Universitat de les Illes Balears
Departament de Biología

THRESHOLDS AND POINTS OF NO RETURN FOR OCEANIC PHYTOPLANKTON: EFFECTS OF POLLUTION ON PHYTOPLANKTON COMMUNITIES

Tesi doctoral

Programa de Doctorat de Biología

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Abstract

Since the Industrial Revolution, significant amounts of natural and synthetic compounds have been released to the environment, interacting with the natural biogeochemical cycles. In oceans, these cycles are partly controlled by phytoplankton. Phytoplankton is the primary producer in the ocean, responsible of inorganic carbon fixation and its transformation and incorporation to the ecosystem as organic carbon. The high mortality observed in the last times by phytoplankton implies that the carbon incorporated cannot be transferred either to the trophic chain nor sedimented with the cells to the ocean bottom, highly influencing the carbon fluxes in the ocean. Among the different factors inducing phytoplankton mortality, exposition to toxic pollutants has been observed as one of the factors causing it. Through multiple experiments with metals and Persistent Organic Pollutants (POPs), several pollution thresholds to marine phytoplankton have been determined during this doctoral thesis. Thresholds are the critical values around which the ecosystem flips from one stable state to another, becoming points of no return when crossing again the same value in the opposite direction does not restore the initial state. Thus, the objectives of this doctoral thesis were to quantify those thresholds, to determine the parameters controlling their sensitivity to marine pollution and to analyze the joint action of mixtures of pollutants and the joint action of these pollutants with environmental factors. This doctoral thesis is the result of these experiments and their findings.

Resum

Desde la Revolució Industrial, quantitats significatives de compostos naturals i sintètics han estat alliberats al medi ambient, interactuant amb els cicles biogeoquímics naturals. En els oceans, aquests cicles són controlats en part pel fitoplàncton. El fitoplàncton és el principal productor de l'oceà, responsable de la fixació de carboni inorgànic i la seva transformació i incorporació als ecosistemes com carboni orgànic. L'elevada mortalitat observada en els darrers temps pel fitoplàncton implica que el carboni no pugui ser transferit ni a la cadena tròfica ni sedimentada amb les cèl lules al fons de l'oceà, influint en els fluxos de carboni en l'oceà. Entre els diferents factors que indueixen la mortalitat del fitoplàncton, l'exposició als contaminants tòxics s'ha observat com un dels factors que el causen. A través de múltiples experiments amb metalls i contaminants orgànics persistents (POPs), s'han determinat nombrosos llindars de contaminació per al fitoplàncton marí durant la tesi doctoral. Els llindars són els valors crítics al voltant dels quals l'ecosistema passa d'un estat estable a un altre, convertint-se en punts de no retorn quan creuar de nou el mateix valor en la direcció oposada no restaura l'estat inicial. Així, els objectius d'aquesta tesi doctoral van ser quantificar aquests llindars, determinar els paràmetres que controlen la sensibilitat a la contaminació marina i analitzar l'acció conjunta de mescles de contaminants i l'acció conjunta d'aquests contaminants amb factors ambientals. Aquesta tesi doctoral és el resultat d'aquests experiments i les seves troballes.

Resumen

Desde la Revolución Industrial, cantidades significativas de compuestos naturales y sintéticos han sido liberados al medio ambiente, interactuando con los ciclos biogeoquímicos naturales. En los océanos, estos ciclos son controlados en parte por el fitoplancton. El fitoplancton es el principal productor del océano, responsable de la fijación de carbono inorgánico y su transformación e incorporación a los ecosistemas como carbono orgánico. La elevada mortalidad observada en los últimos tiempos por el fitoplancton implica que el carbono no pueda ser transferido ni a la cadena trófica ni sedimentada con las células al fondo del océano, influyendo en los flujos de carbono en el océano. Entre los diferentes factores que inducen la mortalidad del fitoplancton, la exposición a los contaminantes tóxicos se ha observado como uno de los factores que lo causan. A través de múltiples experimentos con metales y contaminantes orgánicos persistentes (POPs), se han determinado numerosos umbrales de contaminación para el fitoplancton marino durante esta tesis doctoral. Los umbrales son los valores críticos en torno a los cuales el ecosistema pasa de un estado estable a otro, convirtiéndose en puntos de no retorno cuando cruzar de nuevo el mismo valor en la dirección opuesta no restaura el estado inicial. Así, los objetivos de esta tesis doctoral fueron cuantificar dichos umbrales, determinar los parámetros que controlan la sensibilidad a la contaminación marina y analizar la acción conjunta de mezclas de contaminantes y la acción conjunta de estos contaminantes con factores ambientales. Esta tesis doctoral es el resultado de estos experimentos y sus hallazgos.

A mi familia

y amigos

General Introduction

In the latter part of the eighteenth century, the Northern Hemisphere experienced its own Industrial Revolution, forcing a new geological epoch dominated by humankind, the so-called Anthropocene (Crutzen, 2002). Since then, thousands of natural and synthetic compounds have been introduced into the environment, interacting with the natural biogeochemical cycles. The analysis of air trapped in polar ice showed the beginning of increasing concentrations of pollutants even in regions far away the contaminant sources, showing that contamination was not a local issue, but a global issue with global implications.

Thus, pollutants reach pristine areas such as polar ecosystems, alpine lakes or open Oceans, not only through direct runoff of industrial products used by humans (e.g. petroleum spills, Page et al. 1988), but also through the World's Atmospheric Transport, which can transport contaminants long far away of the source of contamination (e.g. Bidleman, 1988; Duce et al., 1991; Guieu et al., 1991; Paytan et al., 2009) and deposit by diffusive air-water exchange (e.g. Bidleman, 1988; Dachs et al. 2005).

Among the anthropogenic pollutants incorporated in the natural biogeochemical cycles, and according to their chemical nature, persistent organic pollutants (POPs) and trace metals are considered two of the most toxic for organisms (Kennish, 1997; MacDonald et al., 2000; Rockström et al., 2009). In the case of POPs, their high toxicity is due to their hydrophobic and lipophilic nature, properties that allow them to partition into lipids and become stored in fatty tissue in organisms; while in the case of toxic metals, their toxicity is because they displace nutrient metals from their metabolic sites and enter into cells through the same transport systems, where they denature protein molecules (Gipps and Coller, 1980; Brand et al., 1986; Bruland et al., 1991; Sunda and Huntsman, 1998). The metabolism of POPs is slow, becoming persistent and

accumulating in food chains (Baker and Hites, 1999; Skei et al., 2000; Jurado et al., 2004; Lohmann et al., 2007), also known as bioaccumulation process (Jones and de Voogt, 1999).

In oceans, phytoplankton controls the chemistry and cycling of these pollutants (Dachs et al., 2002; Jurado and Dachs, 2008; Lohmann et al., 2007). Responsible of about half of the organic matter production in the world, oceanic phytoplankton plays a key role in the sustainability of marine ecosystems, representing the base of the marine food web (Berger et al., 1989; Falkowski and Woodhead, 1992; Field et al., 1998). Most part of this primary production is done by pico-sized (<2 μm diameter) phytoplankton (Campbell and Nolla, 1994; Li, 1994), which dominates the oligotrophic oceans (Agawin et al., 2000; Alonso-Laita and Agustí, 2006).

Although exposition to toxic pollutants has been observed as one of the factors inducing phytoplankton mortality in oceans (i.e., Mann et al., 2002), scarce studies have been performed in oceanic areas. Traditionally, single species laboratory tests have been run, under ideal environmental conditions, to evaluate the biological responses of phytoplankton to pollutants. However, laboratories are far from being considered "real environments", since parameters like nutrients, light or temperature are controlled and optimized. Besides, as generally these tests are performed with individual species, ecological issues such as competition for limiting nutrients or grazing are avoided. Therefore, it is of especial interest to consider this fact, since most part of toxicity tests with phytoplankton have been (and are still) performed in laboratory conditions, which may underestimate the toxicity of pollutants.

Size matters

Despite in some cases tolerances between the different phylum and kingdom are quite similar (Patrick et al., 1968), generally phytoplankton is more sensitive to pollutants than other aquatic organisms (Genter et al., 1987; Pratt et al., 1987; Niederlehner and Cairns, 1990), probably due to the most apparent characteristic, e.g., its lower body size. Body size has been observed to rule the ecology and evolution of organisms (LaBarbera, 1989), and so it does with phytoplankton,

which size ranges from less than 1 μm diameter, for some cyanobacteria, to over 1 mm in length for some diatoms, dinoflagellates and chlorophytes (Sournia, 1982).

Growth (Eppley and Sloan, 1966; Banse, 1976), sinking rates (Smayda, 1970; Walsby and Reynolds, 1980), susceptibility to zooplankton grazing (Parsons and Lebrasseur, 1970), maximal abundance (Agustí et al., 1987), specific metabolic rates (Geider et al., 1986) or generation times (Banse, 1976) depend on cell size. Phytoplankton sensitivity to pollutants is also cell size dependent (Del Vento and Dachs, 2002; Fan and Reinfelder, 2003). As a consequence of a higher surface to volume ratio, which increases pollutants uptake, the smallest phytoplankton cells are expected to be the most sensitive to pollutants toxicity, being the largest the most resistant.

Looking for signals: Thresholds in the environment

As POPs and metals exert a negative effect to oceanic phytoplankton, it is of special concern to establish "safe" boundaries to protect phytoplankton from pollution. Boundaries are human-determined values set at a "safe" distance from a dangerous level for processes with unknown thresholds... or from its global thresholds. In nature thresholds are defined as the critical values of different variables around which the ecosystem flips from one stable state to another (Muradian, 2001) (examples of thresholds, Fig. 1). Ecosystems can then respond in different manners to perturbations, for example resisting the damage and recovering quickly (also known as resilience) or "memorizing" the perturbation and responding when this perturbation becomes stronger (also known as hysteresis). Thresholds become points of no return when crossing again the same value in the opposite direction does not restore the initial state.

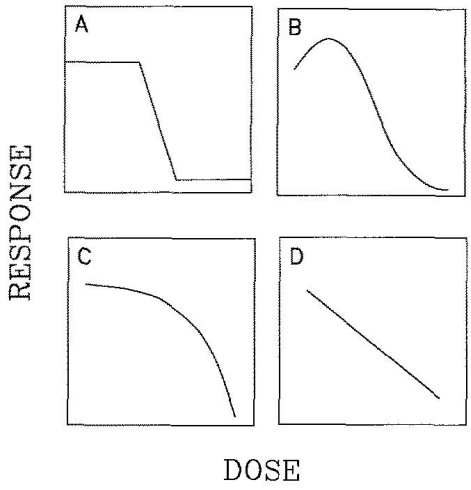

Fig. I.1. From Cairns (1992). Types of dose-response curves. Curve A shows an abrupt change in response with dose, i.e. a threshold. Curve B shows a subsidy at low doses that may serve as a practical threshold. Curve C is asymptotic with a practical threshold. Curve D shows no threshold.

Despite the need of establishing these "safe" boundaries, few toxic thresholds for marine phytoplankton were identified before the consecution of this PhD, mostly obtained from laboratory tests with cultured species, and being scarce those performed with natural communities, especially in open seawaters. It became therefore necessary to find the toxic thresholds to oceanic phytoplankton of the different pollutants occurring in seawater, to frame the global impacts of contamination in the marine planktonic community.

The most commonly used threshold in environmental toxicology is the 50% Lethal Threshold (LC50), where half of the organisms die or are otherwise affected at a certain concentration of a chemical for a particular time of exposure under specified environmental conditions (Cairns, 1992). However, as a 50% reduction of the phytoplankton community would have dramatic consequences for life in oceans, during the following chapters the LC10s, the pollutants concentrations inducing death to a 10% of the population, will be also considered, since a 10% reduction of the phytoplankton community would already have significant consequences to the marine food web and the processes dominated by phytoplankton.

Putting the "eco" in the toxicological tests with phytoplankton

In nature, pollutants do not impact individually, but usually complex mixtures of pollutants affect simultaneously natural communities (Dachs and Méjanelle, 2010). These complex mixtures can have different behaviours according to the joint action of the individual components. If two or more contaminants with similar toxic action impact the cells at the same time, an antagonistic effect may be occurring, disturbing the effect of each other and decreasing the expected toxicity for each individual pollutant. If these contaminants do not disturb each other and the toxicity of each contaminant is summed, we found an additive effect of these contaminants, both through Concentration Addition (Loewe and Muischnek, 1926) or through Independent Action (Bliss, 1939). By the contrary, if these contaminants exponentially increase their own toxicities, a synergetic effect may be expected. Moreover, environmental factors such as temperature or UV radiation (UVR) increase pollutants toxicity (Sokolova, 2004; Diamond, 2003; Larson and Berenbaum, 1988; Pelletier et al., 2006). UVR reacts with the POPs present in the environment, increasing their reactivity by photomodification (photooxidation / photolysis / photodegradation) and/or by photosensitization processes (radical formation), in a process named phototoxicity that causes oxidative damage (Grote et al., 2005; Petersen and Dahllöf, 2007).

It is also important to consider ocean basins when carrying out toxic tests. For example, phytoplankton communities among oceans differ widely. Temperate oceans such as the Atlantic or Pacific Oceans are oligotrophic regions dominated by pico-sized phytoplankton (e.g. Agawin et al., 2000; Alonso-Laita and Agustí, 2006), while those of cold areas such the Arctic and Southern Oceans are generally eutrophic waters dominated by larger sized phytoplankton, mostly diatoms (e.g. El-Sayed, 1971; von Quillfeldt, 1997). Moreover, the different oceans on Earth do not receive same amounts of contaminants, which mean that they are not similarly polluted (Lewis and Landing, 1992; Zeri et al., 2000). This fact probably determines phytoplankton sensitivity to pollutants due to lower/higher expositions to contamination, since it has already been observed

that contamination can cause estrange mutations in phytoplankton, inducing more resistant strains (Carrera-Martínez et al., 2010).

