Degree project

UNIVERSITY OF SKÖVDE

Effect of Ibuprofen on the growth of *Pseudokirchneriella subcapitata*

Bachelor Degree Project in Bioscience

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Preface

I performed this work as a Bachelor's Degree Project in Biology at the University of Skövde. Laboratory work was performed during my stay in Sweden. I had a pleasant stay in Skövde where I was able to learn a lot and meet incredible people.

Abstract

Pharmaceuticals are an important class of pollutants in aquatic ecosystems. Detected concentration are typically in the range 1 ng/L – 1 µg/L. Traditional wastewater treatment does not provide a complete removal of these contaminants; hence, they may have a negative impact on the environment. In addition, microalgae are an ecologically-meaningful target group of species for bioindication purposes as well as primary production and oxygen supply. The present work aimed to investigate the effect of Ibuprofen on the green alga *Pseudokirchneriella subcapitata.* Algal cultures were exposed to five different concentrations of the drug (5, 15, 45, 135, 405 mg/L) for four days. Absorbance measured at 680 nm was determined every day and obtained data were transformed into cell concentration (cells/mL) by a previously prepared calibration curve. Specific growth rate, generation time, percent inhibition and effective concentration were calculated. Moreover, one way ANOVA with Tukey's test were applied to observe differences between groups and time periods. Based on this study, all the cultures treated with Ibuprofen had a growth inhibition as well as presenting a lag phase. Increasing the Non-Steroidal Anti-Inflammatory drug (NSAID) concentration reduced the growth rate and consequently, increased the percent inhibition in a concentration-dependent manner. According to this report, new research should be focused on the development of hybrid systems for degradation and removal of pharmaceuticals. NSAID pollution may lead to a reduction in the diversity and number of functional groups of eukaryotic algae. Finally, more research should be devoted to the toxicity of drugs in a variety of test organisms and development of reliable methods for toxicity test at low and chronic exposures to achieve more realistic conclusions.

Key words: *P. subcapitata***,** toxicity test, Contaminants of Emerging Concern, Ibuprofen.

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Introduction

Background

Contaminants of emerging concern (CECs)

Recently, anthropogenic compounds have been observed in wastewater. Moreover, new contamination spots are continuously discovered by the new detection techniques. These chemicals are known as a Contaminants of Emerging Concern (CECs) and are of particular interest to the scientific world. Recent information shows their negative impact on the environment and human health, and they are generally not regulated by the current environmental laws (Batucan et al., 2022).

CECs span natural and artificial compounds and their by-products, such as personal care products (PCPs), pharmaceuticals, flame retardants (FRs), artificial sweeteners (ASWs), pesticides, microplastics, nanoparticles and products which they are transformed into, but also antibiotic resistant bacteria (ARB), antibiotic resistant genes (ARG) and even, recently, the SARS-CoV-2 virus (Pastorino & Ginebreda, 2021). Moreover, these compounds are detected in a concentration range between 1 ng/L - 1 μg/L and rarely up to 100 μg/L (Pal et al., 2014).

To better understand the growing concern about this new type of pollutant, it is necessary to know differences between CECs and priority pollutants (PP). A PP is defined as a substance or group of substances that are toxic, persistent and can cause bioaccumulation. This group of contaminants have been the subject of study for many years, and they are regulated through Directive 2000/60/EC of the European Parliament Legislation. Some of the regulatory measures include the cessation or reduction of leaks, emissions or pollution sources, and the protection of water and its chemical and ecological quality (Schratter-Sehn et al., 1992). However, CECs have different origin and chemical nature, whose presence and consequences in the environment have not been clearly noticed. Moreover, the lack of legislative regulation makes it difficult to set up a joint and planned actions against these compounds. The main characteristics of CECs can be summarized in Table 1.

Table 1. Main characteristics of CECs.

Characteristics of CECs
Lack of knowledge about their presence and impact in different environmental compartments and humans.
Not regulated yet and limited methods availability for their analysis.
High production, consumption and, introduction into the environment.
Harmful effects are difficult to estimate. Both chronic low concentration and punctual high concentration exposures can cause serious problems on the environment.
Higher presence in aquatic ecosystems. The ambient temperature and pressure allow them to dissolve without evaporating.
Detected at concentrations in the range of 1 ng/L - 1 μg/L, although in some cases, concentrations reach up to 100 μg/L.
Lack of knowledge about their behaviour and biodegradability in water bodies.

Pharmaceuticals as water contaminants

Pharmaceuticals are an important class of CECs, which enter in freshwater environments via discharges from wastewater treatment facilities, agricultural and aquacultural activities, landfill leachate, as well as groundwater contaminated by sewage pipe leaks. Pharmaceuticals and derivatives have been found in drinking water recently. Despite the lack of information, they are known to be harmful to human health, especially in sensitive life stages such as foetal development and early childhood (Batucan et al., 2022).

Diclofenac and Ibuprofen are two widely used drugs that are frequently studied. Both are Non-steroidal anti-inflammatory drugs (NSAIDs) used as analgesia and anti-inflammatory (Batucan et al., 2022; González-Naranjo & Boltes, 2014). Briefly, their mechanism of action is the inhibition of two cyclooxygenase enzymes (COX-1 and COX-2) involved in the synthesis of prostaglandin and thromboxane. These compounds lead to inflammation, pain, and fever. Therefore, blocking COX, it is achieved the therapeutic effect (Batucan et al., 2022; Fokunang, 2018).

Ibuprofen it is metabolized mainly in the liver and excreted by the renal pathway. This compound and other NSAIDs are used by humans, but also in animal farming. Consequently, due to the increase in the human population, the excretions of these compounds have not stopped growing in recent years (Świacka et al., 2021).
Some extra information about the physicochemical properties of Ibuprofen is listed in Table 2.

Table 2. Physicochemical properties of Ibuprofen (Thermo Fisher Scientific, 2012).

Property	**Description**
Formula	$C_{13}H_{18}O_2$
IUPAC	(RS)-2-(4-(2-methylpropyl)phenyl)propanoic acid
Formula Weight	206.28 g/mol
Water solubility	0,011 g/L at 25°C
pKa	4.52/4.91
Naturality	Hydrophobic ion
Charge at pH 7	-1
Appearance	Form: solid Colour: Colourless
Melting point/ freezing point	Melting point/range: 75 – 77°C at 1.013 hPa

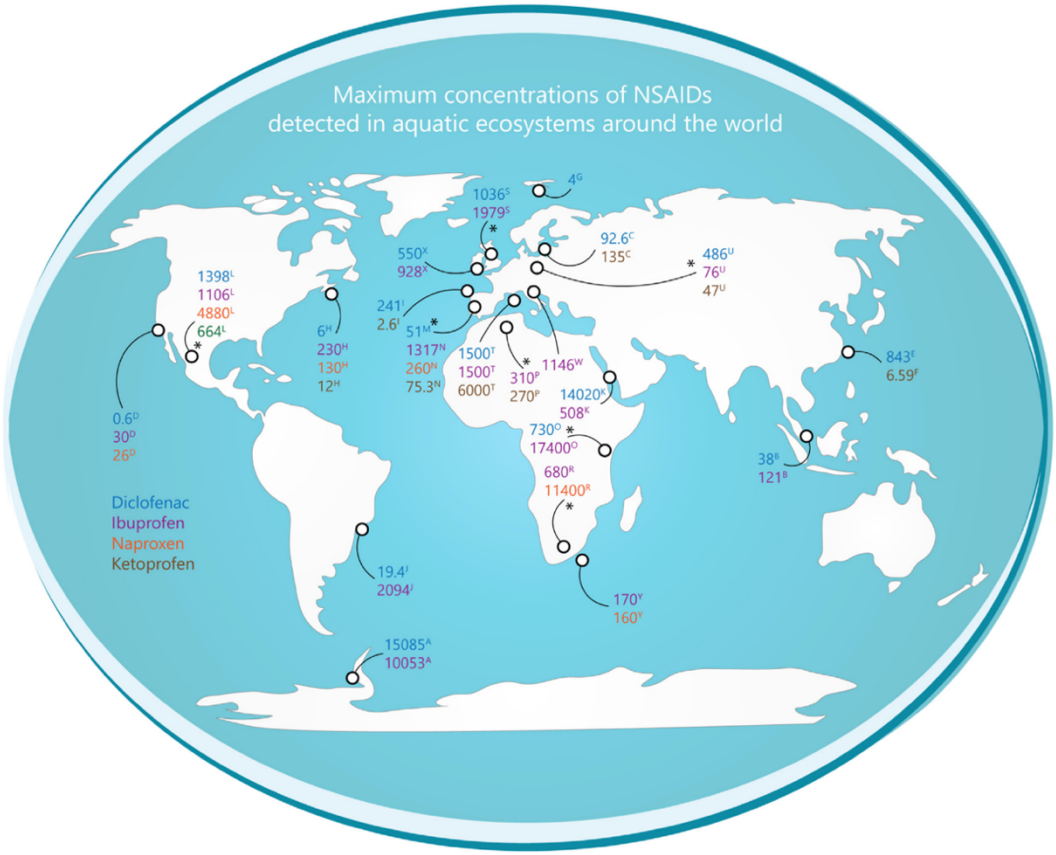
Looking at the properties, Ibuprofen presents a low environmental stability and is easily degradable, especially through aerobic biotransformation and photolysis in surface waters. On the other hand, it is highly insoluble in water (< 1 mg/mL), i.e., hydrolysis does not work on it. However, this drug, as well as other NSAIDs, is constantly detected in different ecosystems. This is mainly due to the fact that its constant inflow into surface waters is greater than its degradation (Świacka et al., 2021). Therefore, its accumulation in water bodies may cause toxic effects on aquatic organisms (Wang et al., 2020).

CECs in aquatic ecosystems

Due to population growth, economic development and changes in consumption patterns, the demand for water has increased at a rate of 1% per year and it is estimated that it will continue growing in the next two decades, especially in industrial and domestic activities. Water quality has worsened since the 1990s, posing a threat to human health, the environment and sustainable development (Schratter-Sehn et al., 1992). Therefore, water pollution is a global concern, as it is an essential natural resource for both humans and the environment (Sousa et al., 2018).

