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Single and combined effects of environmental stressors on the marine microalgae *Tetraselmis chuii*

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Single and combined toxic effects of environmental stressors on the marine microalgae *Tetraselmis chuii*

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Resumo

Os ecossistemas costeiros são dotados de uma ampla biodiversidade, mas vulneráveis devido a várias ameaças de origem antropogénica como a exposição a agentes químicos libertados por diversas fontes. O transporte marítimo de substâncias perigosas de que são exemplo o petróleo, seus derivados e as substâncias perigosas e nocivas, vulgarmente designadas em língua inglesa por "hazardous and noxious substances" (HNS), apresenta-se como uma ameaça de grande relevância atual, devido ao risco de acidentes e consequente libertação do conteúdo transportado. Nos casos de ocorrência de derrames, os organismos aquáticos podem ser expostos a concentrações variáveis das substâncias libertadas durante diferentes períodos de tempo, podendo ocorrer exposições a concentrações elevadas de uma ou várias substâncias durante períodos de tempo curtos. Estudos recentes revelaram que estes acidentes podem ter efeitos consideráveis nos vários níveis tróficos das cadeias tróficas aquáticas. O fitoplâncton constitui a base destas cadeias tróficas, sendo os primeiros organismos potencialmente a influenciarem o destino de vários grupos de poluentes na cadeias tróficas.

Na presente dissertação, pretendeu-se investigar os efeitos tóxicos isolados e em mistura de um hidrocarboneto aromático policíclico (PAH), o fluoranteno, dada a sua toxicidade e por ser um constituinte importante de óleos transportados por via marítima e usados como combustíveis, e uma HNS, a anilina, na microalga marinha *Tetraselmis chuii*. Para esse efeito, realizaram-se ensaios agudos, baseados na inibição do crescimento de culturas desta alga expostas a diferentes concentrações de cada uma das substâncias isoladamente. Pela relevância ecológica que interacções toxicológicas resultantes da exposição simultânea a mais do que um agente químico podem assumir em cenários reais, foram também investigados os efeitos da exposição simultânea à anilina e ao fluoranteno. Os resultados obtidos indicaram que ambos os compostos inibiram significativamente o crescimento de *T. chuii*, com concentrações medianas de inibição do crescimento (CI_{50}) de 0.378 mgL^{-1} para o fluoranteno e de 60.870 mgL^{-1} para a anilina. Os resultados obtidos indicaram ainda um efeito sinérgico na inibição do crescimento da alga quando exposta simultaneamente às duas substâncias. Estes resultados suscitam preocupação relativamente à possibilidade de libertação simultânea das duas substâncias (e.g. na sequência de acidentes com navios que transportem anilina e que resultem na libertação simultânea desta substância e de combustível dos tanques do navio) e reforçam a necessidade de investigar efeitos resultantes da exposição combinada a PAHs e HNS.

Palavras chave: anilina, fluoranteno, misturas, *Tetraselmis chuii*, fitoplâncton marinho.

Abstract

The coastal ecosystems are endowed with a wide biodiversity, but vulnerable, due to several threats of anthropogenic origin, such as chemicals released from several sources. The transportation of oils, other petrochemical mixtures, and hazardous and noxious substances (HNS) by sea is of high concern at the present due to the risk of shipping accidents resulting in spillages of the transported substances in the marine environment. In case of spills, aquatic organisms may be exposed to different concentrations of the released substances for distinct periods of time, and short-term exposure to high concentrations of one or several chemical agents may occur. Recent studies revealed that these spillages may have adverse effects on the several levels of the aquatic food chains. Phytoplankton constitutes the basis of these food chains, being the first group of organisms that could influence the fate of several types of pollutants in the food webs.

The present thesis intended to investigate the effects of a polycyclic aromatic hydrocarbon (PAH), the fluoranthene which is a common component of oils and other petrochemical products used as fuels, and of a HNS, the aniline which is transported at high amounts by sea, on the marine microalgae *Tetraselmis chuii*. For this purpose, 96h acute toxicity tests, based on *T. chuii* growth inhibition, were done with single substances and with mixtures. Both compounds significantly inhibited the microalgae growth with median inhibition concentrations (IC_{50}) of 0.378 mgL^{-1} for fluoranthene and 60.870 mgL^{-1} for aniline. Toxicological interactions were found in the mixture assay namely a synergist effect on *T. chuii* growth inhibition. These results raise concern on the simultaneous spillage of oils and aniline and highlight the need of more research on the combined toxic effects of PAHs and HNS.

Key words: aniline, fluoranthene, mixtures, *Tetraselmis chuii*, marine phytoplankton.

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cells per ml in each treatment. 0 – Control; 0' – Solvent control; * - Significantly different from the control group at 96h ($p<0.05$ Dunnett test).

Figure II.8. – Number of cells of *Tetraselmis chuii* after 96h of exposure to different concentrations of fluoranthene (mgL^{-1}). Values are the mean of three replicates with the corresponding S.E.M bars. 0 – Control; 0' – Solvent control; *- indicate significant differences relatively to the control group ($p<0.05$ Dunnett test, at 96h).

Figure II.9. – Inhibition of *Tetraselmis chuii* growth in the presence of different concentrations of fluoranthene (mgL^{-1}). 0 – Control; 0' – Solvent control; *- indicate significant differences relatively to the control group ($p<0.05$ Dunnett test, at 96h).

Figure II.10. – Inhibition of yeld of the growth of *Tetraselmis chuii* after 96h of exposure to different concentrations of fluoranthene (mgL^{-1}). 0 – Control; 0' – Solvent control; *- significant differences relatively to the control group at 96h ($p<0.05$ Dunnett test).

Figure II.11. – Microalgae growth inhibition after 96h of exposure to a binary mixture (aniline and fluoranthene). 0 – Control; 0' – Solvent control; *- significantly different from the control group ($p<0.05$ Dunnett test); Flu – fluoranthene; Ani – aniline.

Figure II.12. – Second bioassay of the microalgae growth inhibition after 96h of exposure to binary mixtures of aniline and fluoranthene. 0 – Control; 0' – Solvent control; *- significantly different from the control group ($p<0.05$ Dunnett test); Flu – fluoranthene; Ani – aniline.

Table List

Abbreviations' List

AI – Additive Index.

CA – Concentration Addition.

HNS – Hazardous and Noxious Substances.

IA – Independent Action.

IC_{50} – Concentration that caused 50% of inhibition on the microalgae growth.

IMO – International Maritime Organization.

LOEC – Lowest Observed Effect Concentration.

MTI – Mixture Toxicity Index.

NOEC – No Observed Effect Concentration.

PAH – Polycyclic Aromatic Hydrocarbon.

TU – Toxic Unit.

US EPA - Environmental Protection Agency of the United States of America.

CHAPTER I

GENERAL INTRODUCTION

I. 1. GENERAL INTRODUCTION

I.1.1. Coastal Areas

The coastal seas are one of the most precious habitats on Earth and the composition of these ecosystems is extraordinary diverse in all aspects (Jickells, 1998). These areas provide economic benefits from the extraction of goods and in addition supply important services like nutrient recycling, flood control, waste treatment, species refuges, genetic resources and recreational activities (Scavia *et al*., 2002). However, the coastal areas are also vulnerable, being exposed to several threats such as the overexploitation of fisheries resources, deleterious effects of the exposure to chemicals, eutrophication and habitat loss (Jickells, 1998). An example is the Galician coast, a highly productive fishery area, which has a big social and economic importance resulting from the shellfish aquaculture (Saco-Álvarez *et al*., 2008). This area is rich and abundant in marine flora and fauna, being also a stopover for migratory birds, a nesting for resident seabirds and one of the main suppliers of shellfish for the rest of Europe (Carrera-Martínez *et al*., 2010). The main routes of international transport are located just a few tens of miles from this coast and thousands of ships transporting dangerous materials cross those routes every year. Accidents have already happened in the past like *The Prestige* oil spill showing the vulnerability of this area (Saco-Álvarez *et al*., 2008). The NW coast of Portugal is the southern continuation of the Galician coast and like this one is a vulnerable area. Taking into account some particular characteristics of the coast, maritime currents and adverse sea conditions in some periods of the year, the NW coast of Portugal belongs to the “risk” area of the Iberian coast regarding shipping accidents (Vieira *et al*., 2008).

I.1.2. Transportation of Chemical Substances by Sea

Shipping, one of the most important modes of transport for products (e.g. oils and other chemical substances) has increased in the last years (Purnell, 2009; Mamaca *et al*., 2009). Every year thousands of ships transport dangerous goods like oils or chemicals through the main routes of international traffic (Saco-Álvarez *et al*., 2008; Mamaca *et al*., 2009). However, this kind of transport involves risks like spills at the sea whose consequences depend of the type of substances that are spilled (Neuparth *et al*., 2010) and of the amount spilled. The *Prestige* and the *Exxon Valdez* oil spills are examples of accidents occurred in the past with tankers. Accidents like those increase the interest of the scientific community to study the effects on different organisms and on the ecosystems (Carrera-

Martínez *et al*., 2010; Varela *et al*., 2006; Franco *et al*., 2006; Salas *et al*., 2006; Saco-Álvarez *et al*., 2008; Peterson *et al*., 2003; Dean *et al*., 1998; Fukuyama *et al*., 2000; Carls *et al*., 2001).

I.1.3. Hazardous and Noxious Substances (HNS)

One of the most dangerous group of pollutants transported by sea is the one commonly designed by Hazardous and Noxious Substances (HNS), a group of substances that can be defined as any substance other than oil which, if introduced into the marine environment, is likely to induce hazards to human health, to harm living resources and marine life, to damage amenities or to interfere with other legitimate uses of the sea (IMO, 2000). Although the eventuality of an incident involving HNS transport is relatively low, some shipping incidents such as the *MSC Rosa M* in 1997, the *Ever Decent* in 1999, the *Napoli* in 2007 and the *Princess of the Stars* in 2009 have demonstrated that the possibility really exists (Mamaca *et al*., 2009). The investigation of the effects of HNS in the marine environment is relatively scarce and the available toxicity data resulted from laboratory studies using freshwater organisms makes very difficult to predict the effects on marine organisms (Purnell, 2009). Moreover, this group of substances is formed by different compounds that have a very wide range of behaviours. Those behaviours can be seen in Table I.1. (Purnell, 2009).

Aniline, also known as aminobenzene, aminophen, phenylamine or benzeneamine, is classified as an organic compound. It is characterized by a phenyl group to which an amino group is attached (Table I.2.) (Dom *et al*., 2010). This compound belongs to the aromatic amines group which is usually classified as polar narcotics in aquatic toxicology (Verhaar *et al*., 1992). This HNS is an important industrial chemical and is used in a very wide range of processes such as the production of polymers, pesticides, pharmaceuticals and dyes. Several industrial uses, such as production and processing, may lead to the release of aniline into the environment (Wang *et al*., 2007). However, this compound can be also found in the environment due to the partial degradation of xenobiotics, including some azo dyes and herbicides (Wang *et al*., 2007). Aniline is toxic to aquatic species. For example, according to Bhunia *et al*. (2003), the 96h median lethal concentrations (LC_{50}) to *Oreochromis mossambicus* (fish), *Moina micrura* (cladoceran) and for *Branchiura sowerbyi* (oligochaete) are 69.4, 0.6 and 586 mgL^{-1}, respectively. In addition, a 90 days of exposure investigation conducted by the same authors demonstrated that specific growth rate and food conversion efficiency of tilapia were reduced at 0.02 mgL^{-1} and the reproductive functions were also affected at 0.5 mgL^{-1} of aniline. Birge *et al.* (1979) tested the effects of this

compound on the developmental stages of different fish species. The LC_{50} values obtained were 5.6, 10.2 and 47.3 mgL^{-1} for catfish (*Ictalurus punctatus*), goldfish (*Carassius auratus*) and bass (*Micropterus salmoides*), respectively.

Table I.1. – **Behaviour of some chemicals in water (Purnell, 2009).**

Fate	Group	Properties	Examples
Sink	S	Sink	Chlorobenzene
	SD	Sink, Dissolve	Dichloromethane
Dissolve	D	Dissolve rapidly	Some alcohols, glycols and amines
	DE	Dissolve rapidly, Evaporate	Acetone
Float	FD	Float, Dissolve	Butanol
	F	Float	Phtalates, Dipentene
	FED	Float, Evaporate, Dissolve	Butyl acetate, Isobutanol
	FE	Float, Evaporate	Xylene, Toluene
Evaporate rapidly	ED	Evaporate rapidly, Dissolve	Vinyl acetate
	E	Float, Evaporate rapidly	Benzene, Hexane
Evaporate immediately	GD	Evaporate immediately, dissolve	Ammonia
	G	Evaporate immediately	Propane, Butane

I.1.4. Polycyclic Aromatic Hydrocarbons

Petroleum activities may be responsible for negative impacts on the coastal environments (Paixão *et al*., 2007). Many countries are involved in oil extraction and transportation, and because of that, oil spills are common events throughout the world (Cohen and Nugegoda, 2000). Although, in the last decades, accidental discharges have been reduced, due to the development and adaptation of new regulations regarding the safety of tanks and prevention of oil pollution, the increase of the transport of oil by sea and the increasing demand for this product continue to be a threat to the environment due to the potential accidents that can occur (Martínez-Gómez *et al*., 2010). For instance, in April 2010, the largest spill of the U.S. history has occurred after an explosion on a BP licensed platform releasing millions of gallons of oil into the Gulf of Mexico, requiring about three months to stop the spill (Figure I.1.). The environmental and the economic impact on the region were huge, because the oil reached the U.S. Gulf Coast states affecting mainly birds and the aquatic life (Muralidharan *et al*., 2011; Harlow *et al*., 2011).