Therefore, all these issues should not be ignored in a search for the real effects of pollution to oceanic phytoplankton.

Aim of this thesis

As a consequence of the knowledge gap about the real impacts of global pollution in natural phytoplankton communities, the purpose of this PhD thesis was to fill this gap by analyzing the contaminants concentrations affecting these communities and the parameters controlling their sensitivity to them. Thus, three were the main goals pursued:

- To define and quantify the thresholds and points of no return of different POPs and trace metals to marine phytoplankton.
- To analyze the importance of cell size in explaining the variability in induced lethality by pollutants.
- To identify the degree of resistance of natural marine phytoplankton communities to the present levels of pollutants found in the oceans.

By looking for these main goals, other specific objectives were also pursued:

- To compare sensitivities of both phytoplankton cultures and natural phytoplankton communities.
- To identify other parameters controlling phytoplankton sensitivity to pollutants.
- To determine the metal thresholds crossing from a positive to a negative effect.
- To quantify the lethality of simple mixtures of PAHs to marine phytoplankton.
- To identify phototoxicity induced by UVR exposure.

- To identify the synergetic effect of the complex mixtures of organic pollutants found presently in the ocean.

- To quantify the lethality of these natural mixtures to marine photosynthetic plankton.

- To compare these toxicity levels with those obtained for individual pollutants.

- To identify resistant populations exposed to high levels of contamination.

Chronology of this thesis

To achieve all these purposes, several experiments with POPs and trace metals were performed during this PhD. The first approaches were performed with different phytoplankton species growing in cultures exposed to pyrene or phenanthrene, two of the most spread and toxic PAHs. The specific goals of these experiments were to quantify the lethal thresholds of PAHs and to establish the reference levels for future experiments with natural phytoplankton communities, identifying parameters controlling phytoplankton sensitivity to POPs. To test cell size influence in determining phytoplankton sensitivity to pollution, different phytoplankton species covering a wide range of sizes were used.

These experiments were then followed with similar experiments performed in natural communities of the Mediterranean and Black Seas and the Atlantic Ocean. The idea was to quantify the lethal thresholds of these PAHs, in natural communities, allowing us to compare the different sensitivities of cultured and natural marine phytoplankton and to contrast the parameters determining phytoplankton sensitivity to POPs, specially phytoplankton cell size.

Parallel experiments with Cd and Pb, two of the most toxic and abundant trace metals in seawater, were also performed. The purpose of these tests was to quantify the lethal thresholds of Cd and Pb to natural phytoplankton communities, allowing us the comparison of these results with the abundant data present in the literature about metals' toxicity from phytoplankton cultures.

Moreover, low concentrations of both Cd and Pb were also tested to analyze whether these metals could have a positive effect (replacing some nutrients) in natural phytoplankton communities.

After analyzing the effect of individual PAHs, different experiments were performed with a simple mixture of 16 PAHs (pyrene, phenanthrene, anthracene, fluoranthene, etc.) in different trophic level seawaters, i.e. in oligotrophic waters (Mediterranean Sea and Atlantic Ocean) and in eutrophic waters (Arctic and Southern Oceans). The purpose was to quantify the lethality of this simple mixture of PAHs under the hypothesis that increasing complexity of the contaminant added may increase the sensitivity of the phytoplankton community. Moreover, these experiments were performed in the absence or presence of UVR to test the phototoxic effect of UVR to PAHs.

Experiments with Hg and Zn were also performed in phytoplankton communities of the Mediterranean Sea, and the Atlantic, Arctic and Southern Oceans. Moreover, an intense search of literature data on toxicity of trace metals to phytoplankton was conducted, allowing us the comparison of the lethal and sublethal effects of different metals in freshwater and marine phytoplankton species.

Finally, and regarding POPs toxicity to phytoplankton, a series of experiments were performed with complex mixtures of POPs extracted from seawater and the air, from different locations, using the methodology previously developed (Dachs and Bayona, 1997; Gioia et al., 2008; Nizzetto et al., 2008). These experiments gave us a real view of the actual effects of the present mixture of POPs found now in the ocean seawater to marine phytoplankton. This allowed us the comparison of the synergetic effects of these complex mixtures in respect of the effects of simple mixtures and individual pollutants observed in previous experiments.

In summary, the specific goals pursued within each chapter of this PhD thesis were the following:

Chapter 1: Cell size dependent toxicity thresholds of polycyclic aromatic hydrocarbons to natural and cultured phytoplankton populations	The lethality of increasing levels of pyrene and phenantrene to marine phytoplankton growing in cultures and natural communities from the coastal and open Mediterranean Sea and the Atlantic Ocean was examined.
Chapter 2: Cell size dependence of additive versus synergetic effects of UVR and PAHs on oceanic phytoplankton	The lethality of different concentrations of a mixture of 16 PAHs to natural phytoplankton of the Mediterranean Sea and the Atlantic, Arctic and Southern Oceans was examined in the absence or presence of natural levels of ultraviolet radiation (UVR).
Chapter 3: Decrease in the abundance and viability of oceanic phytoplankton due to trace levels of complex mixtures of organic pollutants	In natural phytoplankton communities of the Atlantic Ocean we tested the effect of different complexities of POPs, from additions of individual pollutants (pyrene and phenantrene), to simple mixtures of pollutants (a mixture of 16 PAHs), to real complexity of pollutants mixtures presently found at the ocean. The real complex mixtures of pollutants were extracted from the Atlantic Ocean seawater.

Chapter 4: Toxic thresholds of cadmium and lead to oceanic phytoplankton: cell size and ocean basins dependent effects	The toxicity of different levels of Cd and Pb to natural oceanic phytoplankton communities of the Mediterranean Sea, the Black Sea and the Atlantic Ocean, was examined.
Chapter 5: Dissimilar sensitivities of Arctic and Southern Ocean phytoplankton to Cd, Pb and Hg	The effects (lethality and growth, among other aspects) of different concentrations of Cd, Pb and Hg to natural oceanic phytoplankton communities of the Arctic and Southern Oceans were assessed and the differences in sensitivity examined.

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Chapter 1

Cell size dependent toxicity thresholds of polycyclic aromatic hydrocarbons to natural and cultured phytoplankton populations

by

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Abstract

The toxicity of pyrene and phenanthrene to phytoplankton was studied by analyzing the effect on the growth, abundance and cell viability of cultured species and natural communities of the Atlantic Ocean and the Mediterranean Sea. A decrease in cell abundance and growth rate was observed as concentration of PAHs increased, with catastrophic cell mortality induced at the highest PAHs concentration tested. A strong positive linear relationship was observed between the LC50 (the PAH concentration at which cell population will decline by a half), and the species cell volume, for both phenanthrene and pyrene. Natural communities were however significantly more sensitive to PAHs than cultured phytoplankton, as indicated by the lower slope (e.g. 0.23 and 0.65, respectively, for pyrene) of the relationship LC50 vs. cell volume. The results highlight the importance of cell size in determining the phytoplankton sensitivity to PAHs identifying the communities from the oligotrophic ocean to be more vulnerable.

1. Introduction

Policyclic Aromatic Hydrocarbons (PAHs) are ubiquitous environmental contaminants derived from biogenic sources (forest and prairie fires, natural petroleum seeps, etc.) or anthropogenic sources from incomplete combustion of fossil-fuel (e.g. Hites and Biemann, 1975). PAHs can reach and impact oceanic environments after ongoing atmospheric transport and subsequent deposition by diffusive air-water exchange and dry/wet scavenging (Del Vento and Dachs, 2007). Direct spillage of petroleum and its refined products is another potential source of PAHs to ecosystems (e.g. Page et al., 1988). PAHs are always found in the environment as complex mixtures of dozens of different chemicals with a number of aromatic rings ranging from 2 to up to 10 (Hylland, 2006).

PAHs are considered toxic for organisms and have been described as mutagenic, teratogenic and carcinogenic (Lehr and Jerina, 1977; Gelboin, 1980). The toxicity of PAHs arises from their strong adsorption affinity for particulate surfaces (e.g. Meador et al., 1995) and their interference with cellular membranes (Neff, 1979). Moreover serious damages to DNA (Gelboin, 1980), some cell processes like growth, photosynthetic oxygen evolution, dark respiration and pigment composition are also altered by PAHs (Singh and Gaur, 1988).

Toxicity and lethality of PAHs have been quantified for terrestrial organisms and for a variety of aquatic organisms as copepods, amphipods and other zooplankton, macroalgae, sandworms, crabs, shrimps, and some fish species like salmon or sheepshead minnow and many others (Kennish, 1997; Weber Scanell et al., 2005). Although toxicity of PAHs on phytoplankton has been addressed (Djomo et al., 2004; Grote et al., 2005), the degree of lethality of PAHs to phytoplankton has been quantified only in a few cases, i.e. *Nitzschia palea*, a freshwater diatom, and

Selenastrum capricornutum, a freshwater green algae (Millemann et al., 1984). Moreover, most toxicity experiments have been carried out in laboratory conditions, with cultures, while few of them have been performed in natural communities.

The goal of this study was to quantify the toxicity of 2 different Polycyclic Aromatic Hydrocarbons (PAH), pyrene and phenanthrene, to marine photosynthetic plankton by analyzing the effect of different concentrations on the growth and cell death of a variety of phytoplankton species growing in cultures as well as in natural communities from different seas. Our purpose was to compare the sensitivities of cultured and natural phytoplankton to address whether differences in sensitivities between natural communities and cultured organisms could be significant.

The experiments were carried out in the coastal and open Mediterranean Sea and in the Atlantic Ocean, which are oligotrophic waters dominated by pico-sized phytoplankton: *Prochlorococcus*, *Synechococcus* and picoeukaryotes (e.g. Vaulot et al., 1990; Alonso-Laita and Agustí, 2006). Pyrene and phenanthrene were chosen for these experiments as they are 2 of the most abundant PAHs in the oceans (Neff, 2002; Nizzeto et al., 2008) and, besides, may have differences in their toxicity to marine phytoplankton because they have 4 and 3 aromatic rings, respectively. The resulting cell death-PAHs relationships observed will be used to define the thresholds and points of no return of PAHs to marine phytoplankton. Moreover, these thresholds will be related to the cell volume because it is expected that sensitivity to PAHs will vary with phytoplankton cell size since it has been predicted and measured that PAH accumulation in phytoplankton depends on cell size (Del Vento and Dachs, 2002; Fan and Reinfelder, 2003).

2. Methods

2.1. Experiments with phytoplankton cultures

Laboratory experiments were performed with unspecific phytoplankton cultures of *Prochlorococcus marina* (CCMP1375), *Synechococcus* sp. (CCMP833), *Micromonas pusilla* (CCMP487), *Chlorella* sp., *Phaeodactylum tricornutum* and *Thalassiosira* sp. The cultures grew in large volume (5 L) batch cultures under optimal temperature of 18ºC (21ºC for *Synechococcus* and *Prochlorococcus*), and under continuous light conditions, in a nutrient-rich F/2 medium, except *Prochlorococcus marina*, which grew in Pro-99 medium. When populations entered the exponential growth stage, a variable volume of concentrated Pyrene and Phenanthrene solutions were added to the cultures after dispensing in 250 ml bottles to reach a duplicated gradient of 6-7 final concentrations ranging from 5 to 1000 μg L^{-1} (5-10-50-100-500-1000 μg L^{-1}). In the experiments with *Prochlorococcus marina*, the final concentrations ranged from 0.5 to 100 μg L^{-1} (0.5-1-5-10-50-100 μg L^{-1}) and in those performed with *Thalassiosira* sp., the final concentration gradient ranged from 1 to 1000 μg L^{-1} (1-5-10-50-100-500-1000 μg L^{-1}). Two replicated 250 ml bottles, without adding PAHs, were run as controls, and two more replicates were run as solvent controls to test the lethal effect of the PAHs' solvent used, Dymethil Sulfoxide (DMSO). DMSO was selected as the solvent since other solvent candidates such as acetonitrile and methanol were observed to be toxic at the highest PAHs concentrations used (thus highest solvent concentrations). The concentration of DMSO added to the solvent control was equivalent to the concentration of DMSO contained in the highest PAHs concentration treatment. Concentrated pyrene and phenanthrene solutions of 1000 μg ml^{-1} in DMSO were prepared. The evolution of the population during the experiments was followed from a total of 4 days

(*Prochlorococcus marina, Synechococcus* sp., *Micromonas pusilla* and *Chlorella* sp.) to a maximum of 7 (*Phaeodactylum tricornutum*) or 11 days (*Thalassiosira* sp.), depending on the population response. Cultures were sampled daily, except *Phaeodactylum tricornutum* and *Thalassiosira* sp. that were sampled every two-three days, to analyze the changes in cell abundance. The lethal effect of pyrene was tested in all the cultures, while phenanthrene was tested only in *Prochlorococcus marina* and *Synechococcus* sp. For picophytoplankton communities (*Prochlorococcus* sp., *Synechococcus* sp. and Eukaryotic picophytoplankton) changes in the abundance and viability of cells were quantified by using duplicated 1 ml fresh samples counted in a FACSCalibur Flow Cytometer (Becton Dickinson). An aliquot of a calibrated solution of 1 μm diameter fluorescent beads (Polysciences Inc.) was added to the samples as an internal standard for the quantification of cell concentration. The red, green and orange fluorescence, and forward and side scattering signals of the cells and beads were used to detect different populations and to differentiate them from the fluorescent beads (Marie et al., 2000).