NSAIDs belong to one of the most important drug groups worldwide. These medicines are purchased without prescription, i.e., their consumption is expected to be high. Therefore, NSAIDs are found in detectable concentrations in the environment due to their urinary and fecal excretion and the improper disposal of surplus or expired products.
Focusing on Ibuprofen, previous works reported concentrations between 1 – 3.35 µg/L in sewage, 0.01 – 0.5 µg/L in river water and 1 – 6 ng/L in drinking water (Cleuvers, 2003, 2004). However, the highest concentrations observed in water bodies reach several µg/L (Fig. 2) (Świacka et al., 2021). These concentrations may seem low, but the detrimental effect they have on ecosystems and human health is still unknown. Moreover, toxicity works on a dose-dependent way, so it is important to quantify CECs in ecosystems and evaluate it on model organisms.

Figure 1. Maximum concentrations of Diclofenac, Ibuprofen, Naproxen and Ketoprofen (ng/L) detected in aquatic ecosystems worldwide. Asterisks indicate data obtained from freshwater reservoirs (Świacka et al., 2021).



Traditional wastewater treatment systems

Traditional wastewater treatment comprises three common phases. First, a primary treatment is developed where the solid waste substances (solids, plastics, oils, fats, sand, grit, etc.) are separated by mechanical processes such as filtration and sedimentation. This process is common to most urban wastewater treatment plants (WWTP). Afterward, a second treatment is performed by biological degradation (aerobic or anaerobic) of the organic compounds, substances, or nutrients. These treatments may vary on different plants. The most used process is the activated sludge in which, under appropriate conditions, nitrogen and organic compounds are removed by forming biological flocs using dissolved oxygen. Finally, a tertiary treatment removes phosphorus by precipitation and filtration. This process is not common in all the treatment plants (Sousa et al., 2018).
In addition, some effluents undergo disinfection processes using UV irradiation or chlorination before being discharged into the environment. Despite this, a complete removal is not assured.

Therefore, other techniques such as activated sludge must be used to remove pharmaceuticals or other CECs which are resistant to degradation (Gogoi et al., 2018).
Summarizing, these traditional methods do not provide a complete removal of contaminants; in addition, they may transform these compounds into substances more toxic than the original (Schlüter-Vorberg et al., 2015).

Algae role in emerging pollution

Microalgae are eucaryotic unicellular organisms capable of transforming light energy into chemical energy with a greater efficiency than plants. This group has special relevance because it is a primary producer in the trophic chain and also, generate atmospheric oxygen supply (Wang et al., 2020; Xin et al., 2021). Cyanobacteria proliferation in algal blooms can significantly alter the native structure of the algae and the entire community. As a consequence, ecosystems would be modified and trophic cascades and geochemical cycles would be impacted (Bácsi et al., 2016). Despite this, there is a very limited number of studies on effects of CECs and specifically, NSAIDs on isolated strains of microalgae.

Pseudokirchneriella subcapitata (*P. subcapitata*), also known as *Raphidocelis subcapitata* or *Selenastrum capricornutum* is a unicellular alga that inhabits in oligotrophic and eutrophic aquatic systems with an optimal lighting. Cells in culture are solitary except during cell division when they perform a kind of cluster. Furthermore, these cells have a helical shape, usually semicircular. Reproduction is carried out by division of a mother cell into 2, 4 or 8 daughter cells.

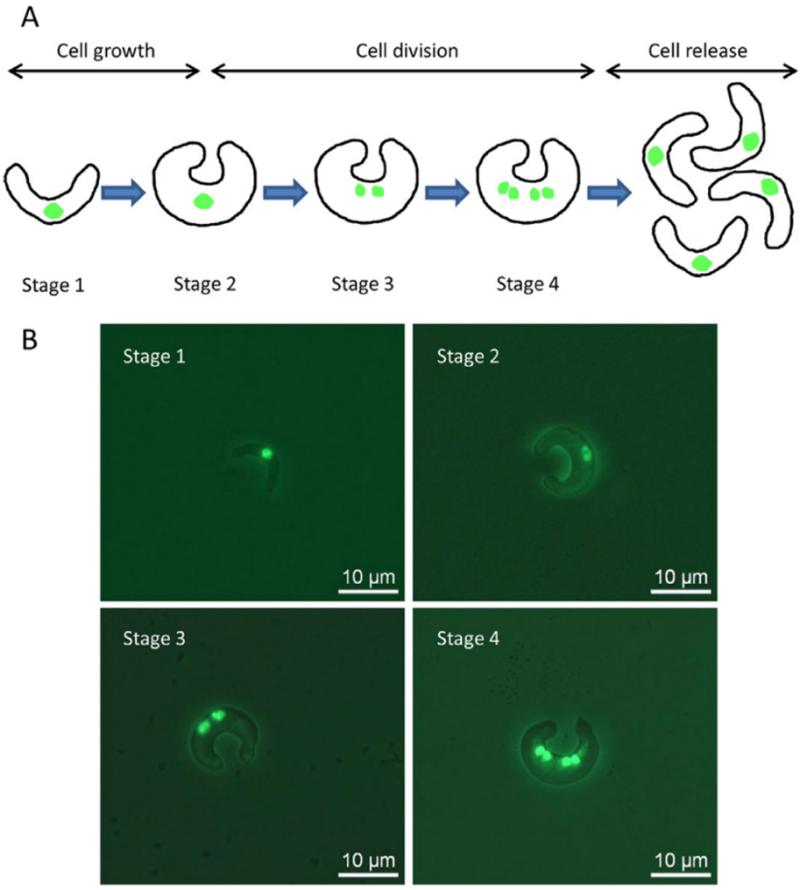


Figure 2. Cell proliferation in P. subcapitata. (A) Diagrammatic representation of the cell cycle of the alga, which comprises of three phases: (1) growth of mother cell; (2) cell division; (3) release of daughter cells (autospores). (B) Photomicrographs of fluorescence plus phase contrast images of algal cells at different stages. For visualization of nucleus, cells are permeabilized with 1-pentanol (70%, v/v) for 1 h, and subsequently stained with 0.5 µmol L^{-1} SYTOX Green for 40 min (Machado & Soares, 2014).

P. subcapitata is commonly used as a bioindicator in ecotoxicological assessments of freshwater ecosystems due to its high growth rate, high sensitivity and high reproducibility (O'Neill et al., 2019). Through this, microalgae assays can be miniaturized, allowing experiments to be carried out at a lower cost (less space is required, and fewer amounts of products are used).

Project's aim

The present work aimed to investigate the effect of Ibuprofen on aquatic organisms using *P. subcapitata* as a model organism. Therefore, the impacts of this NSAID on the algal growth were evaluated.

Research questions and hypothesis

This work intended to answer the following questions and hypothesis:

1. Does Ibuprofen have effects on the growth of *P. subcapitata*?
2. Does Ibuprofen have effects on the cell concentration (cell/mL) of *P. subcapitata*?
3. Are there differences between the growth of *P. subcapitata* when applying different concentration of Ibuprofen?

The following predictions were tested during the project:

1. Ibuprofen inhibits the growth of *P. subcapitata*.
2. Ibuprofen decreases the cell concentration (cell/mL) of *P. subcapitata.*
3. Higher concentrations of Ibuprofen show greater growth inhibition on *P. subcapitata*.

Objectives

This work intended to achieve the following objectives:

1. To maintain algae in algal test medium, prepare pre-cultures and prepare cultures.
2. To perform algal inhibition tests at different Ibuprofen concentrations.
3. To develop a methodology to determinate cell concentration (cells/mL) from absorbance measurements.
4. To measure the Ibuprofen's effect on *P. subcapitata* growth.
5. To resume new effective treatments against CECs' pollution.
6. To make people aware about the CECs' contamination.

Materials and methods

Strain, culture conditions, laboratory experimental setup, and sample processing

In this work, the freshwater green alga *P. subcapitata* (strain 278/4) was used. The original strain was obtained from the Culture Collection of Algae and Protozoa (CCAP), UK.

Growth medium solutions were prepared, sterilized and stored according to OECD guidelines (OECD, 2004).

The starter cultures were prepared by inoculating a loop of algal cells in 1 L OECD medium, in 1 L Erlenmeyer flasks. The cells were incubated, at 22°C, on a Biosan orbital shaker at 150 rpm under continuous "cool white" fluorescent light, with an intensity of ~ 9000 lux at the surface of the flask, verified using a Mastech illumination meter (Machado & Soares, 2014).

The experimental cultures were carried out in triplicates, under the same conditions as the starter cultures, in 60 mL flasks with a final volume of 50 mL of OECD medium and an initial cell concentration of ~ 7,50 x 10^4 cells mL^{-1}. Five nominal concentrations of Ibuprofen (5, 15, 45, 135

and 405 mgL^{-1}) were selected as exposure concentrations. Ibuprofen was diluted in 50 mg Ibuprofen/mL Ethanol (EtOH). All experimental cultures were applied 8 mL of Ethanol at different concentration of Ibuprofen. Cultures without the addition of the diluent and NSAID served as controls. To check the possible effect of the diluent, cultures containing EtOH without Ibuprofen were also applied (Bácsi et al., 2016).
At four intervals of time (24, 48, 72, 96 h), samples were withdrawn and, the absorbance at 680 nm was determined using a DeNovix DS-11 Spectrophotometer. Algal cell concentration (cells/mL) was evaluated, indirectly, by measuring the absorbance at 680 nm and using a calibration curve (cell concentration (cells/mL) versus absorbance). A 10 mm light path cuvette was used (Machado & Soares, 2014).

Calibration curve

A calibration curve was constructed to represent the relation between cell concentration (cells/mL) of the algal samples and the optical density measured at 680 nm (Abou-Shanab et al., 2011). This mathematical relation evaluates the growth rates of algae through spectrophotometry measurements.

