrations of the dilution series were 1.106, 0.553, and 0.28 μg/mL. Each concentration was triplicated, therefore there were six samples in total and one control to measure. All bacteria were activated according to the above-mentioned procedures. The tests on each bacteria supplier were performed twice.

The procedure for sample preparation was based on the same table values as for DCF (Table 4), and the bioluminescence measurement was done the same way as for the previously tested chemicals. The measured values for both 15 and 30 min exposures were recorded into the Excel sheet separately for each of the three suppliers.

4.3 Data assessment and calculations

All the results were available on the instrument display as '*gain*' or '*loss*' in percentages, representing stimulation or inhibition of light emission correspondingly. The values were recorded in the Excel Sheet table in the format of '+%' (for loss) and '–%' (for gain), and they were used to do further calculations.

To obtain the EC_{50} value for each of the chemicals and suppliers, the average values for 15 and 30 min exposure were calculated in Excel. Also, the standard deviation values were calculated to estimate the accuracy of the recorded results.

The average values were further used to plot graphs representing the dependence of the average effect (Y–axis) on concentrations of the chemical (X–axis). The trendlines were added to the graphs as well as the equations and R–squared values (Figure 1). The R–square value was used to choose the fittest trendline (exponential, polynomial, logarithmic, etc). The closer the R–squared value to 1, the more precise the experimentally obtained equation.

The equations, which fit the best, were used to calculate EC_{50} values. The Y value in each of them was equal to 50 because the target concentration corresponded to the average effect of 50%. When the equations were prepared and EC_{50} values were obtained, they were recorded and compared to already-known table values.

5. Results

5.1 Validation of the tested bacteria

According to the mentioned procedure on calculations, the data for experiments on potassium dichromate were proceeded. Average values for 15 and 30 min exposure and standard deviations (SD) of the results were also included in the data analysis as in the example below (Table 6). The same datasets were obtained for SOLO Microtox® and LUMIStox bacteria.

Table 6. The experimentally obtained luminescence measurements for potassium dichromate tested on Biolight bacteria with a concentration of 0.331 mL/mL

	K2Cr2O7 [mg/L]									
	0.0488	**0.0977**	**0.19531**	**0.391**	**0.7813**	**1.5625**	**3.125**	**6.25**	**12.5**	**25**
15 min	–17	–19	–25	–18	–20	20	–4	30	16	37
30 min	–33	–57	–67	–80	–19	41	0	51	39	56
15 min	1	–9	–8	19	36	–8	–6	24	30	67
30 min	–61	–87	–53	6	12	–34	–27	23	15	70
15 min	–2	6	1	9	10	–1	1	9	45	28
30 min	–1	–8	–7	26	27	20	19	36	71	63
Average 15 min	–6.00	–7.33	–10.67	3.33	8.67	3.67	–3.00	21.00	30.33	44.00
Average 30 min	–35.00	–50.67	–42.33	–16.00	6.67	9.00	–2.67	36.67	41.67	63.00
SD 15 min	9.64	12.58	13.20	19.14	28.02	14.57	3.61	10.82	14.50	20.42
SD 30 min	25.06	39.88	31.39	56.32	23.46	38.69	23.12	14.01	28.10	7.00

After a series of experiments conducted on potassium dichromate using different luminescent bacteria sources, the EC_{50} concentrations were calculated and recorded (Table 7). Based on these results, it was possible to choose the optimum concentrations for each of the suppliers.

Table 7. The experimentally obtained EC_{50} values for potassium dichromate

Supplier	Bacteria concentration [mL/mL]	EC_{50} ($K_2Cr_20_7$) [mg/L]	
		15 min	30 min
SOLO Mictotox®	**0.238 - low dilution**	18.17	15.56
	0.333 - slightly toxic	25.68	24.01
Biolight	0.091	14.01	31.49
	0.130	40.86	33.63
	0.331	25.57	19.92
LUMIStox	0.098	21.60	19.58
	0.125	18.21	19.08
	0.357	49.89	20.60

Note.: Concentrations in bold represent the bacteria values that are the closest to the standardized EC_{50} value (i.e., 18.71 mg/L) for potassium dichromate.

5.2 Verification of DCF and TCS

After all the calculations were done and representative graphs with average effect were plotted for each of the chemical and bacteria suppliers as in the example (Figure 5), the final EC_{50} values for each bacteria supplier were compared.