The proportion of living and dead cells in the different populations was followed by applying a cell membrane permeability test, the cell digestion assay (Agustí and Sánchez, 2002). This test consists on the digestion of the membranes of the cells, which are permeable when exposed to an enzymatic cocktail (DNAse and Trypsin). The cell digestion assay was applied to duplicated 1 ml samples of the cultures, by adding 200 µl of DNAse I solution (400 μg ml^{-1} in HBSS (Hanks' Balanced Salts)) to 1 ml sample of each treatment, followed by 15 minutes incubation at 35°C in a Digital Dry Bath. After this time, 200 μl of Trypsin solution (1% in HBSS) were added, followed by 30 minutes incubation at 35°C. At the end of this time, samples were kept in ice in order to stop the enzymatic cell digestion process.

After the incubation, samples were counted using the flow cytometer, as described above.

The cells counted after the cell digestion assay represented the living cells in the population, whereas the cells counted in untreated samples represented the total population (living and dying cells). The percentage of living (or viable) cells was calculated as the ratio between the concentration of cells after the enzyme digestion, and the cell concentration of untreated samples, which represented the total (dead plus living) cell population.

The cell volume of the different species was calculated by approximation to the nearest simple geometric shape, from the dimensions (at x1000) of ca. 20 measured cells at the transmission microscope. The cell diameter of *Prochlorococcus marina* cells was estimated in samples analyzed by Scanning Electron Microscopy.

After the addition of the gradient in PAHs concentration, the cultures in the different treatments were sampled until the decrease of the population was detected, or after several days observing no changes in the population size. For that period, the growth rates (μ, d^{-1}) of the different species at the different treatments were calculated on the changes of the natural logarithm of the cell abundance.

2.2. Experiments with natural communities

Experiments to analyze the lethal thresholds of pyrene and phenanthrene on natural communities of phytoplankton were performed with coastal Mediterranean plankton, sampled at the field station of Far Cap Ses Salines, Mallorca Island, on August 2007; and on open sea plankton, sampled during the oceanographic cruises THRESHOLDS-1 (from June 3 to July 5, 2006) on board the RV Garcia del Cid, along the Mediterranean and Black Seas, and RODA-1 (from August 17 to September 5, 2006) on board the RV Hespérides, along the Atlantic Ocean.

Surface water (5 m) of the Mediterranean Sea and Atlantic Ocean used in the experiments was sampled by using Niskin bottles attached to a rosette-CTD system. In the experiments carried out in Far Cap Ses Salines, surface water was directly collected using an acid clean plastic carboy on the border of the Mediterranean Sea (See Table 1.1 for coordinates).

Campaign	Experiment	Coordinates	Date	Chlorophyll *a* (mg m^{-3})
Thresholds	Pyrene	35º 42' N - 19º 41' E	06/26/2006	0.20
	Phenanthrene	37º 53' N - 10º 38' E	07/01/2006	0.28
RODA	Pyrene	27º 21' N - 17º 11' W	08/30/2006	2.06
	Phenanthrene	27º 19' N - 15º 25' W	08/31/2006	0.47
FARO	Pyrene	39º 16' N - 3º 3' E	08/26/2007	0.50
	Phenanthrene	39º 16' N - 3º 3' E	08/26/2007	0.50

Table 1.1. Position of the stations where the natural phytoplankton communities were sampled, and the date of sampling, indicating the abundance of phytoplankton (as Chlorophyll *a* concentration) found at each station, and whether the experiments were performed with Pyrene or Phenanthrene. Thresholds and FARO campaigns were carried out in the open Mediterranean Sea and coastal Mediterranean Sea, respectively. RODA campaign was carried out in the subtropical North Atlantic Ocean.

Experiments began with the distribution of sampled water into 250 ml acid clean Pyrex bottles. After this gathering, contaminants were inoculated at different concentrations of 5, 10, 50, 100, 500 and 1000 μg L^{-1} for both pyrene and phenanthrene during THRESHOLDS-1 cruise; and 5, 10, 50, 100, 250 and 500 μg L^{-1} for both pyrene and phenanthrene experiments of RODA-1 cruise and FARO campaign. After PAHs addition, bottles were incubated on deck under natural solar radiation in a tank with seawater surface running system to keep "in situ" temperature conditions. Bottles were covered with a neutral net to simulate 5 meters light conditions. For the coastal experiments, a similar incubation system was used, with

tanks placed in the exterior of the field station in air free area, and a surface seawater running system.

As described for the cultures, the lethality of pyrene and phenanthrene was tested in duplicated bottles while duplicated bottles without chemical additions were also run as controls. Daily (RODA-1 and THRESHOLDS-1 cruises) and every two days (coastal Mediterranean community) sampling was performed in the experiments for as long as 4 days.

For picophytoplankton communities (*Prochlorococcus* sp., *Synechococcus* sp. and Eukaryotic picophytoplankton) changes in the abundance and viability of cells were quantified by Flow Cytometry, as described before. The proportion of living and dead cells in the picophytoplankton communities along the experiments were also followed by applying the same cell membrane permeability test, the cell digestion assay (Agusti and Sanchez, 2002), as described before. The changes in the abundance of nano and microphytoplankton communities were analyzed at the beginning and the end of the experiment by using epifluorescence microscopy. In order to have a representative number of cells, 50 ml of water were sampled from each replicated treatment bottle and filtered onto policarbonate 2 μm pore diameter black filters. During the filtration, with the last ml to be filtered, 2 ml of glutaraldehyde (25%) were added to the sample in order to fix it. Once filtered, filters were frozen until their analysis in order to preserve them. Cells were then counted in a Zeiss Axioplan Imaging epifluorescence microscope. Cells were classified into 3 groups: small eukaryotes (3-4 μm size cells), nanophytoplankton (5-20 μm size cells) and microphytoplankton (larger than 20 μm). The cell volume was calculated by approximation to the nearest simple geometric shape, while the cell diameter of *Synechococcus* and *Prochlorococcus* populations was assumed to be the same as

those obtained in the cultures.

Moreover, changes in total phytoplankton abundance were followed by analyzing Chlorophyll *a* concentrations. For this estimation, 50 ml samples were filtered onto 25 mm diameter Whatmann GF/F filters from each bottle on the day 0, day 2 and last day (day 4). After filtration, filters were placed in tubes with 90% acetone for 24 hours for the extraction of the pigment. Then, the fluorescence of the Chlorophyll *a* was measured in a Shimadzu RF-5301 PC spectrofluorimeter and calibrated with pure Chlorophyll *a* as described in Parsons et al. (1984).

2.3. Statistical analysis and calculations

Phytoplankton growth rates (μ, d^{-1}) were calculated from changes in cell density (D, cells ml^{-1}) with time (t, d) for each group considered using the equation:

$$\mu = \ln (D_t/D_o) / t \qquad \text{(eq. 1.1)}$$

The cell death rate of the populations during exponential growth periods was calculated by using the abundance of dead and living cells as indicated in Brussaard et al. (1997), following the equation:

$$\delta_b = \frac{\ln x_t - \ln x_0}{t \cdot \left(\frac{(x+y)_t - (x+y)_0}{y_t - y_0} - 1 \right)} \qquad \text{(eq. 1.2)}$$

where δ_b is the cell death rate (d^{-1}), x is the concentration of living cells, (x+y, cells ml^{-1}) is the total concentration and y is the concentration of dead cells (cells ml^{-1}). The total concentration of cells at time t is represented by $(x+y)_t$ and the concentration of dead cells at time t by y_t.

Half lives ($t_{1/2}$) of the different species were calculated by applying the formula

$$t_{1/2} = \ln 2 / \mu \qquad \text{(eq. 1.3)}$$

where μ is the slope of the ln of the decay of cell abundance with time in days.

The lethal thresholds of pyrene and phenanthrene for each species tested was calculated as the PAHs concentration at which the cell population will be decreased by a half, applying the equation

$$LC50 = \ln 2 / \Omega \qquad \text{(eq. 1.4)}$$

where Ω is the slope of the relationship between the ln of the decay of cell abundance and the PAH concentration ($\mu g\ L^{-1}$) reached at the end of the experimental treatments.

The significant differences observed between treatments were analyzed by using the t-student test.

3. Results

3.1. Phytoplankton cultures

The analysis of the relationship between cell concentration and PAHs concentration showed the decrease in cell abundance as concentration of PAHs increased (Fig. 1.1). For *Prochlorococcus marina* and *Synechococcus* sp., a strong decrease in cell concentration was observed at the highest PAHs concentration for both pyrene and phenanthrene (Fig. 1.1), while *Chlorella* sp., *Micromonas pusilla* and *Phaeodactylum tricornutum* showed a smoother decrease on their populations, with a non clear decrease of *Thalassiosira* sp. populations detected even at the highest concentrations added (Fig. 1.1).

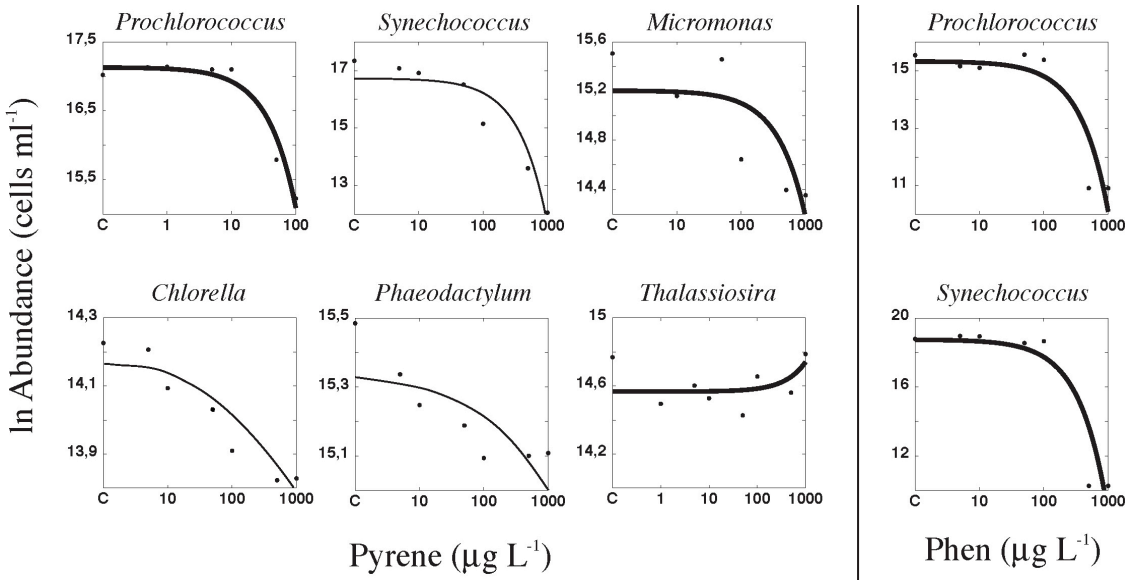

Figure 1.1. Decrease in the cell abundance (cells ml^{-1}) at the end of the experiments, of the different phytoplankton species growing in cultures, with the increase in pyrene ($\mu g\ L^{-1}$) and phenanthrene (Phen, $\mu g\ L^{-1}$) concentration. The lines represent the lowess fit.

A similar relationship was observed between growth rate and PAHs concentration, for the smaller species, showing the decrease in growth rate as PAHs concentrations increased (Table 1.2). For *Prochlorococcus marina* and *Synechococcus* sp. there was a progressive decay of the growth rate until catastrophic cell death occurred, showing negative growth rate at the highest PAHs concentrations, while for *Micromonas pusilla*, *Chlorella* sp. and *Phaeodactylum tricornutum* the decay in growth rate was more moderate (Table 1.2). As observed for cell concentration, *Thalassiosira* sp. did not show any decrease on its growth rate with increasing pyrene concentration (Table 1.2). Thus, for the smallest species, the addition of the highest PAHs concentration tested was lethal. For those species where catastrophic cell death was not detected, the induction of cell death was analyzed by measuring the increase in the proportion of dead cells within the populations. For *Chlorella* sp. and *Phaeodactylum tricornutum*, the proportion of dead cells increased significantly at the two highest pyrene concentration treatments (500 and 1000 $\mu g\ L^{-1}$)

to respect to that observed at lower concentrations (t-student, P< 0.001). However, for *Micromonas pusilla* and *Thalassiosira* sp. the proportion of dead cells did not vary significantly among treatments.

	PYRENE							
	Volume (μm^3)	LC50 ($\mu g\ L^{-1}$)	Growth rate (μ, d^{-1})					
			5 ($\mu g\ L^{-1}$)	10 ($\mu g\ L^{-1}$)	50 ($\mu g\ L^{-1}$)	100 ($\mu g\ L^{-1}$)	500 ($\mu g\ L^{-1}$)	1000 ($\mu g\ L^{-1}$)
P. marina	0.1	35 ± 5	0.25	0.23	-0.07	-0.16	-	-
Synecho. sp.	0.9	135 ± 25	0.83	0.8	0.72	0.4	-0.03	-0.38
M. pusilla	8.4	675 ± 320	0.81	0.79	0.85	0.66	0.55	0.56
Chlorella sp.	10.96	2060 ± 1315	0.61	0.58	0.58	0.55	0.53	0.54
P. tricornutum	42.76	3195 ± 3800	0.47	0.46	0.47	0.42	0.44	0.42
Thalass. sp.	2267.7	19640 ± 7080	0.2	0.21	0.19	0.17	0.17	0.25
	PHENANTHRENE							
	Volume (μm^3)	LC50 ($\mu g\ L^{-1}$)	Growth rate (μ, d^{-1})					
			5 ($\mu g\ L^{-1}$)	10 ($\mu g\ L^{-1}$)	50 ($\mu g\ L^{-1}$)	100 ($\mu g\ L^{-1}$)	500 ($\mu g\ L^{-1}$)	1000 ($\mu g\ L^{-1}$)
P. marina	0.1	77 ± 13	0.3	0.3	0.51	0.46	-0.58	-4.05
Syn. sp.	0.9	40 ± 4	1.63	1.64	1.56	1.53	-1.54	-2.3

Table 1.2. Values of cell volume (μm^3) of the different phytoplankton species used in the laboratory experiments and the LC50 ($\mu g\ L^{-1}$), and growth rates (μ, d^{-1}) values obtained when exposed to different concentrations of pyrene and phenanthrene. The LC50 for *Chlorella* sp., *Phaeodactylum tricornutum* and *Thalassiosira* sp. represent concentrations above the solubility of pyrene in water.

