INSTITUTO SUPERIOR DE ENGENHARIA DO PORTO

MESTRADO EM ENGENHARIA QUÍMICA

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# Avaliação de ecotoxicidade usando microalgas como complemento de estudos para a remoção de poluentes emergentes usando nanomateriais

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RESUMO

O aumento do consumo de fármacos é uma realidade na sociedade atual a nível mundial que contribui para a contaminação dos recursos hídricos, pois a maioria dos compostos farmacêuticos não são completamente removidos nas estações de tratamento de águas residuais (ETAR) e a sua contínua introdução no meio ambiente constitui um potencial risco tanto para organismos aquáticos bem como para a saúde humana.

O desenvolvimento e aplicação de nanomateriais (NMs) em processos de tratamento de águas avançados como a fotocatálise, poderá ser uma alternativa para responder aos desafios que se colocam atualmente no sentido de aumentar a eficiência da remoção de compostos farmacêuticos durante o tratamento de águas. No entanto, numa primeira fase, é necessário averiguar os principais riscos de toxicidade desses NMs e os seus potenciais efeitos na saúde humana.

Neste trabalho foram realizados ensaios de toxicidade de acordo com o teste de inibição de crescimento de algas (OECD 201 Guideline). A microalga *Raphidocelis subcapitata* foi o organismo teste para a avaliação da toxicidade do antidepressivo fluoxetina, e dos NMs GF_N8 e $C_3N_4$, que foram concebidos para a remoção de compostos farmacêuticos da água através de processos de fotocatálise.

O valor 72h-$EC_{50}$ estimado neste estudo para a fluoxetina foi de 9,0 µg $L^{-1}$. Nos períodos iniciais do teste de toxicidade, verificou-se a ocorrência de um aumento do crescimento como resposta ao stress induzido pela fluoxetina. Com o intuito de avaliar o impacto da fluoxetina em baixas concentrações, como as que ocorrem no ambiente aquático, no crescimento da microalga, foi realizado um ensaio ecotoxicológico com 35 ng $L^{-1}$. Neste caso, constatou-se também uma estimulação do crescimento após 24 horas e uma redução do crescimento, comparativamente à do controlo, de 10% e 2% respetivamente após 72 e 96 horas. Estes resultados demostram a toxicidade, mesmo em baixas concentrações, dos fármacos para os organismos aquáticos.

Relativamente aos nanomateriais, a avaliação ecotoxicológica revelou que o $C_3N_4$ tanto na forma exfoliada como em *bulk* apresenta baixo risco para o ambiente. Por outro lado, para GF_N8 foi estimado um valor de 72h-$EC_{50}$ de 45,3 mg $L^{-1}$ o que mostra o seu efeito prejudicial no crescimento da microalga.

Foi também averiguado se para além das propriedades fotocatalíticas, estes NMs possuiriam propriedades como adsorventes, que contribuiriam para um aumento da eficiência destes NMs na remoção de fármacos. GF_N8 mostrou ser um bom adsorvente. Os dados de cinética foram ajustados aos modelos de pseudo-1ª ordem, pseudo-2ª ordem, difusão

intraparticular e Elovich. O modelo Elovich demostrou a melhor correlação com os dados experimentais, no entanto, os parâmetros estimados eram desprovidos de significado estatístico ou estavam em desacordo com a caracterização do GF_N8. Em relação ao equilíbrio, os modelos de Freundlich e Sips foram os que mostraram melhor ajuste e a capacidade de adsorção observada mais elevada foi de 23,5 mg $g^{-1}$ (concentração equilíbrio 2,8 mg $L^{-1}$).

A exfoliação do $C_3N_4$ melhorou as suas propriedades de adsorção, aumentando a percentagem de remoção de fluoxetina de 4% para 9% e a correspondente capacidade de adsorção de 0,20 mg $g^{-1}$ (concentração equilíbrio 3,7 mg $L^{-1}$) para 0,96 mg $g^{-1}$(concentração equilíbrio 4,4 mg $L^{-1}$). Os resultados mostram que a cinética de adsorção da fluoxetina no $C_3N_4$ exfoliado pode ser descrita pelo modelo de Elovich. No entanto, as baixas percentagens de remoção evidenciam que $C_3N_4$ não possui propriedades como adsorvente.

**Palavras-chave**: adsorção, ecotoxicidade, fluoxetina, microalga, nanomateriais.

# ABSTRACT

The increasing consumption of pharmaceuticals is a trend in society worldwide that contributes to the contamination of water resources, seeing that almost all pharmaceutical compounds are not completely removed by wastewater treatment plants (WWTPs) and its continuous input into environment may constitute a potential risk for aquatic organisms as well as for human health.

The development and application of nanomaterials (NMs) in advanced water treatment processes such as, photocatalysis, may be an alternative approach to the current challenges related to improvement of the yield of removal of pharmaceutical compounds during the water treatment. However, firstly, it is necessary to assess the main toxicity risks of NMs and its potential effects on human health.

In this study, the toxicity experiments have been carried out based on the algal growth inhibition test OECD 201 Guideline. The freshwater microalgae *Raphidocelis subcapitata* was the test organism for toxicity assessment of the antidepressant fluoxetine and the NMs GF_N8 and $C_3N_4$, which were designed for the removal of pharmaceuticals from water by photocatalytic process.

The estimated 72h-$EC_{50}$ value of fluoxetine in this study was 9.0 µg $L^{-1}$. During the initial times of the ecotoxicological assessment, it was verified a stimulatory effect in growth as response to the stress induced by fluoxetine exposure. In order to determine if low concentrations, like in aquatic environment, of fluoxetine have impact in microalgae growth, it was also conducted an ecotoxicological assessment with 35 ng $L^{-1}$. In this case, it was also observed growth stimulation after 24 hours of exposure and 10% and 2% reduction in growth relative to the control respectively after 72 and 96 hours. These results evidence the toxicity, even at low concentrations, of pharmaceuticals to aquatic organisms.

The ecotoxicological assessment of nanomaterials revealed that $C_3N_4$ in exfoliated and bulk forms present low risks to the aquatic systems even at high concentrations. On the other hand, for GF_N8 it was estimated a 72h-$EC_{50}$ value of 45.3 mg $L^{-1}$, showing its negative effect on microalgae growth.

Apart from their photocatalytic proprieties, it was also investigated the potential as adsorbents of these NMs, which would contribute to an increase in the removal efficiency of pharmaceuticals. GF_N8 showed to be a good adsorbent. The kinetic data was fitted to the pseudo-first order, pseudo-second order, Elovich and intraparticle diffusion models. Elovich kinetic model provided the best correlation to the experimental data however, the estimated parameters did not have a statistical meaning or were in discordance with characterization

analyses. Regarding equilibrium, Freundlich and Sips were the best fit for the fluoxetine adsorption onto GF_N8 and the highest adsorption capacity observed was 23.5 mg $g^{-1}$ (2.8 mg $L^{-1}$ equilibrium concentration).

The exfoliation of $C_3N_4$ enhanced its adsorption proprieties increasing percentage removal of fluoxetine from 4% to 9% and the corresponding adsorption capacity from 0.20 mg $g^{-1}$ (3.7 mg $L^{-1}$ equilibrium concentration) to 0.96 mg $g^{-1}$ (4.4 mg $L^{-1}$ equilibrium concentration). The results show that the kinetic of adsorption of fluoxetine onto exfoliated $C_3N_4$ may be described by Elovich model. Due to the low removal efficiencies observed, $C_3N_4$ was found to have irrelevant adsorption ability.

**Keywords:** adsorption, ecotoxicity, fluoxetine, microalgae, nanomaterials.

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LIST OF ABBREVIATIONS

A.I.C: Akaike information criterion

AF: Assessment factor

$C_3N_4$: Carbon nitride

EC: Effective concentration

Eg: Gap energy

ERA: Environmental risk assessment

EQS: Environmental quality standard

FTIR: Fourier transform infrared spectroscopy

GF_N8: Graphene doped with nitrogen

GHS: Globally Harmonized System

HPLC: High performance liquid chromatography

INFARMED: Portuguese National authority of medicines and health

Koc: organic carbon soil adsorption coefficient

Kow: Octanol-water partition coefficient

LC: Lethal concentration

LOD: Limit of detection

LOEC: Lowest observed effect concentration

LOQ: Limit of quantification

MEC: Measured environmental concentration

NM: Nanomaterial

MW: Molecular weight

NOEC: No observed effect concentration

PEC: Predicted environmental concentration

PHS: Priority hazardous substance

pKa: acid dissociation constant

PNEC: Predicted no effect concentration

PS: Priority substance/group of substances

REACH: Registration, Evaluation, Authorization, and Restriction of Chemicals

ROS: Reactive oxygen species

rpm: Rotation per minute

RQ: Risk Quotient

SEM/EDS: Scanning Electron Microscopy with Energy Dispersive Spectroscopy

SSRI: Selective serotonin reuptake inhibitor

VB: Valance band

XPS: X-ray photon spectroscopy

XRD: X-ray diffraction

WFD: Water Framework Directive

WWTP: wastewater treatment plant

# 1. Introduction

## 1.1. Frame and objectives

Pharmaceuticals were not considered as pollutants, however, nowadays they constitute a group of great concern among emerging contaminants, due to their increased consumption worldwide and, subsequently, continuous input into environment. These compounds are only partially removed during conventional wastewater treatment and their occurrence is reported worldwide. As biological compounds are designed to have a specific mode of action and their presence may constitute a potential risk for non-target organisms as well as for human health. Recognising that, pharmaceutical compounds have been included on the watch list under the Water Framework Directive (WFD).

Therefore, the development of effective and sustainable treatments for removal of these emerging contaminants is a priority. Among several treatment options, photocatalysis using nanomaterials (NMs) has been pointing to have great potential as it may be capable of total mineralization of diverse organic compounds even at trace concentrations. Apart of photocatalytic capacity, NMs have also high adsorbing proprieties due to its large surface area. This characteristic can also be exploited for removal of pharmaceutical compounds from water through adsorption processes.

This study is divided in two distinct parts. First, it was evaluated the ecotoxicity of an antidepressant, fluoxetine, as well as some NMs designed for photocatalytic processes towards the freshwater microalgae *Raphidecelis subcapitata*. Secondly, it was investigated the adsorption process of fluoxetine onto these NMs. The study of kinetics provides an insight into the possible mechanisms of adsorption while the equilibrium study allows to test the capacity of these NMs for fluoxetine adsorption.

## 1.2. Outline of the dissertation

The present dissertation is structed into 8 chapters. Chapter 1 gives a general introduction followed by the objectives and the dissertation outline. Chapter 2 is structed to highlight the consumption, detection and fate of pharmaceuticals in aquatic systems. Chapter 3 briefly brings legislation regarding monitorization of wastewater discharge as well as evaluation of adverse effects on the environment caused by chemical substances. Chapter 4 introduces the context of ecotoxicology and environmental risk assessment. It also gives an overview of the physical-chemical proprieties of fluoxetine to understand its environmental behaviour and its interaction with biota. At last, it is focused the published literature regarding ecotoxicological studies of

fluoxetine using microalgae as test organism. Chapter 5 presents briefly the water treatments for pharmaceutical compounds removal applied nowadays and it gives a general introduction to nanotechnology with the view of water treatment. It focuses the adsorption and the photocatalytic potential of NMs. Beyond that, it shows literature knowledge about potential risks of nanomaterials to the environment. Chapter 6 gives a short introduction to adsorption and it presents adsorption kinetics models and isotherms. Chapter 7 describes the experimental procedures followed for the ecotoxicology assessment of fluoxetine and NMs and for the adsorption experiments. Chapter 8 presents and discusses the results obtained from the experiments. Chapter 9 is the concluding chapter, it provides a summary of the whole work with future perspectives.

# 2. Contamination of aquatic ecosystem

Water is crucial for life on Earth and essential to support livelihoods of human society [1]. Although, it is known that about one-fifth of the world's population still live without the access to a safe water source and two-fifth lack basic sanitation which causes critical health problems. More than 2 million people die due to polluted water and unsanitary living conditions. Because of human development and improvement of living standards, it is noticeable an increase of chemical water pollution. In fact, more than one-third of the Earth's accessible renewable freshwater is used for agriculture, industrial and domestic proposes and these activities generate wastewater containing synthetic and natural chemical compounds, which flows to rivers, lakes, groundwater, or to coastal seas [2].

The uncontrolled discharge of such compounds into the environment, even at trace concentration (in a range of microgram to picogram per litre) lead to the accumulation of some of them in aquatic compartments, with potentially detrimental effects to aquatic ecosystem and thus, directly or indirectly, human health. Moreover, the use of insecticides and herbicides in agriculture also contribute greatly to the presence of their residues in groundwater resources. Even when industrial and urban effluents reach the wastewater treatment plants (WWTPs) most of the micropollutants, such as pharmaceuticals, hormones, biocides, surfactants and other industrial chemicals, are not completely removed. As a result, the WWTPs are also a way to introduce these compounds into the aquatic ecosystem [3].

Micropollutants can make thousands of miles from the source by air and water currents without being degraded or may form toxic (bio) transformation products. Therefore, micropollutants may enter on human organism via drinking water, food chain or wastewater reuse for household purpose [4]. Due to the lack of knowledge on the middle and/or long-term effects of micropollutants to ecosystems and human health it is required to reinforce precautionary actions, like controlling the main sources of pollution, as well as developing new wastewater treatment options. As a matter of fact, preventing the contamination of surface and groundwater resources improve ecosystem health as well as the production of safe food for human consumption [2,5].

## 2.1. Consumption of pharmaceutical drugs

The extent of life expectancy and improvement of population's quality of life are a result of investment in health care system, advances in research and the development of new drugs. At the moment, there are around 12 000 human pharmaceuticals and 850 different active compounds, reaching an global average consumption of pharmaceuticals of 15 g/capita/annum

[4,6]. The 10 most commercialized pharmaceuticals in Portugal over the year of 2014 with accordance with INFARMED (Portuguese National Authority of Medicines and Health) are listed in Table ***2.1***.

According to the OECD report of 2011, the consumption of antidepressants, namely, selective serotonin reuptake inhibitors (SSRIs) such as sertraline, fluoxetine and venlafaxine, increased 60% all over the world the last decade [7]. At the year of 2014, sertraline was the SSRI with more packages sold in Portugal followed by fluoxetine and venlafaxine respectively [8]. SSRIs are widely prescribed to treat depression, compulsive behaviour as well eating disorders. These substances bind and block serotonin reuptake which lead to increased levels of this neurotransmission in the nerve synapses [7,9].

**Table 2.1 –** Pharmaceutical compounds ordered from the highest to the lowest sales amount in Portugal over the year of 2014. Adapted from [8].

| Ranking | Pharmacotherapeutic group and subgroup | Active substance | % of the Active Substance | Packages |
|---|---|---|---|---|
| **1** | Antilipemics | Sinvastatin | 97,5% | 3 660 155 |
| **2** | Antacids and Anti-Ulcerous | Omeprazole | 95,9% | 2 422 330 |
| **3** | Insulins, Oral Antidiabetics and Glucagon | Metformin | 78,2% | 2 421 800 |
| **4** | Antilipemics | Atorvastatin | 93,0% | 2 116 181 |
| **5** | Psychodrugs | Alprazolam | 78,9% | 1 894 824 |
| **6** | Antacids and Anti-Ulcerous | Pantoprazole | 94,3% | 1 721 161 |
| **7** | Anticoagulants and Antithrombotics | Clopidogrel | 85,3% | 1 444 322 |
| **8** | Anti-Hypertensives | Losartan + Hydrochlorothiazide | 88,3% | 1 356 236 |
| **9** | Non-Steroidal Anti-Inflammatory Agents | Ibuprofen | 56,4% | 1 209 674 |
| **10** | Antibacterial Drugs | Amoxicillin + Clavulanic acid | 47,4% | 1 197 147 |

## 2.2. Impact of pharmaceuticals on water quality

In recent years, advances in instrumentation, especially in the mass spectrometric analytical methods, allowed the reduction of the detection limit values reporting the existence of pharmaceutical compounds in environment. As shown in Figure *2.1*, more than 100 different

pharmaceutical compounds have been identified all over the world in surface water, groundwater, and/or tap/drinking water [10]. Although these compounds are found at low concentrations, their continuous input and persistence in aquatic system constitute a potential hazard inducing biological changes and death of living organisms [11]. One example of biological change is the feminization of fishes discovered in the last 1990s as consequence of presence of oestrogen in effluents [3]. More recently, in 2004, diclofenac, a usual anti-inflammatory drug, was responsible for the high death rate of three different species of vulture in India and Pakistan [12]. Additionally, there are some studies that stablish a direct correlation between the bacterial antibiotic resistance and the existence of pharmaceutical compounds in effluents [13]. Besides the ecological point of view, human health might also be at risk through long term consumption of drinking water containing biological active compounds [14].

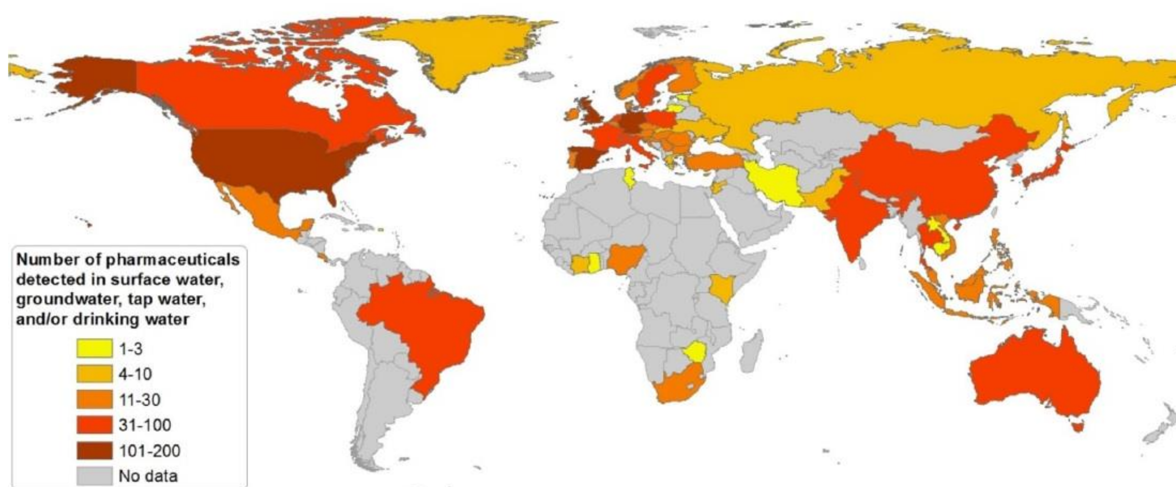



**Figure 2.1**- Number of pharmaceutical substances detected in surface water, groundwater or tap/drinking water in several countries [10].

