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DEPARTMENT OF APPLIED ECOLOGY

ASSESSMENT OF FRESHWATER ALGAE MAINTAINANCE ON ECOTOXICITY TESTING

BACHELOR THESIS

Supervisor: Ing. Lenka Wimmerová, MSc., Ph.D.

Student: Nadija Čehajić

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BACHELOR THESIS ASSIGNMENT

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Thesis title

Assessment of Freshwater Alga Maintenance on Ecotoxicity Testing

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Objectives of thesis

The overall aim of the bachelor work is to assess influence of two maintenance way of a freshwater alga (a living culture vs. an alginate form) on validity of ecotoxicity testing. The theoretical part of the work will focus on the current state of the art in the freshwater algae ecotoxicity measurement, available standardized testing protocols and relevant environmental legislation. Furthermore, applicability of various freshwater algae for growth inhibition tests will be discussed. The practical part of the work will be performed with the standardized freshwater alga, Raphidocelis subcapitata (Selenastrum capricornutum), applied as the living culture and in the alginate form, on selected freshwater pollutants.

Methodology

The bachelor work is experimental. Methodologically, it will be formed as a comprehensive literature review followed by a practical part carried out in laboratory conditions in compliance with relevant ISO and OECD standards.

The proposed extent of the thesis

50-60 pages incl. appendixes

Keywords

Alga, living culture, alginate, ecotoxicity, growth inhibition, water, pollutant, validity

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Recommended information sources

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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The Bachelor Thesis Supervisor

Ing. Lenka Wimmerová, MSc., Ph.D.

Supervising department

Department of Applied Ecology

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prof. Ing. Jan Vymazal, CSc.

Head of department

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prof. RNDr. Vladimír Bejček, CSc.

Dean

Prague on 13. 03. 2021

Bachelor thesis author's statement

I hereby declare that I have independently elaborated the bachelor/final thesis with the topic of: Assessment of freshwater algae maintenance on ecotoxicity 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. I am aware that my bachelor/final thesis is subject to Act No. 121/2000 Coll., on copyright, on rights related to copyright and on amendments of certain acts, as amended by later regulations, particularly the provisions of Section 35 of the act on the use of the thesis. I am aware that by submitting the bachelor/final thesis I agree with its publication under Act No. 111/1998 Coll., on universities and on the change and amendments of certain acts, as amended, regardless of the result of its defence. With my own signature, I also declare that the electronic version is identical to the printed version and the data stated in the thesis has been processed in relation to the GDPR.

In Prague, date: ……. ……

Signature

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Abstract

This bachelor thesis focuses on assessing the validity of two maintenance ways of freshwater algae for ecotoxicity testing. The theoretical part of the work deals with the contemporary standardized testing protocols, taking relevant environmental legislations in consideration. In the practical part, tests on a living free culture and in an alginate immobilized form of freshwater algae *Raphidocelis subcapitata (Selenastrum capricornutum)* are compared on selected freshwater pollutants.

Based on the results from the experimental part, where ČSN EN ISO 8692 methodology is followed, the immobilized algae are more sensitive to potassium dichromate (used as a reference substance) and to triclosan, therefore the alginate immobilized algae cannot replace the living free algal culture when testing triclosan. On the other hand, the immobilized and living free algae have a comparable sensitivity to diclofenac, so the alginate immobilized algae can replace the living free algal culture, but a certain deviation has to be considered. The immobilized algae have a great potential in ecotoxicity testing, however further research should be carried out.

Key words

Alga, living culture, alginate, ecotoxicity, growth inhibition, pollutant, water, validity

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List of abbreviations

API – Active pharmaceutic ingredients

ATP – Adenosine triphosphate

CCALA – Culture Collection of Autotrophic Organisms

CV – Coefficient of variation in the control

ČSN – Czech technical norm

DCF – Diclofenac

EC – European commission

EN – European norm

EU – European Union

IC_x – Inhibition concentration

ISO – The International Organization for Standardization

K_{ow} – Octanol-water partition coefficient

LOEC – Lowest observed effect concentration

NOEC – No observed effect concentration

OECD – Organization for Economic Cooperation and Development

PPCPs – Pharmaceuticals and personal care products

RNA – Ribonucleic acid

TCS – Triclosan

U.S. EPA – United States Environmental Protection Agency

WFD – Waste Framework Directive

1. Introduction

The geological epoch we now live in is called Anthropocene, based on the significant impact human activity has on Earth's geology, ecosystem, balance and climate. These impacts are mostly negative and cause degradation of all spheres (from atmosphere to biosphere), pollution, extinction, exploitation and climate change. One problem which cannot be overlooked is the production of waste and the toxic impact it has on the environment and its organisms.

Ecotoxicology is a science which studies the effects of chemicals, including those found in waste, on living organisms and their environment. Ecotoxicology is based on tests done on representative living organisms, where their vitality, behaviour, growth and the overall effect of a certain chemical is monitored. Based on the results, ecotoxicologists gain from their scientific findings and testing, agencies and decision makers can interpret that data and run their policies further. Data collected contribute to decision-making of resource management (Sparling, 2016).

With rising changes and pollution, more monitoring and better technologies will be needed. Monitoring and predictions can be done through ecotoxicology testing, which requires further development and improvement. Tests need to become more efficient, simple and reproducible. Alternative ways of testing should be advanced and later on accepted as a standard. An example of such test is an ecotoxicity test with the immobilized freshwater algae.

2. Objectives of the thesis

The overall aim of the bachelor work is to assess the influence of two maintenance ways of a freshwater alga (a living culture vs. an alginate form) on validity of ecotoxicity testing. The theoretical part of the work will focus on the current state of the art in the freshwater algae ecotoxicity measurement, available standardized testing protocols and relevant environmental legislation. Furthermore, applicability of various freshwater algae for growth inhibition tests will be discussed. The practical part of the work will be performed with the standardized freshwater alga, *Raphidocelis subcapitata (Selenastrum capricornutum)*, applied as the living culture and in the alginate form, on selected freshwater pollutants.

The main purpose of the experimental part of this thesis was to compare the difference of sensitivity of the immobilized alga (Figure 1) and the living free alga (Figure 2) cultures of *Raphidocelis subcapitata* to widely applied toxic chemicals used in personal care products and in medical drugs.

Figure 1 Immobilized algae

Figure 2 Living free algae: a) ampoule b) inoculum

3. Literary research

3.1 Ecotoxicology

Ecotoxicology is a field of study that combines the science of ecology and toxicology. Ecology (*oikos*, Greek for house) is a study of relationships between living organisms and their environment. It represents a multidisciplinary way of approach which prioritizes concerns of biological problems. With Industrial revolution and increased production of chemicals, including the toxic ones, pollution and disturbances became complex and manifold on various levels of ecosystems. Toxicology is a science studying substances which cause disturbances and changes in the functions of living organisms, eventually resulting in harmful effects.

Due to detected adverse effects caused by human activity after the Second World War and further agricultural development, a new study branched from toxicology – ecotoxicology. Ecotoxicology studies the effects of chemicals on living organisms and their environment. Its main focus is to assess the relationship between the exposure of the living organisms to the specific chemical and the effect it has on them. The term "ecotoxicology" was firstly mentioned by a French toxicologist René Truhaut, in 1969, who is in fact the originator of the discipline. He also defined basic ecotoxicological terms like concentration, dose/response relationship, chronic and acute toxicity, effect etc. (Truhaut, 1975). Big emphasis in the study of ecotoxicology is put on the fate and transport of the contaminants. It focuses on their degradation and movement from one compartment into another. Ecotoxicologists are interested in the effect of contaminants on various levels, starting from individuals, to populations, communities and finally ecosystems (Sparling, 2016).

Ecotoxicology test, as an experimental method, is the main tool in ecotoxicology. A tested organism is exposed to a certain chemical and its reaction is then monitored. Tests can be divided according to:

1. Exposure time:
    - Acute (24-96 h);
    - Subacute (28-90 days);
    - Chronic (long term, through one or more generations of the organism);
2. Medium:
    - Aquatic (organisms living in aquatic environments);
    - Terrestrial (organisms living in terrestrial environments);
3. Trophic level of the organism:
    - Producer;
    - Consumer;
    - Decomposer;
4. Method of evaluation (depending on the organism):
    - Lethal effect;
    - Behavioural;
    - Growth inhibition;
    - Reproduction;
    - Mutagenesis, teratogenesis, carcinogenesis;
5. Chemical type:
    - Homogenic (one chemical);
    - Heterogenic (a mixture of chemicals).

From 1970s, until now, ecotoxicologists have been developing and improving standard tests. This way an accurate, reliable and reproducible system of testing can be manifested in the world. Standardized methodologies for ecotoxicology tests are

developed by international organizations like OECD (*Organization for Economic Cooperation and Development*) (*OECD.org - OECD*), ISO (*The International Organization for Standardization*) (*ISO - Standards*), U.S. EPA (*United States Environmental Protection Agency*) (*US EPA*) and others. These standardized tests are implemented in regulations, directives and protocols around the world.

In this Bachelor Thesis, the main focus is put on an acute aquatic toxicity test done on freshwater algae, where growth inhibition is monitored.

3.2 Test organism

Selection of the test organism is an element that determines the test procedure, which estimates bioavailable chemical contaminants to which the organism is exposed.

Throughout the time and development of the standardized tests, the selection of the tested species became more important. Pollutants appearing in a specific environment are often easily transported into other areas and matrices. So, the question was if it is adequate to use an indigenous species, which represents the environment it grows in. It is stated in (Rojíčková and Maršálek, 1999) research that it is more effective to use a highly sensitive and abundant species for toxicity testing, rather than using an indigenous one.