From the agitated sample of *P. subcapitata,* dilutions of 10 mL, in duplicate, were performed under the following dilution factors: 1, 0.5, 0.25, 0.125, 0.625 and 0.03125. Cell concentration (cells/mL) for each dilution was determined by cell counting using a Neubauer chamber. To perform the cell count, 8 µL of each dilution was deposited at each end of the Neubauer chamber and cell counting was performed using a compound microscope at 40 X magnification. Optical density was calculated using the following equation (Bastidas, 2012):

$$Concentration\left(\frac{cells}{mL}\right) = \frac{total\ cells\ counted \cdot 160000}{number\ of\ squares\ counted}$$

At the same time, optical density for each dilution was evaluated by spectrophotometry. A 1.5 mL volume of each dilution was placed in 1.5 mL cuvettes and, its absorbance was measured at 680 nm by spectrophotometry. This wavelength corresponds to the Chlorophyll A wavelength (Wang et al., 2020).

Sampling, dilutions production, cell count and absorbance measurement were performed on three different occasions. Obtained data was used to establish a calibration curve describing the best relation between optical density and cell concentration (cells/mL).

Calculation of specific growth rate

The specific growth rate for specific period was calculated as the logarithm increase in the biomass from the equation for each single vessel of controls and treatments (OECD, 2004):

$$\mu_{i-j} = \frac{ln\,X_j - ln\,X_i}{t_j - t_i}\ (day^{-1})$$

where:
μ_{i-j} is the average specific growth rate from time *i to j*;
X_i is the biomass at time *i;*
X_j is the biomass at time *j.*

Time ranges were one day obtaining the following periods: 0-1 day, 1-2 days, 2-3 days, 3-4 days.

Calculation of generation time

The time it takes for the algal population to double in number (doubling time or generation time) (g) was calculated using the following equation:

$$g = \frac{\ln 2}{\mu}$$

where:
g is the doubling time or generation time;
μ is the average specific growth rate.

Calculation of the percent inhibition in growth rate

The percent of inhibition in growth rate for each treatment replicate was calculated by the following equation:

$$\%I_r = \frac{\mu_c - \mu_t}{\mu_c} \; x \; 100$$

where:
$\%I_r$ is the percent inhibition in average specific growth;
μ_c is the mean value for average specific growth rate (μ) in the control group;
μ_t is the average specific growth rate for the treatment replicate.

Effective concentration (EC) values were obtained by linear interpolation of the percent inhibition in growth rate (%) and the logarithm of the test concentration (mg/L). Dose – effect curve parameters were expressed as a EC_{10}, EC_{20} and EC_{50}.

Data analysis

To perform a calibration curve, the correlation between cell concentration (cells/mL) and absorbance was tested using the Pearson's test and the degree of correlation (R^2).

Analysis of variance (one way ANOVA) with Tukey's post-hoc was applied to evaluate differences of cell concentration among control groups.

Analysis of variance (one way ANOVA) with Tukey's post-hoc was applied to evaluate differences of cell concentration among control and treated cultures. One way ANOVA was also applied to assess differences of cell concentration among different time periods (24, 48, 72, 96 h) for each group.

To check the ANOVA requirements (homoscedasticity and normality), Kolmogorov - Smirnov and Leven's test were performed to the dependent variable.

Results

Calibration curve

Data obtained show a linear and proportional relationship between absorbance and cell concentration (cells/mL). The degree of correlation between the two variables was high (R^2 = 0,998) and reproducible (CV = 0,11%). Accordingly, the next equation was used to transform absorbance data into cell concentration (cells/mL): $Y = 4{,}43 \cdot 10^7 X - 1{,}48 \cdot 10^5$.
In Figure 3, the result is observed graphically by a scatter plot. In Table 7, it is presented the absorbance and cell concentration (cells/mL) data for the construction of the interpolation line.

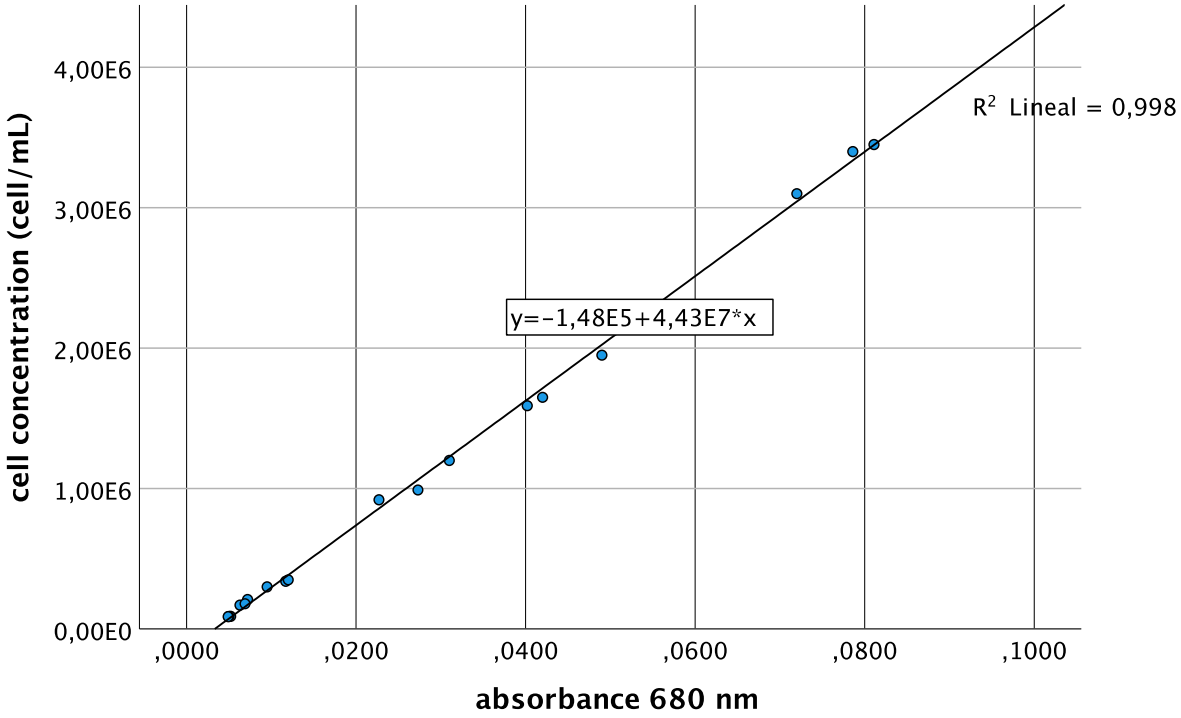


Figure 3. Correlation between cell concentration (cells/mL) and absorbance (measured at 680 nm). R^2 obtained was 0,998 and the equation of the straight line was $Y = 4{,}43 \cdot 10^7 X - 1{,}48 \cdot 10^5$.

Average growth rate, generation time and percent inhibition

The average specific growth rate for the specific period was calculated for each control and treatment group (data available in Table 10). In Table 3, the average specific growth rate (h^{-1}) over the entire test duration is shown. Moreover, in Table 4, data is transformed into generation time (hour).
Control cultures (no Ibuprofen added) grew exponentially with an average specific growth rate (μ) of 0,0263 – 0,0267 $hour^{-1}$, which corresponded to a generation time of ~ 26 hours.
Experimental cultures have a lower growth rate, this being proportional to the concentration of Ibuprofen added, except for the groups 45 - 135 mg/L, which have almost the same rate (μ = 0,0076 – 0,0077 h^{-1}). Cultures with 405 mg/L of Ibuprofen had the lowest rate (μ = 0,0025 h^{-1}), which corresponds to a doubling time of ~ 273 hours.

Table 3. Average specific growth rate (hour^{-1}) data for control and treatment groups.

Treatment	Average specific growth rate (hour^{-1})
Control	0,0267
Control + EtOH	0,0263
5 mg/L	0,0180
15 mg/L	0,0131
45 mg/L	0,0076
135 mg/L	0,0077
405 mg/L	0,0025

Table 4. Generation time (hours) data for control and treatment groups.

Treatment	Generation time (hours)
Control	25,95
Control + EtOH	26,35
5 mg/L	38,52
15 mg/L	53,11
45 mg/L	90,71
135 mg/L	89,56
405 mg/L	273,60

The percent inhibition in growth rate for each treatment was calculated using its respective growth rate and the solvent control data. In Table 5, percent inhibition of the average growth rate is collected for each treatment group.
The growth inhibition percentages were proportional to the drug concentration of each treatment. Thereby, cultures with 5 mg/L added had the lowest inhibition (32%) and 405 mg/L group, in contrast, had the highest (90%). Moreover, 135 and 45 mg/L groups reached the same percentage (71%). In Figure 4, percentage of inhibition is plotted against the logarithm of the concentration of the test substance.

Table 5. Percent inhibition of growth rate for each treatment group.

Ibuprofen concentration	Percent inhibition of growth rate (%)
5 mg/L	32 %
15 mg/L	50 %
45 mg/L	71 %
135 mg/L	71 %
405 mg/L	90 %

Figure 5 shows the linear interpolation method used to determinate the ECs parameters. Dose – effect curve parameters were expressed as a EC_{10}, EC_{20} and EC_{50} with 95 % confidence interval. R^2 obtained was 0.961, indicating a good linear correlation. In Table 6, EC values are expressed. Data was obtained through the next equation: $Y = 3{,}39 + 33{,}35\ X$.

Table 6. Dose – effect relationship parameters for the toxicity test.