## 2.3. Sources of pharmaceuticals in the environment

The widespread use of pharmaceuticals in human, veterinary medicine and agricultural field and the consequent continual release into environmental have been brought serious concerns in last years. After intake, from 30 to 90% of the pharmaceutical compound is metabolized and others substantial fractions of the original compound are excreted with urine and/or faeces, as unmetabolized form or as metabolite, following the wastewater pathway until WWTPs [5,15]. However, many pharmaceutical compounds have poor biodegradability and up to 90% of drug resides are incomplete removed from the water after treatment. So, treated effluents containing pharmaceutical compounds are being discharge in rivers, lakes, estuaries, and consequently, contaminating them directly and, indirectly, groundwater and drinking water

[15]. Otherwise, veterinary drugs are more likely to contaminate soils and groundwater through deposition of manure on agriculture land and subsequent runoff after rainfall [16]. On the other hand, undesirable and expired medicines have improper disposal via the sink/toilet or in household waste that has as destination landfill sites. In this way, the landfill leaching will transfer the pharmaceuticals from the solid waste to the groundwater and, consequently, drinking water (Figure 2.2) [16].

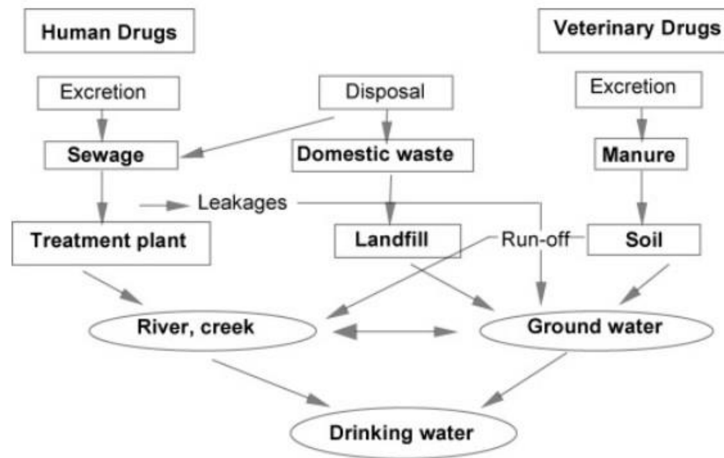



**Figure 2.2** – Main sources of pharmaceuticals in the environment [16].

Beyond that, hospital wastewater, wastewater from pharmaceutical industries and aquaculture are others possible emission pathways of pharmaceuticals into environment [10,12].

# 3. European legislation

Although the lack of legal discharge limits for micropollutants into the environment, some regulations have been published in the last few years. Since the year 2000, Directive 2000/60/EC of the European Parliament establishing framework for Community action in the field of water policy, known as EU Water Framework Directive (EU WFD), defines goals and the approach to ensure the protection and prevents further deterioration of inland surface waters, transitional waters, coastal waters and groundwaters. Article 16 of the EU WFD states that EU Commission shall identify priority substances/group of substances (PSs) that represent a substantial risk to or via the aquatic environment and, subsequently, propose EU Environmental Quality Standards (EQS). According to article 2, EQS is “the concentration of a particular pollutant or group of pollutants in water, sediment or biota which should not be exceeded in order to protect human health and the environment” [17].

In 2001, Decision nº 2455/2001/EC established the first list of 33 PSs having in account the recommendations referred to in article 16 of Directive 2000/60/EC. Some of the PSs have been marked as priority hazardous substances (PHSs) defined as “substances or groups of substances that are toxic, persistent and liable to bio-accumulate” pose a significant risk to the aquatic environment. This decision amended EU WFD and the list was included in Directive 2000/60/EC as Annex X [18]. The EQS for the PSs and other pollutants in the list already mentioned were launched in Directive 2008/105/EC of 16 of December of 2008 [19].

Directive 2008/105/EC was transported to the national legislation by the Decree-law nº 103/2010 of 24 September of 2010, which included the list of PSs as well the PHSs in Annex I, the list of other pollutants in Annex II and the respective EQS in Annex III [20].

Five years after the publication of Directive 2008/105/EC, Directive 2013/39/EU was adopted by European Parliament and the Council of the European Union on August of 2013 containing the first review of the list of PSs. Directive 2013/39/EU updated the list of PSs, set out EQS for those newly identified substances, refined EQS for some substances of the first list in line with scientific and technical progress and set biota EQS for some newly and existing PSs. Additionally, Directive 2013/39/EU included three pharmaceutical compounds (the non-steroid anti-inflammatory diclofenac, the synthetic hormone 17-alpha-ethinylestradiol (EE2), and natural hormone 17-beta-estradiol (E2)) in watch list to gather monitoring data to support future reviews to the list [21]. Directive 2013/495 was transported to national legislation by the Decree-law nº 218/2015 of 7 October of 2007 amending the Decree-law nº 103/2010 [22].

The watch list was published in the Decision 2015/495/EU of 20 March 2015 contemplating ten substances/group of substances: the three substances above mentioned, three macrolide

antibiotics (erythromycin, clarithromycin and azithromycin) together with other natural hormone (estrone (E1)), some pesticides, a UV filter and an antioxidant typically used as food additive. Thus, there are six pharmaceutical compounds for which Union-wide monitoring data need to be gather to support future prioritization of chemicals and contemplate the EU data base [23].

Overall, the EU WFD is aimed to simply the monitoring of the state of the waters having in concern emission limit values and EQs instead of prevent potential environmental impacts [17,24]. For this reason, the European Parliament and the European Council implemented the European Union's REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) on 18 December of 2006. REACH demands that all substances produced or imported into Europe above one ton per year must be evaluated for their adverse effects on the environment. To this point, REACH ensure that toxic substances are used properly and inform users about the hazardous properties of chemicals [25].

The Council Regulation EC 440/2008 of 30 May of 2008 [26] which establishes the regime that governs the classification, packing and labelling of dangerous substances for human health or for the environment is currently applied at the national level through the Decree Law nº 98/2010[27].

# 4. Ecotoxicology: aquatic toxicology

Aquatic ecosystem is a dynamic system which relies on interaction between species of the population in its physical environment. The presence of mixture of contaminants in water released by anthropogenic activities disturb the natural balance of the system due to deterioration of the water quality which can lead to unpredictable changes in the ecosystem [28].

Ecotoxicology has been defined as an environmental natural science that studies the source, transport, fate and impact of toxic substances on biosphere. The main goal of these studies is to explain and predict the effects of potential toxic substances on natural ecosystem with the aim to stablish the “safe” or “non-effective” concentration of these substances on non-target organisms [29–31].

Bioassays are valuable tools to understand the mechanisms of action of chemicals as well as its ecological impact. Unlike chemical analysis, bioassays qualify and quantify the toxic effects of a substance on a living organism. A sufficient level of exposure in artificial situation causes physiological effects on an organism, namely, on growth, metabolism and reproduction level. The potential toxicological effects observed in bioassays are extrapolated to all species representing that trophic level in the environment [31–33].

Overall, ecotoxicity assessments towards freshwater organisms are an important alternative for the evaluation of aquatic contamination and to assess the risk of chemicals, such as pharmaceuticals and its metabolites, on human health and on aquatic life [34–36].

## 4.1. Types of ecotoxicity tests

Toxicity tests measure lethal and/or sublethal effects and may be classified as short or long-term test, depending on the time exposure of the test, life spans of organism and the final substance’s effect.

In general, short-term tests provide indications about acute effects, lethal effects, in organisms after short periods of exposure to relatively high concentration of test substances. In some cases, death is hard to determine unequivocally. So, other effects are evaluated, such as, immobility. At the end, results can be analysed comparing percent effect or percent mortality for organisms exposed to contamination and those exposed to uncontaminated medium. Additionally, the results are used to determine lethal concentration ($LC_{50}$) and effective concentration ($EC_{50}$). Meanwhile $LC_{50}$ express the concentration of substance used to cause 50% of mortality of exposed organisms in test, $EC_{50}$ refer the concentration of substance needed to cause an adverse effect in 50% of exposure organism [12,28,32,35,37].

Another way to characterize the toxicity potential of a substance is as the lowest observed effect concentration (LOEC), which refer to the lowest concentration that cause a visible alteration distinguishable from control organisms of the same species and strain, and as the no observed effect concentration (NOEC), which express the highest concentration of toxicant resulting in no effects on organisms [29,30]. However, many objections have been raised against the use of NOECs to express toxicity, because of the fact that if it does not occur any statically significant effect it does not mean that there is no effect. As an alternative for the NOEC, OECD Guideline 201 suggests to use $EC_{10}$ or $EC_{20}$.[38]

The standard short-term exposure time is normally between 24 to 96 hours. According to OECD Guidelines, ecotoxicity assessment takes 72 h for algal growth inhibition test, 48 h for *Daphnia* immobilization test and 96 h for fish toxicity test [12,28,32,35,37].

On the other hand, long-term tests consider the whole life cycle of an organism and, normally, they provide indications about chronic effects. In contrast to short-time tests, the organism is exposed to a relatively low toxic concentrations for weeks, months or years and is evaluated not only mortality, but also changes in physiological processes like as growth reduction, nerve function atrophied, development of structural, behaviour of individuals and reproductive impairment [28,30]. Typically, for evaluation of chronic effects, the tests are carried out with a set of species of different trophic level and different stage of development [39].

The long-term test responses are analysed comparing the percent effect in organism exposed to contaminated media and those exposed to uncontaminated media and, thus, determine the $EC_{50}$, $EC_{20}$ and $EC_{10}$ [30].

In this study, it was assessed the acute effects on algal growth and the tests were carried out for 72 hours. As the test duration was beyond the life spam of the test organism, the test is classified as long-term.

When the logarithm concentration of the test substance is plotted versus the cumulative response is obtained a curve known as dose-response curve. The major purpose of that curve is the analyse of the effect of the test substance on biota at many exposure concentrations with view to stablish $EC_{10}$, $EC_{20}$ and $EC_{50}$ values.

Generally, the curve has a sigmoidal shape (Figure 4.1 a) where the increase of exposure to a toxicant lead to a greater effect achieving the maximal effect level [35,37]. Occasionally, due to a non-monotonic response, the curve appears as an inverted-U, in which the maximal effect level is situated between low and high toxicant concentrations ( Figure 4.1b)) [35,40].

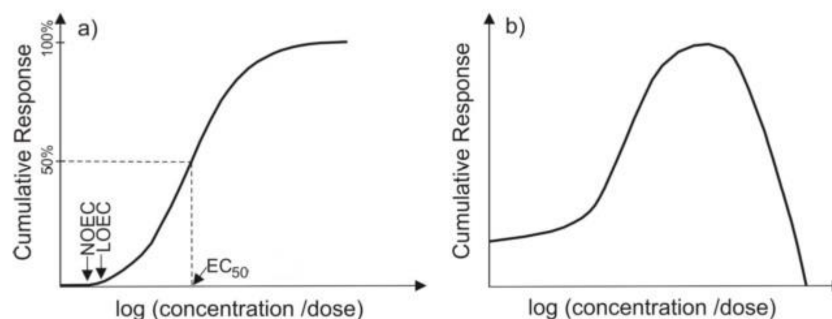



**Figure 4.1** - Hypothetic dose-response curve: **a)** example of a classical dose response curve and **b)** example of a non-monotonic curve. Adapted from [35].

## 4.2. Environmental risk assessment

An environmental risk assessment (ERA) is a stepwise process which allows to assess the likelihood of a compound causing undesirable environmental effects. It therefore requires adequate data of both environmental and ecotoxic potencies of the compound. From a regulatory standpoint, ERA must be performed for marketing authorization of new medical products for human use or in case of increase environmental exposure [41,42].

The first tier is based on the estimation of environmental exposure. If there is lack of adequate data on environmental exposure of the compound, it can be estimated using calculation based on consumption or sales figures, fate in the environment, metabolism and excretion in organism and its physico-chemical properties [41,42]. If the predicted environmental concentration (PEC) or the measured environmental concentration (MEC) value are lower than 10 ng $L^{-1}$ it is presumed that the pharmaceutical compound does not represent any concern for the environment. Otherwise, if the PEC or MEC values are equal or higher than 10 ng $L^{-1}$ , a second tier corresponding to toxicity testing is required [42,43].

In second tier, predicted no effect concentration (PNEC) is derived by applying an appropriate assessment factor (AF) to the lowest relevant effect value obtain from ecotoxicity tests ($LC_{50}$, $EC_{50}$, $EC_{10}$). These AFs are used in rage of 1 – 1000 and they represent the uncertainty of extrapolation from ecotoxicology tests data to the real environmental situation. Evidently, AFs are inversely proportional to availability and quality of toxicity data [44,45].

Then, the pharmaceutical compound is characterized with the risk quotient (RQ) which is calculated as the ratio between PEC or MEC and PNEC [43]. If the RQ is below 0.1, it implies that the compound poses no environmental risk to the aquatic environment. In contrast, if RQ is between 0.1 and 1 indicates a medium risk and if RQ is above 1 suggests high toxicity to the aquatic environmental [3,42].

## 4.3. Test organisms

Toxicity of aquatic systems confine the development of organisms from different trophic levels and, for that reason, organisms may perform as biological indicators of pollution in water. The test organisms applied in bioassays are aquatic organisms from four distinct groups: bacteria, algae, invertebrates and fishes [46]. Table 4.1 summarizes the most commonly considered species for each one group in bioassays to characterize toxicity of water and wastewater, the respective method and some examples of application.

**Table 4.1** - Toxicity tests typically used in assessing risks to human health and aquatic life after water and wastewater treatment. Adapted from [35,46].

| Trophic level | Group and organism | Method | Application |
|---|---|---|---|
| Decomposer | Bacteria<br>*Vibrio fischeri* | ISO 11348, 2007 [47] | Urban waste water treatment [48] |
| Primary producer | Microalgae<br>*Scenedesmus subspicatus*<br>*Raphidocelis subcapitata** | ISO 8692, 2012 [49] | Industrial wastewater treatment [50] |
| Primary consumer | Invertebrate<br>*Daphnia magna* | EPA [30]<br>ISO 6341, 2012 [51] | Industrial wastewater treatment [52] |
| Secondary consumer | Fish<br>Zebrafish (*Danio rerio*) | ISO 7346-1, 1996 [50] | Industrial wastewater treatment [50] |

* *Raphidocelis subcapitata* was formerly known as *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata.*

### 4.3.1. Algae

Algae plays an important role in ecosystem, because, as primary producers in food chain, ensure nutrients to organisms at the upper position in food chain and oxygen in aquatic system as well as in atmosphere though photosynthesis. Therefore, any change in the structure and productivity of the algae community will adversely affect the total aquatic ecosystem. Beyond their high sensibility to changes in environment, algae are found almost everywhere, are easy to collect and grow quickly what make them ideal for laboratory testing [53–55]. As a matter of fact, despite the lack of toxicity data in literature, algae are more sensible to chemicals, specially to organic compounds like pharmaceutical compounds. This implies that algal bioassay are reliable and may be an strategy to avoid fish toxicity testing [54,56,57].

The algae species selected to use in bioassays should be sensible to the compound for which the test is conducted, well-known nutrient requirement, good taxonomic characterization of strains, low genetic and phenotypic variability, available to obtain and cultivate and easy to handle in laboratory [58]. Both macroalgae or microalgae are used as organism test, however, microalgae have been used more frequently for this purpose because toxicity tests with unicellular organisms have a greater reproducibility, repeatability and robustness than tests with multicellular organisms [53]. *Chlorella vulgaris*, *Scenedesmus subspicatus* and *Raphidocelis subcapitata* (Figure 4.2) are some examples of green microalgae commonly used as test organism [37]. *R. subcapitata* stands out as one of the most sensitive and it is wildly used to evaluate toxicity as recommended in the OECD and EPA [30,38,59,60]. The algal cells are a helical shape, which is often to appear as a semicircle shape when in vegetative state [60].

**Figure 4.2** – *Raphidocelis subcapitata* culture [61].

## 4.4. Aquatic ecotoxicity of fluoxetine

The exact metabolic fate of fluoxetine is not yet fully elucidated. Following oral administration, fluoxetine is metabolized by cytochrome P-450 to norfluoxetine, its active metabolite, and other metabolites. Excretion occurs primarily via urine with less than 11 % as unchanged parent compound as well as norfluoxetine, 5.2 – 7% as conjugated fluoxetine, 8 – 9.5% as conjugated norfluoxetine, around 20% as hippuric acid and trace amounts as p-trifluoromethylphenol [62].

As a widely prescribed antidepressant, fluoxetine is detected in aquatic compartments and it is recognized as one of the human pharmaceutical compound with the highest acute toxicity toward non- target organisms [63]. The average treated wastewater concentration globally have been found from 13 to 99 ng.L$^{-1}$ [64]. In Portugal, fluoxetine was reported in Arade river at concentration not exceeding 2 ng L$^{-1}$[65], in WWTP influent at Fernão Ferro, Seixal, at maximum

concentrations of 3465 ng $L^{-1}$[66] and in Lis river with a concentration in the range of 2.01 - 10.0 ng $L^{-1}$[67].