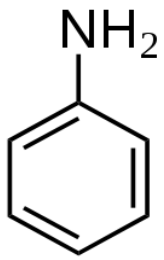
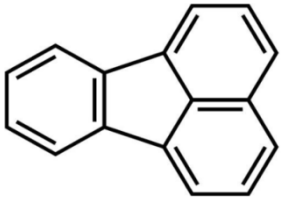
Figure I.1. – Images of the oil spill occurred in the Gulf of Mexico in 2010 (www.nationalgeographic.com).

The aromatic fraction of the oil and polycyclic aromatic hydrocarbons (PAHs) in particular, is usually responsible for the most relevant toxic effects (Albers, 2003). In fact, PAHs are a group of chemicals characterized by the presence of multiple benzene rings and they are considered ubiquitous environmental contaminants (Liu *et al*., 2006; Wang and Zheng, 2008). Moreover, these compounds are considered priority pollutants by the United States Environmental Protection Agency (US EPA) because of their high frequency occurrence rate toxicity and potential human exposure (Palmqvist *et al*., 2006). The compounds that belong

to the list are the ones for which US EPA must establish effluent limitations as well as ambient water quality criteria. PAHs are released into the environment by natural and anthropogenic sources. Natural sources include forest and rangeland fires, oil seeps, volcanic eruptions and exudates from trees (Manoli *et al*., 2004; Haritash and Kaushik, 2009), among others. There are several anthropogenic sources such as petroleum spills and discharges, solid waste incineration, use of lubricating oil and oil filters and burning fossil fuel, coal tar and garbage (Carls *et al*., 2001; Franco *et al*., 2006; Haritash and Kaushik, 2009) and this kind of actions have contributed to the accumulation of these compounds in the environment (Liu *et al*., 2006). PAHs may result from the thermal decomposition of organic molecules and their recombination. This group of compounds is characterized by their low solubility in water, high melting and boiling points and low vapour pressures (Haritash and Kaushik, 2009). They are adsorved by organic and inorganic particulate matter and become available to be accumulated by aquatic biota thanks to their location in rivers, estuaries and coastal waters. They are incoporated in animal and plant tissues, because of their lipophilic nature, by passive diffusion or active metabolism (Riznyk *et al*., 1987). These substances are recognized as mutagens and carcinogens and several studies have already demonstrated it (Bispo *et al*., 1999; Kammann *et al*., 2001). PAHs cause the growth inhibition of both fresh and saltwater algae species (Wang *et al*., 2008; Šepic *et al*., 2003; Liu *et al*., 2006), and adverse effects on other aquatic organisms such as mussels and sea urchins, has also been demonstrated (Bellas *et al*., 2008). In addition, several authors also observed negative effects of these compounds on fish growth (Hanna *et al*., 1982; Heintz *et al*., 2000), reproduction (White *et al*., 1999), survival (Heintz *et al*., 2000) and oxidative stress (Vieira *et al*., 2008).

The US EPA created a 16 PAHs priority list (see Table I.3.) which includes fluoranthene which is a four-ring PAH with pirogenic origin and one of the most abundant PAHs in the environment (Table I.2.) (Šepic *et al*., 2003). This compound was also classified as one of the 68 priority pollutants in China (Liu *et al*., 2006).

Table I.2. – Characteristics of aniline and fluoranthene ([1]US EPA, 1994; [2] US EPA, 1980; [3]Liu *et al*., 2006).

	Aniline	Fluoranthene
Chemical characteristics		
Structure		
CAS number	62-53-3	206-44-0
Molecular Weight	93.12[1]	202.26[2]
Log K_{ow}	0.90[1]	5.33[3]
Water solubility(mgL^{-1})(25 ℃)	35000 [1]	0.265[2]

In the literature, several effects of fluoranthene in wildlife were described. For example, in a work by Suedel and Rodgers (1996), significant effects of fluoranthene after 48 h and 10 days of exposure were observed in *Daphnia magna* (Cladoceran)*, Hyalella azteca* (Amphipod)*, Chironomus tentans* (Midge), and *Stylaria lacustris* (Worm), with *D. magna* and *H. azteca* being more sensitive (LC_{50} of 105.7 and 92.2 μgL^{-1}, respectively) than *C. tentans* and *S. lacustris* (LC_{50} of 220 and 250 μgL^{-1}, respectively) after 48h of exposure; regarding the 10 day exposure, *D. magna* was less sensitive than *H. azteca* and *C. tentans*. The same study revealed a LC_{50} of 102.6 μgL^{-1} for *D. magna* whereas the LC_{50} for *H. azteca* and *C. tentans* were 30.3 and 37.8 μgL^{-1}, respectively. The results showed a considerable difference in the sensitivity of the tested species after 48h and 10 days. Although the LC_{50}s determined for *D. magna* were similar after 48h and 10 days, the LC_{50}s for *H. azteca* and *C. tentans* after 10 days of exposure were much lower than the LC_{50}s after 48h, showing that the 48h exposure is not sufficient to demonstrate the toxic effect of this PAH for these species. This study emphasises the importance of test duration in order to determine the adverse effects of a compound like fluoranthene to aquatic organisms. Šepic *et al*. (2003) studied the toxicity of fluoranthene and its biodegradation metabolites to some aquatic organisms and according to the obtained results, the compound was not toxic to the bacteria's *Pseudomonas putida* cells

neither to the crustacean *Thamnocephalus platyurus* but was toxic to *Daphnia magna*. Its primary metabolites were not toxic to *Pseudomonas putida* cells but were toxic to *Daphnia magna*. The toxicity of this PAH has been also studied under different types of light. Spehar *et al*. (1999) assessed the acute toxicity of fluoranthene to different freshwater and saltwater species under fluorescent and ultraviolet light in order to evaluate the sensitivity of the different species to this PAH under both types of light. Acute toxicity was tested in 21 species in both conditions. Under fluorescent light, LC_{50}s were lower than 100 μgL^{-1} for *Hydra Americana* (hydra), *Hyalella azteca*, *Ampelisca abdita* (amphipods) and *Mysidopsis bahia* (mysid) and between 108 and 142 μgL^{-1} for *Gammarus pseudolimnaeus* (amphipod), *Daphnia magna* (cladoceran) and *Palaemonetes* species (grass shrimp). For several species, including *Oncorhynchus mykiss* (rainbow trout), *Pimephales promelas* (fathead minnow), *Arbacia punctulata* (sea urchin) and *Cyprinodon variegates* (sheepshead minnow) fluoranthene was not lethal in the range of concentrations tested. Under ultraviolet light fluoranthene was much more toxic than under fluorescent light being the LC_{50}s lower than 159 μgL^{-1} for 15 of the 21 species tested).

Table I.3. – List of the 16 priority polycyclic aromatic hydrocarbons of the Environmental Protection Agency of the United States of America (US EPA, 1982).

Polycyclic Aromatic Hydrocarbon	Structure (number of rings)
Naphthalene	2
Acenaphthene	3
Acenaphthylene	3
Anthracene	3
Phenanthrene	3
Fluorene	3
Fluoranthene	4
Benzo(a)anthracene	4
Chrysene	4
Pyrene	4
Benzo(a)pyrene	5
Benzo(b)fluoranthene	5
Benzo(k)fluoranthene	5
Dibenz(a,h)anthracene	6
Benzo(g,h,i)perylene	6
Indeno[1,2,3-cd] pyrene	6

I.1.5. Mixtures

The majority of published works investigate the effects of isolated substances on aquatic life. However, in natural ecosystems, the organisms are usually exposed to complex mixtures of chemicals and the effects caused by those alone are likely to be different from the effects caused by the mixture (Fei *et al*., 2010). The interaction of the chemicals in a mixture can produce antagonistic, synergistic, additive (Barata *et al*., 2006; Fei *et al*., 2010) and potentiation effects. An additive effect occurs when two chemicals interact and the result corresponds to the sum of the effects caused by individual chemicals when tested isolate. When two chemicals interact and the resultant effect is much greater than the sum of the effects of each agent alone, it is classified as a synergistic effect. When a chemical reaction between two compounds results in a less toxic product than the sum of the effects of each agent alone, the effect is called antagonism. If one chemical alone does not cause any toxic effect but when added to another chemical increases its toxicity, that effect is classified as potentiation (Eaton and Klaassen, 1996). In Ecotoxicology, the effects of mixtures of chemicals have been classified using the toxic unit (TU) concept, (Marking and Dawson, 1975), the additive index (AI) (Mayer and Hamelink, 1977) and the mixture toxicity index (MTI) (Könemann, 1981). However, there are two concepts used in Ecotoxicology to predict the toxicity of artificially designed multi-component mixtures, the concentration addition (CA) and the independent action (IA). The CA concept is used when the mixture components have the same molecular target site because it assumes that the mixture components have a similar toxicological mode of action (Backhaus *et al*., 2004). The IA concept is the alternative concept which assumes the mixture components having a dissimilar mode of action interacting with different molecular target sites, inducing by distinct chains or reactions a common toxicological endpoint (Backhaus *et al*., 2004). It is possible to find in the literature, several studies that report or predict the effects of binary mixtures of PAHs, PAHs with other class of compounds and aromatic compounds (Ge *et al*., 2010; Erickson *et al*., 1999; Lu *et al*., 2007). There are also studies that evaluate the effects of mixtures on producers, including algae (*e.g.* Lu *et al*., 2007; Walter *et al*., 2002; Ge *et al*., 2010; Hsieh *et al*., 2006). Therefore, considering the food web position of phytoplankton, previous statements highlight clearly the importance of understanding the joint action of chemical mixtures on those organisms, which is very useful for the risk assessment of chemicals in aquatic environment.

I.1.6. Plankton and Microalgae

Planktonic organisms are of fundamental importance in marine ecosystems because of their function as primary producers being the basis of the food chains (Domingues *et al*., 2008), and because most aquatic life forms are dependent upon the energy and oxygen that they provide. They include small autotrophic organisms (phytoplankton) and animals (zooplankton) that passively drift belong. These organisms movement depend of the ocean currents (Hays *et al*., 2005). Phytoplankton, as mentioned above, belongs to this group of organisms and can be classified as a microscopic group of photosynthetic organisms and most of them are single-celled eukaryotes that live mainly in the top layer of water and moves mainly according water currents and other water movements (Falkowski *et al*., 2004). This group of organisms has a critical function as primary producers and is responsible for providing energy, oxygen and food to sustain other life forms. Phytoplankton is undoubtedly the food source of a large aquatic biomass, which includes several kinds of fish, shrimps and shellfish (Lai *et al*., 2009; Costa and França, 1998). They are rich sources of proteins, carbohydrates and especially essential fatty acids (Ghezelbash *et al*., 2008). In addition to that, they have a tremendous impact on the water quality and play other major roles in many ecosystem processes (Figure I.2.) (Domingues *et al*., 2008). For example, these organisms play a fundamental role on biogeochemical processes, being involved in the transformation and cycling of key elements (Los and Wijsman, 2007).