Effect concentration	Ibuprofen concentration (mg/L)
EC_{10}	1,28
EC_{20}	2,56
EC_{50}	20,42

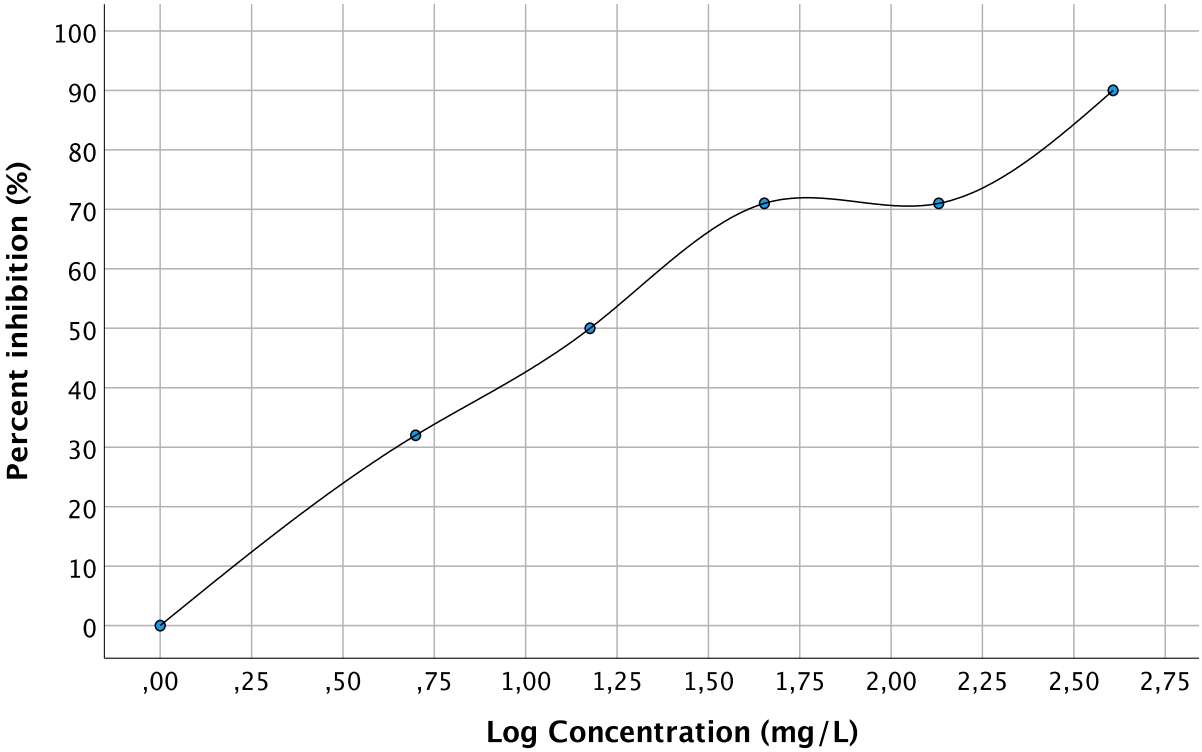


Figure 4. Dose – response curve for inhibition of the average growth rate (%) versus log concentration (mg/L).

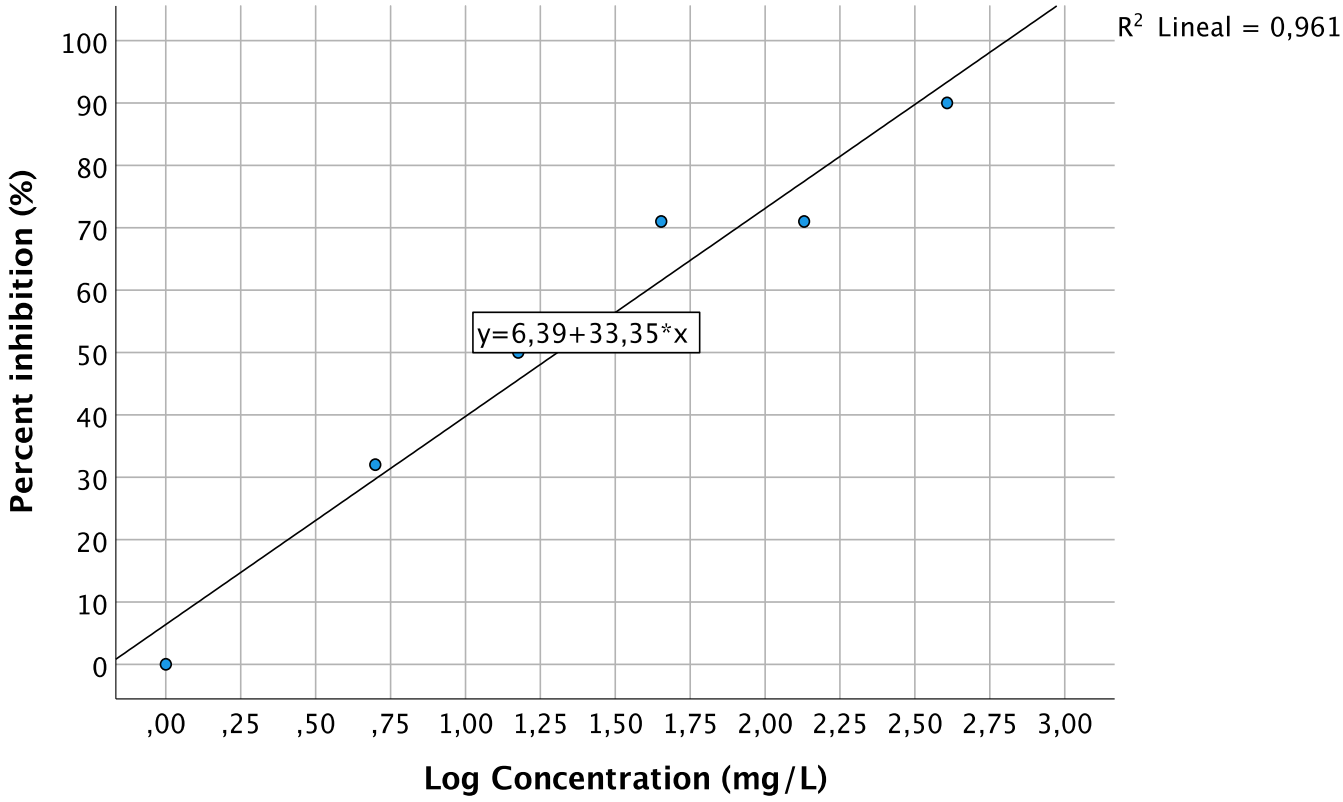


Figure 5. Linear interpolation for the inhibition of the growth rate (%) versus the logarithmic of Ibuprofen concentration (mg/L).

Algal density

Control groups (with or without EtOH) show similar density parameters ($p > 0,05$). Therefore, there are no differences of cell concentration (cells/mL) between both groups.

Significant growth inhibition occurred in the presence of Ibuprofen in all the treated cultures ($p < 0,05$). The lowest cell concentration data (cell/mL) was measured in cultures with 405 mg/L of Ibuprofen. Nonetheless, no significant differences were found comparing it with 45 and 135 mg/L groups ($p > 0,05$).
In addition, the groups of 5 and 15 mg/L, as well as those of 15, 45 and 135 mg/L did not show significant differences ($p > 0,05$).

In terms of time (hours), no significant differences were found in the periods from 0 to 24 hours ($p > 0,05$). On the other hand, after 48 hours, cell concentration remains partially stable ($p > 0,05$). Finally, between 24 and 48 hours, there is a large increase in cell concentration in all groups ($p < 0,05$).

In Table 6, growth curves are represented on a logarithmic scale. Control groups reach their maximum cell concentration at 72 hours. Both groups have a typically growth curve where an exponential growth is observed between 0 and 48 h. Finally, from 48 h it is reached a stationary phase (See Figure 6 and 7).
Treatment groups present a strong lag phase on the first day, specially the 45, 135 and 405 mg/L groups. Moreover, from 45 mg/L, the growth seems to be very low, i.e., it is almost the same as the initial concentration. 5 and 15 mg/L groups have higher growth, but it is still limited. Finally, all treatment groups also stabilize their concentrations from 48 hours.

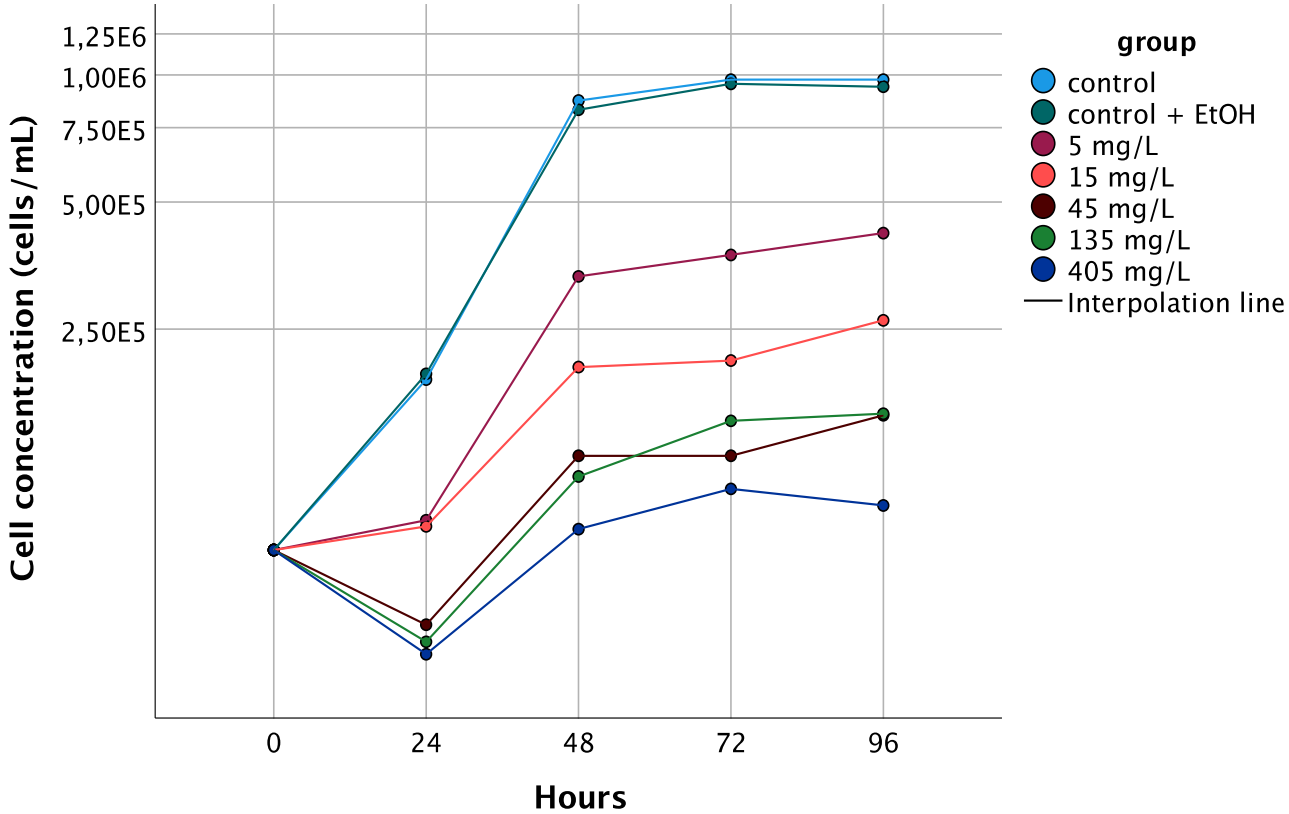


Figure 6. Growth of P. subcapitata on the basis of cell concentration (cells/mL) during the different treatments with Ibuprofen. Mean values are plotted (n = 3) in a semi-logarithmic scale.