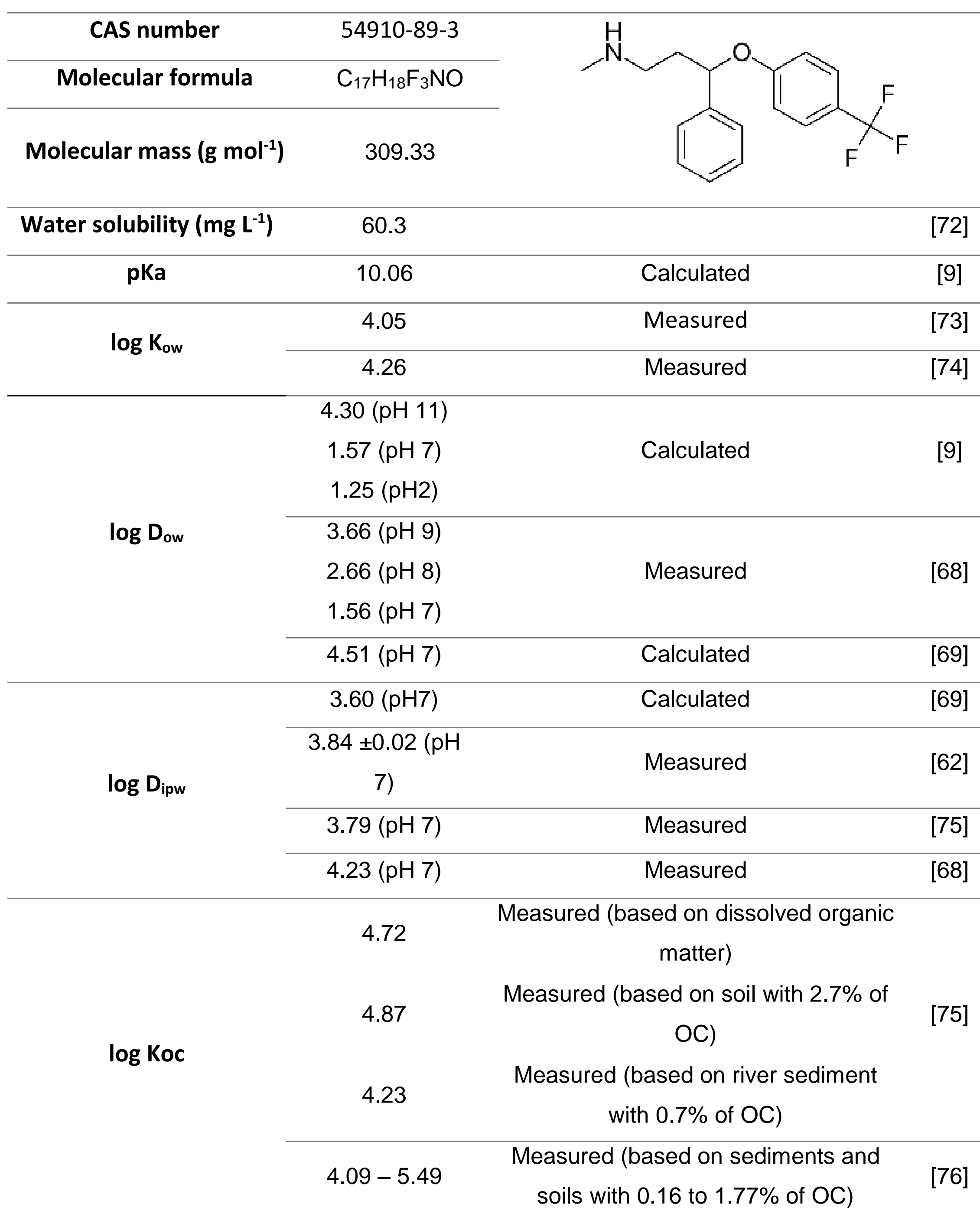
Knowledge of the physical-chemical properties of fluoxetine outline its environmental behaviour. The log $K_{ow}$ (octanol-water partition coefficient) value is a measure of hydrophobicity, which is related with the tendency to be adsorbed in aquatic sediments, and also of lipophilicity, describing the tendency of a molecule, at uncharged form, to permeate through the lipid domain of a membrane cell [29,42]. According with OSPAR Convention, pharmaceutical compounds with a log $K_{ow}$ values higher than 4.5 are of greater concern, because they tend to be persistent, toxic and they may have the potential of bioconcentrate in living organisms [63]. As shown in Table 4.2., measured log $K_{ow}$ values do not exceed this limit, indicating that fluoxetine has a low affinity for the lipids as well as a low tendency to bioaccumulate.

However, at natural environment, fluoxetine will be present mostly in the ionized form (pKa = 10.06) [63]. The ionized form is presumably more easily dissolved in water and, thus, $K_{ow}$ would be higher than a nonionized forms [68]. On the other hand, norfluoxetine, a transformation product of fluoxetine, is more hydrophobic than its parent compound and, consequently, has a higher tendency to bioaccumulate. That is to say that bioconcentration potential of fluoxetine should not be predicted solely based on log $K_{ow}$. For ionized compounds, like in this case, it is recommended to used liposome-water partition coefficient ($D_{lipw}$) at pH 7 [63,69]. Liposome are made of phospholipids, a main constituent of membrane, and, for that reason, liposome model is a closer simulation of a biological membrane [68].

All log $D_{lipw}$ values exposed in Table 4.2 are higher than 3, pointing out to a low to moderate tendency to bioaccumulation of fluoxetine in living organisms [63]. Recent studies have shown the capacity of aquatic organisms to bioaccumulate fluoxetine. Brooks *et al.* collected three species fishes downstream from the effluent discharge and detected 0.11, 1.34 and 1.58 ng $g^{-1}$ of fluoxetine in muscle tissue, liver tissue and brain, respectively [70]. Also Chu and Metcalfe detected fluoxetine in fish from a stream impacted by discharges of WWTP and industry using whole fish tissue [71]. Additionally, the high values of log $D_{lipw}$ indicates that fluoxetine may interact with membrane cells disturbing the membrane-protein interfaces which may be associated with the toxicity of this compound [62].

Also, the water-organic carbon partition coefficient ($K_{oc}$) may be used to predict bioaccumulation potential of sediment and soils. The log $K_{oc}$ values reported in literature (summarized in Table 4.2) are in range from 4.09 to 5.49. Seeing that all log $K_{oc}$ values are higher than 3, fluoxetine is likely to leach or occur as surface runoff to contaminate groundwater [63].

**Table 4.2 –** Physical chemical properties of fluoxetine.



| Property | Value | Method | Reference |
|---|---|---|---|
| **CAS number** | 54910-89-3 | | |
| **Molecular formula** | $C_{17}H_{18}F_3NO$ | | |
| **Molecular mass (g mol$^{-1}$)** | 309.33 | | |
| **Water solubility (mg L$^{-1}$)** | 60.3 | | [72] |
| **pKa** | 10.06 | Calculated | [9] |
| **log $K_{ow}$** | 4.05 | Measured | [73] |
| | 4.26 | Measured | [74] |
| **log $D_{ow}$** | 4.30 (pH 11)<br>1.57 (pH 7)<br>1.25 (pH2) | Calculated | [9] |
| | 3.66 (pH 9)<br>2.66 (pH 8)<br>1.56 (pH 7) | Measured | [68] |
| | 4.51 (pH 7) | Calculated | [69] |
| **log $D_{ipw}$** | 3.60 (pH7) | Calculated | [69] |
| | 3.84 ±0.02 (pH 7) | Measured | [62] |
| | 3.79 (pH 7) | Measured | [75] |
| | 4.23 (pH 7) | Measured | [68] |
| **log Koc** | 4.72 | Measured (based on dissolved organic matter) | [75] |
| | 4.87 | Measured (based on soil with 2.7% of OC) | |
| | 4.23 | Measured (based on river sediment with 0.7% of OC) | |
| | 4.09 – 5.49 | Measured (based on sediments and soils with 0.16 to 1.77% of OC) | [76] |

log $K_{ow}$ = logarithm of octanol – water partition coefficient; log $D_{ow}$(pH) = logarithm of octanol – water distribution ratio at a given pH value; log $D_{lipw}$ (pH 7) = logarithm of liposome – water distribution coefficient ratio of a species; log $K_{oc}$ = logarithm water – organic carbon partition coefficient (L/kg C); OC = organic carbon content.

Overall, contamination of water compartments by fluoxetine is of great concern due to its environmental persistence, acute toxicology to non-target organisms, and unique pharmacokinetics associated with a readily ionisable compound. Indeed, among SSRIs,

fluoxetine is the most studied and toxic antidepressant compound which has strong adverse effects, particularly, on microalgae [72].

Brooks *et al.* assessed the impact of fluoxetine in five freshwater species and concluded that *R. subcapitata* was the most sensible among the tested species. After 120 hours of exposure to fluoxetine, the response of algae growth was measured in terms of changes in cell density and turbidity. It was reported $EC_{50}$ values of 39 µg $L^{-1}$ and 24 µg $L^{-1}$, respectively. Furthermore, changes in cell shapes was also observed. Cells appeared shrivelled and, in some case, the sickle-shaped was not recognisable. The mechanism by which fluoxetine induce cell shape alteration in still unclear, however, may be correlated with its antimicrobial proprieties that inhibits cellular efflux pumps [77]. Likewise, it was reported by Neuwoehner *et al.* that fluoxetine has a pronouncer impact on cell volume growth of microalgae *Scenedesmus vacuolatus* described with an $EC_{50}$, for 24 h, of 93 µg $L^{-1}$. Neuwoehner *et al.* , also proved through photosynthesis inhibition tests with *R. subcapitata,* that fluoxetine is more toxic than its metabolites, norfluoxetine and p-trifluoromethylphenol, with an $EC_{50}$ value (24 h) of 90 µg $L^{-1}$ [62].

In other study, it was demonstrated that fluoxetine was more toxic to algae *R. subcapitata* than other SSRIs compounds (citalopram, paroxetine, fluvoxamine and sertraline) with an $EC_{50}$ value, for 48 h, of 27 µg $L^{-1}$[64]. However, Johnson *et al.* performed a similar laboratory growth inhibition assay with *R. subcapitata* during 96 hours and it was found that sertraline was the most toxic SSRIs compound with $IC_{50}$ of 12 µg $L^{-1}$ followed by fluoxetine with $IC_{50}$ of 45 µg $L^{-1}$ [78].

The higher $EC_{50}$ value of fluoxetine found in literature using the microalgae *R. subcapitata* as test organisms was 200 µg $L^{-1}$ [79].

However, aquatic organisms are typically exposed not to a single compound but, typically to a wide array of substances, indicating that exposure assessment should focus on mixtures rather than one single chemical. Therefore, it is predicted that a mixture of SSRIs produce addictive toxicities effects, because these compounds share the same mechanism of action [63]. Minguez *et al.* studied the interaction between different groups of antidepressants and investigate the joint and single effect on growth of *R. subcapitata* after 72h of exposure. For this purpose, all the compounds were tested individually at the same concentrations as in the mixture. Minguez *et al.* have reported that the mixture effect is higher than the individual toxicities of its components. Identical results had already been reported by other authors towards aquatic test organisms from binary to more complex mixtures of various chemical contaminants [12,80,81]. Then, the antidepressants with a primary/secondary amine (fluvoxamine, fluoxetine, sertraline, paroxetine and duloxetine) were separated from those with tertiary amine (venlafaxine, clomipramine, amitriptyline and citalopram) forming two groups. The toxicity of these two groups of compounds was assessed at the same concentrations as

previously for the mixture of nine compounds. The results showed that the combined toxicity of the antidepressants with presence of primary and secondary amines function results in antagonistic effects, whereas the group of antidepressants with tertiary amine function indicated strong synergistic effects [82].

**Table 4.3** – Acute toxicity data to fluoxetine for microalgae species.

| Microalgae specie | Acute toxicological endpoint | Endpoint | Acute toxicological data | Reference |
|---|---|---|---|---|
| ***Raphidocelis subcapitata*** | $EC_{50}$ (120 h) | Cell density as a measure of growth inhibition | 39 µg $L^{-1}$ | [77] |
| | $EC_{50}$ (120 h) | Turbidity as a measure of growth inhibition | 24 µg $L^{-1}$ | [77] |
| | $EC_{50}$ (96 h) | Growth inhibition | 24 µg $L^{-1}$ | [9] |
| | $EC_{50}$ (72 h) | Chlorophyll fluorescence as measure of growth inhibition | 200 µg $L^{-1}$ | [79] |
| | $EC_{50}$ (48 h) | Growth inhibition | 27 µg $L^{-1}$ | [64] |
| | $EC_{50}$ (24 h) | Yield of photosynthesis as a measure of growth rate | 90 µg $L^{-1}$ | [62] |
| | $IC_{50}$ (96 h) | Growth inhibition | 45 µg $L^{-1}$ | [78] |
| | $IC_{10}$ (96 h) | Growth inhibition | 31 µg $L^{-1}$ | [78] |
| | LOEC (96 h) | Growth inhibition | 14 µg $L^{-1}$ | [9] |
| ***Skeletonema marinoi*** | $EC_{50}$ (72 h) | Growth inhibition | 43 µg $L^{-1}$ | [79] |
| ***Scenedesmus vacuolatus*** | $EC_{50}$ (24 h) | Cell volume growth | 93 µg $L^{-1}$ | [62] |
| ***Chlorella vulgaris*** | $IC_{50}$ (96 h) | Growth inhibition | 4339 µg $L^{-1}$ | [78] |

| | | | | |
|---|---|---|---|---|
| ***Scenedesmus acutus*** | $IC_{50}$ (96 h) | Growth inhibition | 91 µg $L^{-1}$ | [78] |
| ***Scenedesmus quadricauda*** | $IC_{50}$ (96 h) | Growth inhibition | 213 µg $L^{-1}$ | [78] |

As shown previously in Table 4.3, adverse effects of fluoxetine are observed in aquatic toxicity tests at µg $L^{-1}$ levels. Based on results of standardized ecotoxicology tests using *R. subcapitata* as a test organism, Brooks *et al.* achieved an estimated hazard quotient below 1 showing that little risk to aquatic compartments may be expected from such fluoxetine exposure levels [9]. Likewise, Webb *et al.* came to the same conclusion after a refinement in the risk assessment. PEC was recalculated having in account the dilution in surface water and efficiency removal in WWTPs resulted in a refined PEC/PNEC ratio of 0.12, indicating negligible risk of an adverse environmental effect [83]. However, it was noted that fluoxetine, at lower exposure levels, affect fish on non-traditional endpoints, namely, at estradiol levels and the number of malformations warranted further study [9].

In fact, these preliminary risk assessments for fluoxetine were based on traditional acute survival assessments, but, such data alone may not be suitable to evaluate ecosystem changes and risk assessment. Because pharmaceutical compounds are continuously released into the environment at low concentration, chronic tests are needed for an accurate environmental risk assessment [9,63].

More recently, it was determined PNEC for fluoxetine through standard chronic test on algae and the results evidence a PEC/PNEC ratio ≥ 1 for some European countries, suggesting that fluoxetine poses potential risk for the aquatic compartment [63].

# 5. Removal of pharmaceuticals from wastewater

It is widely reported that recalcitrant pharmaceuticals are not completely removed by conventional water and wastewater treatment techniques. The increasing demand to ensure water quality due to the self-aware of the potential risks of pharmaceuticals in the environment, requires the development of advanced techniques capable to remove this persistent contaminates from water. For that propose, various treatment options have been investigated including chemical oxidation processes (e.g. ozonation), biological treatments (e.g. activated sludge process), membrane filtration (e.g. reverse osmosis), and adsorption using activated carbon [11].

The application of ozone to treat drinking water is a promising technique with great results in terms of removal efficiencies. Ozone is a reactive oxidant with potential to transform pharmaceutical compounds through either the direct reaction or by decomposition through the formation of the oxidizing agent $\cdot OH$. It was reported that when applied an ozone dose of 15 mg $L^{-1}$, almost all pharmaceutical compounds, namely carbamazepine, diclofenac, indomethacin, sulpiride and trimethoprim were eliminated to up to 95%. However, this technique was unsuccessful in removal of bezafibrate. [4]. Besides the ineffective removal of some pharmaceutical compounds, other disadvantages of this technique is the requirement of high energy and the possibility of formation of by-products [4,11,84].

Biological treatment was found to be unsuccessful in the removal of the pharmaceuticals in water, because many pharmaceutical compounds are neither biodegradable nor can be adsorbed by sewage sludge. For example, carbamazepine and some sulphonamides are compounds found in WWTPs that are not totally decomposed by biological agents [84,85].

Membrane filtration, namely, the reverse osmosis shows great potential to remove pharmaceutical compounds with low molecular weight, but, is not efficient for all compounds. The retention of pharmaceutical compounds is influenced by its physicochemical properties and membrane's characteristics. The main drawback of the membrane filtration prosses is the membrane fouling that limit its engineering application as well as the requirement of disposal of membrane retentate [11,84].

Another option for removing pharmaceutical residues from water is by adsorption processes. Adsorption has advantages over the other methods in terms of simplicity of design, low energy consumptions and excellency of performance [86]. It involves the accumulation of impurities present in water at the interface of two different phases via physical and/or chemical interactions. The forces that bring about physical adsorption are principally weak electrostatic forces such as dipole induced dipole interactions, van der Waals forces and hydrogen bonding.

In this case, compounds are weakly bound to surface sites through reversible and non-specific interactions which act at longer distances. On the other hand, chemical adsorption results from covalent or electrostatic chemical bond between atoms with shorter bond length. Substances adsorbed are hardly removed because of the specificity of the bond to surface sites or to functional groups [87].

The adsorbents generally used are activated carbons, due to their large specific surface areas (500 to 1500 $m^2$ $g^{-1}$), high pore volume values (higher than 0.5 $cm^3$ $g^{-1}$) and its economic viability [88]. In addition, function groups on the carbon play a key role on adsorption. Both the pore size distribution and chemical surface activity depends on the source of raw material as well the parameters of the activation process [87].

The activated carbons are very efficient for the removal of a broad type of organic and inorganic components, however, these materials have some limitations for removal of pharmaceutical compounds dissolved in water. The activated carbons have a better affinity for non-polar molecules, meanwhile, many pharmaceutical compounds and its metabolites are indeed polar substances [11]. Further, this process transfer organic compounds from water to the adsorbent and, for that reason, it is necessary to regenerate the adsorbent which increase the costs of the process and to treat the solid waste, when no further regeneration is possible [89]. A brief introduction to adsorption theory will be presented in the next chapter.