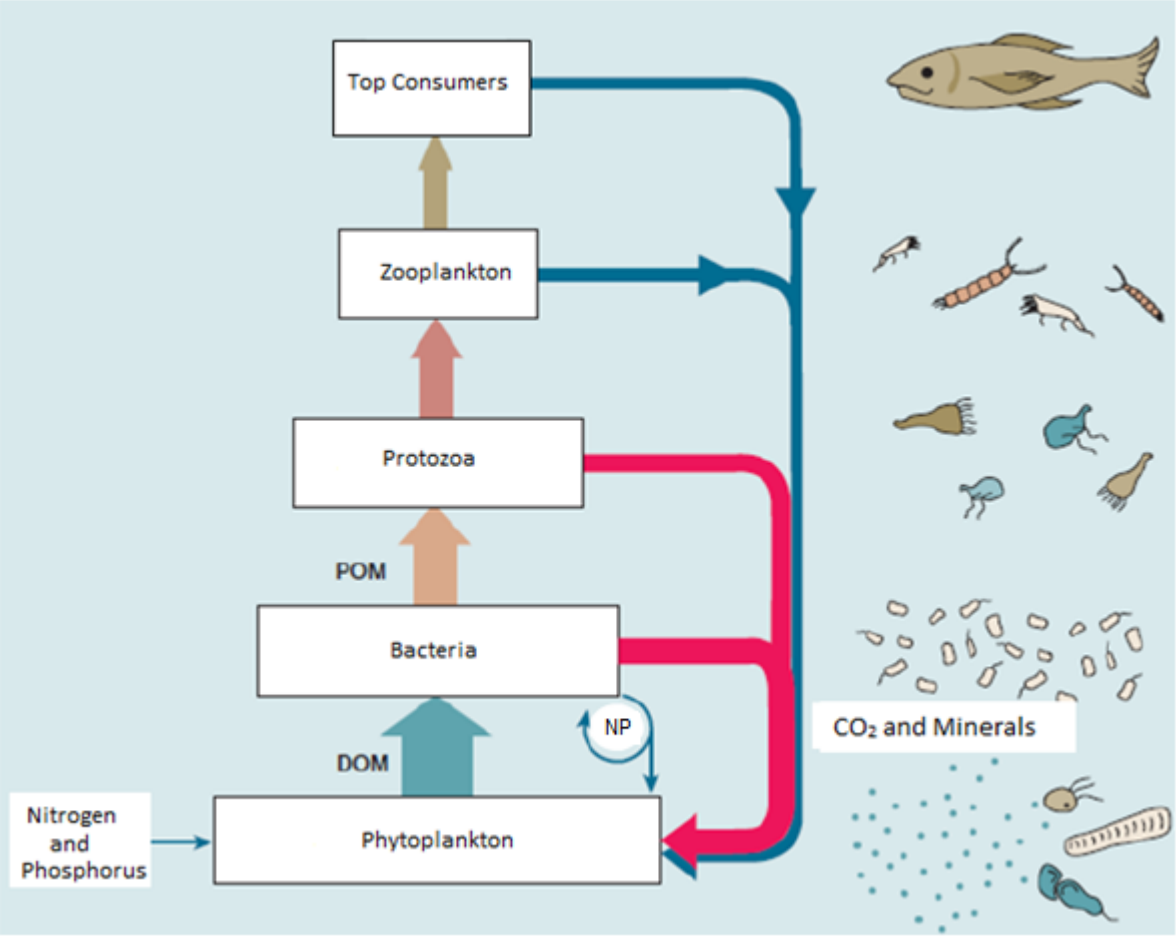


Figure I.2. – *Microbial loop* (adapted from Prescott *et al*., 2002). DOM – Dissolved organic matter; POM – Produced organic matter.

The exposure to contaminants by microalgae may cause the bioaccumulation of those contaminants, including PAHs and may lead to food chain transfer and biomagnification (Okay *et al*., 2000; Rumampuk *et al*., 2003). The use of these organisms in bioassays is very important because if these organisms are affected by toxic compounds, directly or indirectly, the entire ecosystem may be also affected by the lack of food (Wang and Zheng, 2008). Several studies have been done to evaluate PAHs effects on producers (Riznyk *et al*., 1987; Wang and Zheng, 2008; Liu *et al*., 2006), including fluoranthene. Liu *et al*. (2006) studied the effect of fluoranthene on *Cyclotella caspia* and observed a 96h EC_{50} of 0.2 mgL^{-1}. The toxicity of this PAH was also tested using a marine diatom and the 72h IC_{50} obtained was 0.103 µg mL^{-1}. The exposure to this compound caused growth inhibition of the population and ultrastructure damage of the cells (Wang and Zheng, 2008). Šepic *et al*. (2003) reported the toxicity of fluoranthene and its metabolites for the algae *Scenedesmus subspicatus* and the results showed that all of the metabolites were between 37 and 3000 less times toxic than the parental compound. In the literature is also possible to find reports

about the effect of some HNS, such as ammonia, acrylonitrile, styrene and aniline, exposure on algae (Källqvist and Svenson, 2003; Cushman *et al*., 1997; Tong and Hongjun, 1997; Maas-Diepeveen and Leeuwen, 1986). The toxicity of aniline was evaluated using *Chlorella pyrenoidosa* and according to Maas-Diepeveen and Leeuwen (1986) the 96h IC_{50} obtained was 94 mgL^{-1}.

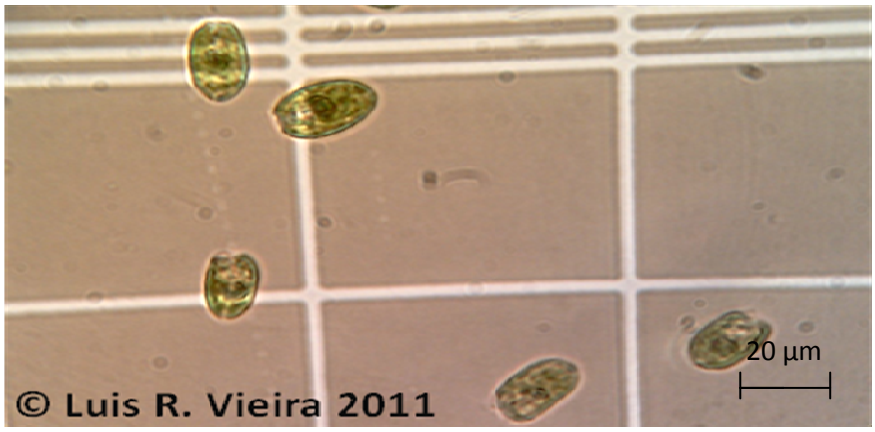


Figure I.3. – **The marine unicellular alga *Tetraselmis chuii* (Luis R. Vieira, 2011).**

The genus *Tetraselmis* can be found as a non motile cell attached by a gelatinous stalk or as a flagellate. Looking at flagellate cells it is possible to observe that these cells have four flagella emerging from the bit in two pairs (Sym and Piennar, 1993). The cells are covered by a distinctive wall, which can be called theca, composed of small scalelike particles in a crystalline array. Sometimes flagella are lost because motile cells often stop swimming for long periods. They are usually green, but due to the accumulation of carotenoids they can become red (Sym and Piennar, 1993). Microalgae from this genus are used in aquaculture as a food source for shrimps, shellfish and several kinds of fish. These microalgae are cultured on a large scale and they are easily disseminated in the food chain. Thus, is very important to assess the dissemination of toxic compounds through the food chain because these microalgae cells have the ability to incorporate those compounds (Costa and França, 1998). *Tetraselmis chuii* is a prasinophyceae alga which has cylindrical shape and measures between 8 and 16 µm of length (Cordero *et al*., 2005) (Figure I.3.). This microalga may become a model species to assess the impact of several xenobiotics in the environment since it has a broad distribution in the tropical ecosystems and large use in laboratory cultures (Cordero *et al*., 2005).

I.2. OBJECTIVES

Thus, considering the above mentioned, the main objectives of this study were: (i) to investigate the effects of the PAH fluoranthene and the HNS aniline on the growth of the marine algae *Tetraselmis chuii;* and (ii) to test the null hypothesis that the exposure of *T. chuii* simultaneously to aniline and fluoranthene does not result in toxicological interactions.

I.3. THESIS STRUCTURE

This thesis is organized in three different chapters: the first chapter includes a general introduction to the topic under investigation, the objectives of the study, an explanation of the organization of the Thesis and a reference list; the second chapter follows in general the structure of a paper including the introduction, material and methods, results, discussion, acknowledgements and references; the third and final chapter includes the general discussion and the final conclusions.

The results of the present thesis will be presented as a flash presentation in the 12th Congress of the European Ecological Federation Congress that will be held in Avila from 25 to 29 September 2011. The abstract is included in the Annex II and its reference is:

Azevedo, M., Vieira, L.R., Guilhermino, L. 2011. Single and combined effects of an oil component (fluoranthene) and a hazardous and noxious substance (aniline) on the marine microalgae *Tetraselmis chuii*. 12th European Ecological Federation Congress 2011 accepted as a flash presentation.

Furthermore, a manuscript corresponding to chapter II of the Thesis is in its final phase of preparation and we expect to submit it for publication to an international peer-reviewed journal during the next month.

I.4. REFERENCES

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CHAPTER II

Single and combined effects of an oil component (fluoranthene) and a hazardous and noxious substance (aniline) on the marine microalgae *Tetraselmis chuii*

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Abstract

The transport of oils and hazardous and noxious substances (HNS) by sea raise concern on their potential spills and consequent effects on marine ecosystems. If an environmental accident of this type occurs, marine organisms may be exposed for short periods of time to high concentrations of the released chemicals. Nowadays, there is a considerable lack of knowledge on the effects of mixtures of substances that may be simultaneously spilled during shipping accidents, such as oil components and HNS. For that reason, the effects of fluoranthene, a polycyclic aromatic hydrocarbon (PAH) that is an important component of oils, and aniline, one of the HNS most transported by sea, on the marine microalgae *Tetraselmis chuii* were investigated both isolated and in mixture. For this purpose, based on *T. chuii* growth inhibition, 96h acute toxicity tests were performed, with single substances and with mixtures. Both compounds, when tested separately, significantly inhibited the microalgae growth with median inhibition concentrations (IC_{50}) of 0.378 mgL^{-1} for fluoranthene and 60.870 mgL^{-1} for aniline. In the mixture assay toxicological interactions were found, namely a synergist effect on *T. chuii* growth inhibition. These results raise concern on the simultaneous spillage of oils and aniline and enhance the need of more research on the field of combined toxic effects of PAHs and HNS.

Keywords: aniline, fluoranthene, mixtures, marine microalgae, growth inhibition.

1. INTRODUCTION

One of the most important modes of transport for anthropogenic products (e.g. oils and other chemical substances) is by sea and maritime traffic has increased in the last years (Purnell, 2009; Mamaca *et al*., 2009). Through the main routes of international transport, every year thousands of ships transport dangerous chemicals (Saco-Álvarez *et al*., 2008; Mamaca *et al*., 2009). This kind of transport involves risks like spills in the sea whose consequences depend on the type of substances that are spilled (Neuparth *et al*., 2010). The *Prestige* and the *Exxon Valdez* oil spills are examples of accidents occurred in the past with ships. The investigation of the effects on different organisms and on the ecosystems of accidents like those increase the interest of the scientist community (Carrera-Martínez *et al*., 2010; Varela *et al*., 2006; Franco *et al*., 2006; Salas *et al*., 2006; Saco-Álvarez *et al*., 2008; Peterson *et al*., 2003; Dean *et al*., 1998; Fukuyama *et al*., 2000; Carls *et al*., 2001).

The Hazardous and Noxious Substances (HNS) are considered one of the most dangerous group of pollutants transported by sea and can be considered as a group of substances that can be defined as any substance other than oil which, if introduced into the marine environment is likely to create hazards to human health, to harm living resources and marine life, to damage amenities or to interfere with other legitimate uses of the sea (IMO, 2000). The investigation of the effects of HNS on the marine environment is relatively scarce and the toxicity data available resulted from laboratory studies using freshwater organisms which makes very difficult to predict the effects on marine organisms (Purnell, 2009). Aniline is classified as an organic compound and can be characterized by a phenyl group to which an amino group is attached (Dom *et al*., 2010). The literature reports toxic effects of this compound to several aquatic species, including fish (Birge *et al*., 1979), crustaceans and oligochaetes (Bhunia *et al*., 2003).

Negative impacts on the coastal environments are also caused by petroleum activities (Paixão *et al*., 2007). Oil spills are common events throughout the world, since many countries and companies are involved in oil and petroleum exploration (Cohen and Nugegoda, 2000). The most relevant effects are usually caused by the aromatic fraction of the oil, the one containing polycyclic aromatic hydrocarbons (PAHs) in particular (Albers, 2003). In fact, PAHs are ubiquitous contaminants in the environment and they are a group of chemicals characterized by the presence of multiple benzene rings (Liu *et al*., 2006; Wang and Zheng, 2008). In addition, these compounds are considered priority pollutants by the U.S. Environmental Protection Agency (US EPA) (Palmqvist *et al*., 2006). They are known as being mutagens and carcinogens and several studies have already demonstrated it (Bispo *et al*., 1999; Kammann *et al*., 2001). PAHs were found to cause the growth inhibition of both

fresh and saltwater algae species (Wang *et al*., 2008; Šepic *et al*., 2003; Liu *et al*., 2006) and adverse effects on other aquatic organisms such as mussels and sea urchins have also been demonstrated (Bellas *et al*., 2008). The negative effects of these compounds on fish growth (Hanna *et al*., 1982; Heintz *et al*., 2000), reproduction (White *et al*., 1999), survival (Heintz *et al*., 2000) and on the induction of oxidative stress have already been observed (Vieira *et al*., 2008). A list of 16 PAHs was created by the US EPA which includes fluoranthene, a four-ring PAH with pirogenic origin and one of the most abundant PAH in the environment (Šepic *et al*., 2003). The effects of this PAH to several aquatic species have been documented in the literature, including crustaceans, fish, algae and bacteria (Suedel and Rodgers, 1996; Spehar *et al*., 1999; Šepic *et al*., 2003).

In the aquatic environment, the organisms are usually exposed to complex mixtures of chemicals and the effects caused by those alone are likely to be different from the effects caused by the mixture (Barata *et al*., 2006; Fei *et al*., 2010). In the literature, it is possible to find several studies that report or predict the effects of binary mixtures of PAHs, PAHs with other class of compounds and aromatic compounds (Ge *et al*., 2010; Erickson *et al*., 1999; Lu *et al*., 2007). There are also studies that evaluate the effects of mixtures on producers, more concretely on algae (Lu *et al*., 2007; Walter *et al*., 2002; Ge *et al*., 2010; Hsieh *et al*., 2006).