The species should fulfil criteria that ensure a successful and efficient testing. The criteria are:

- The organism should be easily identified and have a wide distribution range of environment, thus be representative for that environment;
- It should be able to achieve a sufficient population size and should be easily sampled to ensure practicability;
- It should show a visible and measurable dose-effect relationship, by being sufficiently sensitive;

- It should play an important role in the food web or in biogeochemical cycles of the ecosystem, thus acting as a vector of contaminant transfer to higher trophic levels (Sparling, 2016).

Algae are good reference species, based on their high sensitivity and ubiquitous nature they fulfil all above mentioned criteria. Their sensitivity can be explained by their high surface/volume ratio.

The relative sensitivity of seven species of freshwater algae, from the division *Chlorophyta,* were already compared (Rojíčková and Maršálek, 1999). The result showed that the most sensitive species of the freshwater algae is *Raphidocelis subcapitata,* which has shown high sensitivity to all reference toxic chemicals. This is explained by morphology, cytology, genetics and physiology of this algae. For example, in comparison to *Chlorella kessleri*, *Raphidocelis subcapitata* produces less extracellular defense organic substances, when in contact with toxicants. For this reason, *Raphidocelis subcapitata* is a representative and recommended species in aquatic ecotoxicology, which appears in the OECD guidelines ('OECD GUIDELINES FOR THE TESTING OF CHEMICALS', 2011) and the ISO standard (*ISO - Standards*). This alga is reliable, easy to culture and its growth response in toxicity tests is dose dependent, which implies that the tests are easily run and interpreted.

In general algae are highly sensitive species and are a primary food source for most of the freshwater species, hence their health and growth determine the health of a whole freshwater ecosystem. They perform around 40 % of global photosynthesis and play a key role in aquatic systems.

3.2.1 Algae

Algae is an informal taxonomic term used for photosynthetic, O_2-evolving, eukaryotic organisms. The main difference between algae and vascular plants is that algae do not produce embryo and thus do not have differentiated parts of the body (roots, stem, leaves etc.). All cells of an algae are potentially fertile and sterile.

Algae can occur in many habitats, from aquatic, including marine and freshwater, to terrestrial. Their tolerance of pH, temperature, O_2, CO_2 and turbidity are quite broad. Their form differs with species type, from microscopic single cells, macroscopic multicellular forms to filamentous, branched, leafy colonies. Their size can also differ from 0.2 µm to 60 m. Algae can either be planktonic or benthic. Planktonic occur as unicellular species and live suspended in an aquatic environment. Benthic can occur as unicellular and multicellular, creating colonies, because they live attached to some sediment. The planktonic unicellular algae can be motile, depending on the occurrence of flagella. Algae with flagella move according to their needs and algae without flagella are moved by the water stream.

Algae are divided by their nutrition on autotrophic, heterotrophic and mixotrophic. Most algal groups are photoautotrophic, meaning they use sunlight as a source of energy and CO_2 as a source of carbon to produce carbohydrates and adenosine triphosphate (ATP). But in general algae are mixotrophic, since they are competent to use a wide variety of nutrients from the surrounding by which they produce food themselves. By this manner, they combine photoautotrophy and heterotrophy (Barsanti *et al.*, 2008).

Algae have more ways of reproduction, depending on the species and environmental conditions. Reproduction at algae can be vegetative, asexual or sexual. Vegetative and asexual ways are fast, resulting in an economical and rapid growth rate.

The simplest reproduction is by a binary fission, where the parent cell divides into two identical daughter cells.

The asexual reproduction is by formation of flagellated zoospores, or autospores, which have no flagella. These are produced within a vegetative cell or a specialized structure sporangia.

The sexual involves three ways of reproduction. Plasmogamy is the union of cells, karyogamy is the union of nuclei and meiosis. All involve production of gametes and they are morphologically identical to vegetative cells but are haploid instead of diploid.

A growth rate of algae is defined by a curve. The first phase of this curve is called a lag phase, where the algae are getting used to the environmental conditions. The second phase is log phase, with an exponential growth rate. And the last phase is a plateau phase, where the number of algae is stationary and stable.

When in unfavourable conditions, algae produce a thick wall, which enables them to survive and endure harsh conditions in a resting phase. When they find themselves back in favourable conditions, the thick wall germinates into new vegetative cells (Barsanti *et al.*, 2008).

Algae are divided into Rhodophyta, Euglenophyta, Dioflagellata, Cryptophyta, Chromophyta and Chlorophyta. The algae used in the experimental part of the thesis belongs to the Chlorophyta phylum.

The cell of a Chlorophyta algae consists of a cell wall, porous membrane, nuclei, mitochondria, central vacuole and chloroplasts. The chloroplast contains chlorophyll *a* as the main pigment, functioning in photosynthesis. Other pigments can be found, like carotenoids and xantophylls. Chlorophyta can appear as unicellular and colonial structure, which can be motile or non-motile. The main storage production of this phylum is starch, which is further used as an energy source (Kumar and Singh, 1979).

Raphidocelis subcapitata

Raphidocelis subcapitata is a sickle-shaped, freshwater microalgae (

Figure 3) belonging to the phylum Chlorophyta. It is found in unicellular planktonic form without flagella. Therefore, it is freely carried by the water stream. It has been reported that this algae reproduces either by binary fission or asexually with autospores (Yamagishi *et al.*, 2017).

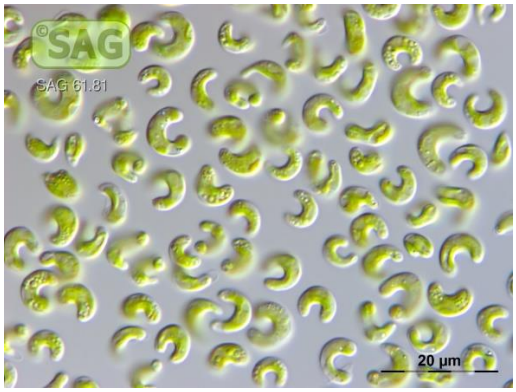

Figure 3 Raphidocelis subcapitata under microscope ('Raphidocelis subcapitata (Korshikov): Algaebase'*)*

Taxonomy of *Raphidocelis subcapitata*:

- phylum: Chlorophyta
- class: Chlorophyceae
- order: Sphaeropleales
- family: Selenastraceae
- genus: *Raphidocelis*
- species: *Raphidocelis subcapitata* (Raphidocelis subcapitata (Korshikov): Algaebase)

3.2.2 Ecotoxicology tests with algae

Freshwater algae toxicity tests are a good and effective way of determination of adverse effects of chemical compounds on living organisms and for that reason it is frequently used for ecotoxicity testing of freshwater sights and wastewater.

Both the standardized test developed by ISO, Water quality – Freshwater algal growth inhibition test with unicellular green algae (ISO 8692:2012) and by OECD, Guidelines for the Testing Chemicals – Freshwater Alga and Cyanobacteria, Growth Inhibition Test (201:2011) are based on the same principle.

Principle

The principle of both methods is to cultivate algae in a defined medium containing a range of concentrations of the test toxicant. It is prepared by mixing appropriate quantities of the growth medium, test toxicant and an inoculum of exponentially

growing algal cells. The test batches are incubated for 72±2 h in identical conditions, after which the cell concentration is measured. The algae response is monitored as an inhibition of growth rate, from which a specific inhibition concentration is deduced, expressed as IC_x (eg. IC_{50}). This methodology is appropriate for chemicals easily dissolvable in water.

Normative references

The following documents are partially or in whole normative references of the ISO, Water quality – Freshwater algal growth inhibition test with unicellular green algae (ISO 8692:2012) and OECD, Guidelines for the Testing Chemicals – Freshwater Alga and Cyanobacteria, Growth Inhibition Test (201:2011):

- ISO 5667-16:2017, Water quality – Sampling – Guidance on biotesting of samples;
- ISO/TR 11044:2008, Water quality – Scientific and technical aspects of algae growth inhibition tests;
- ISO/TS 20281:2006, Water quality – Guidance on statistical interpretation of ecotoxicity data;
- ISO 14442:2006, Water quality – Guidelines for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and wastewater;
- Nyholm N. Sorensen (1992): Statistical treatment of data from microbial toxicity tests;
- OECD:2006 Current Approaches in the Statistical Analysis of Ecotoxicity Data.

Validity

For the results of the testing to be valid the average growth rate of the control should be bigger than 1.4 d^{-1}, the variation coefficient of the growth rate in the control should not be bigger than 5 % and the difference of the pH in the control at the beginning and at the end of the test should not be bigger than 1.5

Apparatus

The apparatus which will come in contact with the test solution should be made of glass or other inert material. It should also be thoroughly washed to prohibit any interference or pollution of the test solution. Additional apparatus which are required are a culturing apparatus (where temperature can be maintained), an apparatus to determine the algal cell concentration (electric particle counter, microscope with a counting chamber, flow cytometer or spectrophotometer), a light measurement instrument, a pH meter and an apparatus for sterilization (either autoclaving or membrane filtration with filters of pore diameter 0.2 µm).

Test organism

This methodology is suitable for a number of non-attached microalgae (*Raphidocelis subcapitata*, *Desmodesmus subspicatus* and *Navicula pelliculosa*) and cyanobacteria (*Anabaena flos-aquae, Synechococcus leopoliensis*).