## 5.1. Removal of pharmaceuticals using nanomaterials

Recent advances in nanotechnology offer opportunities to develop high performed and affordable techniques able to overcome the limitations of conventional water and wastewater treatments. Nanotechnology involves the production, characterization and manipulation of matter in atomic and molecular levels, creating materials and structures that display, at least, dimension between 1 and 100 nm [84]. The small size and large surface to volume ratio of these nanomaterials (NMs) increases their surface energy, reactivity and dissolution. Furthermore, they exhibit high adsorption capacity and photocatalytic properties which have been explored in treatment of water and wastewater [11,90–92].

### 5.1.1. Adsorption using nanomaterials

Adsorption processes using NMs, come up as an effective method for removal of pharmaceuticals and personal care products from aqueous phase.

Among several NMs used as adsorbents, graphene and graphene-based NMs show unique properties with potential application in water treatment [84,92,93]. Graphene is an atomically

thin two-dimensional carbonaceous material arranged in a $sp^2$-bonded aromatic structure [94,95]. It is a building block of graphite and it can be obtained by the exfoliation of graphite flakes [94]. Apart from its exceptional electronic and mechanical proprieties, graphene has the highest specific surface area of all NMs with a theoretical value of 2630 $m^2 g^{-1}$. The large specific surface area provide a large number of surface adsorption sites which is believed to be the main reason for their superior adsorption capacity [84,94]. However, the hydrophobic nature of pure graphene difficult its dispersion in water and the high costs of production limit its application in water treatment processes in its pure form [94,96]. To address this problem and to improve its adsorption performance towards special pollutants, surface structure of graphene may been modified, forming graphene-based NMs [86].

### 5.1.2. Photocatalysis

Adsorption can be used to remove pharmaceutical compounds from the aqueous phase, but this technique does not convert or degrade these compounds and, it still requires a further disposal [94]. In this endeavour, photocatalytic oxidation processes prove to be an effective waste water treatment method capable of degrading diverse organic compounds even at trace concentrations [92].

Sigle metal oxides such as titanium dioxide ($TiO_2$) and zinc oxide (ZnO) are successfully applied as semiconductors to photocatalytic degradation of pharmaceutical compounds, microbial pathogens, organic matter and other anthropogenic contaminants [84,90,92,97].

Figure 5.1 displays a scheme of the photocatalytic process on a semiconductor. When the photocatalyst semiconductors is bombarded with photons from light with energy equal to or greater than their band gap energy Eg ($h\nu \geq Eg$), electrons from valence band (VB) are promoted to the conduction band (CB). The absorption of photons by a semiconductor generate an electron-hole pair that can directly react with organic compounds or indirectly inducing redox reactions [46,84,91,98]. In case of successful degradation, organic compounds are total mineralized to dioxide carbon, water and non-toxic anions. Otherwise, organic compounds are converted to by-products [98].

As a photocatalyst, $TiO_2$ has been the top-choice due to its widely availability, cost efficacy, long term stability, strong oxidising ability and its suitability for the degradation of a large spectrum of contaminants [99]. However, $TiO_2$ has a large band gap energy (3.0 – 3.2 eV) which absorb mostly in UV region (< 390 nm) that is only 4% of solar irradiation [91,99]. In this way, its potential as a sustainable technology to remove emerged contaminants from water cannot be accomplish [100].

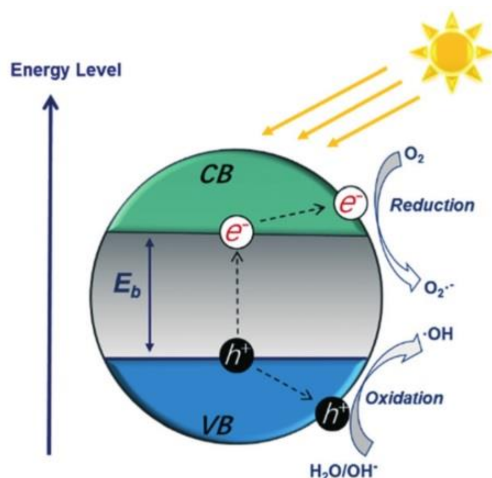



**Figure 5.1** – Schematic representation of photocatalytic mechanism on a semiconductor. Adapted from [84].

Over the last years, ZnO has emerged as an alternative to $TiO_2$ for photocatalyst applications. As matter of fact, ZnO exhibits higher removal efficiency under certain operating conditions and lower costs when compared to $TiO_2$. Nevertheless, similarly to $TiO_2$, ZnO is only active under ultraviolet light irradiation due to it wide band gap energy (3.37 eV) [84,91]. Beyond that, photocorrosion, which leads to a decrease of photocatalytic efficiency due to the loss of toxic $Zn^{2+}$ in water phase, and the fast recombination of the photogenerated electron-hole pairs also makes its application impracticable [84,91,100].

To improve photocatalytic activity under visible light, $TiO_2$ and ZnO have been doped over the surface of graphene. The high electron mobility of graphene accelerates electron transport, which slows the electron-hole recombination reactions and, consequentially, enhances the catalytic activity. Additionally, graphene endows the photocatalytic activity by reduction of band gap energy [94,101].

One of the most comprehensive studies about the photocatalytic transformation of diclofenac, a non-steroidal anti-inflammatory drug, under solar irradiation using $TiO_2$ MNs as a catalysts was carried out by Calza and his co-workers [102]. Calza *et al.* optimized the photocatalytic procedure, identified the intermediates formed and assessed the ecotoxicity of the solution treated by photocatalysis using the marine bacterium *Vibrio fischeri*. It was verified that the photocatalyst efficiency tends to increase when the reaction is performed at small and moderate concentration of $TiO_2$ and it tends to decrease with concentrations of $TiO_2$ between 780–896 mg $L^{-1}$. The highest degradation rate stands for a residual diclofenac percentage of 0.4 % was achieved after 30 min of irradiation with the combination $TiO_2$/diclofenac of 624 and 8.17 mg $L^{-1}$, respectively. Ecotoxicology tests proved that the products formed during the

photocatalytic treatment between 0 and 20 min, are more toxic than diclofenac. However, after 2 hours a decrease in the inhibition percentage to less than 1 % confirms the complete mineralization of diclofenac.

In another study, Teixeira *et al.* compared the photocatalytic activity of $TiO_2$ with ZnO under UV radiation for degrading 14 pharmaceutical compounds present in a WWTP treated effluent with initial concentrations higher than 0.3 µg $L^{-1}$. The results showed that using 1 g $L^{-1}$ of ZnO, it was achieved an average degradation of 95 % after 40 minutes of irradiation, meanwhile, with the same concentration of $TiO_2$ it was required 4 h to reach a similar level of degradation. Additionally, it was found that all pharmaceutical compounds were degraded in a similar way by ZnO. This evidence shows a lower selectivity of ZnO compared to $TiO_2$ which can be explored for removal of pharmaceutical compounds from WWTP effluents [103].

### 5.1.3 Environmental concerns posed by NMs

Despite all the advantages related with the application of NMs, concerns have been arising about the potential undesirable negative impacts on human health and environment. In this scenario, it is important to study the impact of NMs in aquatic organisms through ecotoxicological tests.

Arouja *et al.* have studied the toxicity of ZnO and $TiO_2$ to the green microalgae *R. subcapitata* in accordance with the OCDE Guideline. It was demonstrated that ZnO was more toxic than $TiO_2$, which was evidenced by the 72h-$EC_{50}$ values of 0.042 mg/L and 5,8 mg/L, respectively. In case of ZnO, it was reported equal toxicity in nano and bulk forms, meanwhile, $TiO_2$ in nano form showed a higher toxicity when compared to their bulk counterpart [104]. Similar evidences were found in ecotoxicological tests with marine microalgae [105,106].

Several reviews claim that the insoluble nature and the propensity for aggregation are the key factors in toxicity of NMs. It was reported that nanoparticles of $TiO_2$ tend to aggregate with themselves and with algae cells surface forming clusters. The formation of these $TiO_2$-algae complexes block the light necessary for algal growth and make them settle affecting the availability of food for organisms in higher trophic levels in food chain [104,107,108]. Additionally, it is possible that $TiO_2$ adsorbed on microalgae may cause physical damage of cell wall inducing plasmolysis [105]. The increase of reactive oxygen species (ROS) levels in cells due to the exposure of $TiO_2$ to visible light is another possible mechanism of toxicity. Generation of ROS induces oxidative stress and causes membrane cell damage [104,105,108].

The main toxic pathway attributed to ZnO is the released of zinc ions from particles [104,109,110]. However, a recent study with the yeast *Saccharomyces cerevisiae* suggest that

the zinc ions concentrations formed is not enough to explain the damage caused. The concentration of $Zn^{2+}$ in ZnO suspension was 0.4 mg/L and $LC_{50}$ value for $Zn^{2+}$ was 1.3 mg/L, which evidences that the release of zinc ions is not the only toxicity mechanisms underlying ZnO. Their results show that ZnO can penetrate through *S. cerevisiae* cells and its accumulation induce oxidative stress which may result in cell death. This suggests that the toxic pathway attributed to ZnO is essentially dependent on the adverse effect of nanoparticles themselves, not on the released zinc ions [111]. This evidence was consistent with ecotoxicological tests with *Daphnia magna* when exposure to ZnO suspension with concentration corresponding to $LC_{50}$ [112].

The toxicity of graphene based NMs in aquatic ecosystems is also widely discussed in literature, but only a small fraction part of the investigations is carried out on algae. A study on the green algae *R. subcapitata* reported a decrease of the chlorophyll autofluorescence intensity and, consequently, growth inhibition in the presence of graphene oxide. This phenomenon may be linked with the shading effect caused by the formation of graphene oxide aggregates [95].

Most of the currently available ecotoxicological data regarding NMs are very limited to algae and the toxic effects of such NMs on behaviour of aquatic organisms is still unclear. Additionally, only a few specific NMs have been investigated, so, it is important to expand the ecotoxicological tests.

# 6. Brief introduction to adsorption theory

## 6.1. Adsorption kinetics models

Knowledge of adsorption kinetic and equilibrium are crucial for the full understanding of mechanism of adsorption reactions, surface proprieties of adsorbent, the degree of affinity of the adsorbents and, consequently, for the design and scale-up of an adsorption system [113].

Kinetics consideration should be the first approach in order to evaluate solute reuptake rate as well as the residence time required for the completion of adsorption reaction [114]. There are three main steps in an adsorption process. First, the transport of the adsorbate from bulk solution to the film of solvent around adsorbent surface, followed by intraparticle diffusion of the adsorbate through pores of adsorbent and lastly, the sorption of adsorbate to the active sites of the adsorbent.

Various models have been developed to describe adsorption kinetics considering sorption or diffusion as rate limiting step. In pseudo-first-order model, pseudo-second-order model and in Elovich model, sorption of adsorbate to the active sites of adsorbent is consider the slowest step. On the other hand, in intraparticle diffusion model the slowest step is the mass transfer of adsorbate inside the porous adsorbent [115].

### 6.1.1. The pseudo-first order model

The pseudo-first order describes kinetics adsorption in a liquid-solid system and it considers the adsorption rate of occupation sites to be proportional to the number of unoccupied sites. The pseudo-first order kinetic model is represented by equation 1 [116]:

$$q_t = q_e(1 - e^{-k_1 t}) \qquad (1)$$

where, $q_t$ (mg $g^{-1}$) is the amount of adsorbate adsorbed at time t, $q_e$ (mg $g^{-1}$) is the amount of adsorbate adsorbed at equilibrium, $t$ (min) is the time of contact and $k_1$ ($min^{-1}$) is the pseudo-first order rate constant.

### 6.1.2. The pseudo-second order model

The pseudo-second order model provides a better correlation of the experimental data than the pseudo-first order model. The rate limiting step of the pseudo-second order model is the chemical sorption on adsorbent surface involving valency forces through sharing or exchanging electrons between adsorbent and adsorbate. The pseudo-second order model is express by equation 2 [117]:

$$q_t = \frac{{q_e}^2 k_2 t}{1 + q_e k_2 t} \tag{2}$$

where, $k_2$ (g $mg^{-1}$ $min^{-1}$) is the rate constant of the pseudo-second order kinetic equation.

### 6.1.3. Elovich model

The Elovich model is suitable to describe adsorption onto highly heterogenous adsorbents in which rate decreases with time due to an increase in surface coverage. The Elovich kinetic adsorption can be shown as equation 3 [118]:

$$q_t = \frac{1}{\beta}\ln(\alpha\beta) + \frac{1}{\beta}\ln(t) \tag{3}$$

where, α (mg $g^{-1}$ $min^{-1}$) is the initial sorption rate and the parameter β (g $mg^{-1}$) is related with the extent of surface coverage.

### 6.1.4. Intraparticle diffusion model

The intraparticle diffusion model was proposed by Weber and Morris for describing the kinetics of sorption at solid-solution systems controlled by internal diffusion. The intraparticle diffusion model is given by equation 4 [116]:

$$q_t = k_{id}\sqrt{t} + C \tag{4}$$

where, $k_{id}$ (mg $g^{-1}$ $min^{-0.5}$) is the intraparticle diffusion rate constant and C (mg $g^{-1}$) is a constant related with the thickness of boundary layer. The higher the values for C indicate strong boundary effects.

When intraparticle diffusion model alone is the rate limiting step, a plot $q_t$ versus $t^{½}$ should be a straight line that passes through origin with a slope $K_{id}$. In case of *C* different of 0, another mechanism along with intraparticle diffusion is taking place in controlling the adsorption process [119].

## 6.2. Adsorption isotherms

As mentioned previously, adsorption results in removal of solutes from solution and their accumulation on solid phase until the amount of solute remaining at the solution reaches a dynamic equilibrium with that adsorbed on the solid phase. The equilibrium is described by expressing the amount of solute that can be taken up by a solid phase per unit weight of adsorbent ($q_e$) as a function of the concentration of solute remaining on solution.

Several adsorption isotherms may be described the adsorption processes. Among these the Langmuir and Freundlich adsorption isotherms are the most used to describe water and wastewater treatment applications.

### 6.2.1. Langmuir adsorption isotherm

Langmuir adsorption isotherm is based on assumption that the adsorbate surface has a certain number of definite active sites spread homogenously over the surface. Each site is assumed to be likely probable of binding at most one adsorbate molecule forming a monolayer over the surface of the adsorbent when it gets saturated. Beyond that, it is also assumed that there is no interaction between molecules on different sites. The Langmuir equation is expressed by the following equation [120]:

$$q_e = \frac{QK_LC_e}{1 + K_LC_e} \tag{5}$$

where $q_e$ (mg/g) is the amount of adsorbate at the equilibrium, Q (mg/g) is the maximum amount adsorbed per mass of adsorbent when the surface sites are saturated with adsorbate, $K_L$ (L/mg) is an empirical constant which describes the affinity of the binding sites, $C_e$ (mg/L) is the equilibrium liquid phase concentration.

### 6.2.2. Freundlich adsorption isotherm

Freundlich adsorption isotherm presents one of the earliest isotherm models which describe a nonideal sorption on heterogenous surfaces. On contrary with Langmuir adsorption isotherm, this empirical model assumes that adsorption could occur via multilayer. The energy distribution for adsorptive sites follows an exponential type, thereby, the stronger binding sites are occupied first, until adsorption energy is exponentially decreased upon the finalization of adsorption process. The equation of Freundlich adsorption isotherm is present as follows [121]:

$$q_e = K_F C_e^{\frac{1}{nF}} \tag{6}$$

where, $K_F$ ($L^n\ mg^{1-n}\ g^{-1}$) is the Freundlich equilibrium constant which express the adhesion ability of the adsorbate onto the absorbent and nF (dimensionless) is the Freundlich exponent which indicates the adsorption intensity or surface heterogeneity.

### 6.2.3. Sips adsorption isotherm

Sips adsorption isotherm is a combination of Langmuir and Freundlich expressions, deduced for predicting heterogeneous adsorption system considering absence of interaction between molecules adsorbed on different sites. At low adsorbate concentrations, it reduces to

Freundlich isotherm, meanwhile, at high concentrations, it predicts a monolayer adsorption capacity characteristic of the Langmuir isotherm [122]. The Sips equation is defined as:

$$q_e = \frac{Q_{máx} K_s C_e^{\frac{1}{nS}}}{1 + K_s C_e^{\frac{1}{nS}}} \quad (7)$$

where, $K_s$ (L mg$^{-1}$)$^{1/n}$ is the Sips equilibrium constant and nS (dimensionless) is the Sips exponent, which is regarded as a heterogeneity factor.

# 7. Materials and Methods

## 7.1. Ecotoxicity assessment

The ecotoxicological tests were carried out regarding the algal inhibition test described in Council Regulation EC 440/2008 of 30 May of 2008 [26], which was based on the OECD Test Guideline 201 updated in 2011 [38] and on the US EPA guideline [30].

The essays were prepared under aseptic conditions using an autoclave (AJC, model uniclave 88, Portugal) and a laminar flow chamber (Faster, model two 30, Italy) which was pre-sterilized with ultraviolet light at least for 20 minutes. All the glass flasks used during the experiments were cleaned with diluted acid nitric (10% v/v) and then washed three times with deionized water.

As recommended by US EPA, the growth of the algae was evaluated by the determination of the variation of the fluorescence at the beginning and after 72 h of incubation. The fluorescence chlorophyll reflects the concentration of this pigment in suspension which it is proportional to cell density.

### 7.1.1. Organism test

The ecotoxicity tests were carried out with the freshwater unicellular green algae *Raphidocelis subcapitata.*

The organism test was cultured in laboratory under aseptic conditions. A new culture was started weekly by aseptically transferring 10 – 20 mL of stock culture to a 50 – 100 mL of fresh culture medium (the nutrient medium is described below), in order to algae adapt to the test conditions and ensure that the culture is in exponential growth phase when it is used to inoculate the test solutions.

Occasionally, the culture was concentrated and washed with fresh medium, in order to control bacterial contamination and exclude dead cells. Centrifugation was performed at 1800 rpm for 5 minutes.

The stock cultures were kept at 21 ±2 °C, under cold white fluorescent lighting. The stock culture was examined with an optic microscope, Nikon Alphaphot-2 YS2, to ensure that there was no contamination.

### 7.1.2. Growth medium

The growth medium was prepared in accordance with OECDE Test Guideline 201 adding the required nutrients from four different sterile stock solutions, as described in Table 7.1, to

sterile deionized water. It was prepared 500 mL of stock solution nº 1 and 250 mL of stock solutions nº 2, 3 and 4.