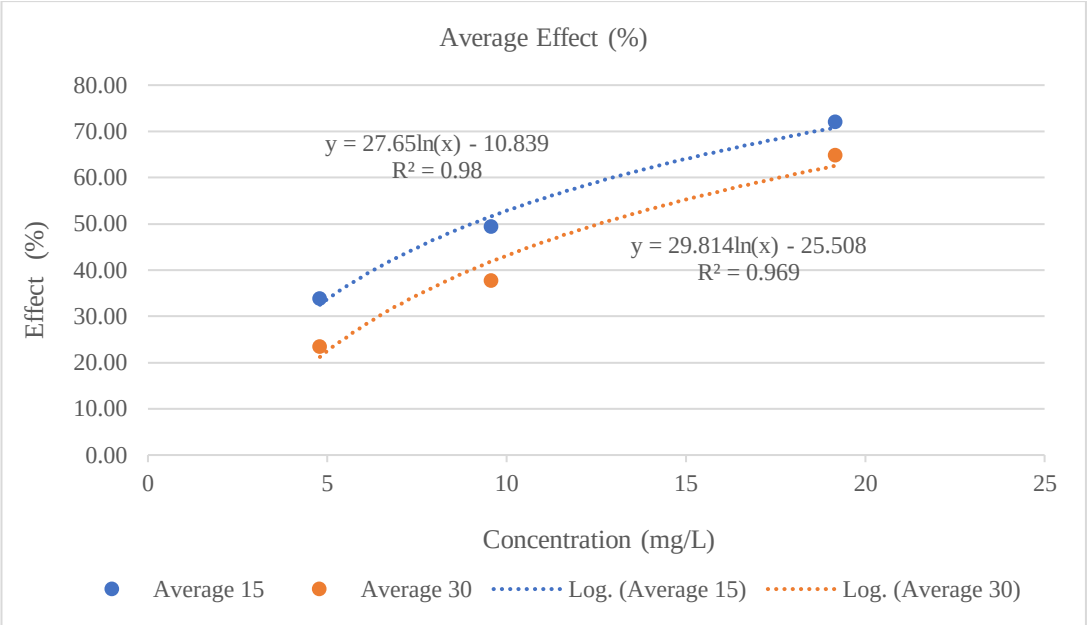

Figure 5. Average Effect of DCF on Hach® LUMIStox bacteria

The EC_{50} values for DCF (Table 8) and TCS (Table 9) were recorded. From the obtained values it can be observed that Biolight and Hach® LUMIStox bacteria provided more precise results as they are the closest to the known EC_{50} values measured for *A. fischeri* (DCF 11.45 mg/L for 30-min exposure and TCS 0.28 µg/mL for 15 min exposure). Microtox® bacteria tested at the chosen concentration did not provide such precise values.

Table 8. The experimentally obtained EC_{50} values for DCF

Supplier	**Bacteria Concentration [mL/mL]**	**EC50 (DCF) [mg/L]**	
		15 min	**30 min**
SOLO Mictotox®	0.238 – low dilution	7.86	4.15
Biolight	0.331 – low dilution	25.98	9.80
Hach® LUMIStox	0.125 – medium dilution	9.03	12.58

Table 9. The experimentally obtained EC_{50} values for TCS

Supplier	Bacteria Concentration [ml/ml]	EC50 (TCS) [µg/mL]	
		15 min	30 min
SOLO Mictotox®	0.238 – low dilution	-*	-*
Biolight	0.331 – low dilution	0.89	0.88
Hach® LUMIStox	0.125 – medium dilution	0.75	0.76

**The values for SOLO Microtox® supplier were not recorded because it was not tested on triclosan due to the limited number of vials available*

5.3 Economic evaluation

Comparing the price of bacteria reagents including shipping costs, it is possible to conclude that the most cost-effective supplier is Hach Lange (Germany) as the price for each sample and the shipping cost is the lowest. On the other hand, Modern Water (U.S.A.) seems to be the costliest among other suppliers, as well as the price for shipping as samples travel the longest distance to reach the laboratory. The Microbiotest supplier (Belgium) can be estimated as quite cost-effective too, although its expenses are higher compared to the Hach Lange (Table 10).

Table 10. Comparison of total price per a measured sample for the tested bacteria suppliers

	Price (EUR)	Pck	Shipping (EUR)	Total including shipping (EUR/pck)	Samples per pck	Total price including shipping (EUR per sample)	Total price without shipping (EUR per sample)
SOLO Microtox®	588.41	50	450.3	20.77	3	6.92	3.92
Biolight	537.00	50	120.00	13.14	2	6.57	5.37
Hach® LUMIStox	804.53	12	41.15	70.47	68	1.04	0.99

6. Discussion

The method of bioluminescent testing conducted on *A. fischeri* has been getting more common for acute toxicity assessment, therefore toxic effects and EC_{50} values of many different pollutants were obtained from some previous ecotoxicology studies. One of them did measurements and calculations of 50% luminescence inhibition after exposure to triclosan and its derivatives. The results showed that the EC_{50} value for the standard solution of triclosan exposed to the bacteria samples for 30-minute exposure was 0.28 μg/mL (Farré et al., 2008). Other studies also estimated the EC_{50} value of triclosan testing on *A. fischeri* but obtained a much different result which was equal to 0.668 ± 0.08 μg/mL (Gorenoglu et al., 2018). The final value of the practical part achieved during my work was 0.88 μg/mL for 30 min exposure to the same substance. The difference between the obtained values is not significant comparing all the studies, and some slight variability of results can be explained by different bacteria sources and its storage and activation procedures. Also, triclosan is known to be highly unstable and easily degraded under UV-light exposure, therefore the final concentration used for testing could differ and be lower in the case of all mentioned studies, and the values could vary.