The initial concentration of the test organism should not exceed 0.5 mg/l of dry weight. So, there are recommended cell concentrations (e.g. for *Raphidocelis subcapitata*: 5 x 10^3 cell/ml).

Test substance

The concentration of the test substance should be determined according to range-finding tests. According to the results of the range-finding test a more precise concentration range is determined and used in the actual test. For the final test at least five concentrations should be used. These concentrations should be arranged in a geometric series, whose factor should not exceed 3.2. The concentration series should cover 5 – 75 % inhibition of growth rate.

As a reference substance 3,5-dichlorophenol or potassium dichromate can be used.

Stock solution

Test sample can either be aqueous (e.g. wastewater) or non-aqueous (chemical substance or a mixture of chemicals). If aqueous, then a possible filtration and pH adjustment can

be considered, depending on the nature of the test sample. If non-aqueous a stock solution preparation is necessary. Stock solutions are prepared by dissolving the test sample in growth medium. If the test sample does not readily dissolve, modifications are required in accordance with ISO 14442:2006, Water quality – Guidelines for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and wastewater and ISO 5667-16:2017, Water quality – Sampling – Guidance on biotesting of samples. If the test solution is drastically acidic or alkaline, the pH should be adjusted with 1 mol/l hydrochloric acid or 1 mol/l sodium hydroxide solution.

Inoculum culture

An inoculum culture is prepared 2-4 days prior to the test. It is incubated in the same conditions in which the test cultures will be incubated, in order to adjust the algae to the conditions. The main purpose of an inoculum culture is to allow an exponential growth of the algae and use it in that phase.

Growth medium

Firstly 4 growth mediums are prepared according to the Table 1:

Table 1 Growth mediums

Chemical	Amount
Medium 1	
NH_4Cl	1.5 g
$MgCl_2.6H_2O$	1.2 g
$CaCl_2.5H_2O$	1.8 g
$MgSO_4.7H_2O$	1.5 g
KH_2PO_4	0.16 g
Medium 2	
$Na_2EDTA.2H_2O$	100 mg
$FeCl_3.6H_2O$	64 mg
Medium 3	
H_3BO_3	185 mg
$MnCl_2.4H_2O$	415 mg
$ZnCl_2$	3 mg
$CoCl_2.6H_2O$	1.5 mg
$CuCl_2.2H_2O$	0.01 mg
$Na_2MoO_4.2H_2O$	7 mg
Medium 4	
$NaHCO_3$	50 g

The final growth medium is prepared by adding 500 ml of distilled water into a 1 l volumetric flask, adding 10 ml of growth medium 1 and adding 1 ml of growth mediums 2, 3 and 4. The rest is filled with distilled water.

Before use, the prepared growth medium should be equilibrated by leaving it overnight in contact with air, or by bubbling filtered air through it for 30 min.

If necessary, the pH of the growth medium should be adjusted to 8.1±0.2 with 1 mol/l hydrochloric acid or 1 mol/l sodium hydroxide solution.

Incubation

After the preparation of test solutions containing the initial algal concentration, a specific amount of tested chemical (toxicant) and the growth medium, they are incubated.

The vessels should be capped with air-permeable stoppers. In the culturing apparatus the vessels should be shaken in order to facilitate CO_2 transfer. The cultures should be maintained at a temperature ranging between 21 to 24°C, controlled at ±2°C, and receive a uniform fluorescent illumination of 400-700 nm. The test duration is 72±2 h.

The test design includes three replicates at each concentration.

Measurements

Measurements of the cell concentration should be done at least every 24 h during the test period; mix each test vessel before measurement. Measurement of the pH of the solutions should be done at the beginning and at the end of the test. If the analysis of the test substance is required, it is necessary to separate the algae from the medium by low g-force centrifugation.

Data and reporting

The plotting of growth curves is based on the tabulated cell concentration in test cultures and controls together with the concentrations of the test material. Logarithmic scales are mandatory and give good precision during the growth period. Using plot data, control cultures should be examined, if they grow exponentially at the expected rate. This gives an overview on possible errors that could appear due to an incorrect inoculation or improper cleaning.

Average growth rate is a logarithmic increase in biomass during time. Formula to calculate the average growth rate:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \qquad \text{[Eq. 1]}$$

Where:

μ_{i-j} - average specific growth rate from time *i* to time *j*

X_i –biomass at time *i*

X_j – biomass at time *j*

unit used to express the growth rate is reciprocal days (day^{-1}=1/d)

A significant lower specific growth rate from the first day compared to the average specific growth rate could indicate a lag phase. After this phase the algae could recover, or the exposure could be reduced due to loss of test material. Therefore, it is advisable to measure the specific growth rate every day and at the end measure the amount of the test material left in the vessel.

Percent inhibition of growth rate is calculated through:

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \qquad \text{[Eq. 2]}$$

Where:

$\%\ I_r$ – percent inhibition in average specific growth rate

μ_C – mean value for average specific growth rate μ in the control group

μ_T – average specific growth rate for the treatment replicate

Yield is calculated as a difference of biomasses at the beginning and at the end of the test. It is done through an equation:

$$\%I_y = \frac{(Y_C - Y_T)}{Y_C} \times 100 \qquad \text{[Eq. 3]}$$

Where:

$\%\ I_y$ – percent inhibition of yield

Y_c – mean value for yield in the control group

Y_T – value for yield for the treatment replicate

Plotting of the response curve can be either done by eye or by computerized interpretation. This is done to get an idea of the concentration-response relationship.

For a **statistical procedure** a number of methods and programs can be used. To obtain a quantitative a concentration-response relationship by regression analysis most common methods are Probit, Logit or Weibull units.

Test report

The test report must include:

1. Test substance: physical properties, chemical properties and chemical identification data;
2. Test organism: species, origin, number of the specific algae, type of cultivation;
3. Test conditions:
    - Duration, replicates;
    - Test design (test vessels, culture volumes, biomass density at the beginning of the test);
    - Composition of the medium;
    - Preparation of test solutions;
    - Culturing apparatus;
    - Light intensity and temperature;
    - Concentrations tested;
    - Deviations from this Guideline;
    - Method for determination of biomass.
4. Results:

    – pH values at the beginning and at the end of the test at all treatments;

    – Biomass for each flask at each measuring point;

– Growth curves;

– Calculated response variables for each treatment replicate, with mean values and coefficient of variation for replicates;

– Graphical presentation of the concentration/effect relationship;

– Estimates of toxicity for response variables e.g., EC_{50}, EC_{10}, EC_{20} and associated confidence intervals. If calculated, LOEC and NOEC and the statistical methods used for their determination.

3.2.3 Alternative ecotoxicity tests

Alternative ecotoxicity tests are test which are used as a replacement, since they can be easier, quicker, more applicable or more efficient in ecotoxicity testing.

Based on both ISO and OECD guidelines for ecotoxicology testing with freshwater algae, the process of preparation of the algae takes time and effort. Firstly, algal cultures have to be adequately prepared as an inoculum, according to the guidelines.

Free algal samples are bought from a producer in a form of a very concentrated algal ampoule, which is further diluted and prepared as a stock culture.

The price of the free algae samples depends on the ordered volume, varies between 100 to 1000 CZK. Survival of the culture depends on the treatment and handling but can last up to 12 months. Usually, one algal ampoule contains 2 ml of the stock. For each new stock, a several dozen µl of the ampoule stock is used, so approximately 15-20 stocks can be made.

Stock cultures are small algal cultures that are transferred to fresh medium at least once every month. It has to be inoculated and fed, under sterile conditions, to prevent contamination or failure of growth. From this stock culture several dozen µl are used for the preparation of a pre-culture. A pre-culture needs to be grown two to four days before the start of the test, in order to be in the exponential phase of growth when starting the test. It is incubated in the same conditions, like the test cultures to allow the algae to

adapt to the test conditions. Both stock culture and pre-culture are grown in mediums that are prepared according to standards, which also require preparation.

During frequent ecotoxicology testing, time and effectiveness are crucial. For that reason, the preparation of living algae is time and material consuming. It is also effort consuming since the living culture needs to be taken care of, to keep it alive and healthy. Therefore, the immobilized algae for ecotoxicity aquatic testing have been introduced as an alternative method. In the ISO 8692:2012 immobilized algae beads are mentioned and described for usage in the B.3.2 paragraph. In Annex B it is stated that a method with immobilized algae beads can be applied to testing of pure chemicals as well as to effluents, waste waters and other environmental aqueous samples. The precision of this testing is not mentioned.

3.3 Immobilized algae

An immobilized cell is defined as a cell that is by natural or artificial means prevented from moving independently to any parts of the aqueous system it is in (Kaparapu and Geddada, 2016).

Immobilization of green algae can be done by various techniques, including active and passive, based on the material used. Active techniques include flocculant agents, chemical attachments and gel entrapment. Gel entrapments can be performed by synthetic polymers, proteins or natural polysaccharides like agars, carrageenans or alginates. Out of those, the most used and the cheapest is the active technique of gel entrapment of living cells by polysaccharide alginate. Alginate is extracted from dry weight of brown algae or from specific bacteria, where this substance plays a structural role. Alginates are formed out of copolymer mannuronic acid and guluronic acid. Alginate is a preferable technique for immobilization because the living cells do not suffer physical or chemical condition changes, while immobilized. It is also very important that the algae are immobilized when in the phase of exponential growth. Alginate is a natural, non-toxic permeable gel that is most commonly formed by Ca^{2+} cations. The gel formation is quick and it dissolves in water at slow pace. The

sufficiency and the stability of alginate immobilized cells depend on the species, but the algae *Raphidocelis subcapitata* shows high stability (Moreno-Garrido, 2008).