Phytoplankton, a microscopic group of photosynthetic organisms (Falkowski *et al*., 2004) has a critical function as primary producers and is responsible for providing energy, oxygen and food to sustain other life forms. Microalgae exposure to contaminants may lead to bioaccumulation, food chain transfer and biomagnification (Rumampuk *et al*., 2003). *Tetraselmis chuii* is a prasinophyceae alga which has a cylindrical shape and measures between 8 and 16 µm of length and this microalga is a model species to assess the impact of several xenobiotics in the environment since it has a broad distribution in the tropical and temperate ecosystems and is widely use in laboratory cultures (Cordero *et al*., 2005).

Overall, there is little information available on the literature regarding the effects of HNS or PAHs exposure on marine phytoplankton, including the genus *Tetraselmis* and nowadays, there is yet a considerable gap of knowledge regarding the effects of mixtures of compounds that may be spilled simultaneously during accidents involving oils and HNS substances. Therefore, the aim of this study was to assess the effects of aniline and fluoranthene isolated and in mixtures on *T. chuii* cultures growth.

2. MATERIAL AND METHODS

2.1. Chemicals

The tested substances, aniline ($C_6H_5NH_2$, CAS No. 62-53-3, purity ≥99.5%) and fluoranthene ($C_{16}H_{10,}$ CAS No. 206-44-0, purity 98%) and all the reagents used to prepare the algal cultures were purchased from Sigma–Aldrich Chemical (Steinheim, Germany).

2.2. Algae Culture and Maintenance

The green unicellular marine algae *T.chuii* was cultured under standardized abiotic conditions at 20±1℃ with continuous aeration and c ontinuous cool-white fluorescent light (100µE/m^2/s). Culture medium was F2 medium prepared with Atlantic seawater with sodium chloride in a concentration of 30 gL^{-1} and supplemented with $NaH_2PO_4.2H_2O$, $NaNO_3$ and a trace metal solution previously prepared (as described in Annex I) in a 1:1000 proportion. The F2 medium was prepared in full strength Atlantic seawater. The water was collected in the NW coast of Portugal, filtered with cellulose nitrate filters (mean pore diameter of 50, 10, 5 and 1 µm) and sterilized with ultraviolet radiation. After that, the seawater was re-filtered using a vacuum pump (KNF Neuberger, Germany) and glass microfiber discs (MGC type, mean diameter pore 47 mm). Finally, the salinity of the seawater was measured using a salinity probe (WTW Multi 340i) and if the value was higher than 30 gL^{-1} of sodium chloride, distilled water was added to reach the concentration of sodium chloride of 30 gL^{-1}. At this point, the medium was sterilized at 121℃ for 35min and after that a vitamin solution (see Annex I) was added in a 1:2000 proportion. In addition, all material used including glass apparatuses, medium, plastics (falcons, eppendorfs, pipette tips), supplements and air were, also, previously sterilized. In order to obtain exponentially growing cultures, a previous algal culture (in exponential phase) was inoculated in the sterilized medium. This green unicellular marine algae was kindly provided by the Instituto de Ciencias Marinas de Andalucía (CSIC). All the inoculation procedure was effectuated using a Bunsen burner. The inoculation was performed in 5000ml glass Erlenmeyer flasks with perforated rubber stoppers with aeration systems. In each flask, on the perforations was introduced one glass tube for aeration and two silicone tubes to withdraw the samples and for air purge. The air was sterilized using nitrocellulose filters (0.2 µm mean per diameter) (Figure II.1.).

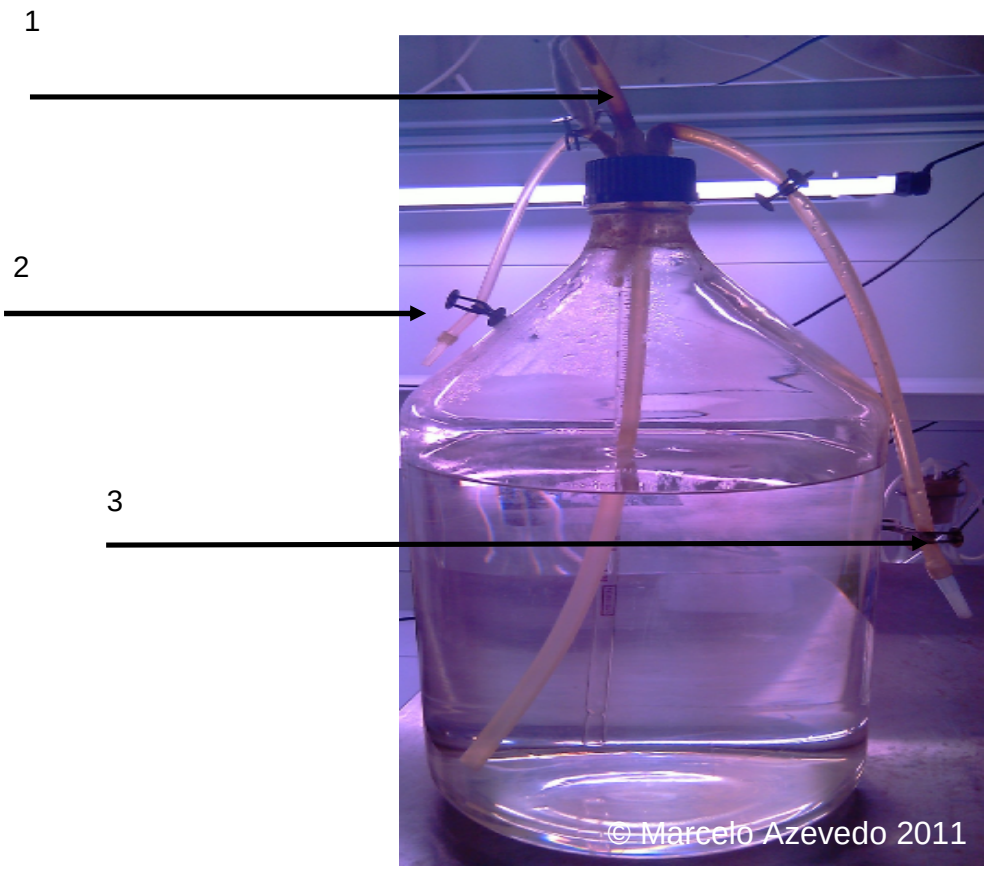

Figure II.1. – Erlenmeyer flasks were the algae inoculation was performed. 1- Air exit; 2- Filtered air entrance; 3- Silicon tube for withdraw samples.

When the cultures reached the exponential phase (usually after two weeks), the medium was replaced aseptically to fresh media, with one day interval, in order to maintain the cultures in logarithmic growth phase. This replacement consisted of taking out half of the culture volume and added the same volume of freshly prepared medium. The medium was previously prepared and sterilized and all the procedure was performed using the Bunsen burner. In all these process, all cultures were monitored by microscopic observation (Leica DM 2000) in order to verify cell growth and a normal and healthy condition of *T. chuii*.

2.3. Acute Tests with Single Substances

Growth inhibition tests were done to determine the concentration causing 50% of inhibition (IC_{50}) of *T. chuii* growth following the OECD Guideline 201 "Freshwater Alga and Cyanobacteria, Growth Inhibition Test" (OECD, 2006). Algal tests were performed in 500 ml Erlenmeyer flasks using the same system described above. Some modifications were made to the guideline which included a longer testing time (96h exposure) and a different testing

volume (400 ml). Erlenmeyer flasks were filled with F2 medium (prepared as described in 2.2.) and then sterilized in the autoclave for 35 minutes at 121° before inoculation of the culture. For each treatment three replicates were used. Stock solution of fluoranthene in acetone in a concentration of 20 mgL^{-1} was prepared because this compound has low water solubility (see Table I.2). Aniline was pipetted directly into the medium because this compound is soluble in water (35 gL^{-1} at 25℃, see Table I.2.). The nominal concentratio ns of the compounds tested were 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 mgL^{-1} for fluoranthene and 4.7, 9.4, 37.5, 75, 150, 300 and 600 mgL^{-1} for aniline.

Test cultures were inoculated with exponential growing algal cultures in a final concentration of 1 x 10^4 cells mL^{-1}. Before this step, the health of the culture (the natural movement of the cells and any sign of contamination of the medium) was verified at the microscope. To know the exact volume that was necessary to introduce in the Erlenmeyer flasks to have the 1 X 10^4 cells mL^{-1}, the algal concentration was determined using a Neubauer Improved bright-line chamber (PRECICOLOR HBG, Germany). Test cultures were maintained at the same conditions as the initial culture (continuous aeration, 20±1℃ and continuous light exposure (24h)). Growth was monitored by cell counts using a Neubauer Improved bright-line chamber (PRECICOLOR HBG, Germany), at 0, 24, 48, 72, and 96 h, using a microscope. During the test, medium temperature and pH were monitored every 24 h and the samples were withdrawn from the Erlenmeyer flasks under positive pressure to avoid contamination. After the withdrawal of the samples, lugol solution was added in the proportion of 1:10 to avoid the natural movement of the *T. chuii* cells. Considering the high mobility of the *T.chuii* cells, this step was fundamental to count the cells in the Neubauer Improved bright-line chamber (PRECICOLOR HBG, Germany) because the cells have to be fixed to make the count possible.

2.4. Acute Toxicity of Binary Mixtures

In order to prepare the binary mixture with aniline and fluoranthene, a stock solution of fluoranthene was prepared as described above, and for aniline a dilution of 1:100 with ultra pure water was done. Using the IC_{50}, IC_{20}, IC_{10} and IC_5 values determined in the tests with isolated substances (section 2.3.), a first mixture was prepared and the Table II.1. shows the test combinations used. Following the recommendations of Jonker *et al*. (2005), Gomez-Eyles *et al*. (2009) and Martin *et al*. (2009) a prior characterization of the dose-response relationship for the isolated substances was done first, and the mixture assay included control treatments without any of the test substances, treatments with fluoranthene only, treatments with aniline only, and binary mixtures of the two substances. In a second test, the IC_{50} of aniline was combined with the IC_{50} of fluoranthene, the IC_{20} of one compound

with the IC_{20} of the other one, and the same thing was done with the IC_{10} and IC_5 of both compounds. To perform the test, the exposure technique and procedures were the same described in the previous section, for isolated substances.

Table II.1. – Concentrations of aniline and fluoranthene tested in the mixture assay. The IC_{10} values correspond to the concentration of the compound that caused 10% of *Tetraselmis chuii* growth inhibition in the assay with single substances, while IC_{50}s are the corresponding concentrations causing a 50% of growth inhibition and zero indicates the absence of the substance in test medium. Three replicates were used per treatment.

Aniline / **Fluoranthene**	**0**	**IC_{10} = 17.007 mgL^{-1}**	**IC_{50} = 60.870 mgL^{-1}**
0	0+0	0+IC_{10}	0+IC_{50}
IC_{10} = 0.066 mgL^{-1}	IC_{10}+0	IC_{10}+IC_{10}	IC_{10}+IC_{50}
IC_{50} = 0.378 mgL^{-1}	IC_{50}+0	IC_{50}+IC_{10}	IC_{50}+IC_{50}

2.5. Data Analysis

2.5.1. Method for Growth Inhibition and Yeld determination

Following the OECD Guideline 201 “Freshwater Alga and Cyanobacteria, Growth Inhibition Test”, average growth rate, the percent inhibition of growth rate and the percent inhibition of yeld were calculated.

The average growth rate was determined using the formula:

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

where $\mu_{i\text{-}j}$ is the average specific growth rate from time I to j, X_i the biomass at time i and X_j the biomass at time j.

For each test the percentage of culture growth inhibition was calculated following the formula:

$$\%I_{\mathrm{r}} = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

where % I_r is the percent inhibition in growth rate, μ_c is the mean value for average growth rate in the control group and μ_T is the average specific growth rate for the treatment replicate.

Yeld was calculated following the formula:

$$\%I_{\mathrm{y}} = \frac{(Y_c - Y_t)}{Y_c} \times 100$$

where the I_y is the percent inhibition of yeld, Y_c is mean value for yeld in the control group and the Y_T is the average yeld for the treatment replicate.

2.5.2. Method of Determining Mixture Effects

To assess the type of interaction between the two compounds tested, the following formula was used (Bocquené *et al*., 1995):

$${}_{x}TU_A + {}_{y}TU_B = 1\ TU_{(A+B)}$$

where, TU is the toxic unit (IC_{50}), A and B are the compounds tested (aniline and fluoranthene) and *x* and *y* are the proportional toxic units of A and B present in the mixture.

A and B are exactly additive if the addiction of half of the concentration of the compound A necessary to produce the IC_{50} and half of the concentration of the compound B to produce that same effect just result in the IC_{50}. But, if that addiction causes more than the IC_{50}, the type of interaction is synergistic. Thus,

if x + y =1, the joint action is additive
if x + y < 1, the joint action is synergistic
if x + y > 1, the joint action is antagonistic

To interpret the type of interactions between the two compounds present in the mixture, the diagram of Gaddum (1948) as modified by Sprague (1970) was used (Figure II.2.). Combinations of two toxicants are illustrated on the diagram with the axes indicating concentrations or toxic units (TU). In this work, the toxic unit corresponds to the IC_{50} of aniline or fluoranthene.