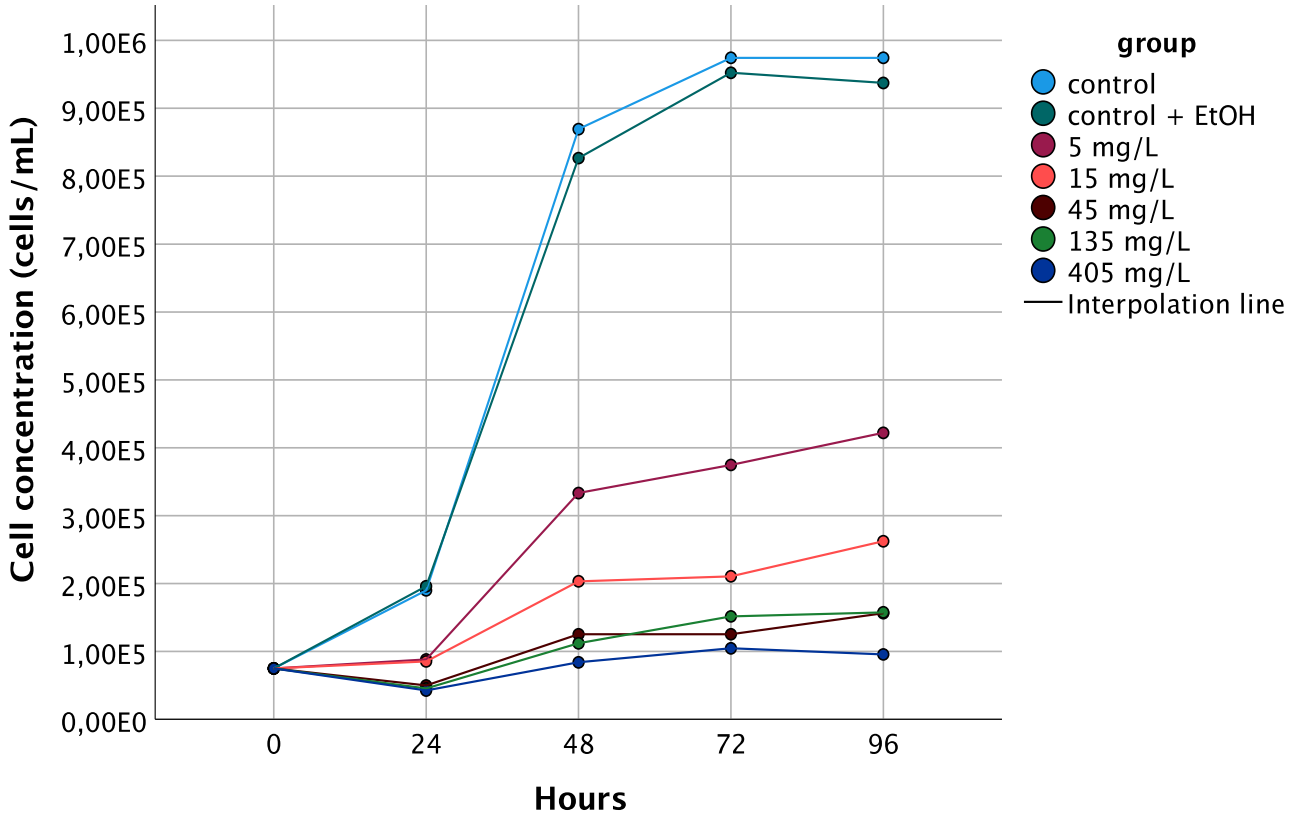


Figure 7. Growth of *P. subcapitata* on the basis of cell concentration (cells/mL) during the different treatments with Ibuprofen. Mean values are plotted (n = 3).

Discussion

Average growth rate, generation time and percent inhibition

Based on this study, experimental cultures had a growth inhibition. The cell concentration in the control cultures increased by a growth rate larger than 0,060 h^{-1} during 48 – 72 h, which is equivalent to a doubling (generation) time of ~ 12 h. According to the "alga growth inhibition test" guidelines, this rate is in agreement with the validation conditions (OECD, 2004). In previous studies, the average specific growth rate for control cultures of *P. subcapitata* was similar (Cleuvers, 2004; González-Naranjo & Boltes, 2014; Machado & Soares, 2014).

The growth rates of experimental cultures (Ibuprofen added) display an inhibition pattern comparing to the control group. Increasing the NSAID concentration reduced the growth rate, and consequently, increased the doubling time in a concentration-dependent manner, except for 45 and 135 mg/L groups, which have the same rate (~ 0,0076 h^{-1}). In addition, no differences were found between 45, 135 and 405 mg/L groups ($p > 0,05$) and consequently, it is assumed that from 45 mg/L and higher, Ibuprofen has a high toxicity.

Percent inhibition in growth rate is commonly used as a proxy measure to determinate the toxicity of contaminants on algae (Wang et al., 2020). In the present study, EC were calculated through a lineal interpolation between the percent inhibition and the logarithmic of the corresponding Ibuprofen concentration. EC_{50}, EC_{20} and EC_{10} obtained were 20.42, 2.56 and 1.28 mg/L, respectively. In previous studies performed on other algae, EC_{50} reported was around 230 - 240 mg/L (Cleuvers, 2004; González-Naranjo & Boltes, 2014). Comparing our data, it is observed a big

difference. Therefore, it is assumed that *P. subcapitata* is more sensitive than other microalgae. Despite that, it would be convenient to repeat the experiments.
According to our EC_{50} value, Ibuprofen could be classified as harmful to aquatic organisms according to the criteria of Regulation (EC) No. 1272/2008 (González-Naranjo & Boltes, 2014).

Effects of Ibuprofen on the algal density

Significant growth inhibition occurred in the presence of Ibuprofen. Significantly lower cell concentrations were measured in the 45, 135 and 405 mg/L groups ($p < 0,05$). In addition, from 45 mg/L, there is a strong inhibition in the growth rate.

Treatment groups present a lag phase on the first 24 hours. It means, there is a significant lower specific growth rate on day one than the total average specific growth rate. This phase may indicate an adaptation process to the new media or a recovery after initial toxic stress. (OECD, 2004). Compared to controls, the toxic effect of ibuprofen is assumed to induce cultures into a lag phase. Moreover, this response is proportional to the NSAID concentration, even observing cell death for 45, 135 and 405 mg/L groups. From 24 hours, cultures experienced a log phase where the cell population increase exponentially. Finally, from 48 - 72 hours, cultures seem to reach a stationary phase. In this stage, growth ceases but cells remain metabolically active (Duque, 2017).

New treatments in CECs removal

Nowadays, most of our drinking water, and wastewater treatment plants (WWTP) follow the old linear model in which products reach a certain lifetime and then, should be rejected in a safe place (somewhere that do not cause any harm). In this strategy, the creation of pollution points sources and their chronic effects on the environment have the least priority. On the other hand, circular strategies consider a completely recycled process achieving less waste production. This method has been implemented on the plastic and metal treatments getting great results. Therefore, new treatments in WWTPs should be evaluated (Taheran et al., 2018).

In Figure 8, it is shown a circular and a linear model graphically. Focusing on the first one, its product can come back to the drinking water network or also, can be used for irrigation if it meets the quality criteria for agriculture or industry. Furthermore, it is necessary to maintain a closed cycle for the sustainable management of urban water, thus, mitigate the effects of competition between humans and wildlife for fresh water and food (Taheran et al., 2018).
Contrary to the circular approach, the old lineal strategy assumes a complete degradation of the products, thus they are spilled into the surface water. The recycling of products is non-existent, generating greater rates of waste. Additionally, the impacts on ecosystems are even greater. Therefore, it is suggested to change the strategies into the circular model.