Immobilized algae are a growing trend in the scientific world, where their application is spreading. Immobilized algae are used for the removal of unwanted substances in media, like nutrients, metals and organic pollutants. Their ability to uptake nitrogen and phosphorus from wastewater is very promising and is intensely investigated. Immobilized algae can even contribute to the reduction of carbon dioxide and increase bioproduction of hydrogen as an environmentally friendly way of energy production. Besides all these applications of the immobilized algae, we are interested and focused on the usage of immobilized algae in toxicity testing (Kaparapu and Geddada, 2016).

3.3.1 Immobilized algae in ecotoxicology tests

No physical differences between immobilized algae beads and free algae cultures can be detected, in regard of cell shape, size, organelles and starch grains. The more important and questionable feature is the response difference of these two types of algae to specific chemicals.

Previously, ecotoxicology tests were done with an aim to compare the sensitivity of the alginate-immobilized and the free algae *Raphidocelis subcapitata.* In (Bozeman *et al.*, 1989) these two forms of the algae have been tested on heavy metals and herbicides. The results show that the sensitivity of two forms of algae was the same to Cu, Cd and pentachlorophenol (PCP). However, the immobilized form of algae was substantially less sensitive to hydrothol, paraquat and glyphosate. This test showed that immobilized algae can be used successfully for toxicity test of a limited number of heavy metals and they can be used for organics.

The study (Al-Hasawi *et al.*, 2020) showed that the immobilized algae are significantly less sensitive to heavy metals like cobalt, mercury, arsenic and lead. The conclusion of both researches was that the toxicity determinations of heavy metals based of the immobilized algae may be inaccurate.

This state is explained by the chemical properties of alginate, which reacts with certain heavy metals and reduces its toxicity to the immobilized algae. The reason for this is the influence of the chemical properties of chelation agents in the immobilized algae. The radii of some metals and herbicides determines the binding to the chelation agents and thus determines the differences in sensitivity between immobilized and living algae. Hydrothol, paraquat and glyphosate are also hydrophilic, which could influence binding to the immobilized algae resulting in the test lower sensitivity.

The preparation of the alginate immobilized algae for an ecotoxicology test is simpler that the preparation of living algae cultures. The immobilized algae are bought in a form of algal beads, which are diluted in a solution, centrifugated and ready for usage. This preparation process in done before the setting up of the test, the same day, whereas the preparation of free algae cultures has to be done circa three days prior to the test.

A preparation procedure for immobilization of algae in alginate beads is described in Annex C of ISO 8692:2012 standard. An algal culture is prepared in the normal growth medium. When the culture has reached the exponential growth phase, the algae is concentrated by centrifuging the suspension for 5 min at low speed (3 000 r/min). An equal volume of a 30 g/l sodium alginate solution is added to the algae suspension and stirred gently until the algae are evenly distributed. This suspension is put into a syringe and slowly dripped into a 10 g/l calcium chloride solution. It is left to harden for 15 min to 30 min. The beads are separated with a sieve and sensed with distilled water. After this, the beads are transferred into a container with 10 times concentrated algal culture medium and stored in a refrigerator at (4 ± 2) °C until use.

According to the ISO 8692:2012, before usage algal beads need to be de-immobilized with the "de-immobilization medium", i.e. a 20 g/l solution of sodium metaphosphate (in a ratio of 10 to 15 algal beads per 5 ml of de-immobilization medium). The suspension is then shaken till the matrix surrounding the algae is completely dissolved. After this, the suspension is centrifuged at low speed. The algal clot is then resuspended in deionized water and centrifuged again. After the de-immobilizing medium and the rinsing water is removed, the de-immobilized algae are added into the algal growth medium and are ready for use.

It is possible to buy alginate immobilized algae kits, with 5 separate tubes. Each tube is used only once, can last for approximately 6 months and the kit costs from 3 000 to 4 000 CZK ('MicroBioTests').

In general, the alginate immobilized algae are more expensive to use, because of a not widely spread usage and limited production. If the usage became more frequent and the production more common, the price could be lower.

To optimize ecotoxicology tests, they must be more accurate and reproducible. Using the immobilized algae in toxicity test has advantages related to convenience, automation, space, material and time saving.

3.4 Freshwater pollutants

Many types of pollutants, from agriculture, industry and healthcare, produced by humans end up in water systems. These pollutants are categorized based on their origin, the product they were intended for. Products for therapeutic purposes (Bebianno and Gonzalez-Rey, 2015) contain low concentration of active pharmaceutic ingredients (APIs), but when discarded from many sources for years, a decent amount is transferred into the water system. Annually approximately 3000 types of different APIs are used worldwide according to their purpose (antibiotics, antiasthmatics, antidepressants, analgesics etc.).

Pharmaceuticals and personal care products (PPCPs) have a growing consumer's demand and the ecotoxicology information is available for less than 10 % of the used ingredients. Pharmaceutical developers' goal is to produce lipophilic ingredients resistant to metabolic degradation, hence the ingredients withstand wastewater treatment and end up in the ecosystem. Therefore, the release of PPCPs is a rising and serious environmental problem (Ewadh *et al.*, 2017).

The main source of these compounds (Table 2) are households, hospitals and industries. These sources metabolite the compounds incompletely and improperly dispose it in sewage or on landfill. Recently the removal of contaminants has grown as a strategic act,

but it is either insufficiently or not enough. Sewage treatment plants are not able to efficiently remove waterborne PPCPs. Water masses are rapidly fluctuating and therefore are the most exposed medium to anthropogenic impacts (Amiard and Amiard-Triquet, 2015). This thesis assesses two widely used PPCPs, namely triclosan and diclofenac.

Table 2 Sources of PPCPs (Ewadh et al.*, 2017)*

Sources of PPCPs				
Hospitals discharge of waste and expired drugs	Hormones fed to fish and antibiotics	Discharge of drugs into wastewater and septic systems	Companies manufacturing drugs and industrial waste	Landfill leachate

3.4.1 Triclosan (TCS)

Triclosan, (5-chloro-2-(2,4-dichlorophenoxy)phenol), further as TCS, with the formula $C_{12}H_7Cl_3O_2$, is classified as a halogenated aromatic hydrocarbon, containing phenol, diphenyl ether and a polychlorinated biphenyl functional group ('PubChem'). Triclosan is an off-white, odourless, tasteless, crystalline powder. It has a molecular weight of 289.5 g/mol, melting point at 57°, octanol-water partition coefficient K_{ow} =4.76 and is slightly soluble in water.

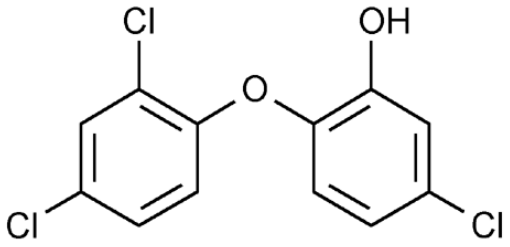

*Figure 4 Molecular structure of Triclosan (*USP Monographs: Triclosan*)*

It is an antimicrobial agent, used since 1968 and widely and extensively used for the last 20 years. It has been incorporated in many personal care products like deodorants, shower gels, handwashes and recently in oral products, for its characteristic of prevention of plaque formation. It dermatologically and topically protects the skin from microbes, where the activity of triclosan is directed against the RNA and protein synthesis in bacteria. It is very effective as a protective chemical against *Escherichia coli, Klebsiella edwardsii, Salmonella* sp., gram-negative, gram-positive bacteria, yeast and dermatophytes (Dann and Hontela, 2011).

TCS has also been used as an antimicrobial packaging material which reduces or inhibits microbial food contamination. It was incorporated into polymeric materials (plastic), which after usage end up in the landfill. If improperly disposed, TCS could leachate into ground water from the landfill (Chung *et al*. 2003).

Various tests were done assessing the triclosan toxicity in late 90's, but only on vertebrates, where no adverse effects have been detected (Dann and Hontela, 2011). Both acute and chronic toxicity tests have been done on animal species, from rodents and dogs to primates, like chimpanzees. The results show that triclosan is not an oral toxicant, not a dermal irritant, does not have a skin sensitizing or photosensitizing effect, is not carcinogenic or mutagenic. In accordance with these results, triclosan has been considered safe for usage and consumption. Due to the fact that triclosan toxicity tests included only vertebrate species, the environmental fate and the adverse effect of microorganisms was neglected. That is the reason for its excessive, unregulated appearance in many personal care products.

Recently, more studies (Orvos *et al.*, 2002) shown that triclosan is becoming a bigger and acute problem for the environment, since it was found to be toxic for aquatic species like algae, invertebrates and some species of fish. TCS is a waterborne compound, transported from household and human usage by sewage into freshwater, soil and further into distanced water bodies. It has been detected in water, sediments, soil and many living organisms. Recently studies show, that TCS is found in different organisms like

fish, vascular plants, and even human breast milk, which should be taken as a health concern (Olaniyan *et al.*, 2016).

Algae are prone to accumulate lipophilic compounds, like TCS, in their organism, which results in high bioaccumulation rate of 1,200 to 1,600 (Roberts *et al.*, 2014). Algae exposed to TCS respond in a decrease of cellular chlorophyll *a* (up to 77 %) and carotenoid content (up to 70 %), where the chloroplast membrane disappears and the spill out of inner stoma occurs. Since green algae are autotrophic and use these pigments for food production, exposure to TCS can dramatically affect their survival and growth. Besides loosing chlorophyll, alternations of inner pH and a protein pattern change were detected. Overall, TCS negatively affects the green alga cell function (Miazek and Brozek-Pluska, 2019).