The cell death rates of the different populations exposed to the different treatments were directly related to their growth rates (Fig. 1.2) except for *Thalassiosira* sp., which showed indeed the smallest cell death rates (Fig. 1.2). For the populations exposed to the highest PAHs concentration tested (500 and 1000 $\mu g\ L^{-1}$) cell death rates increased relative to that expected for their growth rates (Fig. 1.2). Despite this was expected for *Prochlorococcus* and *Synechococcus*, which showed catastrophic death at the highest PAHs concentrations (which represented a threshold

between the last treatment with positive growth rate and the next treatment, with high decay of the abundance), it is interesting that this was also observed in those populations where there was no catastrophic cell death. Thus, *Micromonas pusilla, Chlorella* sp. and *Phaeodactylum tricornutum* showed increased cell death rates (relative to growth rates) at the two highest concentrations treatments (Fig. 1.2).

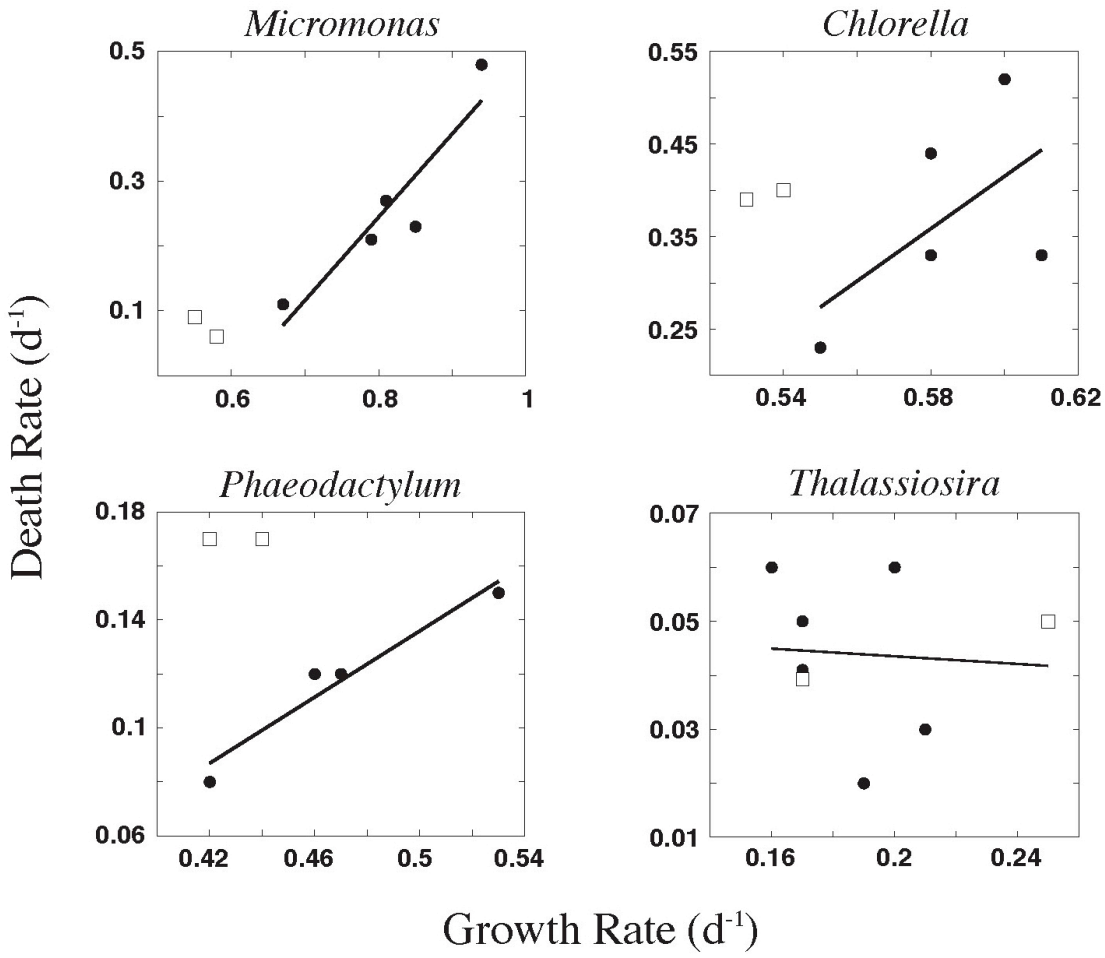

Figure 1.2. The relationship between death rate and growth rate for phytoplankton species growing in cultures. White squares correspond to the highest pyrene concentrated treatments (500 and 1000 μg L^{-1}), while black dots represent treatments with lower pyrene concentrations.

The 50% pyrene Lethal Threshold (LC50), the pyrene concentration at which cell population will decline by a half, varied from 35 μg L^{-1} for *Prochlorococcus* to 19640 μg L^{-1} for *Thalassiosira* sp. (Table 1.2). The lowest LC50 was observed for

Prochlorococcus marina and the highest was the one obtained for *Thalassiosira* sp. For *Chlorella* sp., *Phaeodactylum tricornutum* and *Thalassiosira* sp. the LC50 values obtained were higher than the highest pyrene concentrations added (Table 1.2). We did not include concentrations higher than 1000 μg L^{-1} as the highest reported solubility of pyrene in water is of 1.56 mg L^{-1}, therefore LC50 values higher than these should be considered as pseudo thresholds values. For pyrene, there was a strong and linear relationship between the LC50 and the species cell volume:

$$\ln LC50\ (\mu g\ L^{-1}) = 5.25 + 0.65 \times \ln \text{cell volume}\ (\mu m^3),\ R^2 = 0.96 \qquad \text{(eq. 1.5)}$$

with the 50% Lethal Threshold increasing as the cell volume increased (Fig. 1.3).

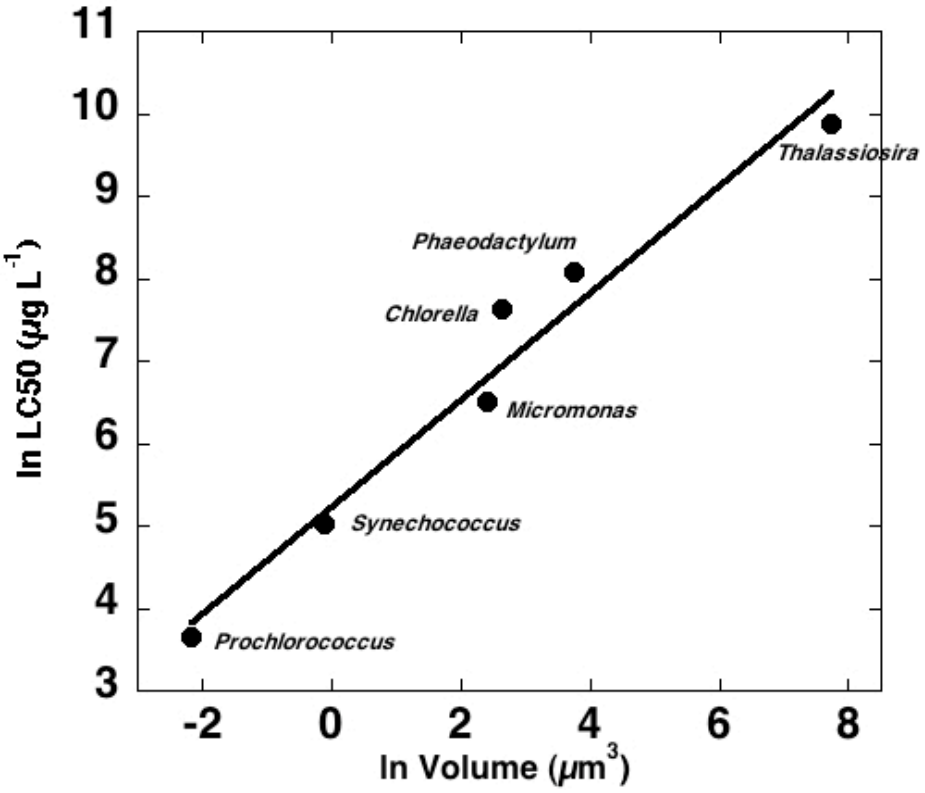

Figure 1.3. The relationship between pyrene LC50 (μg L^{-1}) and Cell Volume (μm^3) obtained for the phytoplankton species growing in cultures.

The lethal concentrations required to reduce the 10% of the population, the 10% Lethal Thresholds (LC10), were however below the solubility of pyrene in water, except for *Thalassiosira* sp., which was still a little bit higher, and were also strongly related to cell size, with the same slope and R^2 (0.65 and 0.96, respectively) observed in the relationship between the LC50 and the cell volume.

For the two species showing catastrophic cell death (*Prochlorococcus marina* and *Synechococcus* sp.) when exposed to PAHs, we were allowed to calculate the half lives values (Table 1.3), which varied from 6.8 days to 0.1 day, showing shorter half lives at the highest PAH concentration, and been lower for phenanthrene than for pyrene (Table 1.3).

		Half Lives (days)			
		50	**100**	**500**	**1000**
PYRENE	*P. marina*	6.8	3.7	-	-
	Synechococcus sp.	Nd	Nd	4.1	0.9
PHEN	*P. marina*	Nd	Nd	0.7	0.7
	Synechococcus sp.	Nd	Nd	0.3	0.1

Table 1.3. Values of half lives calculated for *Prochlorococcus marina* and *Synechococcus* sp. for the highest concentrations ($\mu g\ L^{-1}$) of pyrene and phenanthrene (PHEN) tested. Nd = no decay of the population detected. (-) no treatments tested.

3.2. Phytoplankton Natural Communities

Picophytoplankton, represented by *Prochlorococcus* sp., *Synechococcus* sp. and small eukaryotes, and nanophytoplankton, dominated the phytoplankton communities found in the stations sampled in the Mediterranean Sea open and coastal waters and in waters of the North East Atlantic Ocean (Table 1.3). There were some differences in the community composition of phytoplankton between the areas studied, with *Prochlorococcus* been more abundant in Atlantic waters than in the Mediterranean Sea, where it was often absent. The waters studied were in general

oligotrophic, although phytoplankton communities from the Atlantic Ocean were more abundant than those from the Mediterranean Sea (Table 1.3).

As observed in cultures, natural communities were also strongly affected by the presence of high concentrations of PAHs, and all the populations declined as PAHs concentrations in the treatments increased. The decline with increasing PAHs concentration was observed in the total community, quantified as Chlorophyll *a* concentration (Fig. 1.4) and when considering the individual counts of the different populations conforming the communities (Fig. 1.4).

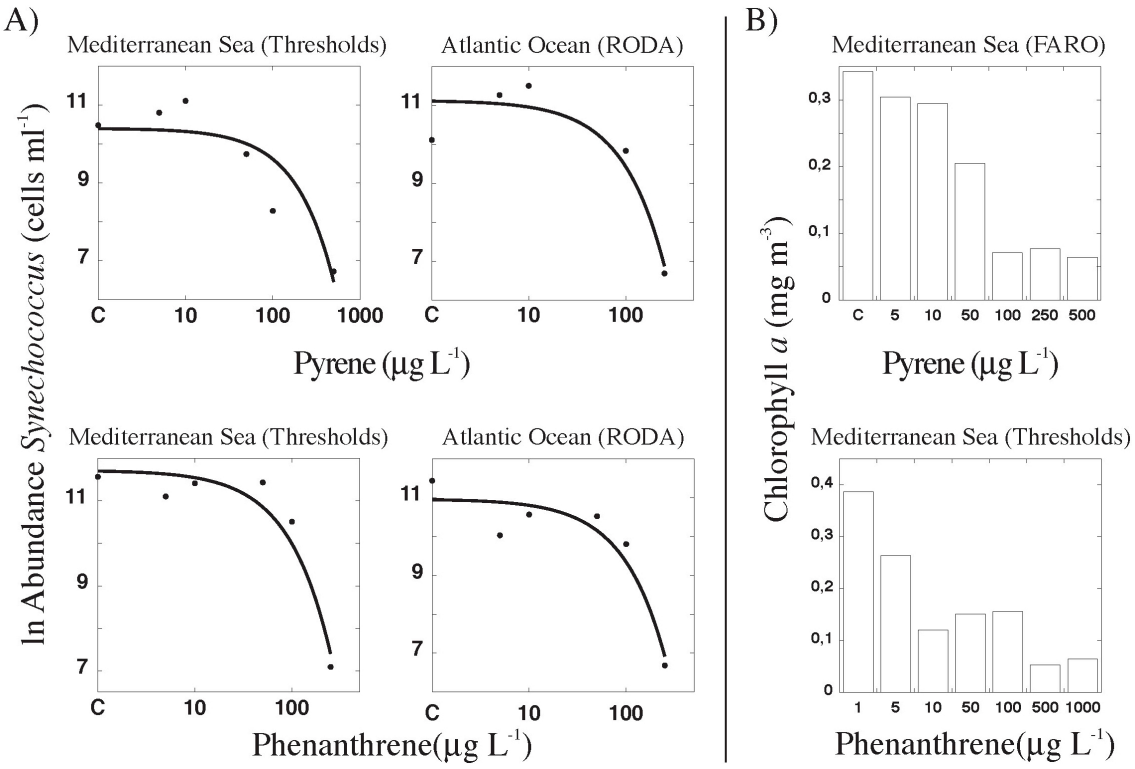

Figure 1.4. Decrease in the phytoplankton biomass with the increase of PAH concentration (μg L^{-1}) observed in some of the experiments carried out with natural communities. Panel A) Decrease of *Synechococcus* cell abundance (cells ml^{-1}) with the increase of PAH concentration (pyrene and phenanthrene, μg L^{-1}) in populations from the Mediterranean Sea (THRESHOLDS cruise) and Atlantic Ocean (RODA cruise). Panel B) Decrease of the chlorophyll *a* concentration with increasing PAH concentration (pyrene and phenanthrene, μg L^{-1}) observed in phytoplankton communities from the Mediterranean Sea, in FARO and THRESHOLDS cruises, respectively.