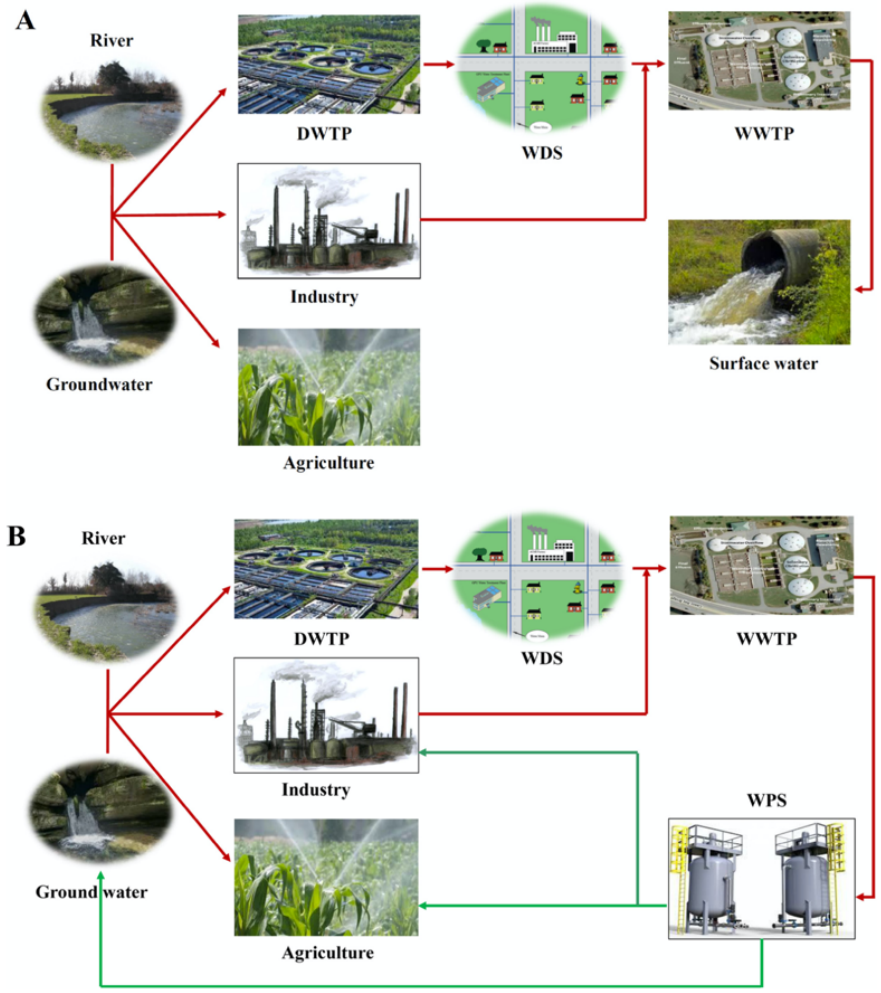


Figure 8. A) Linear and B) circular model for management of water resources (DWTP: Drinking water treatment plant, WDS: Water treatment plant, WDS: Water distribution system, WWTP: Wastewater treatment plant, WPS: Water polishing system (Taheran et al., 2018).

New technology should be integrated to address the growing list of CECs. Technologies for water/wastewater treatment can be classified into physical removal (sedimentation, precipitation, adsorption, filtration, ion exchange, etc.), chemical oxidation/disinfection (chlorination, ozonation, ultraviolet irradiation, etc.) and biological transformation (activated sludge, enzyme reactors, etc.). Each method will be optimal for a specific purpose (Taheran et al., 2018).

Chlorination is an economic method for disinfecting drinking water due to its residual protection power in water distribution systems. Reverse osmosis is an efficient method for desalination of seawater. However, wastewater is a very complex matrix that contains organic matter, metals, microorganisms, organic compounds, pharmaceutical products, etc. Therefore, the use of a single technology would not achieve an optimal disinfection (Taheran et al., 2018).

It should be noted that many CECs have not been detected yet due to the lack of knowledge. Moreover, not much data is known about the fate and damage that can cause the by-products formed after the treatment of their primary substances (Taheran et al., 2018).

The activated sludge method is the most widely used and economical conventional method to reduce carbonaceous organic matter, nitrogenous and phosphorus compounds. However, effluents from treatment plants do not meet the quality criteria for farms and industries because the levels of salts, metals, suspended solids, nutrients, and contaminants present could degrade the soil water and the soil infiltration properties required for the production.
Accordingly, it is necessary a method capable of hybridizing different technologies to achieve a higher quality effluent from WWTPs (Taheran et al., 2018).

An example of hybrid system is the membrane bioreactor. In this method, a microfiltration/ultrafiltration membrane is added to an activated sludge system. In this way, a reduction of suspended solids is achieved, forming large flocs which reduces the mass transfer resistance and moreover, the use of chemicals decreases significantly (García-Muñoz et al., 2019). Besides, these new systems have a high price. Although many tests of hybrid configurations have been developed to investigate their ability to eliminate CECs, techniques are still very expensive, making them difficult to apply. Therefore, more research is needed to find more economical and environmentally friendly methods (Taheran et al., 2018).

Recently, an alternative method developed is the use of the "advanced oxidation processes" that generate oxidizing radicals capable of transforming organic compounds into mineral substances, harmless to the human health and the environment. Two of these processes are photocatalysis and photo-Fenton. In the first method, it is used a solid (photocatalyst) capable of absorbing light and generating the chemical potential necessary to carry out the transformation. In the second, radicals are generated by the decomposition of hydrogen peroxide (H_2O_2) under the presence of iron and light. The great advantage of both methods is the possibility of using sunlight as a source of energy for their activation. Moreover, mesoporous catalysts composed of titanium and iron oxides (pores of 0.2 to 5 nm in diameter) have recently been developed to remove antibiotics. Titanium dioxide (TiO_2) has high photocatalytic activity and, iron oxide (Fe2O3) can carry out the photo-Fenton process without releasing iron into the medium (García-Muñoz et al., 2019).

Finally, successful removal of CECs has been reported using algae-based techniques. These technologies have advantages in improving the removal efficiency and sequestering greenhouse gases.
The action mechanism consists in different ways. Firstly, algae play a biosorbent role. Polysaccharides attached on the cell wall of algae provide sites for sorption of CECs. Secondly, algae participate in the biodegradation of CECs thanks to their metabolizing enzymes. There are three biodegradation phases. Phase – I includes oxidation and hydrolysis, which transforms lipophilic compounds into more hydrophilic compounds. In phase – II, hydrophilic moieties are added to facilitate the CEC's excretion. In phase – III takes place the compartmentation of compound into vacuoles or cell fractions. In addition, algae require illumination to grow. Many CECs can be photodegradable under light irradiation. Therefore, algae also enable to enhance photodegradation by producing free radicals during light illumination (Xin et al., 2021).

According to this report, research should be focused on the development of hybrid systems for degradation and removal of these contaminants from WWTP. However, these techniques are still very expensive making them difficult to apply. Thus, new studies are needed to make them practical and viable.

Knowledge gaps and research needs

Current data are still not conclusive for the comprehensive evaluation of the toxicity of pharmaceuticals to algae. New research work based on the present studies is necessary in the future. Below the knowledge gaps are analysed and research needs are suggested to achieve high realism results.

Gap 1. New research based on drug concentrations measured in the environment is required. Instead of lethal doses, exposure concentration should be like ranges found in ecosystems.
Moreover, experiments should be based on long-term exposures, i.e., test organisms should be exposed to chronic concentrations (Xin et al., 2021).

Gap 2. Research is needed to examine mixture toxicity of pharmaceuticals and other CECs. Ecosystems contain a wider range of pollutants that should be considered together (Xin et al., 2021).

Gap 3. Research is needed to explore environmental factors and their combined impact. New conditions may affect the toxicity of these compounds. For instance, limited studies work on the influence of temperature on pharmaceuticals toxicity. It is necessary more attention since a changing climate may make algae more adaptative or sensitive to increasing pharmaceuticals (Xin et al., 2021).

Gap 4. Research is needed to investigate a wider range of algae. Most of CEC's toxicity works use Chlorophyta and diatoms as a test organism, which are more sensitive species. However, a few studies work on other algal species. For example, dinoflagellates are constituents of phytoplankton. Thus, this group has important relevance on the food chain. New studies using other species are required (Xin et al., 2021).

Gap 5. Research is needed to investigate the toxicity of metabolites and by-products of pharmaceuticals. Algal metabolism produces metabolites and, by-products are produced by physicochemical processes under environmental conditions. Some works noticed the toxicity of transformation products to algae after biodegradation and photodegradation. Moreover, these products may have greater bioaccumulation and be more toxic than the original compound (Xin et al., 2021).

Gap 6. Research is needed to explore algae–based bioremediation techniques to remove pharmaceuticals in WWTP. Previous studies have identified some algal species with biosorbent and degradation abilities. However, cultivation conditions are hard to keep stable. Methods should be improved (Xin et al., 2021).

Ethical considerations

Antibiotic-resistant bacteria are found in multiple environments, even in wastewater ecosystems. The conditions in WWTPs are optimal for the proliferation of bacteria. By exposing them to drugs, resistance genes can be transferred to non-resistant bacteria (Kim & Aga, 2007). In addition, the society assume that there is no need to care about pharmaceutical's adverse effects since their concentrations are very low on the environment. However, the formation of resistant bacteria is achieved at very low concentrations. Moreover, these drug-resistant bacteria can cause infections in humans and animals, making these infections more difficult to treat (Taheran et al., 2018). For this reason, restricting the consumption of certain pharmaceuticals to crucial cases is a possible strategy in short-term. Simultaneously, it is necessary to set new standards for the quality of wastewater treatment plants as well as integrating the water consumers in a closed cycle.

Critical evaluation of this project

In this section, the strengths and weaknesses of the methods and results are going to be discussed. Moreover, a final overview is going to be given.

This study aimed to add information about how affect Ibuprofen in aquatic organisms, in this case, in algae. Firstly, the choice of Ibuprofen as a contaminant agent is relevant because it is one of the most used drugs in the world. I consider its use will continue to increase and therefore, monitoring of this compound is important. Moreover, the concentrations used cover a wide range of quantities. Besides, Ibuprofen is found at a low concentration (in a concentration range between 1 ng/L - 1 μg/L), so there is no representation of this concentration in the experiment. Thus, the experimental method loses realism.

The applied method aimed to be an easy way to perform a toxicity test. So, it is required a spectrophotometer, a compound microscope and a Neubauer chamber, mainly. Conversely, results are inaccurate, and conclusions may vary between different laboratories. Therefore, this method can give us an overview about how affect different Ibuprofen concentrations on the growth. Regarding to the obtained results, the prediction was correct, so increasing the Ibuprofen concentration decreases the growth rate on *P. subcapitata.*

Regarding to the method, it is necessary to mention that algae are exposed chronically to CECs in the environment, so in this experiment would be relevant to keep the time in mind. In the present study, the exposure time was four days. Thus, a short experiment with lethal doses was chosen. Summarizing, it is interesting to research about the amount of the lethal dose in different species but also, on low and chronic exposures.