Reports estimate that around 1 500 t of TCS are used yearly around the world in a range of consumers markets (Olaniyan *et al.*, 2016). It can be found in wastewater at the concentration of 1 000 to 100 ng/l, surface water 10 ng/l and sediment 100 ng/l (Bedoux *et al.*, 2012).

When in the environment, triclosan can thermally degrade or irradiate, converting into its chlorinated derivatives, which are environmentally toxic (Dann and Hontela, 2011). Sorption, degradation and photolytic degradation could be used as a treatment method, which partially removes TCS, but its by-products like chlorinated derivatives are more resistant and also more toxic.

3.4.2 Diclofenac (DCF)

Diclofenac (2-[2-(2,6-dichloroanilino)phenyl]acetic acid), further as DCF, with the formula $C_{14}H_{11}Cl_2NO_2$, is a nonsteroidal benzeneacetic acid derivative with anti-inflammatory activity. (PubChem, no date a) Diclofenac is a crystalline solid. It has a molecular weight of 296.1 g/mol, melting point at 285 °C, octanol-water partition coefficient K_{ow} = 4.51 and is slightly soluble in water. The ionizable carboxyl group results in higher mobility and solubility than is indicated by its K_{ow} (Karaman *et al.*, 2012).

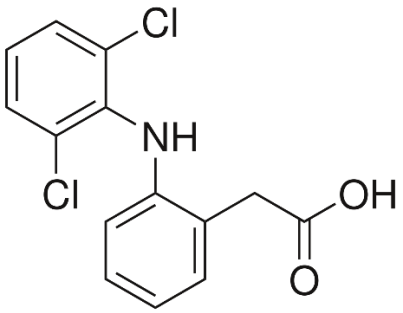

Figure 5 Molecular structure of Diclofenac ('Diclofenac', 2021)

DCF is used as an anti-inflammatory drug for pain, fever and inflammatory disorders. It has been patented in 1965 and a decade later it came into medical use. DCF can be used both by people and on animals (cattle), which means that not only are hospitals and household the source, but agriculture as well (Balmford, 2013).

Approximate annual consumption of diclofenac worldwide is around 940 t (Copolovici *et al.*, 2017). The amount of DCF that can be found differs between countries and proximity to urban areas. In wastewater the concentrations are around 800 ng/l and 350 ng/l in surface water (Huebner *et al.*, 2015).

Diclofenac, when in water bodies, undergoes rapid direct photolysis under sunlight, which is an important removal process. It has a half-life of 39 minutes in natural water and has the ability to absorb maximum of 300 nm. Photochemical processes are responsible for degradation of PPCPs in the environmental systems. Therefore, they are the dominant degradation mechanism for diclofenac, with the significant amount of absorbance in the solar region (Packer *et al.*, 2003).

In a previous research (Rosi-Marshall *et al.*, 2013), the effect of pharmaceuticals on algae has been tested. Similar like TCF, it can alter the composition of algae, growth rate, chlorophyll *a* content and respiration. Diclofenac, when combined with other pharmaceuticals, can significantly reduce concentrations of chlorophyll *a* in green algae, which reduces their biomass. By that, algae lose its functional role in the food chain and its availability as a resource. Algae not only feed the water ecosystem but produce significant amounts of oxygen. Pharmaceuticals reduce respiration of algae up to 50 %.

Although DCF is a toxicant, at low concentrations it can stimulate the algal growth, depending on the species of algae. This happens as the alga uses DCF as a carbon source of food (Miazek and Brozek-Pluska, 2019).

Another study (Doležalová Weissmannová *et al.,* 2018) shows the effect of diclofenac on microbiome of the water ecosystem. DCF interferes with the good microbiome of the environment and thus dangerous pathogens evolve. These facts state that diclofenac, along with other pharmaceuticals, affects nutrient cycle and food webs of water ecosystems.

3.4.3 PPCPs legislation

Micropollutants, or emerging contaminants, include PPCPs, pesticides, industrial chemicals, paints, waterproofing agents and others. They can be found in water bodies in trace amounts, with concentrations μg/l to ng/l. This depends on the consumption per person, rate of production and efficiency of wastewater treatment.

No legislative standards or guidelines exist for most of the micropollutants. Some countries have implemented discharge guidelines for only a few micropollutants, mostly pesticides and a few pharmaceuticals. The Directive 2008/105/EC (European Parliament and The Council, 2008) set quality standards for a small group of micropollutants (DDT, diuron bisphenol etc.) (Ewadh *et al.*, 2017).

Triclosan, as previously mentioned, is used in personal care products (soaps, deodorants, toothpastes etc.), textile industry and plastic food packaging. In the Regulation (EC) No 1223/2009 EU Commission for Cosmetics has stated a maximum limit of TCS concentration in ready for use preparation, which is 0.3 % (*EU law - EUR-Lex*). In 2010 EU Commission made the decision to ban TCS usage in food contact materials. In 2016 Commission implementing decision (EU) 2016/110 did not approve TCS as an active substance for use in biocidal products for product type 1 (disinfectant for personal hygiene, veterinary hygiene, drinking water, food etc.).

The second substance this thesis deals with, diclofenac, is used as an anti-inflammatory drug for humans and animals. However, it has been banned in veterinary use in many countries, like India, due to a sharp vulture (scavengers) population decline (Balmford, 2013). Commission regulation (EC) No 582/2009 established a maximum residue limits of veterinary DCF in foodstuffs (e.g. 0.1 µg/kg in milk and 5 µg/kg in muscle).

When looking into Water Quality Standards from U.S. EPA based on Clean Water Act, EU Directive 2008/105/EC of environmental standards for surface water, or even the amendment of the Czech Water law 544/2020, which came into force in February 2021, there is no mention of triclosan nor diclofenac. It adds information about hazardous substances and their derivates like heavy metals, metalloids, organic substances, biocides, persistent substances etc.

The awareness of the dangers of PPCPs should be increased by direct reduction of disposal, drug management and reclamation, better technologies of wastewater treatment and better industry placement. Additional ecotoxicology research is required, taking antagonistic, additive and synergistic properties into account. A very efficient path of handling micropollutant problem is trough strict regulations and guidelines (Ewadh *et al.*, 2017). During the assessment of water quality (Water Quality Assessments, WHO), the assessment of PPCPs should be included as a regular procedure. This implies especially to triclosan since it is toxic at very low concentrations.

3.5 Waste legislation

According to the definition of the European directive 2008/98/EC, known as the WFD (Waste Framework Directive) waste is '*any substance or object which the holder discards or intends or is required to discard*'. The definitions of waste vary across EU countries, as well as definitions of municipal waste, holder and producer. Czech Republic is a member of EU (Europe Union) and the definition of waste given by the Ministry of Environment is slightly different from EU directive, saying that waste

'consists in every movable thing which the holder thereof discards, or intends to discard or is obliged to discard and which belongs in one of the groups referred to in Annex No. 1 to Act No. 185/2001 Coll.'. In principle, the definitions have the same meaning and are referring to any object which is not in use and therefore discarded. There are multiple types and categories of waste like municipal, industrial, agricultural, hazardous etc. According to the European directive 2008/98/EC each of the numbered types can also be hazardous if it has certain properties and based on those belongs to one or more of 15 categories, where the category H14 is ecotoxic.

According to Commission Regulation (EU) No 1357/2014 the ecotoxic waste is defined as '*waste which presents or may present immediate or delayed risks for one or more sectors of the environment*' and is labelled with number H14. The European directive does not specify the assessment procedure of the hazardous waste nor ecotoxicology tests which should be used for the assessment. Therefore, the directives and legislations of each EU member state can differ.

EUROSTAT is a statistical office of EU which monitors and reports statistical data of every EU member country, including waste data. Approximately 500 kg of municipal waste is produced annually per capita in the EU countries ('Eurostat'). The fact that municipal waste can also be hazardous puts a big responsibility on the 'producer' and the 'holder'. Producer is '*anyone whose activities produce waste*' and a holder is '*the waste producer, or the natural or legal person who is in possession of the waste*', according to the EU definition 2008/98/EC. In the Czech Republic, by the new law 541/2020, a producer is '*a legal entity producing waste in connection with its operation or a natural person authorized to conduct business, who produces waste in connection with his activity*'. If a natural person deposits waste in a designed location, the municipality becomes the producer and the holder of the waste. At this moment the municipality is responsible to deal with the waste, its disposal and processing.

On 1st January 2021 the Ministry of Environment of Czech Republic has published the new law, the Waste Act 541/2020 and on 5th January 2021 the new regulation, the Waste Catalogue and Waste Assessment 8/2021. These documents replaced the previous Waste legislation. The purpose of this amendment is to ensure a high level of protection of the

environment and human health with sustainable use of natural resources by managing waste in accordance with the waste hierarchy.

This regulation incorporates the relevant regulations of EU and manages the Waste Catalogue, the waste classification according to the catalogue, training for the assessment of hazardous properties of waste, requirements for the assessment of hazardous properties of waste, methods and procedures for the evaluation of hazardous properties of waste, additional limit values and criteria for hazardous properties of waste H9, H14 and H15, waste sampling and the methodology of laboratory testing, analyses, ecotoxicological and microbial testing of waste.