Stock solution nº 1 and nº 3 were sterilized by autoclaving at 120 °C for 20 minutes, meanwhile, stock solution nº 2 and nº 4 were sterile-filtered using 0.2 µm filters in laminar flow chamber. For the preparation of growth medium, it was added 9 mL of stock solution nº1 and 0.9 mL of all the other stock solutions to 900 mL of sterile deionized water. The culture medium was stored at dark at 4 °C.

The final pH of growth medium was between 7 and 8. The composition and the respectively concentration of all compounds are shown in Table 7.1.

**Table 7.1** – Composition of growth medium (Adapted from Regulations EC nº 440/2008 [26]).

| Stock solution | Compounds | Concentration in stock solution | Concentration in growth medium |
|---|---|---|---|
| **Solution nº 1 Macronutrients** | $NH_4Cl$ | 1.5 g/L | 15 mg/L |
| | $MgCl_2.6H_2O$ | 1.2 g/L | 12 mg/L |
| | $CaCl_2.2H_2O$ | 1.8 g/L | 18 mg/L |
| | $MgSO_4.7H_2O$ | 1.5 g/L | 15 mg/L |
| | $KH_2PO_4$ | 0.16 g/L | 1.6 mg/L |
| **Solution nº2** | $FeCl_3.6H_2O$ | 64 mg/L | 0.64 mg/L |
| | $Na_2EDTA.2H_2O$ | 100 mg/L | 0.100 mg/L |
| **Solution nº3 Trace elements** | $H_3BO_3$ | 185 mg/L | 0.185 mg/L |
| | $MnCl_2.4H_2O$ | 415 mg/L | 0.415 mg/L |
| | $ZnCl_2$ | 3 mg/L | $3 \times 10^{-3}$ mg/L |
| | $CoCl_2.6H_2O$ | 1.5 mg/L | $1.5 \times 10^{-3}$ mg/L |
| | $CuCl_2.2H_2O$ | 0.01 mg/L | $10^{-5}$ mg/L |
| | $Na_2MoO_4.2H_2O$ | 7 mg/L | $7 \times 10^{-3}$ mg/L |
| **Solution nº4** | $NaHCO_3$ | 50 mg/L | 0.50 mg/L |

### 7.1.3. Test Substances

Ecotoxicity tests were performed with fluoxetine and with some NMs, namely, carbon nitride ($C_3N_4$) in bulk and exfoliated forms, and graphene doped with nitrogen (GF_N8) to stablish concentration-effect relationships.

Fluoxetine was purchased from Sigma-Aldrich as fluoxetine hydrochloride ($C_{17}H_{18}F_3NO.HCl$) in solid form with 99,9 % of purity.

$C_3N_4$ was supplied by Dr. Marta Nunes, Faculty of Sciences of Porto university. Scanning Electron Microscopy with Energy Dispersive Spectroscopy (SEM/EDS) and X-ray diffraction (XRD) analyses indicate that $C_3N_4$ is formed by stacking the conjugated aromatic systems into layered structure. The atomic composition is mainly C, N and O. The identification of functional groups by Fourier transform infrared spectroscopy (FTIR) and the analysis of chemical composition by X-ray photon spectroscopy (XPS) are still being carried out. Specific surface area and interlamellar spaces (and porosity), are higher in the exfoliated form than in bulk form.

GF_N8 was supplied by Dr. Bruno Jarrais, Faculty of sciences of Porto University. Graphene flasks were doped with nitrogen atoms through a mechanochemical treatment with melamine as a precursor followed by thermal treatment at 800 °C in an inert atmosphere. The XPS spectra of GF_N8 indicated the presence of 92.3% of C1 s, 2.6% O1 s, 5.1% of N1 s. The high-resolution N1 s spectrum revealed three peak formations centred at 398.5, 400.1, 401.6 eV corresponding to pyridinic N (55.7%), pyrrolic N (36.7%) and quaternary N (7.6%). Also worth of notice is the inexistence of N-oxides/ nitrates functionalities in this material. From the results of BET surface area measurements, the specific surface area of GF_N8 was 336 $m^2$ $g^{-1}$ leading to 8 layers per crystallite [123].

### 7.1.4. Algae growth inhibition assays procedure

Test solutions of fluoxetine at concentration of 15.2 and 43.3 mg $L^{-1}$ were prepared by dissolving the fluoxetine in powder directly in culture media. The dilutions series was prepared by mixing culture media with the test solutions of fluoxetine and then inoculating a subculture of exponential growing *R. subcapitata*. Agitation was performed manually twice a day and the test flasks position was randomised and changed daily to ensure a uniform exposure to the light. All the experiments were carried out in three replicates and blanks without algae were prepared for each different concentration. Fluoxetine concentration and the blanks were analysed at the beginning and at the end of the experiments to check if there is a significant loss of fluoxetine due to volatilization, degradation or adsorption by the flasks. Besides the same analysis was performed in the other assays (with alga), after centrifugation (Thermo Scientific, Fresco 21) at 13,500 rpm for 10 min to separate it from the solution, in order to verify if there is a mechanism of adsorption onto *R. subcapitata.*

NMs in powder were previous sterilized by autoclaving at 120 °C for 20 min inside the glass flasks. *R. subcapitata* in exponential phase of growth was exposed to medium with different concentrations of NMs. Application of ultrasounds before inoculation and continuous shaking (≈ 100 rpm) over the incubation period were performed to reduce aggregation and settlement of

NMs. The test flasks were incubated for 72 h under the same condition used for culturing. All the experiments were made in triplicate and controls (without test substance) were included. The test conditions are summarised in Table 7.2.

**Table 7.2** – Summary of test conditions.

| **Test conditions of the algal bioassay** | |
|---|---|
| Test type | Static non-renewable |
| Test organism | *Raphidocelis subcapitata* |
| Temperature | 21 ± 2 °C |
| Light quantity | White fluorescent lighting |
| Photoperiod | Continuous illumination |
| Test chamber size | 250 mL |
| Test solution volume | 50 mL |
| Replicate chambers | 3 |
| Stirring | Twice daily by hand (for ecotoxicity assessment of fluoxetine)<br>Continuous shaking at 100 rpm (for ecotoxicity assessment of NMs) |
| Test duration | 72 ± 2 h |
| Endpoint | Growth inhibition |
| Sampling | Test beginning and final |

### 7.1.5. Validity of the test

The acceptability of the bioassay was assessed based on validation criteria explicit in OECD Guideline 201 [38]. It recommends that algal biomass in control cultures should increase exponentially by a factor at least 16 within the 72 hours test period. Moreover, the mean coefficient of variation in control cultures between days 0-1, 1-2 and 2-3, for 72 hours of exposure, must not exceed 35%. The pH of solutions must be measured at the beginning of and at the end of the test and its variation should not exceed 1.5 units. Concentration of the test substance should be analysed to verify the initial concentration and its maintenance during the test, the variation of each solution concentration cannot exceed 20%.

### 7.1.6. Instrumental analysis

The pH was measured using a pH meter Crison 2002 (Spain) at the beginning and at the end of the test.

For the ecotoxicology assessment of NMs, conductivity was also measured using multiparameter analyser Consort C861 (Belgium).

The fluoxetine analysis were performed by High Performance Liquid Chromatography using a HPLC Prominance Shimadzu LC (Shimadzu Corporation, Japan) equipped with a 20AB LC pump, a DGU-20A5 degasser, a SIL 20A automatic injector, a CTO-20AC column oven and a RF-10AXL fluorescence detector. The analytes were separated on a Luna C18 chromatographic column (4.6 × 150 mm, 5 µm particle size) purchased from Phenomenex (USA). As mobile phase, ultra-pure water acidified with 0.1% formic acid (eluent A) and acetonitrile (eluent B) were used. A sample volume of 20 µL was injected. The flow rate of mobile phase was set to 1.0 mL/min. The run time was 16 min, eluent B increased from 10% to 100% within 5 minutes and held for 4 minutes, then decreased to 10% over 1 minute and held for 6 minutes. Fluorescence detection was performed at the excitation wavelength of 230 nm and at the emission wavelength of 290 nm, which is the optimal emission / excitation pair for fluoxetine. Data acquisition was carried out by LCsolution software (Shimadzu Corporation, Japan). The obtained limits of detection (LOD) and quantification (LOQ) for fluoxetine were 1.75 and 5.32 µg $L^{-1}$, respectively (Appendix A).

Biomass growth was estimated by chlorophyll fluorescence *in vivo* analysis as recommended by EPA [30] for its sensibility and simplicity. All essays were run is quadruplicate colleting a suspension volume of 0.25 mL of each sample. The fluorescence was measured with a microplate fluorometer using as excitation wavelength 485 nm and for emission 645 nm (Biotek Synergy HT microplate reader with Gen5 software version 2.0.18, USA) at the beginning of the test and after 72 hours.

## 7.2. Adsorption experiments

### 7.2.1. Kinetic studies

To determine the minimum time required for adsorption equilibrium, the adsorption kinetic experiments were conducted. Kinetic studies were performed at room temperature (20 °C), without pH adjustment, using a magnetic stirrer (Arec X, Heating Magnetic, Stirror, VELP Scientifics). A fluoxetine solution in the concentration range 4 - 5.5 mg/L was prepared in a volume of 250 mL. The experiments were conducted by placing 32 mg of GF_N8, 239 mg of bulk $C_3N_4$ and 111 mg of exfoliated $C_3N_4$ in each vessel. $C_3N_4$ in bulk and exfoliated forms were previously dispersed in 20 mL of deionized water by ultrasounds.

Samples (1 mL) were taken at different times during the experiment, which lasted for 140 minutes and were immediately centrifuged at 13 500 rpm for 10 minutes (Thermo Scientific, Fresco 21) to separate the adsorbent. Then, fluoxetine concentration in the supernatant was measured by HPLC in conditions described in 7.1.6. The pH was measured continuously over the experiment using a pH meter (Consort C861).

The amount of adsorption at time t was calculated by the following equation:

$$q_t = \frac{(C_0 - C_t)}{m} V \quad (8)$$

where $q_t$ (mg $g^{-1}$) is adsorption capacity of the NM, $C_0$ and $C_t$ (mg $L^{-1}$) are concentrations of fluoxetine at the beginning and at time t, respectively, m (g) is the mass of NM tested and V (L) is the volume of solution.

### 7.2.2. Adsorption isoterms studies

In the equilibrium experiments, portions between 1 and 30 mg of GF_N8 were stirred at constant speed for 75 minutes to ensure equilibrium (Arec X, Heating Magnetic, Stirror, VELP Scientifics) in Erlenmeyer flacks (with caps) with 50 mL of fluoxetine aqueous solution of 3.7 mg $L^{-1}$. Experiments were carried out at room temperature and without adjustment of pH. After 75 minutes, 1 mL of sample was taken and centrifuged at 13 500 for 10 minutes (Thermo Scientific, Fresco 21). The concentration of fluoxetine in solution was determined in triplicate by HPLC in conditions described in 7.1.6. The quantity adsorbed at equilibrium was calculated using eq. (8). A similar procedure was followed in the blank, without GF_N8.

### 7.2.3. Validation of kinetic models and isotherms

Kinetic and equilibrium parameters were estimated by non-linear regression using Curve Expert Professional software. Considering that the stirring speed used during the experiments was high enough to eliminate the external resistance to mass transfer, the influence of external film diffusion was not had in account for application of the models.

The applicability and suitability of kinetic and equilibrium models to the experimental data was evaluated by judging the values of the correlation coefficient, $R^2$, the variance, s, and the Akaike information criterion (A.I.C.). $R^2$ and A.I.C. values were calculated using Curve Expert Professional software. The s was calculated from equation (9) and a lower value indicates a better fit between experimental and calculated data.

$$s = \sqrt{\frac{\sum(q_{exp} - q_{cal)}{}^2}{n-1}} \tag{9}$$

Where n is the number of data points, $q_{exp}$ and $q_{cal}$ (mg $L^{-1}$) are the experimental and the calculated adsorption capacity values, respectively.

A.I.C. is a tool used to determine which model fits better the experimental data considering the number of parameters. The model with the lowest A.I.C. value is considered the most adequate to describe experimental data [124].

# 8. Results and Discussion

## 8.1. Ecotoxicity assessment

The toxic effects of fluoxetine as well as some NMs designed for water and wastewater treatment (GF_N8 and $C_3N_4$ in bulk and exfoliated forms) were evaluated based on the *R. subcapitata* growth inhibition. Thus, it was measured the chlorophyll content fluorometrically at the beginning and at the end of the bioassay and determined the growth inhibition using equation (10).

$$I(\%) = \frac{(\Delta Fc - \Delta F)}{\Delta Fc} \tag{10}$$

Where, I is the percentage of growth inhibition at each test substance/material, ΔFc is the variation of average fluorescence of algal control cultures and ΔF is the variation of average fluorescence of algal cultures exposed to the test chemical/material.

All the bioassays conducted with *R. subcapitata* agreed with the validation and acceptability criteria stipulated by OECD (2006) [38]. At the end of 72 hours, the algal growth in the control cultures increased at least 16-fold. For all tests, the coefficient of variation of growth within controls was less than 7%. Furthermore, as shown in Appendix B, the pH recorded at the beginning and at the end of the test did not vary significantly.

In case of the toxicity assessment of NMs it was also measured the conductivity and the beginning and at the end of the growth inhibition test and the results are revealed in Appendix C, showing that there is no consistent increase and therefore no significant leaching of compounds from the NMs.

### 8.1.1. Fluoxetine

Data summarized in Table 8.1 shows that the test substance at higher concentrations remains almost 80% of the initial concentration value, indicating that there was no significance degradation or adsorption of fluoxetine by algae. The significant variations associated at lower concentrations are explained by their proximity to the detection limit of HPLC (Appendix A).

It was not possible to use another analytical method with a lower limit of quantification, such as LC-MS/MS. In preliminary studies, fluoxetine was injected in the LC-MS/MS and some problems have arisen which did not allow to continue using LC-MS/MS for the analysis of fluoxetine in the culture medium. In the electrospray ionization, the solution containing the analyte of interest was dispersed by electrospray with nitrogen into a fine aerosol. As fluoxetine was prepared in culture medium the salts were also concentrated and blocked MS entrance of

the mass spectrometry detector due to the deposit in the probe that will hinder proper ionization. Thus, fluorescence detector was used to analyse fluoxetine**.**

The observed dose-response data were fitted by nonlinear regression to a log-Probit model (equation 11), as suggested by OCDE guideline 201 [38], using CurveExpert Professional software version 2.6.

$$I = \gamma + (1-\gamma)[1 + \mathrm{erf}\left(\frac{\alpha + \beta lnC}{\sqrt{2}}\right)] \tag{11}$$

Where I is the response in terms of growth inhibition, γ, α and β are the parameters of the log-Probit model and C is the concentration of fluoxetine.

**Table 8.1** – Variation of fluoxetine concentration in replicates and blanks.

| Concentration of blanks (μg $L^{-1}$) | | Variation (%) | Mean concentration of replicates (μg $L^{-1}$) | | Variation (%) | Growth inhibition (%) |
|---|---|---|---|---|---|---|
| Initial | Final | | Initial | Final | | |
| 70.6 | 71.9 | 1.8 | 69.8 | 62.9 | -8.0 | 93 |
| 29.1 | 20.0 | -45.6 | 16.6 | 15.3 | -8.7 | 78 |
| 10.1 | 10.6 | 4.4 | 10.3 | 11.4 | 9.4 | 43 |
| 6.62 | 6.87 | 3.6 | 9.6 | 11.6 | 16.7 | 58 |
| 1.1* | 1.0* | -1.8 | 2.7* | 0.78* | -71.5 | 8 |
| 0.84* | 1.4* | 1.4 | 1.1* | 0.59* | -45.2 | 2 |

*estimated values by HPLC, because they are below the quantification limits

The dose-response curve obtained exhibits a good fit with experimental data with a $R^2$ of 0.963 from which were estimated the effective concentration of fluoxetine that inhibits 10, 20 and 50% of algae growth after 72 hours of exposure to fluoxetine (Figure 8.1). It was estimated an $EC_{10}$, $EC_{20}$, $EC_{50}$ values towards *R. subcapitata* growth of 3.3, 4.3 and 9.0 μg $L^{-1}$, respectively. These results evidence that low concentrations of fluoxetine cause significant adverse effects on algal population.

Literature reports regarding the toxicity of fluoxetine to *R. subcapitata* vary considerably as shown in Table 4.3. The reasons for this deviation may be related with the use of different *R. subcapitata* strains in biotests, variations of test conditions (temperature, light, culture medium) among laboratories, divergency in choice of endpoint, analytical method for measurement of

algal biomass as well as exposure times, and application of distinct mathematical models for plotting dose-response curve.

Mingez *et al.* obtained an $EC_{50}$ value of 200 µg $L^{-1}$ towards *R. subcapitata* [79]. Similarly to this study, cell density was measured after 72 hours of exposure and the growth inhibition was calculated based on the differences on chlorophyll fluorescence between the beginning and the end of the test. However, in study carried out by Minguez *et al*., the toxicity test was performed in 96-well culture plates, while in this study, toxicity test was performed in Erlenmeyer flasks. It has been demonstrated that microplates essays generate smaller toxic responses than flasks essays under the same conditions and this may be the reason for the discrepancy between these studies [60].