Regarding the results, no differences were found between control groups. First, the addition of ethanol as a diluent did not affect the growth of *P. subcapitata*. This shows that the growth inhibition results are focused on the Ibuprofen added. So, the data is reliable. Moreover, three replicates were selected for the experiment. On the other hand, more replicas could have been selected to increase statistical validity and, to repeat the experiment in different weeks to assume the temporary pseudo-replication. Nonetheless, the data is representative and meets the scientific needs of the project.

In the growth curves, it was observer a lag phase in the treatment groups. It means, there was a significant lower specific growth rate on day one than the total average specific growth rate. This phase may indicate an adaptation process to the new media or a recovery after initial toxic stress. So, the ideal experiment must avoid this phase to achieve more realistic results. It would be interesting to repeat the experiments to minimize this effect.

Finally, regarding to the *P. subcapitata* sensitive, it was observed a high growth inhibition in this study. In previous studies carried out on other algae, inhibition was lower. It suggests that *P. subcapitata* is more sensitive than other algae or also, the methods are wrong. It would be interesting to repeat the experiments to clear up any doubts.

To sum up, this project adds relevant information about how Ibuprofen affects the growth of *P. subcapitata.* Nonetheless, the method could be more precise and the data more accurate. Moreover, further experiments on low chronic exposures should be considered in the future to achieve a high realism.

Conclusion

We have reported individual data for the effect of ibuprofen on a photosynthetic organism. Our results show that *P. subcapitata* was quite sensitive to Ibuprofen, at any concentration. Generalizing, NSAID pollution may lead to a reduction in the diversity and number of functional groups of eukaryotic algae. However, pharmaceuticals toxicity considerably depends on algal species.
In addition, new methods such as algae-based remediation are emerging technology to prevent the release of pharmaceuticals from WWTPs. More research should be devoted to the toxicity of CECs in a variety of test organisms and development of reliable methods for toxicity test at low and chronic concentrations.

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Appendix A: Data

In Table 7, it is represented absorbance versus cell concentration data used to build the calibration curve. The dilution factors were: 1, 0.5, 0.25, 0.125, 0.0625, 0.03125.

Table 7. Absorbance (measured at 680 nm) and cell concentration (cell/mL) data obtained from dilutions of *P. subcapitata* culture. Dilutions were performed under the next factors: 1, 0.5, 0.25, 0.125, 0.0625, 0.03125.

Dilution factor	Absorbance	Cell concentration (cell/mL)
1/1	0,0786	3,40E+06
	0,0720	3,10E+06
	0,0811	3,45E+06
1/2	0,0420	1,65E+06
	0,0402	1,59E+06
	0,0490	1,95E+06
1/4	0,0227	9,20E+05
	0,0273	9,90E+05
	0,0310	1,20E+06
1/8	0,0117	3,40E+05
	0,0120	3,50E+05
	0,0095	3,00E+05
1/16	0,0072	2,10E+05
	0,0063	1,70E+05
	0,0069	1,80E+05
1/32	0,0052	9,00E+04
	0,005	8,90E+04
	0,0049	8,80E+04

In Table 8, it is represented the absorbance (at 680 nm) data measured every day for the treatment and control groups. For each group and time, there are three replicates; therefore, three absorbance measurements.

Table 8. Daily absorbance data (measured at 680 nm) obtained from the experimental and control samples for one week. Each treatment and control group has three replicates per day.

		Absorbance (measured at 680 nm)						
Time (hours)	**Rep.**	**Control**	**Control + EtOH**	**5 mg/L**	**15 mg/L**	**45 mg/L**	**135 mg/L**	**405 mg/L**
0 h	**1**	0,0061	0,0055	0,0069	0,0061	0,0057	0,0054	0,0063
	2	0,0063	0,0059	0,0061	0,0053	0,0058	0,0064	0,0061
	3	0,0057	0,0069	0,0057	0,0065	0,0065	0,0059	0,0055
24 h	**1**	0,0073	0,0082	0,0063	0,0056	0,0046	0,0046	0,0042
	2	0,0079	0,0079	0,0050	0,0044	0,0043	0,0043	0,0044
	3	0,0077	0,0072	0,0047	0,0058	0,0045	0,0042	0,0043
48 h	**1**	0,0239	0,0220	0,0122	0,0080	0,0059	0,0061	0,0058
	2	0,0222	0,0210	0,0122	0,0080	0,0059	0,0061	0,0058
	3	0,0288	0,0230	0,0099	0,0084	0,0064	0,0057	0,0042

72 h	**1**	0,0257	0,0250	0,0117	0,0081	0,0062	0,0070	0,0057
	2	0,0245	0,0245	0,0119	0,0082	0,0059	0,0065	0,0056
	3	0,0258	0,0250	0,0118	0,0080	0,0064	0,0068	0,0058
96 h	**1**	0,0258	0,0250	0,0131	0,0091	0,0070	0,0070	0,0055
	2	0,0257	0,0245	0,0128	0,0087	0,0069	0,0069	0,0054
	3	0,0245	0,0240	0,0127	0,0100	0,0067	0,0068	0,0056

In table 9, data is transformed into cell concentration (cells/mL) using the lineal equation: $Y = 4{,}43 \cdot 10^7 X - 1{,}48 \cdot 10^5$. From these data, the entire statistical procedure is performed.

Table 9. Cell concentration (cell/mL) data obtained from the transformation of the absorbance data using the next lineal equation: $Y = 4{,}43 \cdot 10^7 X - 1{,}48 \cdot 10^5$. Each treatment and control group has three replicates per day.

		Cell concentration (cells/mL)						
Time (hours)	**Rep.**	**Control**	**Control + EtOH**	**5 mg/L**	**15 mg/L**	**45 mg/L**	**135 mg/L**	**405 mg/L**
0	**1**	$1{,}22 \cdot 10^5$	$9{,}57 \cdot 10^4$	$1{,}58 \cdot 10^5$	$1{,}22 \cdot 10^5$	$1{,}05 \cdot 10^5$	$9{,}12 \cdot 10^5$	$1{,}31 \cdot 10^5$
	2	$1{,}31 \cdot 10^5$	$1{,}13 \cdot 10^5$	$1{,}22 \cdot 10^5$	$8{,}68 \cdot 10^4$	$1{,}09 \cdot 10^5$	$1{,}36 \cdot 10^5$	$1{,}22 \cdot 10^5$
	3	$1{,}05 \cdot 10^5$	$1{,}58 \cdot 10^5$	$1{,}05 \cdot 10^5$	$1{,}40 \cdot 10^5$	$1{,}40 \cdot 10^5$	$1{,}13 \cdot 10^5$	$9{,}57 \cdot 10^4$
24 h	**1**	$1{,}75 \cdot 10^5$	$2{,}15 \cdot 10^5$	$1{,}31 \cdot 10^5$	$1{,}00 \cdot 10^5$	$5{,}58 \cdot 10^4$	$5{,}58 \cdot 10^4$	$3{,}81 \cdot 10^4$
	2	$2{,}02 \cdot 10^5$	$2{,}02 \cdot 10^5$	$7{,}35 \cdot 10^5$	$4{,}69 \cdot 10^4$	$4{,}25 \cdot 10^4$	$4{,}25 \cdot 10^4$	$4{,}69 \cdot 10^4$
	3	$1{,}93 \cdot 10^5$	$1{,}71 \cdot 10^5$	$6{,}02 \cdot 10^5$	$1{,}09 \cdot 10^5$	$5{,}14 \cdot 10^4$	$3{,}81 \cdot 10^4$	$4{,}25 \cdot 10^4$
48 h	**1**	$9{,}11 \cdot 10^5$	$8{,}27 \cdot 10^5$	$3{,}17 \cdot 10^5$	$1{,}80 \cdot 10^5$	$1{,}27 \cdot 10^5$	$1{,}09 \cdot 10^5$	$1{,}05 \cdot 10^5$
	2	$8{,}35 \cdot 10^5$	$7{,}82 \cdot 10^5$	$3{,}92 \cdot 10^5$	$2{,}06 \cdot 10^5$	$1{,}13 \cdot 10^5$	$1{,}22 \cdot 10^5$	$1{,}09 \cdot 10^5$
	3	$8{,}62 \cdot 10^5$	$8{,}71 \cdot 10^5$	$2{,}91 \cdot 10^5$	$2{,}24 \cdot 10^5$	$1{,}36 \cdot 10^5$	$1{,}05 \cdot 10^5$	$3{,}81 \cdot 10^4$
72 h	**1**	$9{,}91 \cdot 10^5$	$9{,}60 \cdot 10^5$	$3{,}70 \cdot 10^5$	$2{,}11 \cdot 10^5$	$1{,}27 \cdot 10^5$	$1{,}62 \cdot 10^5$	$1{,}05 \cdot 10^5$
	2	$9{,}37 \cdot 10^5$	$9{,}37 \cdot 10^5$	$3{,}79 \cdot 10^5$	$2{,}15 \cdot 10^5$	$1{,}13 \cdot 10^5$	$1{,}40 \cdot 10^5$	$1{,}00 \cdot 10^5$
	3	$9{,}95 \cdot 10^5$	$9{,}60 \cdot 10^5$	$3{,}75 \cdot 10^5$	$2{,}06 \cdot 10^5$	$1{,}36 \cdot 10^5$	$1{,}53 \cdot 10^5$	$1{,}09 \cdot 10^5$
96 h	**1**	$9{,}95 \cdot 10^5$	$9{,}60 \cdot 10^5$	$4{,}32 \cdot 10^5$	$2{,}55 \cdot 10^5$	$1{,}62 \cdot 10^5$	$1{,}62 \cdot 10^5$	$9{,}57 \cdot 10^4$
	2	$9{,}91 \cdot 10^5$	$9{,}37 \cdot 10^5$	$4{,}19 \cdot 10^5$	$2{,}37 \cdot 10^5$	$1{,}58 \cdot 10^5$	$1{,}58 \cdot 10^5$	$9{,}12 \cdot 10^4$
	3	$9{,}37 \cdot 10^5$	$9{,}15 \cdot 10^5$	$4{,}15 \cdot 10^5$	$2{,}95 \cdot 10^5$	$1{,}49 \cdot 10^5$	$1{,}53 \cdot 10^5$	$1{,}00 \cdot 10^5$

In Table 10, section-by-section growth rate ($hour^{-1}$) is shown.