3.5.1 Ecotoxicity test of waste according to Czech legislation

As mentioned before ecotoxicity tests are an efficient way for assessment and monitoring of the toxicity of chemicals, as in this case the ecotoxicity of hazardous waste. According to the annex No 3 of Regulation 8/2021 ecotoxicity tests are done with either the liquid waste or aqueous solid waste extract (leachate). The test results can be used both for the evaluation of waste applied on surfaces (agricultural land) and for the evaluation of hazardous properties (H14 Ecotoxicity). One of the testing organisms is algae, namely *Desmodesmus subspicatus* or *Raphidocelis subcapitata* (duration of action 72 hours).

Firstly, the waste is sampled following the ČSN EN 14899:2005 of 1 July 2006 Characterization of waste - Sampling of waste - Principles of preparation of a sampling program and its use. Sampling can be carried out only by an authorized person, a professional certified person or a laboratory worker. All these people have to be certified according to the ČSN EN ISO/IEC 17024:2013 Conformity assessment - General requirements for bodies for the certification of persons. Samples are stored in tightly closed containers in the dark at temperatures up to 4 ° C, in accordance with the instruction 4/2007 – Methodology of Waste Sampling for Ecotoxicity Determination, given by the Czech Ministry of Environment.

Based on the Regulation 8/2021, the preparation of the different types of tested waste stands as following:

- SOLID WASTE: Such concentration of the solid waste is mixed with dry matter of artificial soil, so the total concentration of waste is 10 %
- WASTEWATER: Wastewater is filtrated through a 0.45 μm membrane filter in accordance with the standard ČSN EN 14735:2007 Waste characterization - Waste characterization - Preparation of waste samples for ecotoxicity tests.

The leachate of waste is diluted (at a concentration of 100 ml/l) with the addition of the same nutrients and in the same concentration as in the control, according to the standard ČSN EN 12457:2003. Characterization of waste - Leaching - Verification test for the feasibility of granular waste and sludge.

For ecotoxicology tests done with algae a standard ČSN EN ISO 8692:2012 Water quality - Test for inhibition of growth of freshwater green algae, is used. The protocol of ČSN EN ISO 8692 is based on the previously mentioned standardized test ISO 8692:2012 which has been accepted in the Czech Republic.

4. Methodology

The methodology used in the experimental part of the thesis is in the accordance with the ČSN EN ISO 8692:2012 standards and OECD 201:2011 Guidelines for the Testing of Chemicals – Freshwater Alga and Cyanobacteria, Growth Inhibition Test. The methodology is appropriate for chemicals easily dissolvable in water. It is based on the observance of growth of the concentration of algal cells in the test solution and the growth medium through time.

During the laboratory practice it is necessary to comply with safety measures according to ČSN 01 8003:2017 – Basics of safety for laboratory practice.

The hypothesis of this bachelor work was to replace living free algae in toxicity testing by immobilized algae without changing the sensitivity results of the standardized algae test.

4.1 Material

4.1.1 Chemicals

- Sodium hydroxide, NaOH (Sigma Aldrich, Czech Republic)
- Potassium dichromate, $K_2Cr_2O_7$ (Sigma Aldrich, Czech Republic)
- Hydrochloric acid 35%, HCl (Sigma Aldrich, Czech Republic)
- Ammonium chloride, NH_4Cl (Sigma Aldrich, Czech Republic)
- Magnesium chloride, $MgCl_2.6H_2O$ (Sigma Aldrich, Czech Republic)
- Calcium chloride, $CaCl_2.2H_2O$ (Sigma Aldrich, Czech Republic)
- Magnesium sulphate, $MgSO_4.7H_2O$ (Sigma Aldrich, Czech Republic)
- Potassium dihydrogen phosphate, KH_2PO_4 (Sigma Aldrich, Czech Republic)
- Chelaton III, $Na_2EDTA.2H_2O$ (Sigma Aldrich, Czech Republic)

- Ferric chloride, $FeCl_3.6H_2O$ (Sigma Aldrich, Czech Republic)
- Boric acid, H_3BO_3 (Sigma Aldrich, Czech Republic)
- Manganese chloride, $MnCl_2.4H_2O$ (Sigma Aldrich, Czech Republic)
- Zinc chloride, $ZnCl_2$ (Sigma Aldrich, Czech Republic)
- Cobalt chloride, $CoCl_2.6H_2O$ (Sigma Aldrich, Czech Republic)
- Copper chloride, $CuCl_2.2H_2O$ (Sigma Aldrich, Czech Republic)
- Sodium molybdate, $Na_2MoO_4.2H_2O$ (Sigma Aldrich, Czech Republic)
- Sodium bicarbonate, $NaHCO_3$ (Sigma Aldrich, Czech Republic)
- Demineralized water (with a conductivity of less than 10 μS/cm), production in PURELAB flex 1 (ELGA LabWater, Great Britain)
- Sodium metaphosphate, NaO_3P (matrix dissolving medium for de-immobilization, MicroBioTests, Gent, Belgium)
- Potassium dichromate $K_2Cr_2O_7$ (Sigma Aldrich, Czech Republic)
- Triclosan $C_{12}H_7Cl_3O_2$ (Sigma Aldrich, Czech Republic)
- Diclofenac sodium salt $C_{14}H_{11}Cl_2NNaO_2$ (Sigma Aldrich, Czech Republic)

4.1.2 Equipment

All equipment in contact with the test medium had to be made of glass or other inert material. Equipment was prior to the use sterilized for one hour on 150 °C.

- Beaker
- Erlenmeyer flasks
- Volumetric flask (1 l and 100 ml)
- Volumetric flask stoppers
- Funnel

- Spatula
- Cuvette (for the spectrophotometer, P-Lab, Czech Republic)
- Automatic pipette Research plus 0.5-5 ml (Eppendorf, Germany)
- Cellulose stoppers for Erlenmeyer flask

4.1.3 Apparatus

- air-conditioned box / room with fluorescent white lamp (Duckweed light unit, MicroBioTests, Gent, Belgium)
- spectrophotometer with appropriate calibration (Agilent, Czech Republic)

  **spectrophotometer information:*

  Instrument- Cary 60, version 2.00

  **settings of the instrument during the test measurements:*

  Wavelength: 685.0

  Ordinate mode: Abs

  Average time of the measurement: 0.100 s

  Sample averaging- ON

  Weight and volume corrections- OFF

  Fit type: linear

  Minimal R2: 0.9500

  Concentration units: b/ml

  Replicates: 3
- pH meter (WTW, Czech Republic)
- analytical balances (Conrad, Czech Republic)
- laboratory centrifuge machine (Verkon, Czech Republic)

- orbital shaker (Verkon, Czech Republic)
- stainless steel mixing plate (P-Lab, Czech Republic)
- Wall CO_2/thermo detector (Klimafil, Czech Republic)

4.1.4 Test organisms

a) *Raphidocelis subcapitata* immobilized beads

In the experimental part of the thesis alginate immobilized algae were bought from the supplier MicroBioTests in Gent, Belgium ('MicroBioTests'), in a form of algae kits, with 5 separate tubes.

b) *Raphidocelis subcapitata* living free culture

In the experimental part of the thesis the fresh algae samples were supplied from the Institute of Botany CAS, which has the Culture Collection of Autotrophic Organisms (CCALA), Třeboň (*'CCALA'*), Czech Republic.

4.2 Method

4.2.1 Growth medium preparation

Four individual mediums, which are needed for the growth medium, were prepared prior to the test, based on the ISO, Water quality – Freshwater algal growth inhibition test with unicellular green algae (ISO 8692:2012) standard (

Table 1). Each medium was prepared in a sterilized volumetric flask. Specific chemicals for each medium were put into 1 l of distilled water inside the volumetric flask and homogenized, respectively.

After homogenization they were transferred into a glass, tightly closed container, which was marked with the date of the preparation of the medium. They were stored in a dark room at the temperature of 4 ± 2 °C.

4.2.2 Cultivation of living free algae culture

The tested living algae, in order to be used, must be in the exponential phase of growth. Therefore, 72 hours before the beginning of the test a new algal culture was set up with the concentration of algal cells of 10,000 c/ml. In the following 72 hours the exponential phase and the wanted cell concentration were established. The cell concentration should not exceed 2,000 c/µl.

Optimal cultivation conditions were maintained with the temperature 23 ± 2 °C, constant illumination of 6 000 – 10 000 lux and constant shaking on a laboratory orbital shaker with 170 ± 10 rpm.

4.2.3 De-immobilization of alginate immobilized algae

One of the tubes containing the immobilized algal beads was taken and the liquid they were placed in was poured out. Then 5 ml of the "Matrix dissolving medium" was put into the tube and shaken for approximately 2 minutes, until the immobilized algal beads were dissolved. The tube was put into a lab centrifuge for 10 min at 3,000 rpm. After the centrifugation the algae were concentrated in one place of the tube and the supernatant was poured out. 10 ml of deionized water was added into the tube and closed. It was shaken until the solution became homogenized. The tube was centrifugated for another

10 min at 3,000 rpm. The supernatant was poured out and replaced by 10 ml of algal culturing medium. The algae were re-suspended by shaking and by that a pre-culture was made.

4.2.4 Stock solution preparation

A potassium dichromate stock solution was prepared by adding 1,000 mg of potassium dichromate into a 1,000 ml volumetric flask and the rest was filled with distilled water.

A triclosan stock solution was prepared by adding 0.290 mg of dried triclosan into a 200 ml volumetric flask and the rest was filled with distilled water.

A diclofenac stock solution was prepared by adding 0.200 g of dried diclofenac into 200 ml volumetric flask and the rest was filled with distilled water.