The concentration of the 50% Lethal Thresholds (LC50) varied from 14.8 μg L^{-1} for *Prochlorococcus* to 137.6 μg L^{-1} for picoeukaryotes in the case of pyrene and from 20.8 μg L^{-1} for *Synechococcus* to 189.9 μg L^{-1} for picoeukaryotes in the case of phenanthrene (Table 1.4). The lethal concentrations required to reduce the 10% of the population, the LC10, were lower than those of the LC50 but still high (Table 1.4), being only on average, 6.5 times lower.

PYRENE		**Volume (μm^3)**	**LC50 ($\mu g\ L^{-1}$)**	**LC10 ($\mu g\ L^{-1}$)**
Mediterranean Sea (Thres)	*Synechococcus*	0.9	89.5 ± 34.9	13.6 ± 5.3
	Picoeukaryotes	18	137.6 ± 146.1	20.9 ± 22.2
	Nanoplankton	214.4	88.9 ± 16.4	13.5 ± 2.5
	Chlorophyll *a*		145.1 ± 45.4	22.1 ± 6.9
Atlantic Ocean (RODA)	*Prochlorococcus*	0.1	14.8 ± 6.9	2.3 ± 1.1
	Synechococcus	0.9	40.7 ± 10	6.2 ± 1.5
	Chlorophyll *a*		165.7 ± 77.1	25.2 ± 11.7
Mediterranean Sea (Faro)	*Synechococcus*	0.9	37.8 ± 12.7	6.1 ± 1.9
	Picoeukaryotes	22.4	88.2 ± 113.5	13.4 ± 17.3
	Nanoplankton	156.6	141.6 ± 95.9	21.5 ± 14.6
	Chlorophyll *a*		121.6 ± 19.8	18.5 ± 3
PHENANTHRENE		**Volume (μm^3)**	**LC50 ($\mu g\ L^{-1}$)**	**LC10 ($\mu g\ L^{-1}$)**
Mediterranean Sea (Thres)	*Prochlorococcus*	0.1	30.2 ± 12.9	4.6 ± 2
	Synechococcus	0.9	42 ± 14.1	6.4 ± 2.1
	Chlorophyll *a*		117 ± 85.9	17.8 ± 13.1
Atlantic Ocean (RODA)	*Prochlorococcus*	0.1	36.3 ± 8.9	5.5 ± 1.4
	Synechococcus	0.9	42.5 ± 10.5	6.5 ± 1.6
	Picoeukaryotes	20.6	189.9 ± 105.9	28.9 ± 16.1
	Nanoplankton	356.3	165.7 ± 50.3	25.2 ± 7.7
	Chlorophyll *a*		154.3 ± 50.9	23.5 ± 7.7
Mediterranean Sea (Faro)	*Synechococcus*	0.9	20.8 ± 7.6	3.3 ± 1.2
	Picoeukaryotes	18	158.4 ± 90.7	24.1 ± 13.8
	Nanoplankton	156.6	179.5 ± 162.7	27.3 ± 24.7
	Chlorophyll *a*		163.1 ± 60.5	24.8 ± 9.2

Table 1.4. Cell volumes (μm3) of the different groups forming the natural phytoplankton communities examined, and the Pyrene and Phenanthrene LC50 and

LC10 (μg L-1) values obtained for each phytoplankton group in all the experiments carried out.

The consideration of LC10 is relevant because a decrease of 10% in population would already have a dramatic effect on the ecosystem and marine carbon cycle. As observed for phytoplankton cultures, there was a strong and linear relationship between the LC50, for both phenanthrene and pyrene, and the species cell volume, with the 50% Lethal Threshold increasing as the cell volume increased (Fig. 5), as described in the equations:

ln LC50 (μg L^{-1} phenanthrene) = 3.88 + 0.27 x ln cell volume (μm^3), $R^2 = 0.78$ (eq. 1.6)

ln LC50 (μg L^{-1} pyrene) = 3.78 + 0.23 x ln cell volume (μm^3), $R^2 = 0.66$ (eq. 1.7)

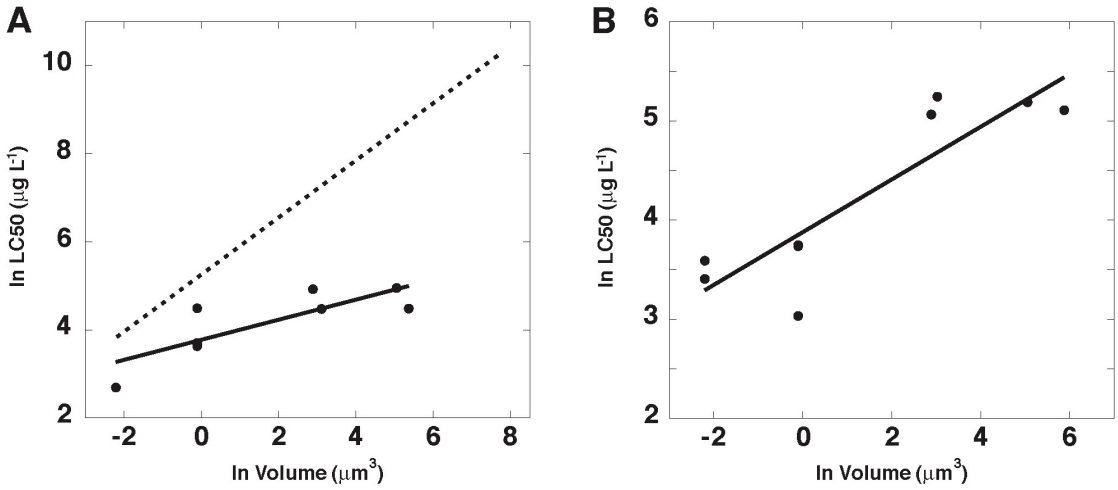

Figure 1.5. The relationship between PAH LC50 (Panel A- pyrene; Panel B- phenanthrene) and phytoplankton cell volume obtained for the natural phytoplankton communities. The continuous lines represent the fitted linear regressions (eq. 1.7, Panel A; eq. 1.6, Panel B). The discontinuous line represents the relationship obtained between pyrene LC50 and cell size for cultured phytoplankton (eq. 1.5).

The slope of the relationship between pyrene LC50 and cell volume of the natural populations (eq. 1.7) was significantly smaller (t-student, $P< 0.05$) than that

observed between the pyrene LC50 and cell volume obtained for phytoplankton cultures (eq. 1.5, Fig. 1.5).

The half lives obtained under the different treatments, calculated for *Prochlorococcus* sp. and *Synechococcus* sp., the two species which suffered catastrophic cell death in the natural communities examined (Table 1.5), varied from 9 days to 0.1 days, showing shorter half lives at the highest PAH concentration (Table 1.5), comparable to the values observed in cultures (Table 1.2). For larger phytoplankton populations we could calculate half values in few of the experiments, for the higher concentration treatments, showing values between 1.4 and 0.2 for picoeukaryotes, and between 2.4 and 0.3 days, for nanophytoplankton.

PYRENE		**Half Live (day)**		
		100	**250**	**500**
Mediterranean Sea (Thresholds)	*Synechococcus*	2	-	0.5
Atlantic Ocean (RODA)	*Prochlorococcus*	0.5	0.25	0.25
	Synechococcus	8	0.9	0.75
Mediterranean Sea (Faro)	*Synechococcus*	0.5	0.4	0.4
PHENANTHRENE		**Half Live (day)**		
		100	**250**	**500**
Mediterranean Sea (Thresholds)	*Prochlorococcus*	1.2	0.5	0.25
	Synechococcus	9	0.8	0.36
Atlantic Ocean (RODA)	*Prochlorococcus*	1.7	0.4	0.1
Mediterranean Sea (Faro)	*Synechococcus*	2.6	0.5	0.35
	Synechococcus	0.85	0.4	0.4

Table 1.5. Values of half lives calculated for *Prochlorococcus* sp. and *Synechococcus* sp. for the highest concentrations of pyrene and phenanthrene (μg L^{-1}) tested in the experiments performed with natural communities. (-) no treatments tested.

In the treatments where the concentrations were smaller than those causing catastrophic cell death, the induction of cell death was analyzed by measuring the increase in the proportion of dead cells within the populations. In almost all the

experiments, the proportion of dead cells in both *Prochlorococcus* sp. and *Synechococcus* sp. increased as the pyrene or phenanthrene concentration treatments were increased at concentration levels below the LC50 (Fig. 1.6). These increases on the percentage of dead cells were sometimes higher than 20% with respect to the control (Fig. 1.6), but even in some cases, i.e. *Synechococcus* of the Mediterranean Sea in FARO campaign, for both pyrene and phenanthrene (Fig. 1.6), an increase in the percentage of dead cells was also detectable at the lowest PAH concentration (Fig. 1.6).

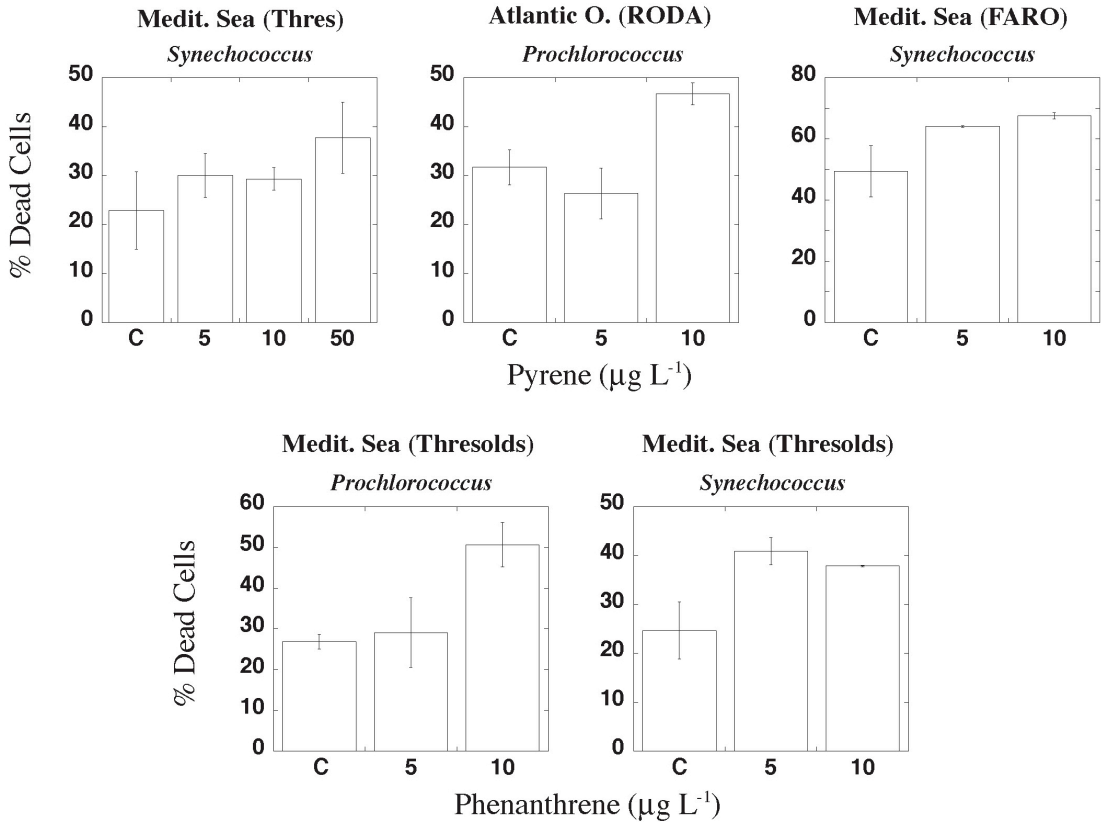

Figure 1.6. Changes in the proportion of Mediterranean and Atlantic *Synechococcus* sp. and *Prochlorococcus* sp. dead cells observed in the control (c) and low concentration PAHs (pyrene or phenanthrene) treatments.

4. Discussion

Previous studies analyzing the toxicity of PAHs to phytoplankton have been focused mostly on the physiological responses at the sublethal level (e.g. growth, photosynthetic oxygen evolution, dark respiration, etc. (Singh and Gaur, 1988)) and the present study contributes with new data to the few data describing the lethal levels of PAHs to phytoplankton (Weber Scanell et al., 2005). The results of cell death induction obtained revealed a high variability in the sensitivity of phytoplankton to the two PAHs tested. We found small differences in the degree of toxicity for phytoplankton between phenanthrene and pyrene. Pyrene has one aromatic ring more than phenanthrene, for which toxicity may be higher (e.g. Millemann et al., 1984), however, PAHs of high molecular weight are reported to reduce toxicity due to a concomitant decline in solubility, that may explain the small differences between phenanthrene and pyrene toxicity (Kenish, 1997) and permeability restrictions to enter into the phytoplanktonic cell (Del Vento and Dachs, 2002).