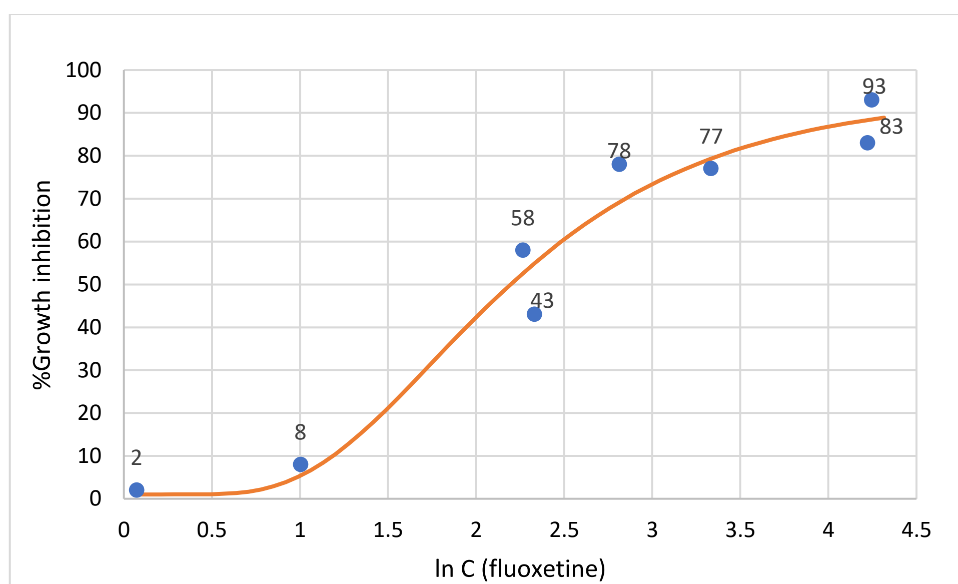



**Figure 8.1** – Dose-response curve of *Raphidocelis subcapitata* to fluoxetine after 72 hours of exposure.

Toxicity data derived using OECD Guideline may be used to classify substances for the hazards they present to the aquatic environment according to Globally Harmonized System (GHS). Toxicity levels of substances are classified based on the acute toxicity data in which an $EC_{50}$ < 1 mg $L^{-1}$ would entail the classification *very toxic to aquatic life* (category 1), from 1 to 10 mg $L^{-1}$ *toxic to aquatic life* (category 2) and from 11 to 100 mg $L^{-1}$ *harmful for aquatic life* (category 3). On the basis of this scheme, and based on these results for one single species, the acute toxicity of fluoxetine is category 1 – very toxic for aquatic life [125].

Fluoxetine has been measured in treated wastewater at concentrations in range 13-99 ng $L^{-1}$ [64] which is significantly less than the $EC_{50}$ of 9.0 µg $L^{-1}$ obtained in this study. The $EC_{10}$ of 3.3 µ $L^{-1}$ is approximately 34 times higher than the reported environmental concentration. To confirm the little effect that environmental concentration of fluoxetine poses to phytoplankton

population, it was also performed an ecotoxicological assessment with 35 ng $L^{-1}$ of fluoxetine (Figure 8.2). In this case, no significant adverse effect on *R. subcapitata* growth was observed. After 72 and 96 hours of exposure, growth inhibition was -10% and -2%, respectively. However, it should be noted that at environmental conditions (pH, temperature and interaction with other toxicants) may enhance the toxicity of fluoxetine.

In order to better understand the toxicity mechanism of algae to fluoxetine it was assessed the growth inhibition of *R. subcapitata* daily, for 6 days, and results are shown in Figure 8.2. After 1 day of inoculation it was noticed a stimulation of the algal growth when exposed to 0.035 and 69.8 µg $L^{-1}$ of fluoxetine. The same response was visible later, after 2 days of inoculation, for concentrations from 1.1 to 5.9 µg $L^{-1}$ reaching a stimulation between 105 and 86%. These results may be indicative of protective mechanism of *R. subcapitata* against external stress induced by fluoxetine at the initial stage of exposure. This phenomenon is known as hormesis and it is characterized by low concentration stimulation and high concentration inhibition [60]

After 3 days of exposure, the concentration response function reveals the same toxic pattern for all concentrations, an increasing of growth inhibition of algae to increasing concentrations of fluoxetine. At the end of the bioassay, after 6 days of exposure, it was verified that concentrations of fluoxetine between 0.035 and 2.7 µg $L^{-1}$ did not have a significant impact in algal growth while 5.9 and 69.8 µg $L^{-1}$ caused 23 and 99% of growth inhibition, respectively.

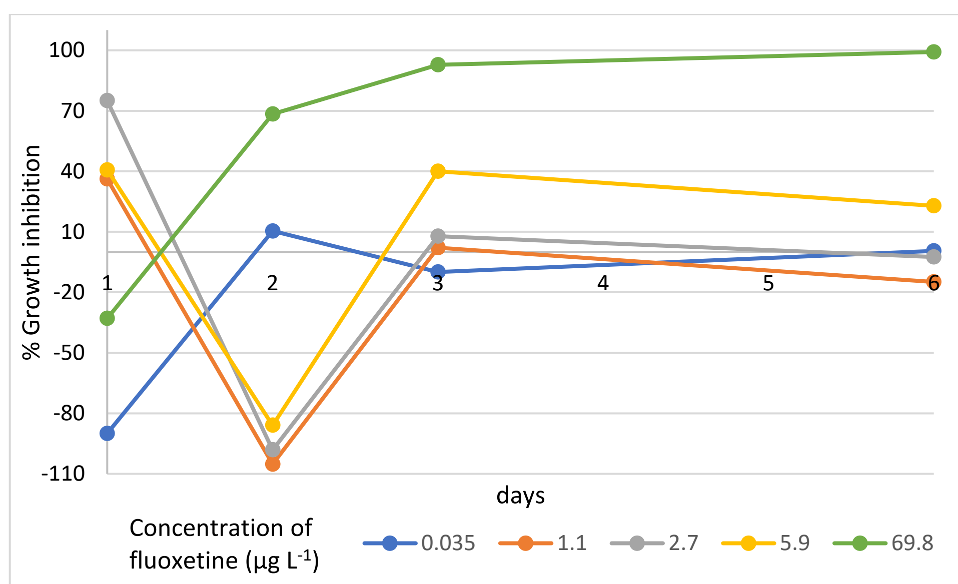



**Figure 8.2** – Dose-response relationship curve from growth inhibition test of fluoxetine after 1, 2, 3 and 6 days.

### 8.1.2. GF_N8

Hill equation was chosen to fit the results of the ecotoxicity tests by non-linear regression, which allowed the estimation of the concentrations of GF_N8 that induce 10, 20 and 50% growth inhibition. The mathematical form of the Hill model used by the Curve Expert Professional software is explicit in equation 12 and its plotting is shown in Figure 8.3. Responses reported with 2 and 3 mg $L^{-1}$ of GF_N8 (red points in Figure 8.3) were excluded from the regression, because these concentrations suggest a different pattern of toxicity.

$$I = \alpha + \frac{\theta C^{\eta}}{\kappa^{\eta} + C^{\eta}} \quad (12)$$

Where I is the response in terms of growth inhibition, α, $\theta$, $\kappa$ and $\eta$ are the parameters of the Hill model and C is the concentration of fluoxetine leading to growth inhibition.

Based on dose-response curve shown in Figure 8.3 the $EC_{10}$, $EC_{20}$ and $EC_{50}$ for GF_N8 were found to be 34.6, 39.7 and 45.3 mg $L^{-1}$ for 72h of exposure. The highest tested concentration (80 mg $L^{-1}$) resulted in 90% growth inhibition.

It was visible that GF_N8 MNs has a strong tendency to aggregation, mostly in higher concentrations (16 – 80 mg $L^{-1}$), demonstrating that aggregate formation may be concentration-dependent. There is a general hypothesis that ecotoxicity of NMs are related with its surface area. Aggregation phenomena lead to a reduction of contact surface area of NMs and, subsequently, ecotoxicity [45]. As shown in Figure 8.3, the smallest concentrations tested leaded to a growth inhibition higher than expected that may be explain by the absence of aggregation.

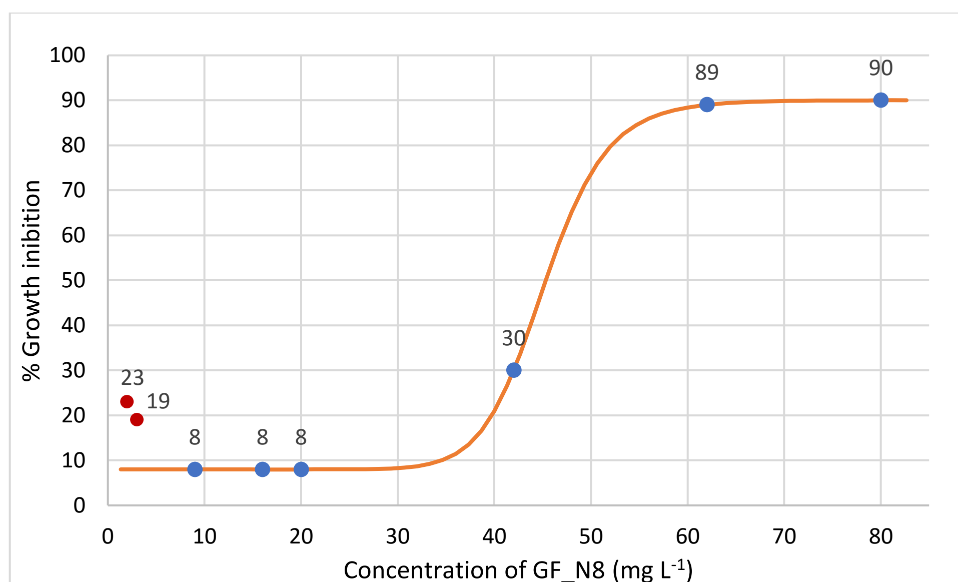



**Figure 8.3** – Dose-response curve of *Raphidocelis subcapitata* to GF_N8 NMs for 72 hours of exposure.

### 8.1.3. Bulk $C_3N_4$

*R. subcapitata* in exponential phase growth was inoculated into fresh culture medium with bulk $C_3N_4$ NMs with concentrations ranging from 9 to 285 mg $L^{-1}$. From the results exposed in Figure 8.4, no recognizable dose-response relationship pattern was observed. At the lowest concentration tested (9 mg $L^{-1}$) it was reached 10% of growth inhibition, while at 25 and 101 mg $L^{-1}$ no significant inhibitory effects on the 72 hours growth of *R. subcapitata* were observed. When the tested concentration of bulk $C_3N_4$ was increased almost three times (285 mg $L^{-1}$) it was reached 15% of growth inhibition, indicating the low toxicity of this material towards *R. subcapitata* even at high concentrations.

These results show that toxicity decreased with increasing of concentrations of bulk $C_3N_4$, but only until a certain level, which may be related with aggregation phenomena. Probably at lower concentrations, less particles aggregate, and these non-aggregated particles seems to be more toxic than aggregated nano-size particles. At higher concentrations, the likelihood of aggregation increases which lead to a decrease of available specific surface area of bulk $C_3N_4$ and, consequently, a decrease of its toxicity. The highest growth inhibition measured at very high concentrations of bulk $C_3N_4$ shown in Figure 8.4 may be because an overloading of this NM in the cells and/or due to shading effects in culture. These evidences were already reported by some ecotoxicological studies with other types of NMs [95,126].

Although it is not possible to calculate the 72h-$EC_{10}$ value due to the atypical dose-response relationship, it probably lays in the range 25-285 mg $L^{-1}$. It was made an attempt to estimate $EC_{10}$ value using a linear regression model excluding the results for 9 and 102 mg $L^{-1}$ (Appendix D), which originated a 144 mg $L^{-1}$ value.

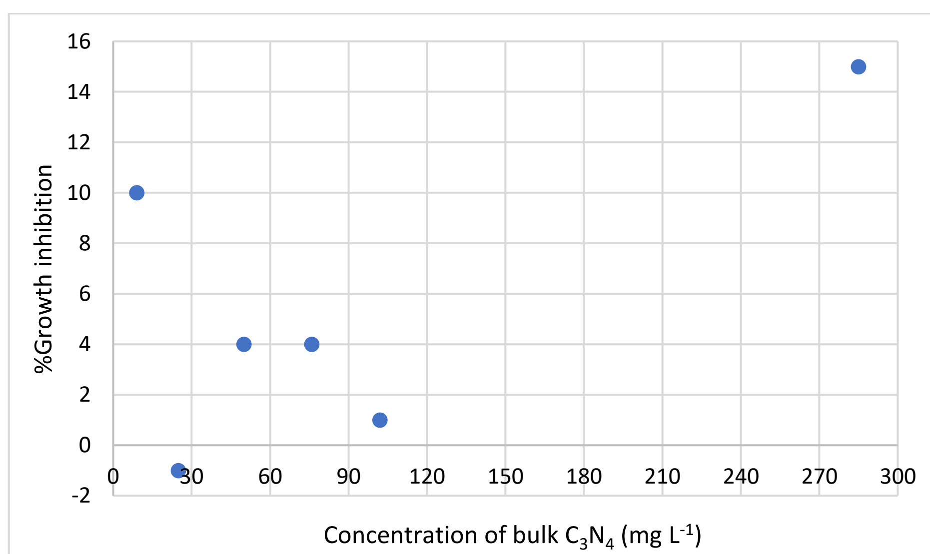



**Figure 8.4 -** Dose-response curve of *Raphidocelis subcapitata* to bulk $C_3N_4$ NMs for 72 hours of exposure.

### 8.1.4. Exfoliated $C_3N_4$

Figure 8.5 shows the 72 hours growth inhibition of *R. subcapitata* caused by different concentrations of $C_3N_4$ in exfoliated form. Except for 41 mg $L^{-1}$ of exposure concentration, it was visible a slight increase in toxicity towards *R. subcapitata* with the increasing of concentration of $C_3N_4$ reaching 19% growth inhibition when cells were exposed to 101 mg $L^{-1}$. Furthermore, it was also observed a slight stimulation of algae growth at the lowest tested concentration which may be attributed to an adaptive response of algae to toxicants exposure [105].

$C_3N_4$ in exfoliated form is clearly more toxic towards *R. subcapitata* than the bulk counterpart. For concentrations between 9 and 101 mg $L^{-1}$ of $C_3N_4$ the highest inhibition growth achieved was 10% in case of bulk form and 19% for the exfoliated form. These results support previous findings that toxicity towards microalgae are also related to the size of NMs. Lower toxicity are observed for NMs possessing a larger particle size [105,127].

Due to the response obtained for a 41 mg $L^{-1}$ concentration, it was not found a typical dose-response relationship for exfoliated $C_3N_4$ and, and for that reason it is not possible to calculate $EC_{10}$ and $EC_{20}$. However, rejecting the result for 41 mg $L^{-1}$ and using a linear regression it was estimated $EC_{10}$ and $EC_{20}$ values of 27 and 103 mg $L^{-1}$, respectively (Appendix E).

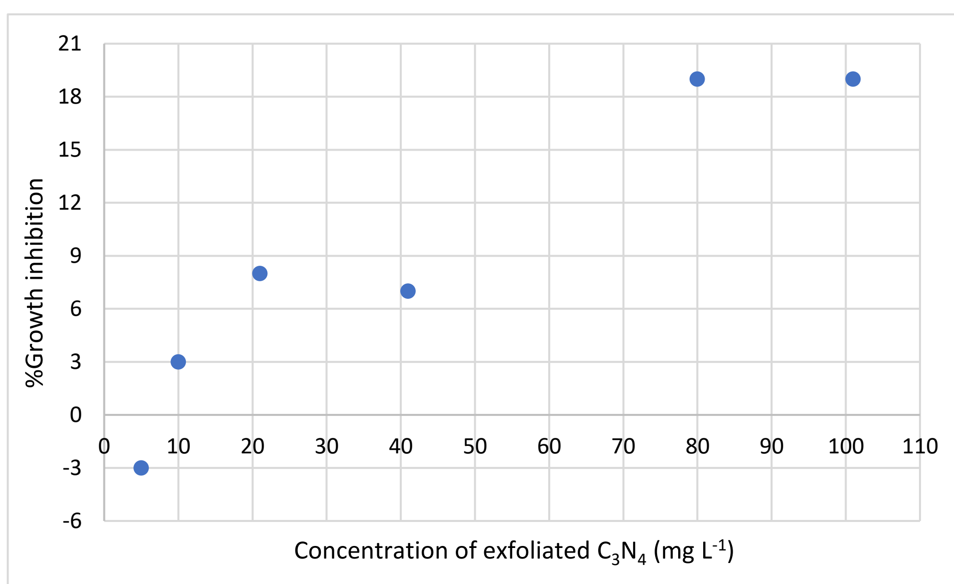



**Figure 8.5 -** Dose-response curve of *Raphidocelis subcapitata* to exfoliated $C_3N_4$ NMs for 72 hours of exposure.

## 8.2. Adsorption of fluoxetine onto NMs

In parallel to the determination of the effects of NMs on *R. subcapitata* growth, it was also investigated the adsorption of fluoxetine onto NMs. Studies of kinetic and equilibrium of

adsorption are an important feature to be considered in aqueous effluent treatments as it provides the time required for attaining equilibrium and the rate-limiting step of adsorption process.

### 8.2.1. GF_N8

- Kinetic study

A Kinetic experiment was carried out using 4 mg $L^{-1}$ fluoxetine solution containing 32 mg GF_N8 for a period of 140 minutes at 295 K. The pH was monitored, from the initial value of 4.5 up to the final value of 5.3.

The variation of the amount of fluoxetine adsorbed on GF_N8 ($q_t$) is shown in Figure 8.6. It is perceptible an initial fast adsorption in the first 9 minutes reaching 68% of removal. With the progress of the experiment, the rate of fluoxetine adsorption gradually slowed down until equilibrium was attained, after 45 minutes. The maximum percentage removal of fluoxetine from the solution by adsorption was 77% corresponding to the equilibrium stage. The rapid adsorption rate at the initial stage may be attribute to the high availability of active sites on the GF_N8 surface which are progressively occupied by fluoxetine molecules leading to a decrease of adsorption rate.

To better understand the controlling mechanism of the adsorption process, pseudo-first order, pseudo-second order and Elovich kinetic models were employed to fit the experimental data. Results are displayed in Figure 8.6 while the fitting parameters of the models estimated using non-linear curve fitting procedure are presented in Table 8.2. The pseudo-first order kinetic model is not shown because this model was not able to fit the experimental values.

From the plots is clear that the data does not fit well to the pseudo-second order model, as can be confirmed by the low value of the $R^2$ (0.575), indicating that chemisorption is not mechanism of adsorption. This conclusion is supported by the XPS spectra of GF_N8 which evidenced the absence of functional groups on the surface [123].

It was found that Elovich model fits better kinetic data than pseudo-second order model for the description of kinetic data, presenting the highest $R^2$, and the lowest s and A.I.C. values. Although Elovich model does not provide any mechanistic evidence about adsorption process, it is an indication of the heterogenous surface of GF_N8 and multilayer adsorption.