4.2.5 Setting up of the test

The laboratory work was done as 6 tests, each repeated for 3 times. Three tests were done with de-immobilized algal cells and three with the living algal cells. Each was exposed to different concentrations of potassium dichromate ($K_2Cr_2O_7$), triclosan (TCS) or diclofenac (DCF) separately. 6 volumetric flasks (100 ml) were filled halfway with distilled water. Different amounts of the chemical were added into the volumetric flasks (Table 3).

Table 3 Setting up of the test

	Test A	Test B	Test C
Flask	Concentration of $K_2Cr_2O_7$	Concentration of TCS	Concentration of DCF
1.(blank)	0 mg/l	0 µg/l	0 mg/l
2.	0.3 mg/l	1.5 µg/l	50 mg/l
3.	0.6 mg/l	4.5 µg/l	100 mg/l
4.	1.2 mg/l	13.5 µg/l	200 mg/l

5.	2.4 mg/l	40.5 µg/l	400 mg/l
6.	4.8 mg/l	121.5 µg/l	800 mg/l

The volume of the inoculum (either de-immobilized or living cells) was determined so the final concentration added into all 6 volumetric flasks was 10,000 c/ml. Each flask was filled with the same amount of the growth medium (1 ml of medium 1 and 0.1 ml of medium 2, 3 and 4) and filled with distilled water till the mark. These solutions were properly mixed and each was put into a 100 ml Erlenmeyer flask. They were closed with cellulose stopper and put on a mixing plate in the cultivation conditions (Figure 6).

Figure 6 Tests set in the cultivation conditions

4.3 Measurements and calculations

4.3.1 Measurement of the pH

The pH measurement was done on each test control sample before and after the growth period. It was done by firstly calibrating the pH meter. The electrode of the pH meter was immersed into a stirred sample and the stabilization of the pH value, shown on the pH meter monitor, was waited out. The pH is not further adjusted, due to toxic effect of

the acid or the base on the algae. The pH adjustment is done only on the cultivation mediums.

4.3.2 Measurement of the temperature

The temperature of the room of cultivation was stable and constantly monitored by a wall thermometer.

4.3.3 Measurement of algal cell concentration

To determine the concentration of algal cells in the sample US/VIS spectroscopy was used. It is a fast and reliable method. The procedure of the measurement is the following:

- The spectrometer was calibrated on 0 – demineralized water was added into a clean and dry cuvette, on the computer program ZERO was pressed;
- 3-4 ml of the monitored solution was added into a clean and rinsed cuvette and the concentration of the algal cells was measured according to the predetermined calibration.

4.3.4 Calculation of volume of the inoculum which will be used in the test

After the preparation of the inoculum (de-immobilization and cultivation of living algae) the concentration of algal cells in the inoculum was measured with spectrophotometer. The result was used in a formula to determine the amount of the inoculum that needs to be added into each flask in a further explained test. The concentration of algal cells in each flask should be around 10 c/µl (10,000 c/ml).

$$V = \frac{10 \times V_{test}}{n} \quad \text{[Eq. 4]}$$

Where:

V – volume of the inoculum that needs to be added into the flask (ml)

V_{test} – volume of the solution in the flask which will be tested (ml)

n – concentration of the algal cells in the inoculum (b/µl)

4.3.5 Calculation of the average growth rate

The average specific growth rate for a period of time is calculated as the logarithmic increase in the biomass through an equation mentioned in the standard [Eq. 1].

4.3.6 Calculation of the percent inhibition of growth rate

The percent inhibition of growth rate is calculated through the equation from the standard [Eq. 2].

4.3.7 Calculation of the variation coefficient of the growth rate

Calculation of the variation coefficient of the growth rate in the control replicates is done in order to check the validity of the testing through an equation:

$$CV = \frac{s_d}{\mu_{avg}} \times 100 \qquad \text{[Eq. 5]}$$

Where:

CV – coefficient of variation in the control

μ_{avg} – average growth rate in the control (1/d)

s_d – standard deviation of the average growth rate in the controls

4.3.8 Estimation of IC_{50}

IC_{50} was calculated with a Probit method using the MS Excel program. For each substance concentration a logarithmical scale was plotted, which was put on the x-axis in the graph. For each inhibition a value was found in the standard table of standard probit values (Říhová Ambrožová *et al.*, 2014), these were put on the y-axis of the graph. If the result points in the graph are too scattered, the mean value of the replicates with the corresponding standard deviations is made.

Regression analysis was used to construct a suitable nonlinear model *probit = kx + q,* for the experimental points. After a specific formula was given, y was put as value of 5 and the result was the logarithm of the IC_{50} concentration.

4.3.9 Calculation of IC_{50}

For the processing of the data and calculation of the IC_{50} a module PROAST, as a part of the programming language R, was used. PROAST is a software package developed by the National institute of the Netherlands, used for the statistical analysis of dose-response data (*'PROAST | RIVM'*). The data was inserted in the module in a specific form and the IC_{50} was calculated through two methods. The first method of calculation is called the exponential method, and the second one is called the Hill method. Since the procedure of calculation differs between the two methods, the results also deviate slightly.

4.3.10 Validity of the test

The validity of the tests was checked by the fulfilment of the following parameters: the average specific growth rate had the minimum value of 1.4 d^{-1}, the pH value and the end of the test did not differ from the starting value more than 1.5 and the calculation of the variation coefficient of the growth rate in the control replicates did not exceed 5 %.

5. Results

After 72±2 h the concentration of algal cells was measured in each sample with a spectrophotometer for 6 times, for a better precision. These concentrations can be found in the Appendix 1. Based on these results the average growth rate, percent inhibition of growth rate was determined, this can be found in the Table 3 for the free algae and the Table 4 for the immobilized algae.

Table 4 Results of tests with the free algae

chemical	concentration	averages of growth rate			percent inhibition of growth rate		
replicate	mg/l (*µg/l)	1	2	3	1	2	3
$K_2Cr_2O_7$	0	1.68	1.84	1.95			
	0.3	1.71	1.79	1.73	-2	3	11
	0.6	1.51	1.48	1.81	10	19	7
	1.2	1.09	1.15	1.7	35	37	12
	2.4	0.59	0.54	0.61	65	71	69
	4.8	0.53	0.52	0.42	68	72	78
TCS	0*	1.71	1.84	1.71			
	1.5*	1.85	1.79	1.84	-8	3	-8
	4.5*	0.73	1.18	0.72	58	36	58
	13.5*	0.53	0.98	0.52	69	46	70
	40.5*	0.37	0.91	0.39	78	50	77
	121.5*	0.46	0.58	0.47	73	69	73
DCF	0	1.67	1.85	1.95			
	50	1.55	1.62	1.63	7	12	16
	100	1.12	0.81	1.59	33	56	18
	200	0.72	0.74	0.92	57	60	52
	400	0.68	0.78	0.57	59	58	71
	800	0.62	0.72	0.58	63	61	70

Table 5 Results of tests with the immobilized algae

chemical	concentration	averages of growth rate			percent inhibition of growth rate		
replicate	mg/l (*µg/l)	1	2	3	1	2	3
$K_2Cr_2O_7$	0	1.9	1.68	1.84			
	0.3	1.76	1.8	1.79	8	-8	3
	0.6	1.38	1.45	1.47	28	13	20
	1.2	0.69	0.69	0.59	64	59	68
	2.4	0.34	0.39	0.36	82	77	80
	4.8	0.28	0.44	0.25	85	74	86
TCS	0*	1.9	1.68	1.84			
	1.5*	1.53	1.74	1.71	20	-4	7
	4.5*	0.78	1.12	0.92	59	33	50
	13.5*	0.53	0.57	0.55	72	66	70
	40.5*	0.38	0.39	0.53	80	77	71
	121.5*	0.36	0.18	0.55	81	89	70
DCF	0	1.78	1.67	1.67			
	50	1.57	1.5	1.76	12	10	-5
	100	1.37	1.39	1.33	23	16	20
	200	0.8	0.81	0.73	55	51	56
	400	0.68	0.74	0.77	62	55	54
	800	0.61	0.65	0.66	66	61	61

The pH measurements before and after the test in the control samples, coefficient variation of growth rate and the IC_{50}, as the main result of the work, are displayed in the Table 5. Since the IC_{50} was measured through two methods, both are displayed in the table and in Figure 7.