Our results show that phytoplankton cell size was an important parameter determining the sensitivity to PAHs, both in cultures and in natural communities, with the smallest pico-cyanobacteria, *Prochlorococcus* and *Synechococcus*, been the most sensitive. The largest phytoplankton species tested (*Thalassiosira* sp.) were however the most resistant and a strong relationship between LC50 and cell volume was found. Cell volume has been identified previously as an important factor to determine persistent organic pollutant accumulation in phytoplankton (Del Vento and Dachs, 2002), because small cells should have a higher capability to incorporate these contaminants into their cells due to their higher surface to volume ratio (Del Vento and Dachs, 2002). Our study strongly confirms this theoretical relationship, since in both cultured and natural phytoplankton communities the variability in the lethal

levels of PAHs was strongly related to phytoplankton cell size. Fan and Reinfelder (Fan and Reinfelder, 2003) confirmed experimentally that phenanthrene adsorption at surface was higher for smaller cells.

The importance of phytoplankton cell size in explaining their sensitivity to PAHs was not restricted to the results found for cultures but extended clearly to the results obtained with natural phytoplankton communities. Despite the experiments were carried out in communities from different waters (open and coastal Mediterranean Sea and the Atlantic Ocean), and despite some differences in community composition characterized the waters sampled, the differences in the LC50 were mostly explained by differences in cell size. For example, the LC50 for phenanthrene was very similar for the Atlantic (36.3 μg L^{-1}) and Mediterranean (30.2 μg L^{-1}) *Prochlorococcus*. For *Synechococcus*, the major difference in LC50 among locations was less than two times for both phenanthrene and pyrene. For other phytoplankton groups as picoeukaryotes and nanoplankton, although taxonomic differences, not discernible with the methods used here, probably existed among communities, differences in cell size strongly explained the variation in LC50, especially for phenanthrene. In consequence, we found not significant differences in the sensitivity of phytoplankton with similar cell size between the waters tested, meaning that location has less influence on the phytoplankton community response to PAHs than the phytoplankton size of cells.

The present results also show that natural phytoplankton was less resistant to PAHs than phytoplankton species growing in cultures. The differences in the pyrene LC50 observed between cultured and natural phytoplankton were in the order of 20-35 times for the nano-sized phytoplankton, as reflected in the difference between *Phaeodactyllum tricornutum* LC50 of 3195 μg L^{-1} for pyrene, while similar sized

nanoplankton of the Mediterranean Sea showed a LC50 of 88.9 μg L^{-1} (Thresholds) and 141.6 μg L^{-1} (Faro) for pyrene. The differences decreased with decreasing phytoplankton cell size, which may represent 15-25 times difference for pico-sized phytoplankton, and in the order of 2-3 times difference between cultured and natural *Prochlorococcus*.

This remarkable difference in the lethal sensitivity to PAHs between cultured and natural phytoplankton is probably the result of a variety of parameters that differ between laboratory conditions and natural growth conditions, which imply the optimization of the growth conditions in the laboratory in comparison with the growth at the sea. Another reason may be derived from the fact that species growing in cultures have been exposed for several generations to an elevated concentration of contaminants, relative to the open ocean, in the laboratory environment, which could have derived in the selection of species showing higher resistance to contaminants. Even though, a specific study should be done to assess this difference.

When the PAHs concentration treatments analyzed here did not result in a substantial decline of the population, the induction of cell death was revealed from our direct estimates of the proportion of living cells within the populations. By doing this analysis, we detected a decrease in the percentage of living cells in some of the cultured species even at the treatments were a decline in the total population was not detected. Also, cell mortality was expected at the levels of PAHs concentration below the LC50, and we effectively observed in the natural populations of picoplancton that, as the concentrations of both pyrene or phenanthrene increased, the proportion of living cells decreased, resulting in decreases of more than 20% of the living cells in the treatments with the concentrations close to the LC50 found. These results are highly original since there are not previous reports of similar data for natural

phytoplankton communities. Moreover, in the case of cultures, cell death was also detected by the observation of an increase in the rate of cell death for those species that did not show catastrophic decline in their populations when exposed to pyrene. Cell death rates in the cultures were related to the growth rates, as were calculated using the changes in the abundance of living, dead, and total population (Brussard et al., 1997). An increase in cell death rate, relative to growth rate, was clearly observed at the highest concentrations levels of pyrene for the largest species tested.

Our results, by providing PAHs LC50 values of phytoplankton, allows the comparison of the sensitivity of phytoplankton with that reported for other organisms. Kenish et al. (1997) compiled data of lethal levels of PAHs for a variety of terrestrial and aquatic organisms, and they reported results for phenanthrene in shrimp and sandworms, showing LC50s of 370 and 600 μg L^{-1} respectively. Although these values are much lower than those observed for phytoplankton cultures (except for those of the pico-sized species) these values are higher than those reported here for the natural phytoplankton. The highest phytoplankton LC50 for phenanthrene obtained here was 165.7 μg L^{-1}, still smaller however than the LC50 reported for aquatic invertebrates (Kenish et al., 1997).

The higher sensitivity of natural phytoplankton, from the oligotrophic waters examined here, with respect of that of larger organisms is also well reflected in the half lives values obtained. Thus, while half lives for grass shrimps and sandworms were described to be of 1 and 4 days, for concentrations in the medium of 370 and 600 μg L^{-1} respectively (Kenish et al., 1997), picophytoplankton species showed, for similar concentrations (250 and 500 μg L^{-1}), much lower half lives, ranging from 0.1 to 0.8 days, showing also lower values, smaller than a day, for picoeukaryotes and nanophytoplankton.

The PAHs lethal concentrations required to reduce the 50% of the population, the LC50 values found here, are concentrations highly above the concentrations found in nature. Hence, the LC10, the lethal concentrations required to reduce the 10% of the populations, which have been previously calculated for marine organisms such as copepods (Barata et al., 2002), amphipods (Page et al., 2002) or fishes (Oris and Bailer, 1997), but not for phytoplankton, were calculated, since a decrease of 10% of the population would already have important consequences in terms of ecosystem function such as carbon fluxes mediated by phytoplankton, which are key processes in the marine carbon cycle. The natural levels for pyrene found in the Mediterranean Sea during the THRESHOLDS cruise were below 0.15 ng L^{-1}, and reached values around 0.5 ng L^{-1} for the subtropical Atlantic ocean during the RODA cruise (Berrojalbiz and Dachs, in prep.), similar to previous values described for these marine regions (Dachs et al., 1997; Nizzeto et al., 2008). The LC50 for *Prochlorococcus* in the experiments performed during the THRESHOLDS and RODA cruises ranged from 15 to 35 μg L^{-1}, which represented more than 10^5 times the natural levels. Even the LC10 values were well above the PAHs concentrations found in those waters, suggesting that phytoplankton communities in these areas should not be affected by the natural levels of individual PAHs. Although these LC50 and LC10 are highly above natural concentrations, these natural concentrations can arise significantly due to special episodes of air deposition or oil spills, which would cause phytoplankton cell death in oligotrophic waters. Furthermore, similar PAHs water concentrations than those reported here could be found in impacted coastal environments. However, it is important to remark that these PAHs do not impact individually on the ecosystem and that there are other environmental factors, like UV (Groth Petersen and Dahllöf, 2007), and the presence of other PAHs or the myriad of organic contaminants

occurring in the water, that may enhance their impact due to synergistic effects that need further attention.

4.1 Conclusions

This study has provided new data to the few data describing the lethal levels of PAHs to marine phytoplankton. Both phytoplankton growing in cultures and in nature were sensitive to high concentrations of pyrene and phenanthrene, as indicated in the experiments by the decay of their populations as the PAHs concentrations increased, been lethal at the highest concentrations tested for most of the species. For phytoplankton growing in cultures, both growth and cell death rates were affected by the PAHs when added at concentrations below the lethal levels. Similarly, for natural communities there was a measurable increase in the percentage of dead cells at sublethal concentrations. Our results show, both for cultures and for natural communities, that phytoplankton sensitivity to PAHs was dependent of cell size, with the smallest pico-cyanobacteria, *Prochlorococcus* and *Synechococcus*, been the most sensitive. However, significant differences between cultured phytoplankton and natural communities were observed, been natural communities more sensitive. These differences ranged from 2 times for *Prochlorococcus*, to 20-35 times for the nano-sized phytoplankton. As a consequence, cell size and not the geographical origin of the Atlantic and Mediterranean communities explained the differences in their sensitivity to PAHs. Our results highlight that these contaminants may have a higher incidence in oligotrophic waters than expected, due to the prevalence of small sized phytoplankton in this environments. In addition, the relationships of LC50 with species and variables such as their cell volume provide evidence of the factors controlling the toxicity of anthropogenic chemicals to phytoplankton.

5. Acknowledgments

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Chapter 2

Cell size dependence of additive versus synergetic effects of UVR and PAHs on oceanic phytoplankton

by

Pedro Echeveste, Susana Agustí and Jordi Dachs

Environmental Pollution. In press

Abstract

Polycyclic Aromatic Hydrocarbons' (PAHs) toxicity is enhanced by the presence of ultraviolet radiation (UVR), which levels have arisen due to the thinning of the ozone layer. In this study, PAHs' phototoxicity for natural marine phytoplankton was tested. Different concentrations of a mixture of 16 PAHs were added to natural phytoplankton communities from the Mediterranean Sea, Atlantic, Arctic and Southern Oceans and exposed to natural sunlight received in situ, including treatments where the UVR bands were removed. PAHs' toxicity was observed for all the phytoplankton groups studied in all the waters and treatments tested, but only for the pico-sized group a synergetic effect of the mixture and UVR was observed ($p=0.009$). When comparing phototoxicity in phytoplankton from oligotrophic and eutrophic waters, synergy was only observed at the oligotrophic communities ($p=0.02$) where pico-sized phytoplankton dominated. The degree of sensitivity was related to the trophic degree, decreasing as Chlorophyll *a* concentration increased.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) have been described as mutagenic, teratogenic and carcinogenic for organisms (Lehr and Jerina, 1977), altering some cell processes like growth, photosynthetic oxygen evolution, dark respiration and pigment composition (Singh and Gaur, 1988). PAHs impact natural ecosystems not only by direct spillage of petroleum and its refined products (Page et al., 1988), but also through long-range atmospheric transport and subsequent deposition, as they do in remote parts of the oceans (Del Vento and Dachs, 2007).

Moreover, there are environmental factors that enhance PAHs' toxicity, such as the presence of ultraviolet (UV) light on ecosystems (Diamond, 2003; Larson and Berenbaum, 1988; Pelletier et al., 2006), which radiation has increased last decades as a result of a decrease of the ozone layer (Weatherhead and Andersen, 2006). UV has been addressed to affect the growth and the productivity of phytoplankton (Holm-Hansen et al., 1993; Neale, 2001) and has been identified as an important factor inducing phytoplankton mortality (Llabrés and Agustí, 2006). Ultraviolet radiation (UVR) affects photosynthetic energy-harvesting enzymes and other cellular proteins and also photosynthetic pigment contents (Häder et al., 1998), producing as well reactive oxygen substances (ROS), which are strong oxidants that cause cell damage as a result of a photolysis of dissolved organic matter (Murphy, 1983; Tyrrel, 1991).

Thus, UV enhances PAHs reactivity by photomodification (photooxidation / photolysis / photodegradation), when organic compounds are structurally altered to a variety of compounds; and/or by photosensitization processes (radical formation), when intracellular singlet-state oxygen and other reactive oxygen species (ROS) are generated, causing oxidative damage (Petersen and Dahllöf, 2007). Moreover, as PAHs also absorb light within the visible range of the spectrum, high intensities of the

Photosynthetic Active Radiation (PAR) may induce to phototoxicity (Cody et al., 1984; Grote et al., 2005).

Many works have studied the toxic effects of individual PAHs to phytoplankton, mainly on freshwater species, under UVR, but mostly regarded at sublethal levels such as algae reproduction (Grote et al., 2005), photosynthetic efficiency (Gala and Giesy, 1992; Marwood et al., 1999) or inhibition of growth rate (Gala and Giesy, 1992; Okay and Karacik, 2007; Petersen et al., 2008). Despite scarce studies have analyzed the lethal levels of PAHs to marine phytoplankton, clear patterns have been shown, identifying cell size as a key property determining the sensitivity of phytoplankton to PAHs (Echeveste et al., 2010a).

The goal of this study was to quantify the lethal action of a mixture of 16 PAHs, when exposed to UV light, to marine photosynthetic plankton from different oceanic regions. For this purpose, four experiments were carried out to observe the lethality of different concentrations of a mixture of PAHs under natural UVR to different phytoplankton communities, focusing in the antagonistic/additive/synergetic joint actions between UVR and the mixture of PAHs. Thus, the experiments were carried out in the coastal Mediterranean Sea and in the open Atlantic Ocean, which are oligotrophic waters dominated by pico-sized phytoplankton: *Prochlorococcus*, *Synechococcus* and picoeukaryotes (e.g. Agawin et al., 2000; Alonso-Laita and Agustí, 2006); and in the Arctic and Southern Oceans, which are more eutrophic waters dominated by micro-sized phytoplankton, mostly diatoms (e.g. El-Sayed, 1971; von Quillfeldt, 1997). The resulting cell death-PAHs relationships observed will be used to define the thresholds and points of no return of PAHs to marine phytoplankton. Moreover, these thresholds may be related to the cell volume, as

sensitivity to PAHs will vary with phytoplankton cell size (Echeveste et al., 2010a; Fan and Reinfelder, 2003).

2. Material and methods

The four experiments to analyze the synergy between PAHs and UV light on natural communities of phytoplankton were performed with coastal Mediterranean plankton, sampled at the field station of Far Cap Ses Salines, Mallorca Island, and on open sea plankton, sampled during the oceanographic cruises RODA-I, ATOS-I and ATOS-II (Table 2.1 for coordinates, Fig. 2.1 for position) performed on board the RV Hespérides.