In spite of the better agreement of experimental data in statistical terms with Elovich model, it is necessary to check if the obtained parameters have a physical meaning. The 1/ β value gives an estimation of the number of sites available for adsorption. As shown in Table 8.2,

β value is low, hence, uptake of fluoxetine onto GF_N8 may have been through functional groups. However, this hypothesis is rejected by XPS analysis which confirms that GF_N8 does not have functional groups. Additionally, the constant α value obtained using this model does not have a statistical meaning due to the high confidence level associated to it.

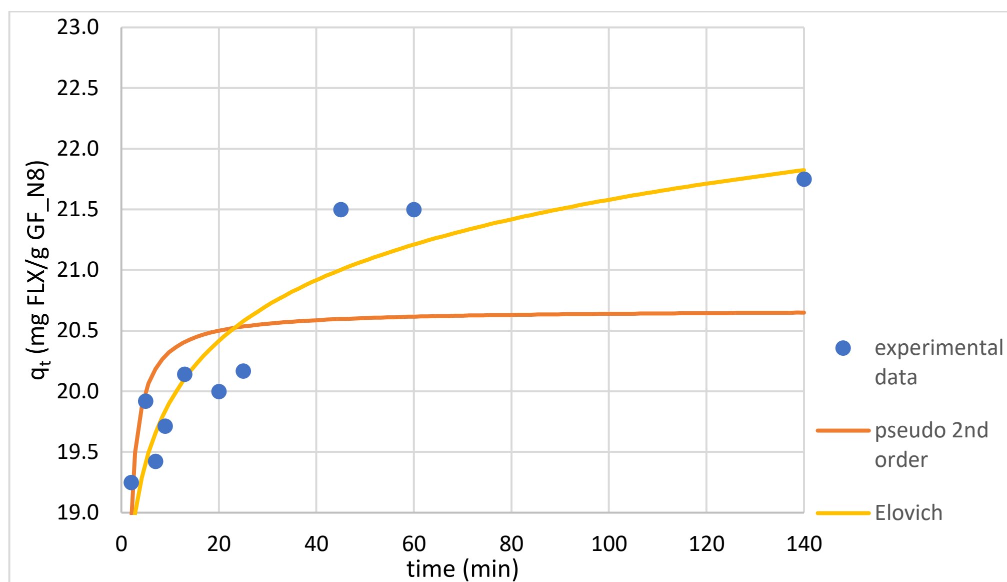



**Figure 8.6** – Comparison between the measured and modelled time profiles for fluoxetine adsorption onto GF_N8.

**Table 8.2** – List of kinetic model parameters values for adsorption of fluoxetine onto GF_N8 with confidence level of 95%.

| Pseudo 2nd order | | Elovich | |
|---|---|---|---|
| **$q_e$** (mg/g) | 20.7 ± 0.3 | **α** (mg/g min) | $6.6 \times 10^{10} \pm 2 \times 10^{11}$ |
| **$k_2$** ($min^{-1}$) | 0.29 ± 0.09 | **β** (mg/g) | 1.4 ± 0.2 |
| **$R^2$** | 0.575 | **$R^2$** | 0.882 |
| **s** | 0.771 | **s** | 0.406 |
| **A.I.C.** | -6.04 | **A.I.C.** | -21.4 |

The possibility of mass transfer resistance affecting adsorption process was explored using the intraparticle diffusion model. Thus, fitting the intraparticle diffusion model to the experimental data was performed by plotting $q_t$ versus the square root of time [122].

Figure 8.7 is the plot of $q_t$ versus $t^{0.5}$ and, the multilinearity obtained confirm that adsorption process occurred in three separate stages which can be attribute to each linear regression. The first stage was the fastest sorption stage and it is assigned to the diffusional process of the fluoxetine on the GF_N8 external surface, which was expected due to high stirring

rate used in the experiments that minimized the external mass transfer resistance. The second stage characterizes the intraparticle diffusional of fluoxetine within GF_N8 pores, which should be the rate-limiting step, and the third stage is ascribed the adsorption of fluoxetine on active sites of GF_N8.

Since the plot did not pass through the origin, intraparticle diffusion was not the only rate-limiting step. The low linearity of the second stage also lend crease to the assertion of this conclusion, suggesting that other processes may control the rate of adsorption. The adsorption of fluoxetine on the available active sites of GF_N8, represented in third stage (Figure 8.7), seems to control the rate of adsorption, as evidenced by the high value of $R^2$, however it should be taken in account that the adjust considers only 3 experimental points.

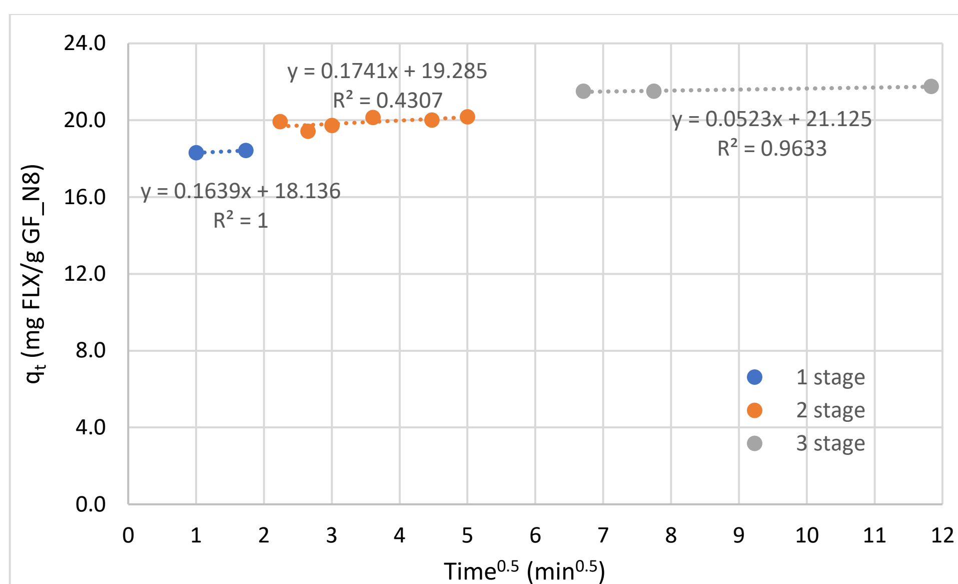



**Figure 8.7** – Plots of intraparticle diffusion model for the adsorption of fluoxetine onto GF_N8.

- Equilibrium study

Equilibrium isotherms are frequently applied to provide some insights into salient features of the adsorption process. For this purpose, the Langmuir, Freundlich and Sips isotherm models were assayed. A comparison of nonlinear fitted curves from experimental data are depicted in Figure 8.8 and the fitted parameters for the best models are listed in Table 8.3. The Langmuir isotherm did not correlate the experimental equilibrium satisfactorily as shown is Figure 8.8.

The highest adsorption capacity observed was 23.5 mg $g^{-1}$ which correspond to 26% of removal. The relatively high value of $R^2$ (> 0.95) of Freundlich and Sips isotherms confirmed its applicability for describing the experimental equilibrium. The good fit of these isotherms to the experimental data indicate multilayer adsorption at low adsorbent concentrations.

Although Sips seems to be slightly better than Freundlich's model, it is not possible to conclude which is the best fitting model, because Freundlich and Sips isotherms have different number of parameters, respectively two and three, and both fittings are statistically similar and almost superposed (Figure 8.8).

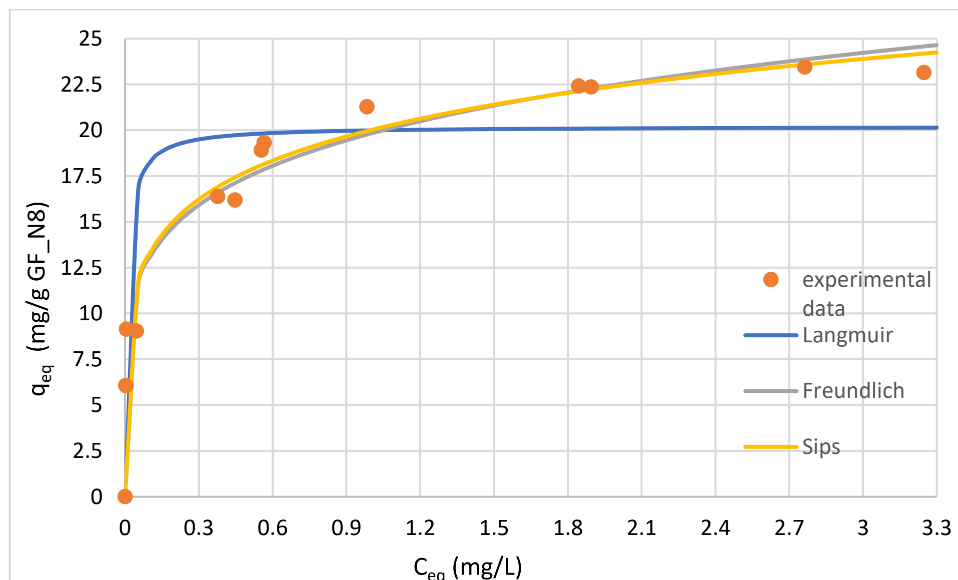



**Figure 8.8** – Adsorption isotherm models fitted to the experimental adsorption of fluoxetine onto GF_N8.

**Table 8.3** - List of isotherm parameters values for adsorption of fluoxetine onto GF_N8 with confidence level of 95%.

| Langmuir | | Freundlich | | Sips | |
|---|---|---|---|---|---|
| **Q** (mg/g) | 20.2 ± 1.1 | **$K_F$** (mg/g (mg/dm$^3$)$^{-1/nF}$) | 19.8 ± 0.4 | **$Q_S$** (mg/g) | 70.4 ± 66.1 |
| **$K_L$** (L/mg) | 91.7 ± 46.2 | **$n_F$** | 5.5 ± 0.4 | **$K_S$** (L/mg) | 0.40 ± 0.53 |
| | | | | **$n_S$** | 4.27 ± 1.15 |
| **$R^2$** | 0.833 | **$R^2$** | 0.967 | **$R^2$** | 0.979 |
| **s** | 3.29 | **s** | 1.25 | **s** | 1.21 |
| **A.I.C.** | 33.5 | **A.I.C.** | 6.02 | **A.I.C.** | 7.03 |

### 8.2.2. Bulk $C_3N_4$

- Kinetic study

The effectiveness of bulk $C_3N_4$ in adsorbing fluoxetine was investigated mixing 239 mg of bulk $C_3N_4$ with 4.2 mg $L^{-1}$ aqueous solution of fluoxetine for 90 minutes at room temperature. It was measured a decreasing of pH value during the experiment from an initial value close to 7 until the final value around 6.

The data gathered in this experiment is present in Figure 8.9. The highest value for $q_t$ was 0.38 mg $g^{-1}$ and it was reached after 5 minutes of the adsorption process. The removal efficiencies achieved was between 2% and 4%. The low removal efficiencies caused large error in $q_t$ results what make results inconclusive.

Results from this experiment reveal that bulk $C_3N_4$ does not have adsorption properties. A characteristic analysis of $C_3N_4$ should be accomplished to reveal its surface and explain the ineffectiveness for fluoxetine adsorption.

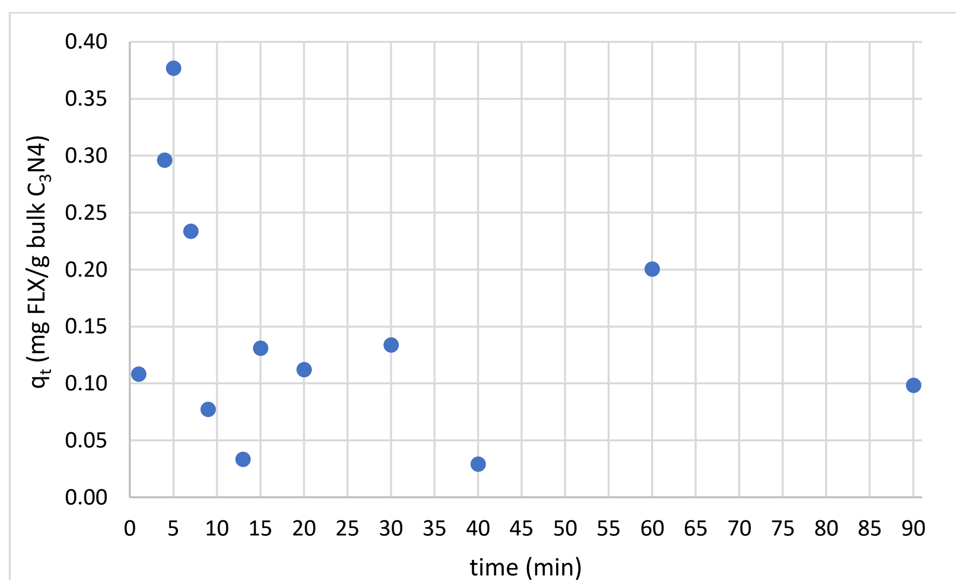



**Figure 8.9** – Adsorbed amount of fluoxetine as function of time on bulk $C_3N_4$.

### 8.2.3. Exfoliated $C_3N_4$

- Kinetic study

Adsorption kinetic experiment were conducted at room temperature adding 111 mg of exfoliated $C_3N_4$ to a solution of fluoxetine with a concentration of 4.85 mg $L^{-1}$. The initial pH was close to 6 which decreased to the final value around 5.

Figure 8.10 shows the variation of the amount of adsorbed fluoxetine onto $C_3N_4$ exfoliated ($q_t$) in function of the time. The plots in the Figure 8.10 shows that the adsorption increased with time until the equilibrium was attained, after 60 min, achieving the highest observed adsorption capacity (0.96 mg $g^{-1}$) and 8.7% of fluoxetine was removed from the solution. In the initial stage was observed a faster adsorption rate due to the higher concentration gradient and the large number of available active sites which make fluoxetine transfer swiftly to exfoliated $C_3N_4$. With the time the number of active sites for adsorbing fluoxetine decrease and after 20 min adsorption rate slowed down.

In order to better understand the adsorption kinetics, pseudo-first order, pseudo-second order and Elovich model were employed to fit experimental data. The kinetic curves are presented in Figure 8.10 and the various parameters obtained from the three kinetic models are listed in Table 8.4. It was verified that $q_e$ value found in pseudo-second order model was very close to $q_e$ experimental with a variation lower than 1.4%. However, statistical analysis suggests that Elovich model fits better the experimental data and it may describe the adsorption process of fluoxetine onto exfoliated $C_3N_4$. The Elovich model does not predict any adsorption mechanism but is useful in describing adsorption on heterogenous adsorbents.

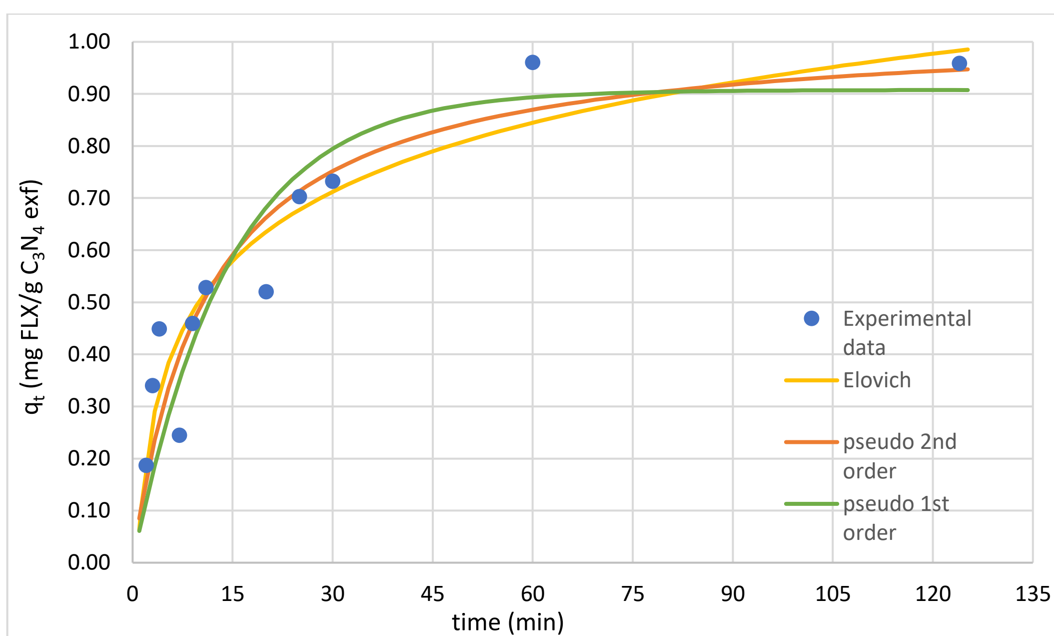



**Figure 8.10** - Comparison between the measured and modelled time profiles for fluoxetine adsorption onto exfoliated $C_3N_4$.

**Table 8.4** - List of kinetic model parameters values for adsorption of fluoxetine onto exfoliated $C_3N_4$ with confidence level of 95%.

| Pseudo 1st order | | Pseudo 2nd order | | Elovich | |
|---|---|---|---|---|---|
| $q_e$ (mg/g) | 0.91 ± 0.09 | $q_e$ (mg/g) | 1.0 ± 0.1 | α (mg/g min) | 0.26 ± 0.65 |
| $k_1$ ($min^{-1}$) | 0.070 ± 0.017 | $k_2$ ($min^{-1}$) | 0.087 ± 0.033 | β (mg/g) | 5.2 ± 0.1 |
| $R^2$ | 0.796 | $R^2$ | 0.854 | $R^2$ | 0.877 |
| s | 0.124 | s | 0.105 | s | 0.0966 |
| A.I.C. | -45.6 | A.I.C. | -49.3 | A.I.C. | -51.2 |

Figure 8.11 shows the plots of intraparticle diffusion model with multilinear profiles. Considering that exfoliated $C_3N_4$ is a material with high specific surface area, the first region, at the first 4 min, shows a fast adsorption due to the adsorption of fluoxetine on the external surface of the adsorbent and lamellar interspaces. At the second stage, where the intraparticle diffusion is the rate-limiting step, it was observed a gradual adsorption. At the last region adsorption in the active sites occurs.