Table 6 CV, pH and IC50 results

Free algae							
Substance	No	CV (%)	pH (t0)	pH (t72h)	IC_{50} (met1)	IC_{50} (met2)	IC_{50} average
$K_2Cr_2O_7$	1	0.2	7.6	7.7	1.5913	1.5538	1.7
	2	0.1	7.6	7.9	1.4756	1.4734	
	3	0.1	7.5	7.9	2.0438	1.9165	
TCS	1	0.6	7.6	7.6	3.9942	3.5948	17.2
	2	0.1	7.6	7.7	24.881	22.995	
	3	0.6	7.5	7.8	24.834	22.953	
DCF	1	0.4	7.6	7.7	171.6	175.3	177.7
	2	0.3	7	8	155.4	155.1	
	3	0.1	7.2	8	203.45	205.25	
Immobilized algae							
Substance	No	CV	pH (t0)	pH (t72h)	IC_{50} (met1)	IC_{50} (met2)	IC_{50} average
$K_2Cr_2O_7$	1	0.4	7.5	7.8	0.9302	0.9436	1.0
	2	0.1	7.6	8	1.0596	1.0306	
	3	0.1	7.2	8.1	0.9355	0.8928	
TCS	1	0.4	7.6	8.1	3.529	3.435	5.4
	2	0.1	7.6	8.5	8.1467	8.3792	
	3	0.1	7.1	8.2	4.507	4.56	
DCF	1	0.4	7.5	7.7	180.64	183.89	189.1
	2	0.9	7.5	7.9	203.2	214.2	
	3	0.9	7.2	8.1	168.7	184	

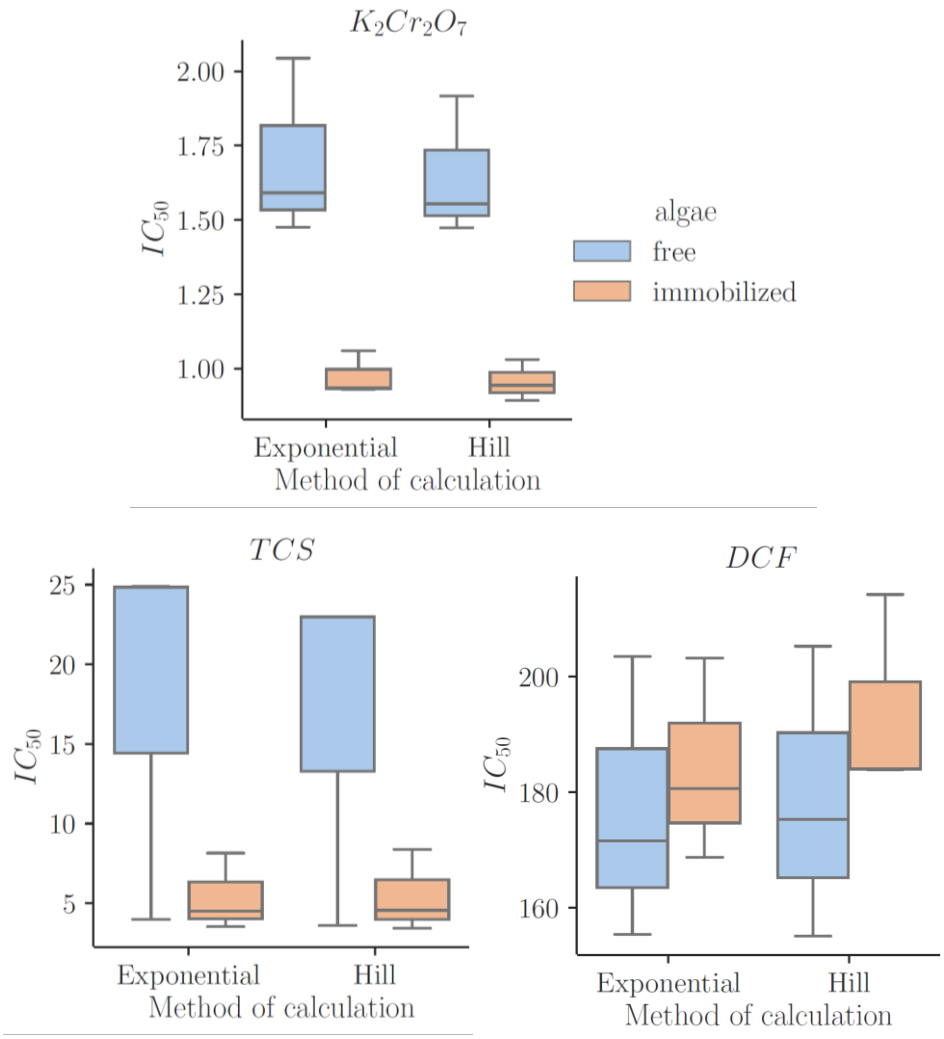

Figure 7 IC50

6. Discussion

The hypothesis of this bachelor work was to replace living free algae in toxicity testing by immobilized algae without changing the sensitivity results of the standardized.

According to the results from previous tables, the growth inhibition differs both among tested substances and algal cultures. The results demonstrate the response of algae to different concentrations of three substances. Each test was repeated for three times for a better precision. Since this test is a biotest, meaning it includes living organisms, the results contain a percentage of uncertainty and deviation. Algal bioassays are very commonly used for their reliability and accuracy, yet algae are living organisms and a slight offset in response is predictable. Even though all algae cultures were incubated in the same conditions, treated the same and the methodology of all tests was the same, the results slightly differ.

Firstly, a positive response is demonstrated in a few samples, where the concentration of the substance is low. Lower concentrations of the substances can stimulate algal growth, but higher concentrations significantly reduce the growth of both living free algae and immobilized algae. "*Sola dosis fecit venum*" (the dose makes the poison) said Paracelsus years ago (Truhaut, 1975), while this is applicable in this case also.

IC_{50} is a measure used in ecotoxicology, which indicates the concentration of a substance which is needed *in vitro* for an inhibition of growth at 50 % of the algal cells.

Potassium dichromate ($K_2Cr_2O_7$) was used as a reference substance and the chosen concentrations were determined based on previous research. According to the research (Santos *et al.*, 2007) the IC_{50} was established at 0.9 mg/l of potassium dichromate. A similar IC_{50} was indicated by immobilized algae, the average 1 mg/l. The living algae growth inhibition was significantly lower, meaning they are less sensitive to the substance. This resulted in a higher IC_{50}, precisely 1.7 mg/l.

Triclosan (TCS) was used as a first test substance and was tested with concentrations in µg/l, since its toxicity is much higher. In a research (Tatarazako *et al.*, 2004) the IC_{50} of

TCS on *Raphidocelis subcapitata* was measured at 4.7 µg/l. A similar IC_{50} was measured at immobilized algae, with the average IC_{50} 5.4 µg/l. The inhibition of growth at free living algae was lower, like in the case of potassium dichromate, meaning free living algae are less sensitive to the substance. IC_{50} of free algae to TCS was 17.2 µg/l.

In the case of diclofenac (DCF), the concentrations which caused IC_{50} were 60 mg/l, according to a study (Doležalová Weissmannová *et al.*, 2018). The IC_{50} concentrations in immobilized and free algae were 189.1 mg/l and 177.7 mg/l, respectively. These results are fairly similar, meaning that the sensitivity of both immobilized and free algae is comparable.

From previous researches, where the sensitivity of immobilized and living algae to heavy metals and pesticides was compared (Al-Hasawi *et al.*, 2020; Bozeman *et al.*, 1989), the immobilized algae either turned out to be less sensitive or comparable. In this thesis the results contradict the previous researches. The (Al-Hasawi *et al.*, 2020) research explains the lower sensitivity of immobilized algae due to the effect of the alginate. Alginic acid, alginate, binds strongly to divalent cations and thus reduces the toxicity of the tested substances. The difference between these previous researches and this thesis is that the immobilized algae were used while still immobilized, but it this thesis' methodology the algal culture was de-immobilized, following the standardized procedure. Through the de-immobilization, alginate was removed. It was treated with the de-immobilization matrix and washed twice with distilled water. There was no reason for the chemical properties of the alginate to be key factors in the final results.

The differences between immobilized algae beds and living algae cultures are recognized in the length of lag period, the period of time necessary for the algae to start growing exponentially after the introduction to a new medium, i.e. the period when the algae are adjusting to the new environment. Immobilized algae have a longer lag period. Thus, after the lag period, the specific growth rate is similar of immobilized and living algae (Moreno-Garrido, 2008). This seems logical, since the immobilized algae are chemically treated to be immobilized and then chemically treated to be de-immobilized. These treatments can affect or even chock the algae. Therefore, they need more time to adjust to the newly introduced conditions. On the other hand, free algae are introduced

to the test conditions for three to four days prior to the test, hence they overcame the lag phase of adjusting. The immobilized algal cells are immobilized in their exponential phase but the de-immobilization procedure and introduction into new conditions can affect the exponential phase.

Another reason for the sensitivity difference could be the fact that in the experimental part of the work the living free algae were bought from a supplier in the Czech Republic and the immobilized from a supplier in Belgium. The method of immobilization with alginate, described in the ISO 8692:2012 does not include unique equipment or an extremely complicated procedure. The immobilization should be considered as a further improvement and a good opportunity in the market. I would personally recommend including immobilized algae beads in the algal suppliers' market in the Czech Republic, so the price of the good can be reduced and become more affordable. With a lower price, immobilized algae could be widely used and favoured as an alternative, rapid and effective toxicity test.

The hypothesis of this bachelor work was partially confirmed. Immobilized algae could replace the living free algae in toxicity testing when diclofenac is used as a tested substance. However, immobilized algae cannot replace the living free algae in toxicity testing when potassium dichromate or triclosan are used as tested substances.

7. Conclusion and contribution of the thesis

The aim of this work was to conclude if the living free algae could be replaced in toxicity testing by the immobilized algae without changing the sensitivity results of the standardized 72±2 h acute algae test, following the ČSN EN ISO 8692:2012, with the freshwater green alga *Raphidocelis subcapitata (Selenastrum capricornum).*

From the achieved results, it is evident that with some substances the immobilized algae can replace the living free algae, and controversially with some not. For instance, the immobilized algae were more sensitive to the reference substance potassium dichromate and to triclosan than free living algae. On the other hand, the sensitivity of both living free algae and immobilized algae was comparable when tested with diclofenac. The difference of sensitivity could be explained by the longer lag period of the immobilized algae.

In general, toxicity determination based only on test results with the immobilized algae affected by potassium dichromate and triclosan may be inaccurate. Toxicity determination could be precise on test results with the immobilized algae for diclofenac.

Based on these conclusions, it is necessary to further investigate accuracy and sensitivity differences between immobilized and living free algae. Suppliers of living free algae in each country should consider the practice of immobilization. By this manner the sensitivity of algae can be increased, along with better precision and more affordable prices.

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