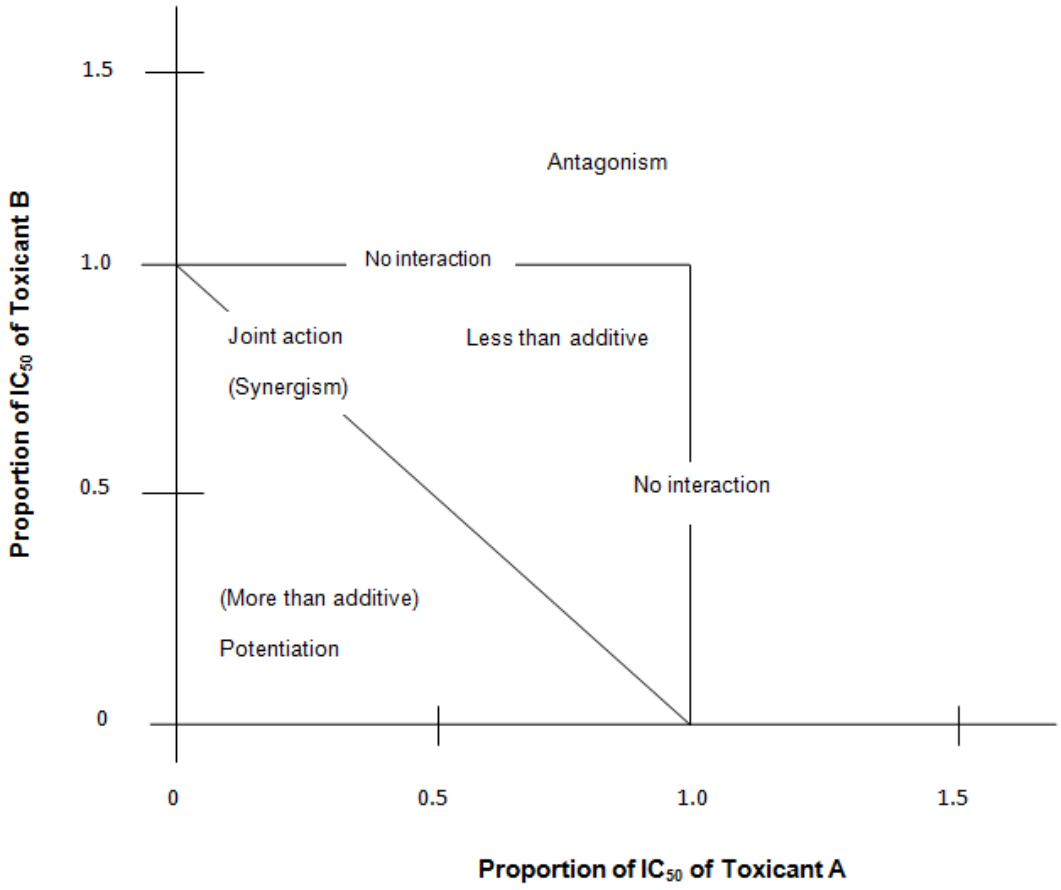

Figure II.2. – Diagram showing the joint action of two toxicants [from Gaddum (1948) modified by Sprague (1970)].

2.5.3. Data Treatment

All data was tested for normality using the Kolmogorov–Smirnov normality test and homogeneity of variance (Levine's test), was also verified (Zar, 1996). One-way analysis of variance (ANOVA) was used to compare different treatments in each algal toxicity test and Dunnet's test was used to determine both lowest observed effect concentration (LOEC) and no-observed effect concentration (NOEC) (Zar, 1996). In order to produce growth curves, the mean values of each treatment were plotted against time. The 10% and 50% Effect Concentrations (IC_{10} and EC_{50}, respectively), values were calculated by probit analysis using the percentage of inhibition values determined before (Finney, 1971).The SPSS© software was used to perform all the statistical analysis of the data and differences were considered statistically significant when $p < 0.05$.

3. RESULTS

The growth of *T. chuii* during the 96 h of exposure to tested fluoranthene and aniline concentrations is represented in Figures II.3. to II.10. The calculated IC_{10} and IC_{50} values of both acute exposures to isolated substances are presented in the Table II.2. In all bioassays no significant differences between control and solvent control were observed.

The evolution of *T.chuii* growth during the exposure to different concentrations of aniline is shown in Figure II.3., and the final number of cells (mean of three replicates at 96h) is represented on Figure II.4. At the end of the acute test with aniline the lowest concentration that caused a significant inhibition of *T. chuii* growth, when compared to the control, was 37.5 mgL^{-1} (Lowest observed effect concentration (LOEC)). No inhibition growth of the algae was observed up to 18.75 mg L^{-1} (No observed effect concentration (NOEC)), while exposure of *T. chuii* cells to 37.5, 75, 150, 300 and 600 mgL^{-1} of aniline caused significant inhibitions ($F_{(8,17)}$ = 88.875, $p<0.05$) of 47%, 52%, 69%, 100% and 100%, respectively (Figure II.5.). Regarding the inhibition of yeld, the lowest concentration that lead to a significant inhibition of this parameter when compared to the control, was 37.5 mgL^{-1} (LOEC). Inhibition of yield was not observed up to 18.75 mgL^{-1} (NOEC), while at 37.5, 75, 150, 300 and 600 mgL^{-1} of the tested HNS, it was obtained a significant inhibition of this endpoint ($F_{(8,18)}$ =49.909, $p<0.05$) of 85%, 89%, 95%, 100% and 100% respectively (Figure II.6).

Figure II. 7 and 8 represented the growth of *T. chuii* within the several tested fluoranthene concentrations and the final number of cells (mean of three replicates), respectively, during the 96h of exposure. Considering the bioassay, with the selected PAH, significant differences among treatments were found ($F_{(8,23)}$ = 49.342, $p<0.05$; LOEC= 0.4mg L^{-1}), with algae growth being significantly inhibited at the higher concentrations; the exposure of the algae cells to 0.2, 0.4 and 0.8 mgL^{-1} of fluoranthene caused a 20%, 35% and 100% inhibition of growth, respectively (Figure II.9.). Regarding the inhibition of yield, a significant inhibition was obtained at concentrations equal or higher than 0.2 ($F_{(8,23)}$ = 15.4, $p<0.05$; NOEC= 0.1mg L^{-1}). At 0.2, 0.4 and 0.8 mgL^{-1} of fluoranthene of the inhibition of yield was 58%, 79% and 100%, respectively (Figure II.10.).

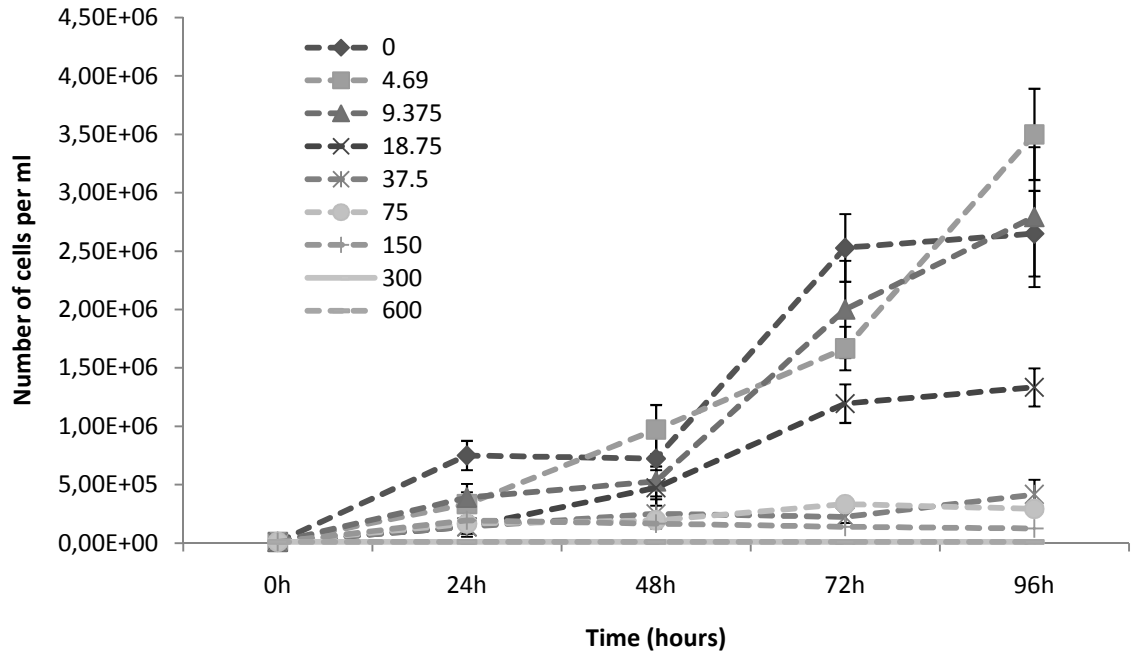

Figure II.3. - *Tetraselmis chuii* growth in the presence and absence of aniline (mgL^{-1}). Values are the mean (three replicates) with corresponding S.E.M. bars. 0 – Control.

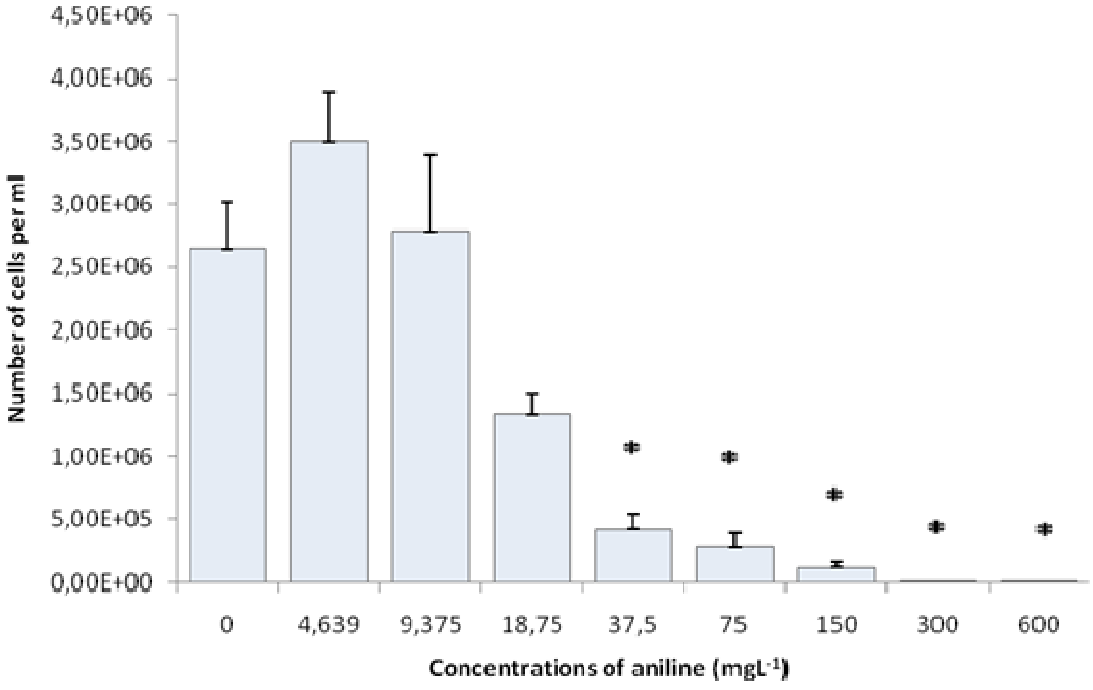

Figure II.4. - Number of cells of *Tetraselmis chuii* after 96h of exposure to different concentrations of aniline (mgL^{-1}). Values are the mean of three replicates with the corresponding S.E.M bars. 0 – Control; * - Significantly different from the control group at 96h ($p<0.05$ Dunnett test).