In figure 8.11 the plots do not pass through the origin, indicating that intraparticle diffusion is not the sole rate-limiting step. However, the high linearity of the second stage shows that intraparticle diffusion has strong influence on adsorption rate.

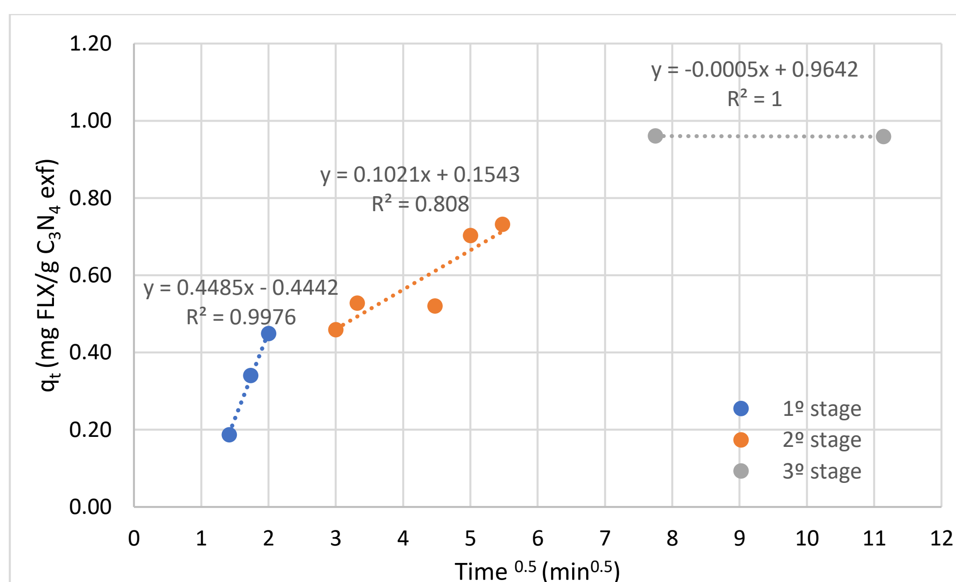



**Figure 8.11** - Plots of intraparticle diffusion model for the adsorption of fluoxetine onto exfoliated $C_3N_4$.

- Equilibrium study

The low removal efficiency (< 10%) as well as the low adsorption capacity at equilibrium (0.96 mg $g^{-1}$) obtained in kinetic experiments show that exfoliated $C_3N_4$ is not a good absorbent. For that reason, equilibrium experiments were not carried out.

# 9. Conclusion and future perspectives

## 9.1. Conclusion

In the presented study, the antidepressant fluoxetine was tested towards the freshwater microalgae *Raphidocelis subcapitata* according to the OECD Guideline 201. Adverse effects of fluoxetine on *R. subcapitata* growth were observed at low µg $L^{-1}$ levels after 72 hours of exposure as evidenced by the $EC_{10}$, $EC_{20}$ and $EC_{50}$ values of 3.3, 4.3 and 9.0 µg $L^{-1}$, respectively. According to the *Globally harmonized system of classification and labelling of chemicals*, fluoxetine may be classified as very toxic to the aquatic life. With the aim to determine if environmental levels of fluoxetine concentration impact microalgae growth, it was also conducted an ecotoxicological assessment with 35 ng $L^{-1}$. In this case, a stimulatory effect in growth was observed after 24 hours of exposure and after 72 and 96 hours of exposure growth inhibition was -10% and -2%, respectively. These results indicate the hazards of pharmaceuticals to the environment and the urgency of developing a new treatment for their removal from water.

Conventional water treatments are not able to entirely degrade fluoxetine and other pharmaceutical compounds that end up in the environment. Photocatalysis processes using NMs may be the solution for resolving this problem. So, it was assessed the potential effect of new NMs (GF_N8 and $C_3N_4$ in exfoliated and bulk forms) on the environment, special in the aquatic compartment, to evaluate if these NMs are appropriate for a safe use in WWTPs.

Evaluation of the toxicity of NMs on microalgae growth revealed that GF_N8 was the most toxic with 72 h-$EC_{50}$ value of 45.3 mg $L^{-1}$, followed by exfoliated $C_3N_4$ and bulk $C_3N_4$. It was not found a typical dose-response relationship for $C_3N_4$, but when microalgae were exposed to 100 mg $L^{-1}$ of $C_3N_4$ in exfoliated and bulk forms 7% and 1% of growth inhibitions were observed, respectively. According to the results obtained, $C_3N_4$ NMs seemed to be the one with less risks to the receiving aquatic system, if accidental releases to the environment occur after being used in WWTPs.

Adsorption of fluoxetine on GF_N8, bulk $C_3N_4$ and exfoliated $C_3N_4$ have been also tested. Elovich equation model provided the best correlation of the experimental data of adsorption kinetic of GF_N8, however, the estimated parameters did not have a statistical meaning and were in discordance with characterization analyses. Kinetic experiments also showed that beyond intraparticle diffusion other mechanisms may control the rate of adsorption. Furthermore, it was needed 45 minutes to reach the equilibrium state. Regarding equilibrium, Freundlich and Sips were the best fits for fluoxetine adsorption on GF_N8 and the highest adsorption capacity observed was 23.5 mg $g^{-1}$ (2.8 mg $L^{-1}$ equilibrium concentration) which correspond to 26% of removal.

Bulk $C_3N_4$ was the NMs with the lowest adsorption capacity removing only 4% of fluoxetine. The exfoliation of $C_3N_4$ enhanced its adsorption proprieties increasing fluoxetine removal from 4% to 9% and the corresponding adsorption capacity from 0.20 mg $g^{-1}$ (3.7 mg $L^{-1}$ equilibrium concentration) to 0.96 mg $g^{-1}$ (4.4 mg $L^{-1}$ equilibrium concentration). The kinetics of adsorption of fluoxetine onto exfoliated $C_3N_4$ can be described by Elovich model suggesting the heterogeneity of the surface of this material. In this case, the intraparticle diffusion had stronger influence controlling the adsorption process, but, likely adsorption onto GF_N8, is not the only rate-limiting step.

Taking this in consideration, GF_N8 has been designed and fabricated to satisfy photocatalytic process and it was visible from adsorption experiments that GF_N8 also possesses adsorption proprieties. However, from the ecotoxicological point of view, GF_N8 may be classified as harmful for aquatic life. On the other hand, $C_3N_4$ benefits from its low toxicity, however, its mechanism of removal of pharmaceuticals is restricted to photocatalysis.

## 9.2. Future perspectives

The results showed that the toxicity of pharmaceuticals as well as NMs should not be neglect, but there are still many topics that need clarification or improvement.

First of all, the toxicity of an effluent after treatment with photocatalysis should be assessed in order to confirm the total mineralization of the pharmaceutical compounds and to evaluate the toxicity of possible transformation products formed during the treatment process.

In future experiments, it would be interesting to confirm the aggregation of GF_N8 in algal culture medium and the possible agglomeration of this NM with algal cells by scan electron microscopy, which could give an insight about the mechanism toxicity of GF_N8. Although $C_3N_4$ NMs did not cause significant adverse effect on the *R. subcapitata* growth, it would be important to confirm that no changes or relevant abnormalities were found in algal cells.

In order to generate toxicity data, studies of chronic toxicity using organisms of different trophic levels would be useful to further study the ecotoxicity of NMs and to investigate the cascading effects of NMs through accumulation along the food chain.

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# Appendix

## Appendix A: Calibration curve of fluoxetine used in HPLC analysis

Calibration curve (Figure A.1) used to determine fluoxetine concentration and its respective determination coefficient ($R^2$), limits of detection (LOD) and limits of quantification (LOQ) are listed above.

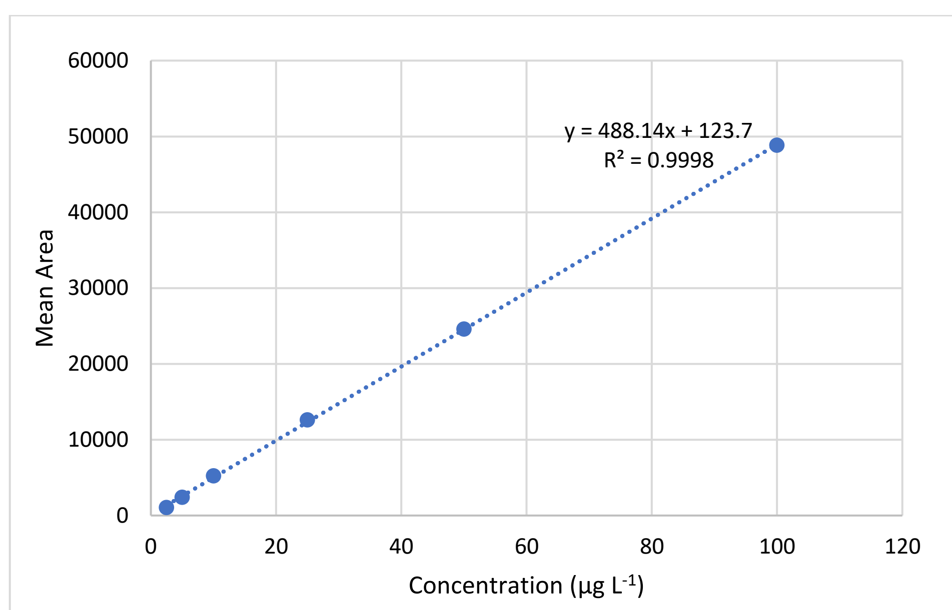


**Figure A.1-** Calibration curve of fluoxetine.

| $R^2$ | LOD | LOQ |
|---|---|---|
| 0.9998 | 1.75 µg $L^{-1}$ | 5.32 µg $L^{-1}$ |

## Appendix B: pH values at the beginning and at the end of the growth inhibition test

**Table B.1-–** pH measurements of growth inhibition test with fluoxetine.

| Concentration of fluoxetine (μg $L^{-1}$) | Initial pH value | Final pH value | Variation |
|---|---|---|---|
| 69.8 | 7.39 | 7.18 | -0.21 |
| 68.1 | 7.70 | 7.32 | - 0.38 |
| 28.0 | 7.70 | 7.28 | -0.42 |
| 16.6 | 7.41 | 7.24 | -0.17 |
| 10.3 | 7.42 | 7.15 | -0.27 |
| 9.6 | 7.43 | 7.18 | -0.25 |
| 2.7 | 7.53 | 7.47 | -0.06 |
| 1.1 | 7.52 | 7.41 | -0.11 |
| 0.035 | 7.71 | 7.60 | -0.11 |

**Table B.2-** pH measurements of growth inhibition test with GF_N8.

| Concentration of GF_N8 (mg $L^{-1}$) | Initial pH value | Final pH value | Variation |
|---|---|---|---|
| 9 | 7.34 | 7.26 | -0.08 |
| 16 | 7.50 | 7.27 | -0.23 |
| 20 | 7.50 | 7.27 | -0.23 |
| 42 | 7.34 | 7.21 | -0.13 |
| 62 | 7.71 | 7.80 | 0.09 |
| 80 | 7.70 | 7.80 | 0.1 |

**Table B.3-** pH measurements of growth inhibition test with bulk $C_3N_4$.

| Concentration of bulk $C_3N_4$ (mg $L^{-1}$) | Initial pH value | Final pH value | Variation |
|---|---|---|---|
| 9 | 7.65 | 7.40 | -0.25 |
| 25 | 7.70 | 7.45 | -0.25 |
| 50 | 7.59 | 7.40 | -0.19 |
| 76 | 7.59 | 7.50 | -0.09 |
| 102 | 7.54 | 7.38 | -0.16 |
| 285 | 7.63 | 7.88 | 0.25 |

**Table B.4–** pH measurements of growth inhibition test with exfoliated $C_3N_4$

| Concentration of exfoliated $C_3N_4$ (mg $L^{-1}$) | Initial pH value | Final pH value | Variation |
|---|---|---|---|
| 5 | 7.42 | 7.07 | -0.35 |
| 10 | 7.39 | 7.01 | -0.38 |
| 20 | 7.42 | 7.41 | -0.01 |
| 40 | 7.40 | 7.09 | -0.31 |
| 80 | 7.40 | 7.03 | -0.37 |
| 100 | 7.43 | 7.01 | -0.42 |

## Appendix C: Conductivity value at the beginning and at the end of the growth inhibition test.

**Table C.1-** Conductivity measurements of growth inhibition test with GF_N8.

| Concentration of GF_N8 (mg $L^{-1}$) | Initial conductivity value (µS $cm^{-1}$) | Final conductivity value (µS $cm^{-1}$) | Variation (%) |
|---|---|---|---|
| 9 | 136.8 | 139.0 | 1.58 |
| 20 | 134.1 | 173.9 | 22.89 |
| 42 | 162.1 | 135.4 | -19.72 |

**Table C.2 -** Conductivity measurements of growth inhibition test with bulk $C_3N_4$.

| Concentration of bulk $C_3N_4$ (mg $L^{-1}$) | Initial conductivity value (µS $cm^{-1}$) | Final conductivity value (µS $cm^{-1}$) | Variation (%) |
|---|---|---|---|
| 9 | 174.5 | 133.7 | -30.5 |
| 25 | 173.5 | 132.0 | -31.4 |
| 50 | 173.3 | 132.9 | -30.4 |
| 76 | 168.9 | 132.0 | -28.0 |
| 102 | 170.2 | 134.1 | -26.9 |
| 285 | 170.9 | 136.8 | -24.9 |

**Table C.3 -** Conductivity measurements of growth inhibition test with exfoliated $C_3N_4$.

| Concentration of exfoliated $C_3N_4$ (mg $L^{-1}$) | Initial conductivity value (µS $cm^{-1}$) | Final conductivity value (µS $cm^{-1}$) | Variation (%) |
|---|---|---|---|
| 5 | 133.4 | 118.6 | -12.5 |
| 10 | 122.5 | 117.7 | -4.08 |
| 20 | 141.3 | 199.0 | 29.0 |
| 40 | 129.4 | 116.6 | -11.0 |
| 80 | 129.4 | 119.4 | -8.4 |
| 100 | 129.0 | 120.9 | -6.7 |

## Appendix D: Linear regression curve for estimation of $EC_{10}$ of bulk $C_3N_4$

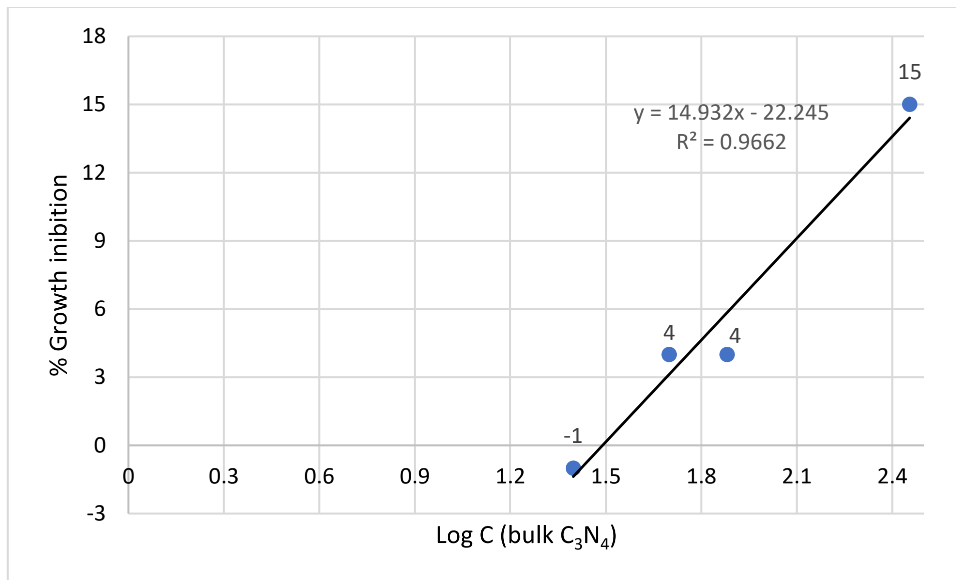


**Figure D.1** – Growth inhibition versus logarithm of bulk $C_3N_4$ concentration.

$$EC_{10} = 144\ mg\ L^{-1}$$

## Appendix E: Linear regression curve for estimation of $EC_{10}$ and $EC_{20}$ of exfoliated $C_3N_4$

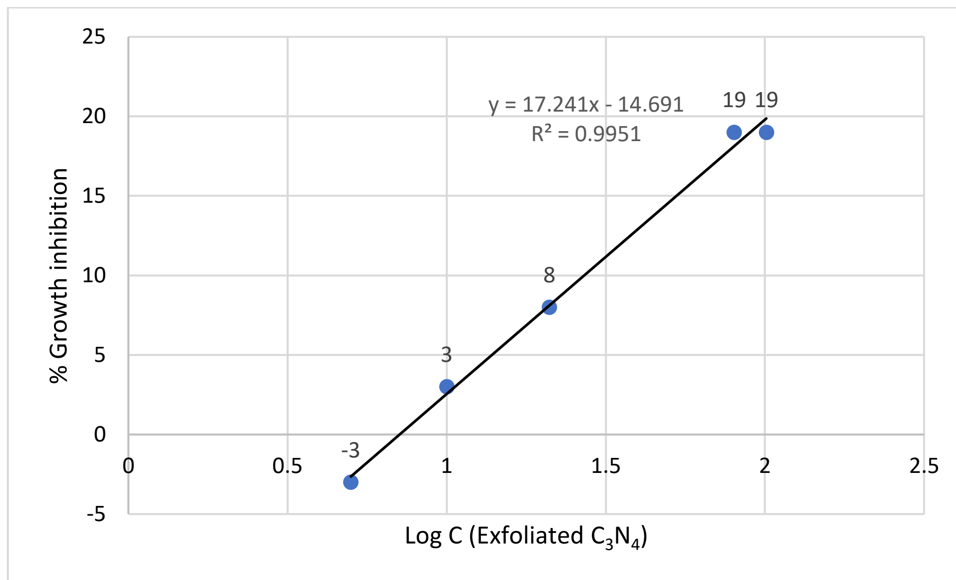


**Figure E.1** - Growth inhibition versus logarithm of exfoliated $C_3N_4$ concentration.

$$EC_{10} = 27\ mg\ L^{-1}; EC_{20=} 103\ mg\ L^{-1}$$