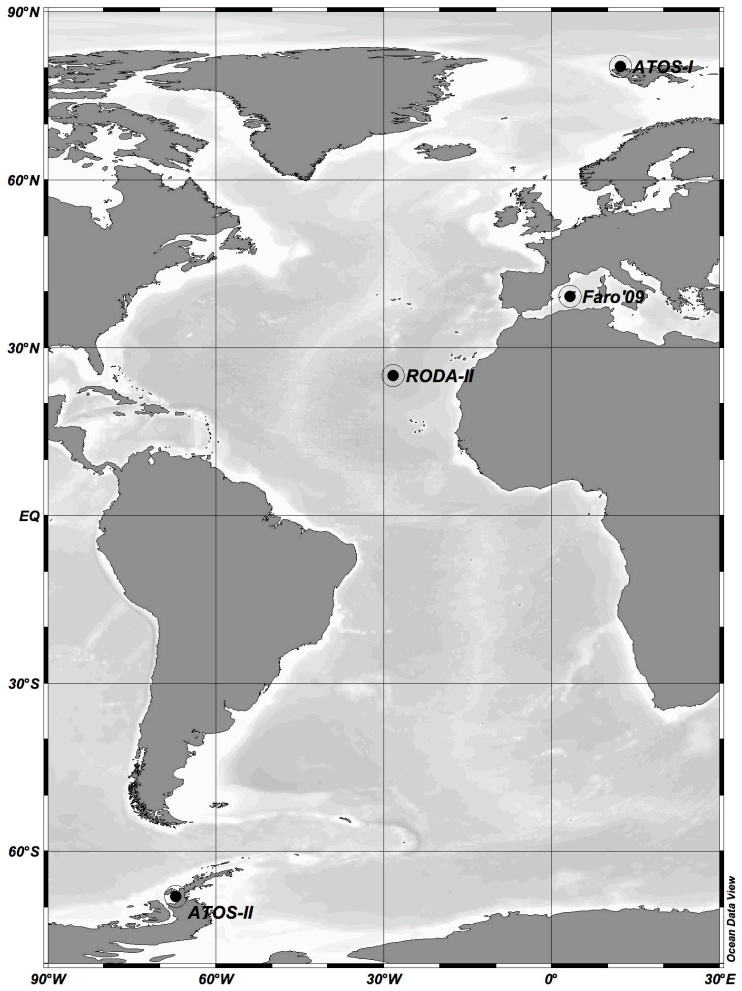

Figure 2.1. Position of the experiments.

RODA-I cruise was held along the subtropical Atlantic Ocean from August 17 to September 5, 2006; ATOS-I along the Arctic Ocean from June 27 to July 27, 2007; and ATOS-II along the Southern Ocean from January 24 to March 2, 2009. The experiment on Far Cap Ses Salines was carried out from 27 to 31 of July 2009. Surface water (5 m) of the Atlantic, Arctic and Southern Oceans used in the experiments was sampled by using Niskin bottles attached to a rosette-CTD system. In the experiments carried out in Far Cap Ses Salines, surface water was directly collected using an acid clean plastic carboy from the border of a rocky shore of the Mediterranean Sea (Table 2.1 for geographical position).

Campaign	**Coordinates**	**Experiments duration**	**Chlorophyll *a* (mg m^{-3})**	**UV index**	**UV doses (KJ m^{-2} day^{-1})**
RODA-II	25º 0' N - 28º 27' W	02/21/2007 - 02/25/2007	0.28	0.3 - 7.7	2.1
ATOS-I	80º 23' N - 12º 26' E	07/19/2007 - 07/25/2007	1.51	0.1 - 4	2.2
ATOS-II	68º 12' S - 67º 12' W	02/18/2009 - 02/24/2009	2.57	0.9 - 2.4	2.3
Faro'09	39º 16' N - 3º 3' E	07/27/2009 - 07/31/2009	0.57	0.3 – 9.4	8.7

Table 2.1. Cruise and position of the stations where the natural phytoplankton communities were sampled. Faro'09 campaign was carried out in the coastal Mediterranean Sea, while RODA-II, ATOS-I and ATOS-II campaigns were carried out in the subtropical North Atlantic Ocean, Arctic and Southern Oceans, respectively. The extent of each experiment is indicated by initial and final dates. The abundance of phytoplankton on the sampling day is indicated as Chlorophyll *a* concentration found at each station. The incident ultraviolet radiation conditions are indicated by the minimum and maximum UV index recorded during the days of the experiments, and by the UVB average daily doses received during the experiments.

The mixture was composed by 16 PAHs: acenaphtene, acenaphthylene, anthracene, benzo[a]anthracene, benzo[b]fluoranthene, benzo(k)fluoranthene, benzo[ghi]perylene, benzo(a)pyrene, dibenzo[a,h]anthracene, chrysene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, pyrene, phenanthrene). PAHs in the mixture were dissolved in cyclohexane representing concentrations of 10 μg mL^{-1} for

each PAH. Experiments began with the inoculation of the mixture of PAHs at different concentrations. Final concentrations in the treatments were 0.1, 0.8, 4 and 10 μg L^{-1} for the Mediterranean communities, 0.5, 10 and 70 μg L^{-1} for Atlantic communities, and 0.1, 0.8, 4 and 20 μg L^{-1} for the Arctic and Southern Ocean communities. Before seawater addition, the dissolvent of the mixtures was let to evaporate for 3 hours in darkness. Then the sampled water was distributed into acid clean glass and quartz bottles, of 100 mL in the case of the Mediterranean Sea and Atlantic Ocean experiments, and of 2 L capacity in the case of the Arctic and Southern Oceans experiments. Glass bottles removed UVR to pass through although quartz bottles allow all the solar spectra to pass (Llabrés and Agustí, 2006). All bottles were incubated on deck under natural solar radiation in a tank with seawater surface running system to keep "in situ" temperature conditions. For the coastal experiment, a similar incubation system was used, with tanks placed in the exterior of the field station in air free area, and a surface seawater running system. Glass and quartz duplicated bottles without chemical additions were also run as controls. Daily sampling was performed in the experiments of the oligotrophic waters for as long as 4 days and every 2 days in the experiments of the eutrophic waters, which lasted for 6 days.

Solar and ultraviolet (UV) radiations were automatically measured by a Weatherlink Vantage Pro. Davis Co. meteorological station located on board the R/V Hespérides and the Far Cap Ses Salines. PAR was measured with the Solar radiation 6450 Davis sensor (from 400-1100 nm) every 5 minutes. In addition, integrated UV (290-390 nm) values in all the wavelengths were obtained every 5 minutes with the UV 6490 sensor. Data of UV was provided as UV Index, which is an irradiance scale calculated by multiplying the Erythema corrected irradiance in Watts m^{-2} by 40

(McKinlay and Diffey, 1987). UVB indexes (UVI) and UVB doses (KJ m^{-2} day^{-1}) were then calculated (Table 1). The UV index is an international standard measurement based on irradiance values (Watts m^{-2}), indicating how strong the ultraviolet (UV) radiation from the sun is. UV doses were calculated by integrating the daily cumulative radiation values received during the experiments.

The effect of both the mixture of PAHs and UV on the different groups forming the phytoplankton communities was determined. For picophytoplankton communities (*Prochlorococcus* sp., *Synechococcus* sp. and Eukaryotic picophytoplankton), changes in the abundance and viability of cells were quantified by using duplicated 1 ml fresh samples counted in a FACSCalibur Flow Cytometer (Becton Dickinson). An aliquot of a calibrated solution of 1 μm diameter fluorescent beads (Polysciences Inc.) was added to the samples as an internal standard for the quantification of cell concentration. The red, green and orange fluorescence, and forward and side scattering signals of the cells and beads were used to detect different populations and to differentiate them from the fluorescent beads (Marie et al., 2000).

The proportion of living and dead cells was also followed by applying a cell membrane permeability test, the cell digestion assay (Agustí and Sánchez, 2002), which allows the counting and identification of living phytoplankton cells. The cell digestion assay was applied to replicate samples, by adding 200 μl of DNAse I solution (400 μg ml^{-1} in HBSS (Hanks' Balanced Salts)) to 1 ml sample of each treatment, followed by 15 minutes incubation at 35ºC in a Digital Dry Bath, for the temperate waters, and at 30ºC for the polar waters. After this time, 200 μl of Trypsin solution (1% in HBSS) were added, followed by 30 minutes incubation at 35 or 30ºC. At the end of this time, samples were kept in cold conditions in order to stop the cell digestion process. Similarly, and to analyze nano- and microphytoplankton viability

in polar waters, the cell digestion assay was applied to replicate samples, by adding 1 ml of DNAse I solution (400 μg ml^{-1} in HBSS (Hanks' Balanced Salts)) to 5 ml sample of each treatment, followed by 15 minutes incubation at 30ºC in a Digital Dry Bath. After this time, 1 ml of Trypsin solution (1% in HBSS) was added, followed by 30 minutes incubation at 30ºC. At the end of this time, samples were kept in cold conditions in order to stop the cell digestion process as described before.

The changes in the abundance of nano and microphytoplankton communities were analyzed at the beginning and the end of the experiments by using epifluorescence microscopy, in the case of the experiments carried out in oligotrophic waters, and by using a submersible flow cytometer and microscope (FlowCAM, Fluid Imaging, Inc., Edgecomb, ME, USA), in the case of the experiments carried out in eutrophic waters. For the microscopic analysis by epifluorescence, and in order to have a representative number of cells, 50 ml of water were sampled from each replicated treatment bottle and filtered onto policarbonate 2 μm pore diameter black filters. During the filtration, with the last ml to be filtered, 2 ml of glutaraldehyde (25%) were added to the sample in order to fix it. Once filtered, filters were frozen until their analysis in order to preserve them. The FlowCam has been recently developed for the monitoring of phytoplankton communities in freshwater and marine environments (See et al., 2005; Sieracki et al., 1998; Sterling Jr. et al., 2004), allowing us to count, image and analyze cells in situ, measuring the size and parameters of the fluorescence waveform generated, and storing a digital image of each passing particle. For this analysis, duplicate water samples were pumped through a glass flow chamber (2x0.1mm) at a rate of approximately 0.3 ml min^{-1} for 3-4 min from each treatment bottle and then cells monitored.

Cells were then counted in a Zeiss Axioplan Imaging epifluorescence microscope. Cells counted in the microscope were classified into 3 groups: small eukaryotes (3-4 μm size cells), nanophytoplankton (5-20 μm size cells) and microphytoplankton (larger than 20 μm). Cells counted in the FlowCAM were collected in "trigger" mode and then classified in 4 different groups: Large diatoms with equivalent Spherical Diameter (ESD) larger than 65 μm, medium diatoms with ESD between 35 and 65 μm, and small diatoms and flagellates with ESD lower than 35 μm.

Moreover, changes in total phytoplankton abundance during the experiments were followed by analyzing Chlorophyll *a* concentration. For this estimation, 50 ml samples were filtered onto 25 mm diameter Whatmann GF/F filters from each bottle on the day 0, day 2 and last day (day 4). After filtration, filters were placed in tubes with 90% acetone for 24 hours for the extraction of the pigment. Then, the fluorescence of the Chlorophyll *a* was measured in a Shimadzu RF-5301 PC spectrofluorimeter and calibrated with pure Chlorophyll *a* as described in Parsons et al. (1984).

2.1. Calculations

Half lives ($t_{1/2}$) of the different species were calculated by applying the formula

$$t_{1/2} = \ln 2 / \mu \qquad \text{(eq. 2.1)}$$

where μ is the slope of the natural logarithm (ln) of the decay of cell abundance with time in days.

The lethal thresholds of the mixture of PAHs for each species or phytoplankton population tested was calculated as the PAHs concentration at which the cell population will be decreased by a half, applying the equation

$$LC50 = \ln 2 / \Omega \qquad \text{(eq. 2.2)}$$

where Ω is the slope of the relationship between the decay ln of cell abundance and the PAH concentration ($\mu g\ L^{-1}$) reached at the end of the experimental treatments.

The significant differences observed between treatments were analyzed by using the t-student test.

In order to analyze whether the exposure to the mixture of PAHs ($\mu g\ L^{-1}$) and to the UVR treatments may show a synergetic effect affecting phytoplankton communities, the decay rates (μ, $days^{-1}$) of the populations observed in the different experiments were analyzed following the equation:

$$\mu = a1 + b1 * T + a2 * UV + b2\ (UV * T) \qquad \text{(eq. 2.3)}$$

where a1 is the intercept on the Y-axis of the linear regression, b1 is the slope of that linear regression, a2 is the variation of the intercept in the presence of UVR and b2 is the variation of the slope in the presence of UVR (more details in Fig. 2.2). Thus, in the absence of UVR, the equation would be $\mu = a1 + b1 * T$, showing the effect of the increasing concentrations of the mixture of PAHs on the decay rates of the different populations, while in the presence of UVR (i.e., UV=1), the equation would be $\mu = (a1 + a2) + (b1 + b2) * T$, showing the joint action of the increasing concentrations of the mixture of PAHs and UVR on the decay rates of the different populations.