Table 10. Section-by-section growth rate ($hour^{-1}$) data for control and treatment groups.

	Section-by-section growth rate ($hour^{-1}$)			
Treatment	**0 – 1 day**	**1 – 2 days**	**2 – 3 days**	**3 – 4 days**
Control	0,039	0,063	0,005	0,000
Control + EtOH	0,040	0,060	0,006	0,000
5 mg/L	-0,007	0,055	0,005	0,005
15 mg/L	0,005	0,036	0,001	0,009
45 mg/L	-0,017	0,038	0,000	0,009
135 mg/L	-0,021	0,038	0,013	0,002
405 mg/L	-0,024	0,028	0,009	-0,004

Appendix B: Statistics

Calibration Curve

The Coefficient of Determination for the Calibration Curve's variables was performed to get a lineal equation relating both variables. Table 11 presents the results where R^2 adjusted was 0,998 and the standard error was 0,0011. Therefore, there is a high correlation degree, and it is strongly reproducible.

Table 11. Results from Coefficient of Determination of the Calibration Curve's variables. R^2 adjusted was 0,998 and the Standard error of the estimate 0,0011.

R	R squared	R squared adjusted	Standard error of the estimate	Change in R squared	Change in F	df 1	df 2	Sig. change in F
0,999	0,998	0,998	0,0011	0,998	10593,269	1	16	<0,001

Table 12. Results from Pearson Correlation of the Calibration Curve's variables. It was significant at level 0,01 (bilateral).

Person Correlation Coefficient	Sig. (bilateral)	Sum of squares and cross products	Covariance	N
0,999	<0,001	528847,633	31108,648	18

Statistics for control groups

To assume the ANOVA requirements, the dependent variable cell concentration (cells/mL) was transformed into logarithmic data.
For each control group (with or without EtOH) was performed the Kolmogorov – Smirnov and Leven's test to check the requirements. Assumptions for one-way ANOVA were achieved, i.e., population has a normal distribution (Normality), and population variances are equal (Homoscedastic). See results in Table 13 and 14.
The ANOVA test performed show no differences between control groups (sig = 0,517 > 0,05). See Table 15.

Table 13. Results from Kolmogorov - Smirnov test of the variable dependent (cell concentration) in control groups. Population has a normal distribution (p = 0,273 > 0,05).

Kolmogorov - Smirnov		
Statistic	df	Sig.
0,950	24	0,273

Table 14. Results from Leven's test of the variable dependent (cell concentration) in control groups. Population variances are equal (p = 0,308 > 0,05).

Levene's test			
Statistic	df 1	df 2	Sig.
1,088	1	22	0,308

Table 15. Results from one-way ANOVA were not significant for the means of the variable between control groups (p = 0,517 > 0,05), that is, there are no differences between control and control + EtOH groups.

Analysis of Variance (ANOVA)		
F	df	Sig.
0,433	1	0,517

Statistics for control and test groups

ANOVA test was also used to see differences in algal growth between control and experimental groups. Moreover, it was also performed to observe differences of cell concentration between the different day periods. Control + EtOH data was used as a control in one-way ANOVA tests.

First, to assume the ANOVA requirements, the dependent variable cell concentration (cells/mL) was transformed into logarithmic data.
For each control and treatment group was performed the Kolmogorov – Smirnov and Leven's test to check the ANOVA requirements. Assumptions for one-way ANOVA were achieved, i.e., population has a normal distribution (Normality), and population variances are equal (Homoscedastic).

ANOVA for control and test groups

Table 16. Results from Kolmogorov – Smirnov test of the variable dependent (cell concentration). Population has a normal distribution (p = 0,073 > 0,05).

Kolmogorov – Smirnov		
Statistic	df	Sig.
0,950	18	0,073

Table 17. Results from Leven's test of the variable dependent (cell concentration). Population variances are equal (p = 0,412 > 0,05).

Levene's test			
Statistic	df 1	df 2	Sig.
1,023	5	66	0,412

Table 18. Results from one-way ANOVA were significant for the means of the variable between different groups (p < 0,001).

Analysis of Variance (ANOVA)		
F	df	Sig.
6,699	5	< 0,001

Table 19. Results from Tukey's test between different groups (No diff. = No significant difference).

Tukey's test	
Groups	Sig.
Control – 5 mg/L	0,005
Control – 15 mg/L	< 0,001
Control – 45 mg/L	< 0,001
Control – 135 mg/L	< 0,001
Control – 405 mg/L	< 0,001

5 – 15 mg/L	0,539 (No diff.)
5 – 45 mg/L	0,004
5 – 135 mg/L	0,004
5 – 405 mg/L	< 0,001
15 – 45 mg/L	0,283 (No diff.)
15 – 135 mg/L	0,278 (No diff.)
15 – 405 mg/L	0,009
45 – 135 mg/L	0,995 (No diff.)
45 – 405 mg/L	0,716 (No diff.)
135 – 405 mg/L	0,723 (No diff.)

ANOVA for time periods

Table 20. Results from Kolmogorov – Smirnov test of the variable dependent (cell concentration). Population has a normal distribution (p = 0,073 > 0,05).

Statistic	df	Sig.
0,100	72	0,073

Table 21. Results from Leven's test of the variable dependent (cell concentration). Population variances are equal (p = 0,615 > 0,05).

Levene's test			
Statistic	df 1	df 2	Sig.
0,604	3	68	0,615

Table 22. Results from one-way ANOVA were significant for the means of the variable between different time periods (p < 0,001).

Analysis of Variance (ANOVA)		
F	df	Sig.
10,974	3	< 0,001

Table 23. Results from Tukey's test between different time periods (No diff. = No significant difference).

Tukey's test	
Groups	Sig.
0 – 24 hours	0,089 (No diff.)
0 – 48 hours	< 0,001
0 – 72 hours	< 0,001
0 – 96 hours	< 0,001
24 – 48 hours	< 0,001
24 – 72 hours	< 0,001
24 – 96 hours	< 0,001
48 – 72 hours	0,926 (No diff.)
48 – 96 hours	0,781 (No diff.)
72 – 96 hours	0,988 (No diff.)

Appendix C: Materials and Equipment

The materials and equipment used for the laboratory work are presented below.

Materials for laboratory work

- Biological material: *P. subcapitata* (strain 278/4) from the Culture Collection of Algae and Protozoa (CCAP), UK.
- Distillated water
- Plastic cuvettes 1,5 mL
- Beakers 5O – 100 mL
- Pippete tips
- Test tubes in rack
- Volumetric Flask 250 mL
- Erlenmeyer flasks 250 mL
- Experimental flasks 50 mL
- Flaks 1000 mL
- Ethanol absolute
- Ibuprofen I4833 Sigma-Aldrich
- OECD medium: NH_4Cl, $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, KH_2PO_4, $FeCl_3{\cdot}6H_2O$ $Na_2EDTA{\cdot}2H_2O$, H_3BO_3, $MnCl_2{\cdot}4H_2O$, ZnCl2 $CoCl_2{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$, $Na_2MoO_4{\cdot}2H_2O$, NaHCO3, $Na_2SiO_3{\cdot}9H_20$

Table 24. Composition of the OECD medium.

Nutrient	**Concentration in stock solution**
Stock solution 1: macro nutrients	
NH_4Cl	1.5 g/L
$MgCl_2{\cdot}6H_2O$	1.2 g/L
$CaCl_2{\cdot}2H_2O$	1.8 g/L
$MgSO_4{\cdot}7H_2O$	1.5 g/L
KH_2PO_4	0.16 g/L
Stock solution 2: iron	
$FeCl_3{\cdot}6H_2O$	64 mg/L
$Na_2EDTA{\cdot}2H_2O$	100 mg/L
Stock solution 3: trace elements	
H_3BO_3	185 mg/L
$MnCl_2{\cdot}4H_2O$	415 mg/L
$ZnCl_2$	3 mg/L
$CoCl_2{\cdot}6H_2O$	1.5 mg/L
$CuCl_2{\cdot}2H_2O$	0.01 mg/L
$Na_2MoO_4{\cdot}2H_2O$	7 mg/L
Stock solution 4: bicarbonate	
$NaHCO_3$	50 g/L
$Na_2SiO_3{\cdot}9H_20$	

Equipment for laboratory work

- Metrohm Ion Analysis
- Systec HX-150 Autoclave
- DeNovix DS-11 Spectrophotometer
- Neubauer chamber
- Micropipettes 10, 100, 1000 μL
- Pipettes 1, 10 mL
- Incubator room
- Biosan Orbital shaker
- “Cool white” fluorescent light
- Mastech Digital Lux Meter
- Compound microscope
- Vortex

Computer programs

- IBM SPSS Statistics 28
- Microsoft Excel
- Microsoft Word
- Microsoft Power Point
- Mendeley

Scientific Database

- Science direct
- Scopus
- SciELO

Appendix D: List of Abbreviations

NSAID: Non-Steroidal Anti-Inflammatory drug
CEC: Contaminant of Emerging Concern
PCP: Personal Care Product
FR: Flame Retardants
ASW: Artificial Sweeteners
ARB: Antibiotic Resistant Bacteria
ARG: Antibiotic Resistant Genes
PP: Priority Pollutants
COX: Cyclooxygenase enzyme
WWTP: Wastewater Treatment Plant
OECD: Organization for Economic Co-operation and Development
EC: Effective concentration
EC_{50}: Half maximal effective concentration