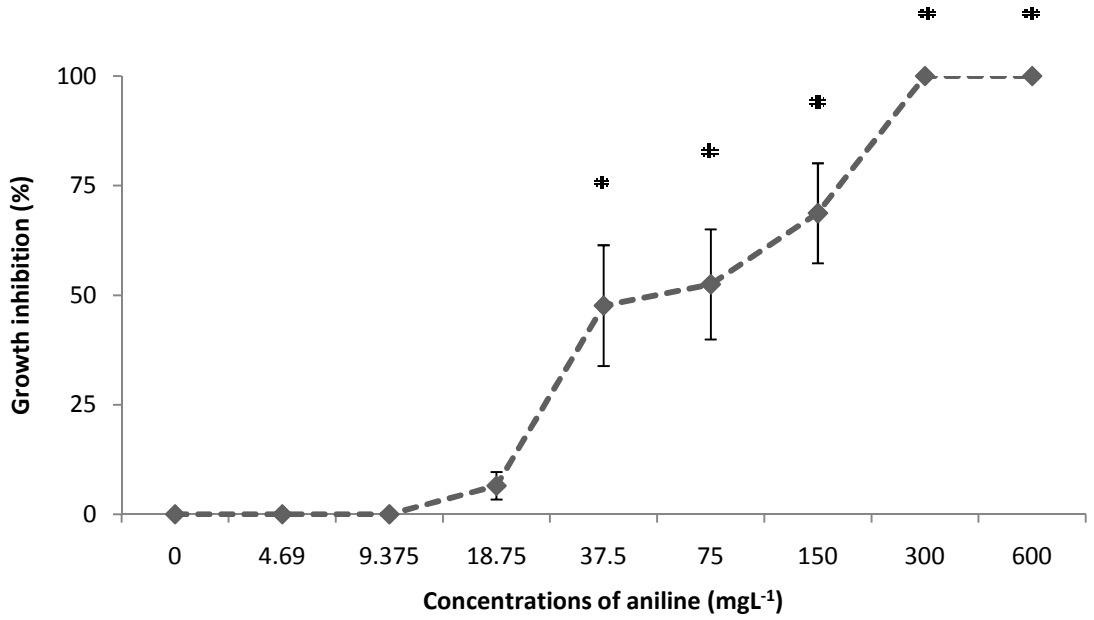

Figure II.5. - Inhibition of *Tetraselmis chuii* growth in the presence of different concentrations of aniline (mgL^{-1}). Values are the mean of three replicates with the corresponding S.E.M bars. 0 – Control; * - Significantly different from the control group at 96h ($p<0.05$ Dunnett test).

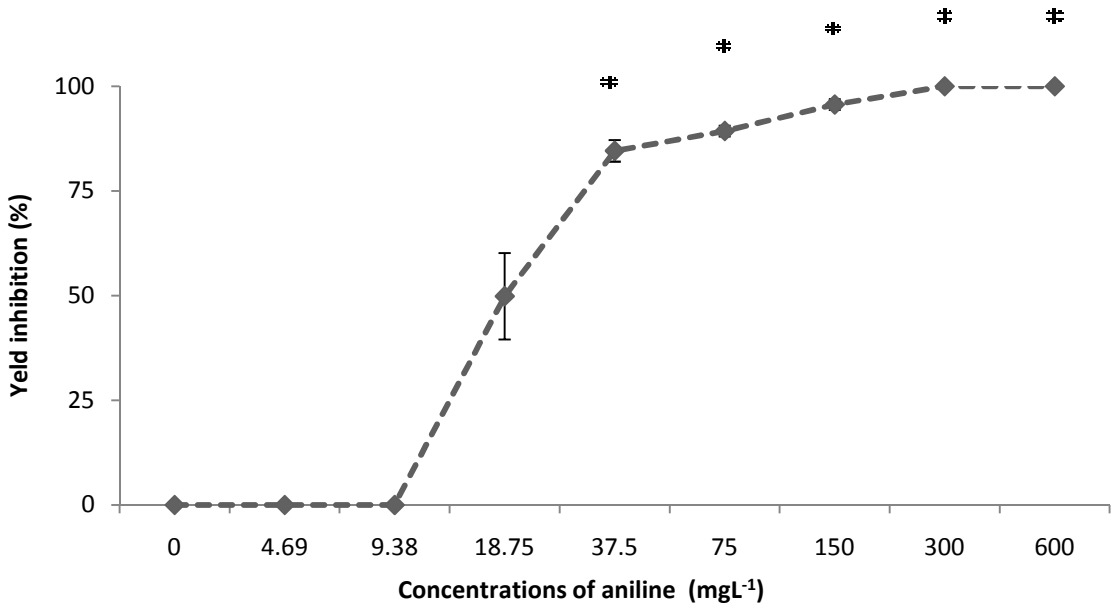

Figure II.6. – Inhibition of yeld of the growth of *Tetraselmis chuii* after 96h of exposure to different concentrations of aniline (mgL^{-1}). Values are the mean of three replicates with the corresponding S.E.M bars. 0 – Control; * - Significantly different from the control group at 96h ($p<0.05$ Dunnett test).

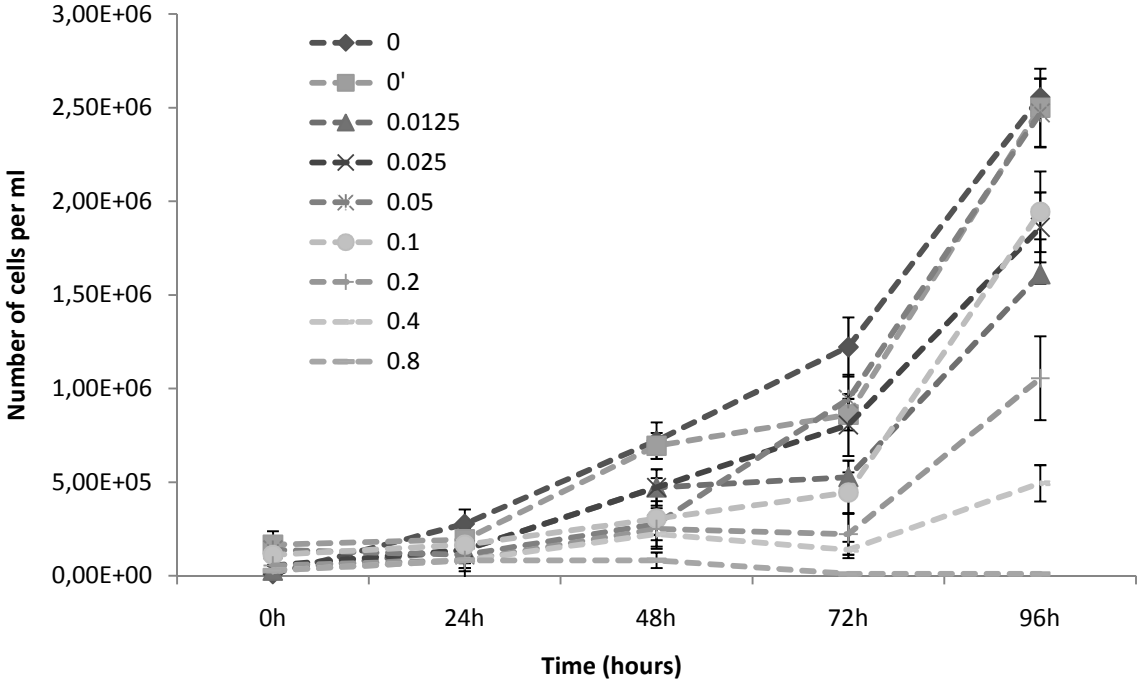

Figure II.7. - *Tetraselmis chuii* 96h growth in the presence and absence of different fluoranthene concentration (mgL^{-1}). Values are the mean (three replicates) of number of cells per ml in each treatment. 0 – Control; 0' – Solvent control; * - Significantly different from the control group at 96h (p<0.05 Dunnett test).

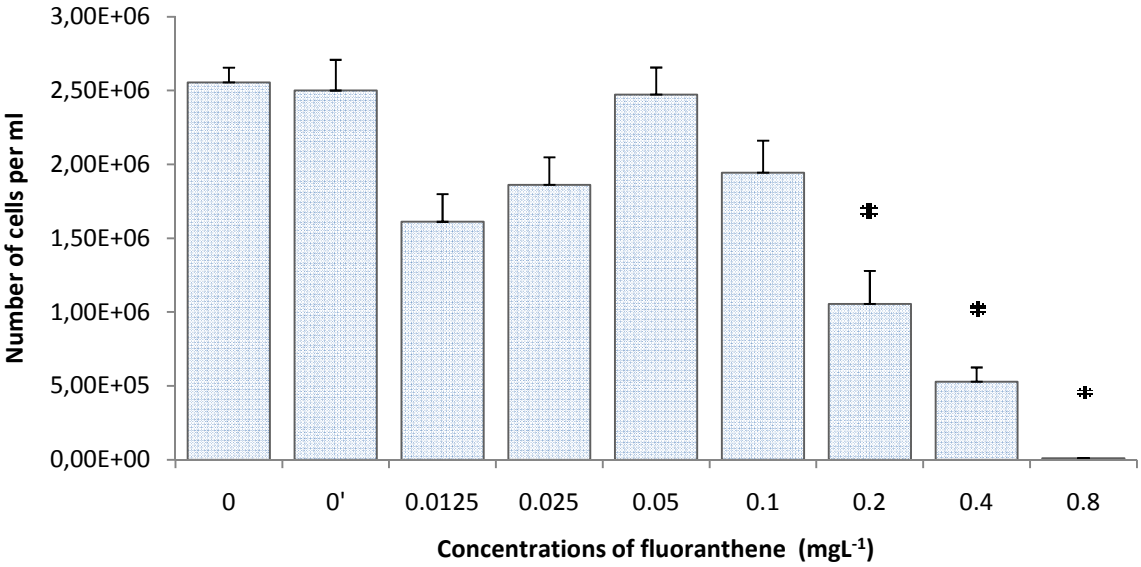

Figure II.8. - Number of cells of *Tetraselmis chuii* after 96h of exposure to different concentrations of fluoranthene (mg L^{-1}). Values are the mean of three replicates with the corresponding S.E.M bars. 0 – Control; 0' – Solvent control; *- indicate significant differences relatively to the control group (p<0.05 Dunnett test, at 96h).

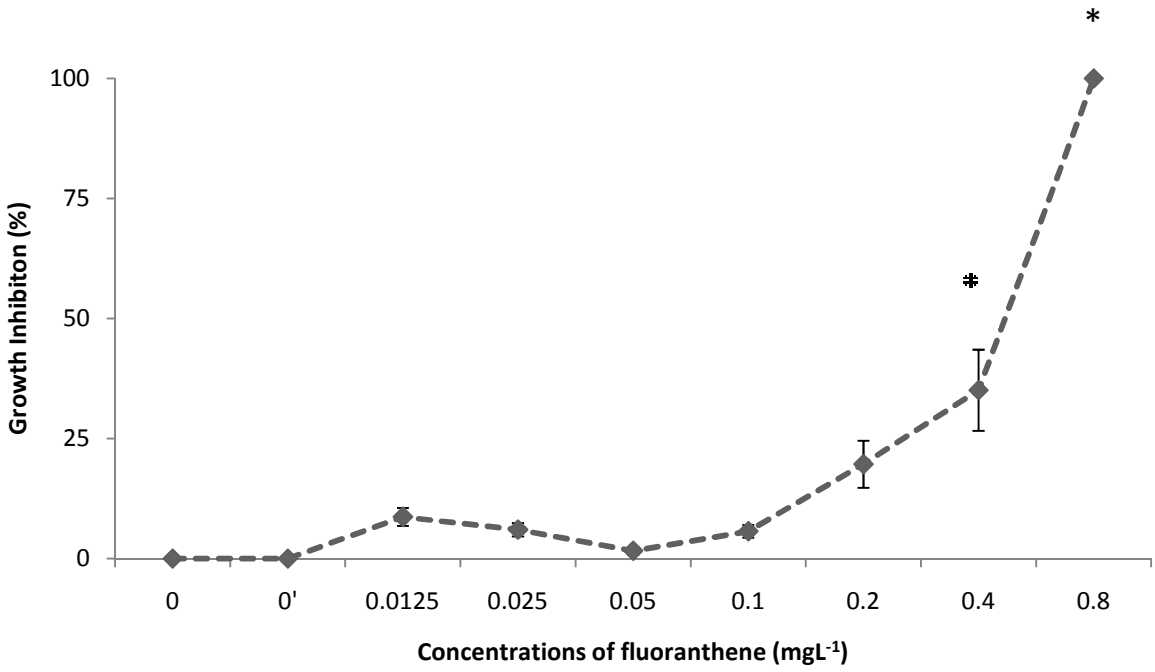

Figure II.9. - Inhibition of *Tetraselmis chuii* growth in the presence of different concentrations of fluoranthene (mgL^{-1}). 0 – Control; 0' – Solvent control; *- indicate significant differences relatively to the control group ($p<0.05$ Dunnett test, at 96h).