Diclofenac and its derivatives were also measured in several previous studies. For example, in one of them, the final EC_{50} value obtained after the 30 min exposure test on *A. fischeri* was equal to 23 ± 4 mg/L. However, in that case, the bioluminescence of bacteria was impacted due to the occurrence of some carbon substances in the medium as a consequence of microbial activity (Grandclément et al., 2020). As a result of another research, in which toxicity of single and combined substances was assessed, the EC_{50} rate of diclofenac was 11.79 ± 1.75 mg/L. Although the results of the calculations were not exactly the same, all studies concluded that diclofenac was toxic for organisms, in some cases, it was the most toxic substance among other tested ones (Dökmeci et al., 2014). The achieved value, which was calculated within my work, is 12.58 mg/L. As it was mentioned before, many different factors can have an influence on the results of an ecotoxicology test, although, in the case of diclofenac, the values did not have such considerable difference as in the case of triclosan.

Continuing the possible explanation of such variety of the values obtained in different studies, it is important to mention the source of bacteria more in detail. There are many suppliers available on the market today, and Microtox®, Biolight, and LUMIStox bacteria suppliers are the most common ones as they have been used a lot for various ecotoxicology research. Microtox bacteria are known to be highly sensitive in general, and it was proven to be predictive for high toxicity in many acute biotests and sublethal effects during wastewater organic extract analysis (Weltens et al., 2014). Other studies showed that LUMIStox bacteria are quite effective in testing the toxicity of chemical substances used in the textile industry (such as dyes and auxiliaries), industrial

effluents, and municipal wastewater (Abbas et al., 2018). Thus, different luminescent bacteria sources can be more accurate for testing depending on the origin of the substance and the sensitivity of the supplier.

It is also important to highlight that not only the source of luminescent bacteria itself but also the conditions at which they were frozen and transported to the destination laboratory can make a difference. In the case of my work, some visible disturbances were noticed in several Microtox© vials with the bacteria, which could be caused by nonadherence to storage conditions while transportation. The frozen samples of bacteria were cracked in some of the vials, and after its activation according to the protocols, those bacteria had a specific consistency and a level of transparency that were slightly different from the other vials. Thus, this could lead to misleading and inaccurate results of the experiment. Therefore it is important to pay attention to such characteristics and features prior to the test conduction and make sure that all the vials are stored properly.

In my personal opinion, despite all the mentioned challenges, the bioluminescence ecotoxicity tests conducted on *A. fischeri* are high-potential and progressive as they offer a fast and easy assessment of the toxic effect of various chemicals. The data and values observed during this type of test can provide with a wider perspective of the toxicity of substances, and the overall impact on different organisms, the environment, and human health can be evaluated. The use of bioluminescence represents toxicity more closely than chemical analysis (Steinberg et al., 1995), provides with a clear picture of the toxic effect, and does not require much time and space for conducting. Moreover, the laboratory conditions necessary for such tests are easy-maintainable, and the overall method of testing is quite affordable and cost-efficient. Also, this type of test allows to assess toxicity in the field, and it can be used for immediate measurements (ibid.). Although bioluminescent testing is advantageous for acute test performance only, and chronic effect assessment cannot be achieved using such tests, I assume that they significantly contribute toward the development of ecotoxicology as a science.

In addition to all above-mentioned, a lot of changes and updates of the laws, legislations, and regulations are regularly made due to ecotoxicology research which assesses the toxic effects of various substances exposed to the environment as a result of anthropogenic activities. It makes ecotoxicology crucial as a science and a tool in terms of water quality and management assessment, waste management, and many other fields related to the environment and human health. That is why it is extremely important to continue the development of this environmental science and search for more precise, fast, and cost-effective ecotoxicity testing techniques to contribute to human health and the environment.

7. Conclusion and contribution of the thesis

The main aim of this bachelor thesis was to analyze and interpret the influence of the supply stock of *Aliivibrio fischeri* (formerly *Vibrio fischeri*) on the validity of the luminescence toxicity testing in line with the ČSN EN ISO 11348-3:2007 standard.

Based on the results obtained, it is possible to assume that the source of the testing luminescent bacteria has a slight effect on the experiment results. The dilution of test biota samples can also cause some interference in the results. The overall toxic effect of chemical substances can be evaluated quite precisely, however, more accurate values of EC_{50} can vary according to the bacteria supply used during the experiment. On the other hand, this difference can be also influenced by tested chemicals, such as quite unstable triclosan.

Depending on the available laboratory equipment and required instructions on the bacteria storage and its activation, it is possible to choose the most suitable supplier of the species for ecotoxicity testing in each particular case. The location of a laboratory also matters as the shipping costs for the bacteria can considerably differ, and the overall price can increase drastically in case the samples have to travel a long distance. This factor is important to consider to be able to stay within the overall budget of the research. In addition to it, long-distance travel can be quite risky for the bacteria as there is a high chance of its damage due to complicated conditions of transportation.

To conclude, the main aim of this work was fulfilled, and the influence of the bacteria supply on the validity of bioluminescent testing was described. However, it is necessary to repeatedly check the sensitivity of the bacteria received from different suppliers. Other chemical substances may be selected for further verification and analysis.

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List of figures

List of tables