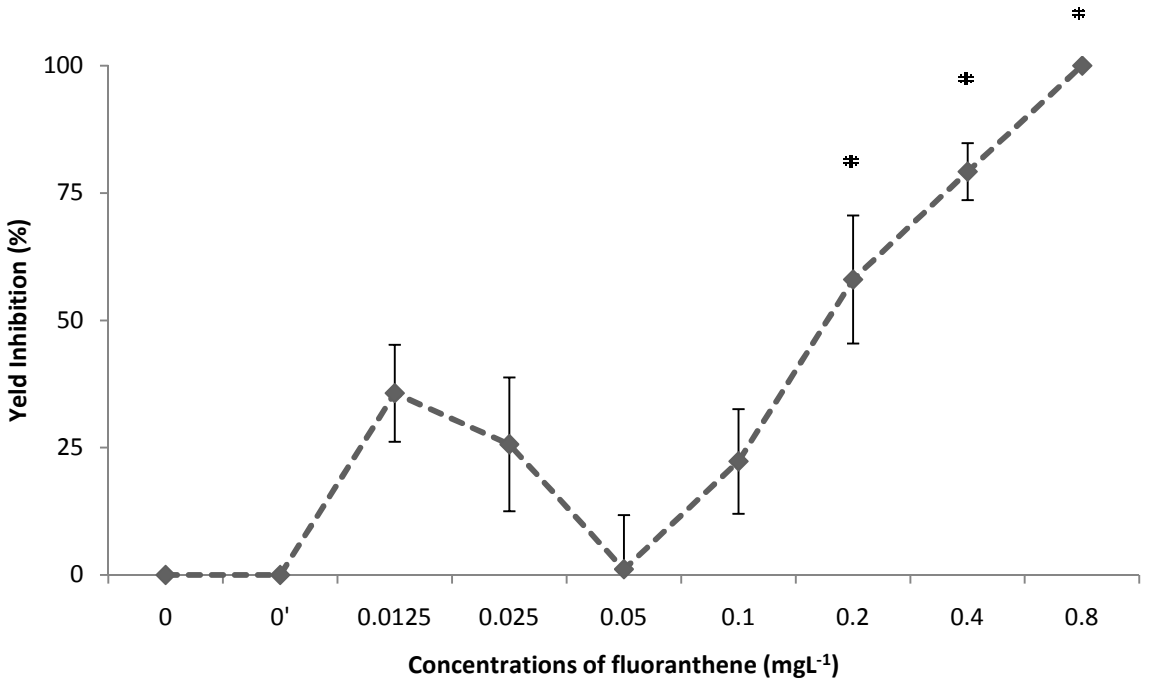

Figure II.10. - Inhibition of yeld of the growth of *Tetraselmis chuii* after 96h of exposure to different concentrations of fluoranthene (mgL^{-1}). 0 – Control; 0' – Solvent control; *- indicate significant differences relatively to the control group at 96h ($p<0.05$ Dunnett test).

Regarding the results obtained for the two bioassays with the binary mixture, all the concentrations tested caused a significant inhibition of *T. chuii* growth when compared to the control (Figures II.11. and II.12.). In the first bioassay, the exposure of the microalgae cells to combinations of IC_{10}s of both compounds, IC_{10} of aniline with IC_{50} of fluoranthene and vice versa and IC_{50} of both compounds result in a 50%, 59%, 58% and 93% of inhibition of the algae growth, respectively (Figure II.11.). Therefore, toxicological interactions seem to have occurred, with the simultaneous exposure to IC_{10} concentrations of both compounds inducing 50% of *T. chuii* growth inhibition. Thus, the type of toxicological interaction can be calculated by applying the formula of section 2.5.2:

Fluoranthene: IC_{10} = 0.066 mgL^{-1}; IC_{50} = 0.378 mgL^{-1} => x = 0.17

Aniline: IC_{10} = 17.007 mgL^{-1}; IC_{50} = 60.870 mgL^{-1} => y = 0.28,

X + Y = 0.17 + 0.28 = 0.45

Since the sum of x and y is lower than 1, the toxicological interaction corresponds to synergism.

As expected, in the second mixture bioassay, all the combinations tested also caused a significant inhibition of *T.chuii* growth when compared to the control, with the sum of two IC_5s, two IC_{10}s, two IC_{20}s or two IC_{50}s causing 20%, 49%, 73% and 100% inhibition of growth respectively. The mixture IC_{50} calculated from this curve was 17.007 mgL^{-1} of aniline + 0.066 mgL^{-1} of fluoranthene.

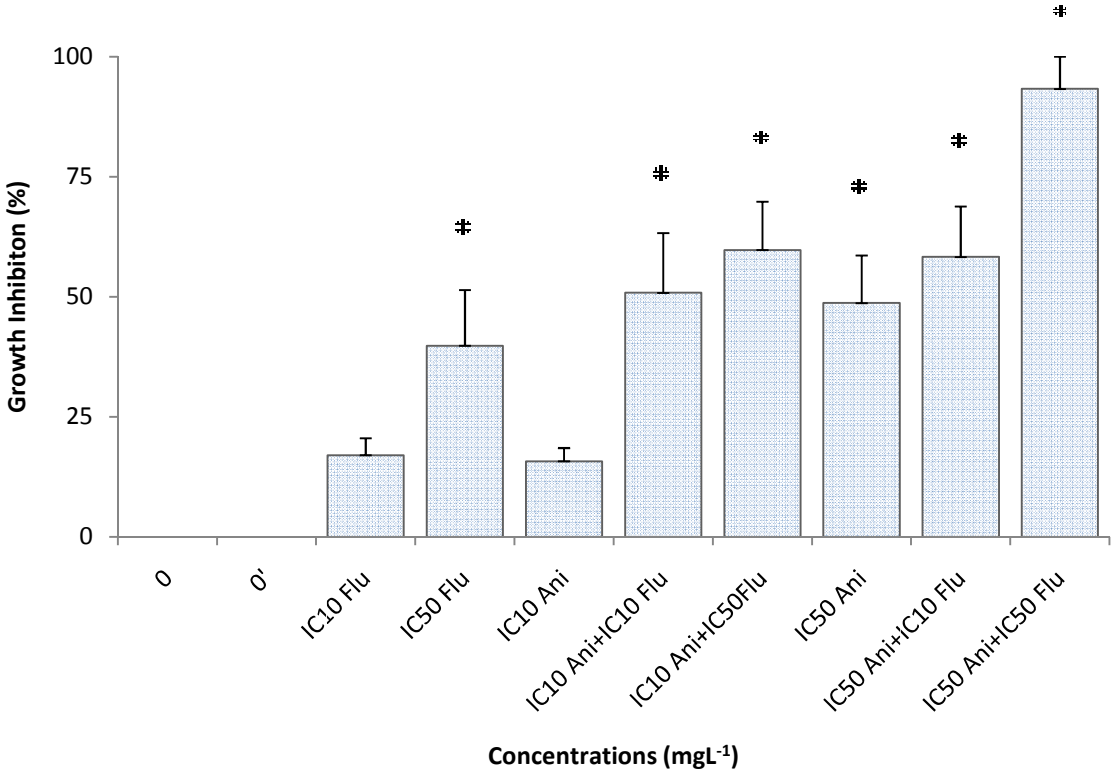

Figure II.11. – Microalgae growth inhibition after 96h of exposure to a binary mixture (aniline and fluoranthene). 0 – Control; 0' – Solvent control; *- significantly different from the control group (p<0.05 Dunnett test); Flu – fluoranthene; Ani – aniline.

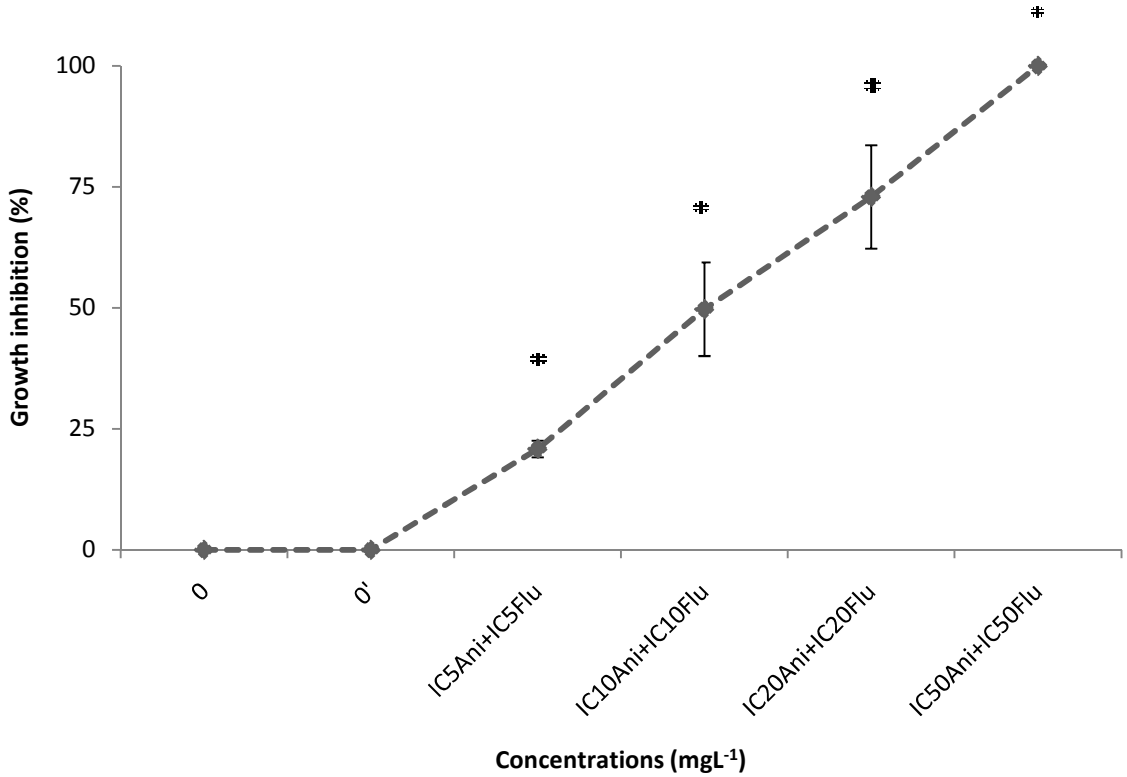

Figure II.12. - Second bioassay of the microalgae growth inhibition after 96h of exposure to binary mixtures of aniline and fluoranthene. 0 – Control; 0' – Solvent control; *- significantly different from the control group (p<0.05 Dunnett test); Flu – fluoranthene; Ani – aniline.

Table II.2. - Median growth inhibition concentration (IC_{50}) and 10% growth inhibition concentration (IC_{10}) (mgL^{-1}) with corresponding 95% confidence intervals of aniline and fluoranthene for *Tetraselmis chuii*.

Compound	IC_{50} (95% CL)	IC_{10} (95% CL)
Aniline	60.870 (46.714-79.505)	17.007 (10.216-23.979)
Fluoranthene	0.378 (0.206-1.166)	0.066 (0.016-0.126)

4. DISCUSSION

4.1. Toxic Effects of Single Substances

After a spill accident with oils, HNS and other common harmful transported chemicals on marine coastal zones, the organisms in the water column are exposed to continuously changing concentrations of these substances. These substances are usually present at higher concentrations near the origin of the spill. Therefore, to investigate the effects of these compounds, at concentrations usually found near the accident, is an issue of interest in Ecotoxicology. When these accidents occur, the aquatic food web may be affected, including the basis of it, the phytoplankton. The exposure to contaminants by microalgae may cause the bioaccumulation of those compounds and lead to food chain transfer and biomagnifications (Rumampuk *et al*., 2003), being these organisms, also, involved in the transformation and cycling of key elements (Domingues *et al*., 2008). The concentrations of the selected HNS (aniline: 4.69 – 600 mgL^{-1}) and PAH, (fluoranthene: 0.0125 – 0.025 mgL^{-1}) are higher than those found in the wild as far as we could find in the literature. For example, in an aquifer near an underground coal gasification site in Wyoming, the level of this compound was measured at a maximum of 36 parts per billion (ppb = μgL^{-1}) (US EPA, 1994). In a shallow aquifer contaminated by coal-tar wastes, aniline has been detected but not properly quantified (US EPA, 1994). Armenta-Arteaga and Elizalde-González (2003) reported a concentration of fluoranthene of 0.09 μgL^{-1} in the water of the Mecoacán Lake, examined after an oil spill. According to Reddy and Quinn (1999) a concentration of fluoranthene of 0.0352 μgL^{-1} was measured in seawater samples collected after the *North Cape* oil spill. However, in order to investigate IC_{50} for selected species, representing the phytoplankton community, there were selected concentrations able to producing damaging effects in organisms, allowing the determination of IC_{10} and IC_{50} values, very useful for the study of binary mixtures and risk assessment programs.

A high volume of chemicals are produced and transported along coastal marine routes every year representing a threat to humans and to the marine environment (Mamaca *et al*., 2005). Chemicals like xylene, toluene, styrene, benzene and ammonia are examples of HNS transported by sea that constitute a threat if an accident occurs. Several studies have been carried out with the intent to understand the effects of the exposure to this variety of chemicals (Mamaca *et al*., 2005; Teuschler *et al*., 2005; Wicks *et al*., 2002; Tatarazako *et al*., 2002; Vidal *et al*. 2001; Cushman *et al*., 1997; Nimptsch and Pflugmacher, 2007). The 96h IC_{50} value obtained to aniline in our study was 60.87 mgL^{-1}, being this value comparable to those described by other authors. For example, *T. chuii* seems to be more sensitive than the

green algae *Chlorella pyrenoidosa* because Maas-Diepeveen and Leeuwen (1986) have reported a 96h EC_{50} of 94 mgL^{-1} to this unicellular green algae. There is limited information about the acute effects of this HNS on marine algae, which highlights the importance of investigate this subject. The toxicity of this compound has been also tested on other aquatic species. Bhunia *et al*. (2003) reported 96h LC_{50} values to the cladoceran crustacean *Moina micrura*, to the tilapia fish *Oreochromis mossambicus* and to the oligochaete worm *Branchiura sowerbyi* of 0.6, 69.4 and 586 mgL^{-1}, respectively. These results showed that *T. chuii* is much more sensitive than *B. sowerbyi* to the exposure to this compound. However, when compared to *M. micrura*, the results shown that this crustacean is much more sensitive than *T. chuii*. The IC_{50} obtained to *T. chuii* is very similar to the value reported to tilapia fish (*O. mossambicus*).

PAHs are the most widespread class of contaminants and are considered priority pollutants by the U.S. Environmental Protection Agency (Palmqvist *et al*., 2006; Riznyk *et al*., 1987). Several studies have been done to evaluate PAHs effects on the producers (Riznyk *et al*., 1987; Wang and Zheng, 2008; Liu *et al*., 2006). In the present work the 96h IC_{50} of fluoranthene to *T. chuii* was 0.378 mgL^{-1}. This value indicates that this compound is toxic to this microalga, being very similar to the result obtained for the marine algae *Cyclotella caspia* of 0.2 mgL^{-1} (Liu *et al*., 2006), to the EC_{50} obtained by Šepic *et al*., 2003 of 0.19 mgL^{-1} and to the 72h EC_{50} obtained by Wang and Zheng (2008) to the marine diatom *Phaeodactylum tricornutum* of 0.103 mg L^{-1}. Spehar *et al*. (1999) reported the toxicity of this PAH to several freshwater and saltwater species. According to the results obtained, the hydra *Hydra Americana*, the amphipod *Hyalella azteca* and *Ampelisca adbita* and the mysid *Mysidopsis bahia* are all more sensitive to the exposure to fluoranthene (LC_{50}s < 100 μgL^{-1}). There are some studies that demonstrate that solar ultraviolet (UV) radiation increases the toxicity of fluoranthene on aquatic organisms (Spehar *et al*., 1999; Wang *et al*., 2008). This study was made in a closed room under fluorescent light and for that reason, the potential of UV radiation for enhance the toxicity is not assessed. Due to this fact, the toxicity of this compound may be underestimated to this marine microalga. Further studies should be performed in order to evaluate that.

Overall results demonstrated clearly that both substances are able to produce significantly ($p<0.05$) growth inhibition to *T. chuii*. Considering the endpoints obtained, 96h IC_{50} values of 60.87 and 0.378 mgL^{-1} to aniline and fluoranthene respectively, it can be concluded that *T. chuii* is much more sensitive to fluoranthene than to aniline, both 96h IC_{50} values in good agreement with data found in the literature to other algae species and to other marine organisms as well.

4.2. Toxic Effects of Binary Mixtures

In the environment, organisms are rarely exposed to one single compound only. Usually, they are in contact with complex mixtures of compounds (Barata *et al*., 2006). The investigation of the effects of mixtures on marine wildlife is a recent subject, however there is a lack of knowledge concerning its effects on marine microalgae. The presence of mixtures of compounds from different sources with different modes of action and chemical structures is frequently on the environment (Barata *et al*., 2006). However, the studies that have been performed concerning the effects of mixtures on different species usually focus on compounds belonging to the same class or with the same mode of action (Erickson *et al*., 1999; Hsieh *et al*., 2006; Wang *et al*., 2011). It is important to assess the effects of mixtures, including compounds of different classes, because in the environment single compounds can often exist below levels predicted to cause any effects or below NOECs (Walter *et al*., 2002; Shuler *et al*., 2009). Despite this, as they do not exist alone in the environment, they still can cause effects by interacting with other compounds (Shuler *et al*., 2009). Walter *et al*. (2002) studied the effect of pollutants at NOEC levels and concluded that the toxicity of the mixtures were higher than expected for the compounds alone, highlighting the importance of this issue on aquatic toxicology research.

In order to evaluate the type of interaction between the two compounds tested, first it was necessary to determine the IC_{50}, IC_{20} and IC_{10} values for the isolated substances. Characterize the dose-response relation for the isolated substances before and the evaluation of each compound isolated simultaneously with the different combinations tested is suggested in the literature and was performed on this study (Jonker *et al*., 2005; Gomez-Eyles *et al*., 2009; Martin *et al*., 2009). Therefore, using the 96h IC_5 ,IC_{10}, IC_{20}, and IC_{50} values determined, a binary mixture was prepared in order evaluate the effect of the simultaneously exposure to both compounds on *T. chuii* growth. The obtained results seem to suggest that this binary mixture has a synergistic effect because the treatment having concentrations corresponding to the IC_{20} of the two toxicants caused a 50% inhibition which is more than the result expected (20%) for an additive effect, with the sum of the proportions concentration tested/IC50 of the two substances lower than 1.

5. CONCLUSIONS

In this study, the toxicity of an oil component, the PAH fluoranthene, and of the HNS aniline to the marine algae *Tetraselmis chuii* was investigated in 96h laboratory assays based on growth inhibition. Both substances were found to inhibit *T. chuii* growth with IC_{50} 60.870 mgL^{-1} and 0.378 mgL^{-1} for aniline and fluoranthene, respectively. Furthermore, in mixture assays, toxicological interactions were found between the two chemical agents resulting in synergistic effects on *T. chuii* growth inhibition, allowing the rejection of the null hypothesis of no increased effects induced by the mixture that was one of the central objectives of this study. Therefore, these findings highlight the importance of testing effects potentially resulting from toxicological interactions, particularly involving oil components and HNS, due to the scarcity of information existing at the present and the risk of shipping accidents resulting in the simultaneous release of HNS and oils.

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CHAPTER III

GENERAL DISCUSSION

1. GENERAL DISCUSSION

In the last years, shipping as a mode of transportation of dangerous goods has increased (Purnell, 2009; Mamaca *et al*., 2009). This fact is also linked with the increase in the risk of accidents like spills. Several accidents have occurred in the past years with big and dramatic consequences on both environmental and economic level, being the *Prestige* oil spill and the BP platform accident two of the most recent examples (Muralidharan *et al*., 2011; Harlow *et al*., 2011; Carrera-Martínez *et al*., 2010; Varela *et al*., 2006; Franco *et al*., 2006; Salas *et al*., 2006; Saco-Álvarez *et al*., 2008).

A group that raises concern because of their negative effects when spilled and that are transported on big volumes is the HNS group. Aniline is an organic compound that belongs to this list. On this study, a 96h acute test was performed with this compound and the results showed that this compound inhibits significantly the growth of *T.chuii* at the concentrations equal or higher than 37.5 mgL^{-1}. The 96h IC_{50} obtained was 60.870 mgL^{-1}, a value that is lower than the result obtained by Maas-Diepeveen and Leeuwen (1986) to the green algae *Chlorella pyrenoidosa* of 94 mgL^{-1} after a 96h test.

Petroleum products are explored and transported on a big scale throughout the world and sometimes accidents occur, being oil spills common events around the world (Cohen and Nugegoda, 2000). PAHs belong to the aromatic fraction of oil and are usually responsible for the most relevant effects caused (Albers, 2003). Fluoranthene is one of the compounds that belong to this group, is among the 16 EPA's PAH priority list and is one of the most common PAHs found in the environment (Šepic *et al*., 2003; Liu *et al*., 2006). The results of the 96h acute test performed on this Thesis demonstrate that fluoranthene has significantly ($p<0.05$) inhibited the growth of the microalgae at concentrations equal or higher than 0.4 mgL^{-1}. The 96h IC_{50} determined was 0.378 mgL^{-1} which demonstrates that this compound is highly toxic to the microalgae *T.chuii.* The observed result is in good agreement with other studies found in the literature such as the result obtained for the marine algae *Cyclotella caspia* of 0.2 mg L^{-1} (Liu *et al*., 2006), the EC_{50} obtained by Šepic *et al*., 2003 of 0.19 mgL^{-1} and to the 72h EC_{50} obtained by Wang and Zheng, 2008 to the marine diatom *Phaeodactylum tricornutum* of 0.103 mgL^{-1}.

Therefore overall results demonstrated clearly that *T. chuii* is much more sensitive to fluoranthene than to aniline, and it can also be concluded that the 96h IC_{50} values determined fit with data found on the literature to other algae species and to other marine organisms as well.

In the ecosystems, the organisms are usually exposed to complex mixtures of chemicals and the effects caused by those alone are likely to be different from the effects caused by the mixture (Barata *et al*., 2006; Hodges *et al*., 2006). In the present dissertation, a binary mixture of fluoranthene and aniline was tested and the results demonstrate a higher inhibition than expected. In order to evaluate the type of interaction between the two compounds tested, first it was necessary to determine the 96h ICs values for the isolated substances. In addition, and has it is suggested in the literature, a prior characterization of the dose-response relation for the isolated substances and the evaluation of each compound isolated simultaneously with the different combinations tested (Jonker *et al*., 2005; Gomez-Eyles *et al*., 2009; Martin *et al*., 2009). Using the method described before to characterize the mixture toxicity, the type of interaction among the compounds was determined and the results obtained suggest a synergistic effect for the mixture. Finally, the assessment of the toxicity of this mixture should be done on other aquatic species and other binary mixtures composed of other PAHs and HNS should be done in order to obtain knowledge about the interaction between these compounds and their respective effects towards different aquatic species.

2. FINAL CONCLUSIONS/ CONSIDERATIONS

Present dissertation demonstrated clearly the potential of aniline and fluoranthene to inhibit significantly the growth of *T. chuii*, accepting the first proposed hypothesis. Considering that this species has been considered by other authors as representative of marine phytoplankton and a model to assess the impact of several pollutants, in the environment since this microalgae has a broad distribution in the tropical ecosystems and large use in laboratory cultures, present results also suggest that concentrations, near the observed LOECs, of both tested substances may affect severally the local aquatic wildlife, by the disruption of aquatic food chain. In addition, following the recent recommendations in marine Ecotoxicology area, the effects of a binary mixture, using the same assessed compounds in the first part of present work, on *T. chuii* was investigated. The obtained results suggest that a synergistic effect on selected microalgae growth, rejecting the proposed hypothesis. Overall results demonstrate that *T. chuii* is a suitable species to be used as test organism in laboratorial bioassays, in good agreement with previous published works.

Present dissertation findings demonstrate the potential of two classes of contaminants to inhibit the growth of an ecological relevant algae, generating important ecotoxicological data (IC_{10} and IC_{50} values), useful to produce information for the evaluation

of the potential impact of these classes of compounds on aquatic system food webs. Considering the increasing importance of more studies concerning the effects of mixtures on biota, this work, also, contributed to the literature with data regarding combined effects of aniline and fluoranthene on the marine algae *T. chuii*. The present work also highlights the need of further investigation on the effects of mixtures of PAHs and HNS.

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ANNEX I

F2 MEDIUM COMPOSITION (Guillard and Ryther, 1962)

$NaNO_3$ (75.0 gL^{-1} dH_2O)	1.0 ml
$NaH_2PO_4 \cdot H_2O$ (5.0 gL^{-1} dH_2O)	1.0 ml
F2 Trace Metal Solution	1.0 ml
F2 Vitamin Solution	0.5 ml
Filtered seawater to	1000 ml

TRACE METAL SOLUTION

$FeCl_3 \cdot 6H_2O$	3.15 g
$Na_2EDTA \cdot 2H_2O$	4.36 g
$CuSO_4 \cdot 5H_2O$ (9.8 gL^{-1} dH_2O)	1.0 ml
$Na_2MoO_4 \cdot 2H_2O$ (6.3 gL^{-1} dH_2O)	1.0 ml
$ZnSO_4 \cdot 7H_2O$ (22.0 gL^{-1} dH_2O)	1.0 ml
$CoCl_2 \cdot 6H_2O$ (10.0 gL^{-1} dH_2O)	1.0 ml
$MnCl_2 \cdot 4H_2O$ (180.0 gL^{-1} dH_2O)	1.0 ml
Distilled water to	1000 ml

VITAMIN SOLUTION

Vitamin B_{12} (1.0 gL^{-1} dH_2O)	1.0 ml
Biotin (0.1 gL^{-1} dH_2O)	10 ml
Thiamine HCl	0.200 g
Distilled water to	1000 ml

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ANNEX II

Abstract

The transport of oils and hazardous and noxious substances (HNS) by sea raise concern on their potential spills and consequent effects on marine ecosystems. In the case of this type of environmental accidents, marine organisms may be exposed for short periods to high concentrations of the released chemicals. At the present, there is a considerable lack of knowledge on the effects of mixtures of substances that may be simultaneously spilled during shipping accidents, such as oil components and HNS. Thus, here, the effects of fluoranthene, a polycyclic aromatic hydrocarbon (PAH) that is a component of oils, and aniline, one of the main HNS transported by sea, on the microalgae *Tetraselmis chuii* that has been used in Ecotoxicology to assess the risks of chemicals for marine producers, were investigated both isolated and in mixture. Acute toxicity tests based on growth inhibition of *T. chuii* cultures were carried out for 96h and ecotoxicological parameters were calculated. The results indicate that both substances were able to reduce the algae growth at the concentrations tested and toxicological interactions occur when the algae is simultaneously exposed to fluoranthene and aniline.

Keywords: aniline, fluoranthene, mixture, marine microalgae, growth inhibition.