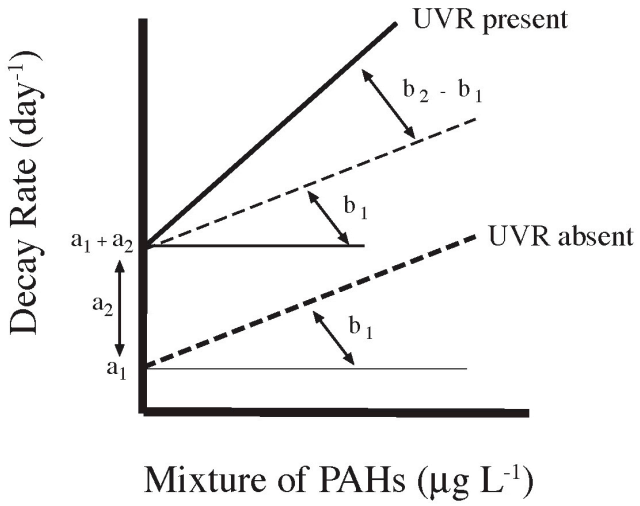

Figure 2.2. Increase in the decay rates (days $^{-1}$) as a result of the joint action of UVR and PAHs. Following equation 1, a1 is the intercept on the Y-axis of the linear regression, b1 is the slope of that linear regression, a2 is the variation of the intercept in the presence of UVR and b2 is the variation of the slope in the presence of UVR.

These models were statistically analyzed using the program JMP. To analyze the model, phytoplankton species and groups were classified according to their cell size (<2 μm-picophytoplankton, 2-15 μm-nanophytoplankton, and >15 μm-microphytoplankton) and trophic characteristic i.e., as oligotrophic when Chlorophyll *a* concentration was < 1 mg m^{-3} or eutrophic waters when Chlorophyll *a* concentration was > 1 mg m^{-3}.

3. Results and discussion

3.1. Phytoplankton communities

The four experiments, run in different waters, were dominated by different phytoplankton communities. In the experiments run in the coastal Mediterranean Sea and in the North East Atlantic Ocean, picophytoplankton, represented by *Prochlorococcus* sp., *Synechococcus* sp. and small eukaryotes, dominated the phytoplankton communities of these oligotrophic waters, although nanophytoplankton was also present. There were some differences in the community composition of

phytoplankton between these two oligotrophic areas, with *Prochlorococcus* been more abundant in Atlantic waters and almost absent in the Mediterranean Sea. On the other hand, the experiments run in the eutrophic waters of the Arctic and Southern Oceans were dominated mainly by micro and nanoplankton, represented by diatoms and flagellates, respectively.

3.2. Phytoplankton response to increased concentrations of PAHs and UVR

However, no matter the phytoplankton community composition was, phytoplankton populations decreased when exposed to high concentrations of the mixture of PAHs. The concentrations tested in the first experiment, performed in Atlantic waters, suggested the use of lower and more accurate concentrations of the mixture, narrowing the range of concentrations used in the subsequent experiments. Thus, the decline with increasing mixture concentrations was observed in the total community, quantified as Chlorophyll *a* concentration (Fig. 2.3) and when considering the individual counts of the different populations conforming the communities (Fig. 2.3). When phytoplankton was exposed to total solar spectra, populations decreased when exposed to high concentrations of the mixture of PAHs as observed in the treatments where UVR was removed (Fig. 2.3).

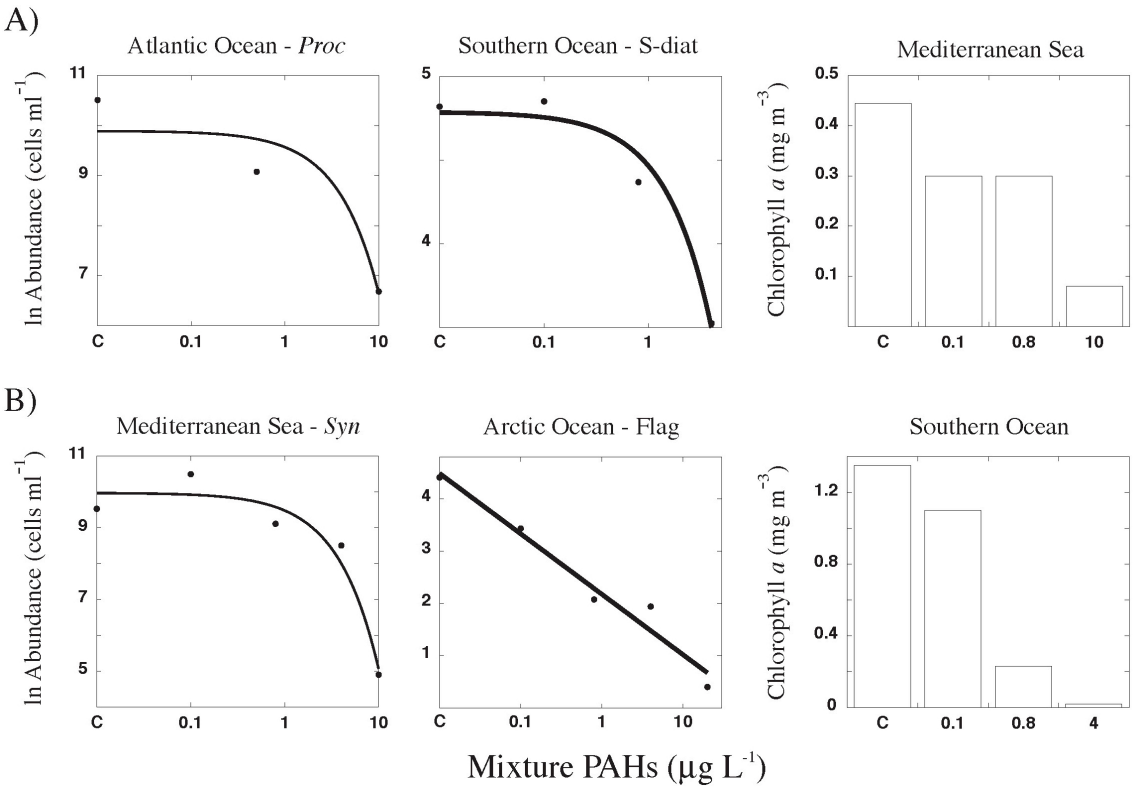

Figure 2.3. Decrease in the phytoplankton cell abundance (cells ml^{-1}) and total biomass (represented by Chlorophyll *a* concentration, in mg m^{-3}) with the increase of the mixture of PAHs concentration (μg L^{-1}). Panel A) UV-b filtered. Panel B) Total solar radiation. (*Proc*) *Prochlorococcus*; (S-diat) Small diatoms; (*Syn*) *Synechococcus*; (Flag) Flagellates.

The concentrations of the mixture of PAHs required to reduce the 50% of the population, the 50% Lethal Threshold (LC50), were calculated for the treatments receiving UVR and not receiving it. LC50s were only calculable for the Mediterranean Sea and the Arctic Ocean (Table 2.2), since the number of concentration treatments was low (limited to 3) in the experiments made in the Southern and Atlantic Oceans. LC50s did not vary much, ranging from 1.94 μg L^{-1} to 6.55 μg L^{-1} (Table 2.2).

		Cell Vol. (μm^3)	Absence of UV		Presence of UV	
			LC50 ($\mu g\ L^{-1}$)	LC10 ($\mu g\ L^{-1}$)	LC50 ($\mu g\ L^{-1}$)	LC10 ($\mu g\ L^{-1}$)
Arctic Ocean (ATOS-I)	Flagellates	3157	4.48 ± 1.44	0.68 ± 0.22	4.14 ± 3.18	0.63 ± 0.48
	Small diatoms	2738	6.55 ± 6.03	1 ± 0.92	6.63 ± 13.73	1.01 ± 2.09
	Med. diatoms	12954	5.66 ± 15	0.86 ± 2.28	7.52 ± 13.17	1.14 ± 2
	Chlorophyll *a*		1.52 ± 0.14	0.23 ± 0.02	1.14 ± 0.37	0.17 ± 0.06
Mediterra-nean Sea (Faro'09)	*Synechococcus*	0.9	1.94 ± 0.27	0.3 ± 0.04	1.41 ± 0.4	0.21 ± 0.06
	Small Euk.	24	3.84 ± 3.03	0.58 ± 0.46	2.35 ± 1.31	0.36 ± 0.2
	Nanoplankton	173.6	3.98 ± 3.07	0.61 ± 0.47	2.55 ± 2.56	0.39 ± 0.39
	Chlorophyll *a*		4.6 ± 1.45	0.7 ± 0.22	1.97 ± 1.17	0.3 ± 0.18

Table 2.2. Cell volumes (μm^3) of the different groups forming the natural phytoplankton communities examined, and the mixture of PAHs LC50 and LC10 ($\mu g\ L^{-1}$) values obtained for each phytoplankton group in the experiments carried out in the Arctic Ocean and Mediterranean Sea. (Med. diatoms) Medium diatoms; (Small Euk.) Small eukaryotes.

The half lives obtained under the different treatments (Table 2.3), varied, depending on cell size, from more than 10 days in the case of the small diatoms of the Southern Ocean to 0.1 days for the *Prochlorococcus* of the Atlantic Ocean, showing shorter half lives at the highest mixture of PAHs concentrations (Table 2.3).

Trtmnt ($\mu g\ L^{-1}$)	UV	Atlantic Ocean			Arctic Ocean			Southern Ocean			Mediterranean Sea			
		P	*S*	P_e	F	S-d	M-d	F	S-d	M-d	*S*	P_e	S-e	Npl
0.8	**X**	-	-	-	nd	nd	nd	8.5	nd	nd	nd	1.0	1.0	1.4
	+	-	-	-	6.7	>10	nd	nd	6.7	8.7	2.0	0.7	2.5	nd
4	**X**	-	-	-	5.5	3.3	3.3	5.1	>10	6.7	>10	0.7	1.1	1.6
	+	-	-	-	5.5	2.9	4.9	9.0	8.2	6.7	3.5	0.4	0.9	1.1
10	**X**	0.3	0.4	0.4	-	-	-	-	-	-	1.1	nd	0.8	1.0
	+	0.1	0.2	0.4	-	-	-	-	-	-	0.6	nd	0.8	1.1
20	**X**	-	-	-	1.5	2.5	7.5	-	-	-	-	-	-	-
	+	-	-	-	1.5	3.1	3.3	-	-	-	-	-	-	-

Table 2.3. Values of half lives calculated for the highest concentrations of the mixture of PAHs ($\mu g\ L^{-1}$) tested. Symbols in the UV column represent treatments in the presence of UVR (+) and when UVR was removed (x). (Trtmnt) Treatment; (*P*) *Prochlorococcus*; (*S*) *Synechococcus*; (F) Flagellates; (S-d) Small diatoms; (M-d) Medium diatoms; (S-e) Small eukaryotes; (Npl) Nanophytoplankton. (-) no treatments tested. (nd) not detectable.

The LC50 values represented concentrations highly above the concentrations found in open sea marine waters. Obviously, the dissolved phase concentrations in the dissolved phase vary depending on the individual PAH, being significantly lower in the polar oceans than in the Mediterranean or subtropical Atlantic. For example, phenanthrene concentrations are about 100 pg L^{-1} in the Arctic and can be an order of magnitude higher (more than 1 ng L^{-1}) in the mid-Atlantic and Mediterranean Sea (Berrojalbiz et al., unpubl.; Echeveste et al., 2010b; Lohmann et al., 2009; Nizzetto et al., 2008). In most cases the presence of UVR increased the toxicity of the mixture of PAHs by decreasing up to 40% the LC50s (Table 2.2). Episodes of atmospheric deposition or oil spills may increase however the concentrations found in water to values close to the LCs reported here (Table 2.2) indicating that for these cases, strong phytoplankton decays will be expected. In addition, experiments performed here for simple mixtures of PAH provide valuable information of the general trends of the factors governing the toxicity of organic pollutants to phytoplankton, which may be extrapolated to the real mixtures found in the environment made of thousands of anthropogenic chemicals (Dachs and Méjanelle, 2010; Echeveste et al., 2010b).

Moreover, in the treatments where the concentrations of PAHs were lower than those causing catastrophic cell death (below the LC50 levels), the induction of cell death was analyzed by measuring the increase in the proportion of dead cells within the populations. At these concentrations cell death was also induced, as the proportion of dead cells increased, almost for all the experiments, as the concentration of the mixture of PAHs increased (Table 2.4, Fig. 2.4). The degree in the increased percentage of dead cells differed, however, between the phytoplankton groups for the same experiment and between experiments (Table 2.4).

	Absence of UV								
Trtmnt (μg L^{-1})	**Atlantic Ocean**		**Arctic Ocean**			**Southern Ocean**			**Med. S.**
	P	*S*	F	S-d	M-d	F	S-d	M-d	*S*
C	11	27	0	0	35	31	39	47	35
0.1	-	-	0	0	64	49	42	57	57
0.5	43	68	-	-	-	-	-	-	-
0.8	-	-	17	19	76	44	49	66	Nd
	Presence of UV								
Trtmnt (μg L^{-1})	**Atlantic Ocean**		**Arctic Ocean**			**Southern Ocean**			**Med. S.**
	P	*S*	F	S-d	M-d	F	S-d	M-d	*S*
C	22	22	Nd	Nd	69	17	19	53	41
0.1	-	-	Nd	Nd	83	37	4	38	37
0.5	28	44	-	-	-	-	-	-	-
0.8	-	-	Nd	Nd	100	71	42	75	50

Table 2.4. Percentage of dead cells for the treatments below the LC50s. (Trmnt) Treatment; (*P*) *Prochlorococcus*; (*S*) *Synechococcus*; (F) Flagellates; (S-d) Small diatoms; (M-d) Medium diatoms; (Med. S.) Mediterranean Sea; (-) no treatments tested; (Nd) Not detectable.

UVR induced cell death in most of the communities, as observed in the increase in the percentage of dead cells in the controls (Fig. 2.4). The degree of sensitivity to UVR exposure depended on the sensitivity of the different species, as for example *Prochlorococcus* was more sensitive to UVR than *Synechococcus* (Fig. 2.4), as previously described (Llabrés and Agustí, 2006). Under UVR exposure, the increasing concentration of PAHs resulted in increased percentages of dead cells and in some cases this effect was highly significant, been even higher than 50% with respect to the control (Fig. 2.4).

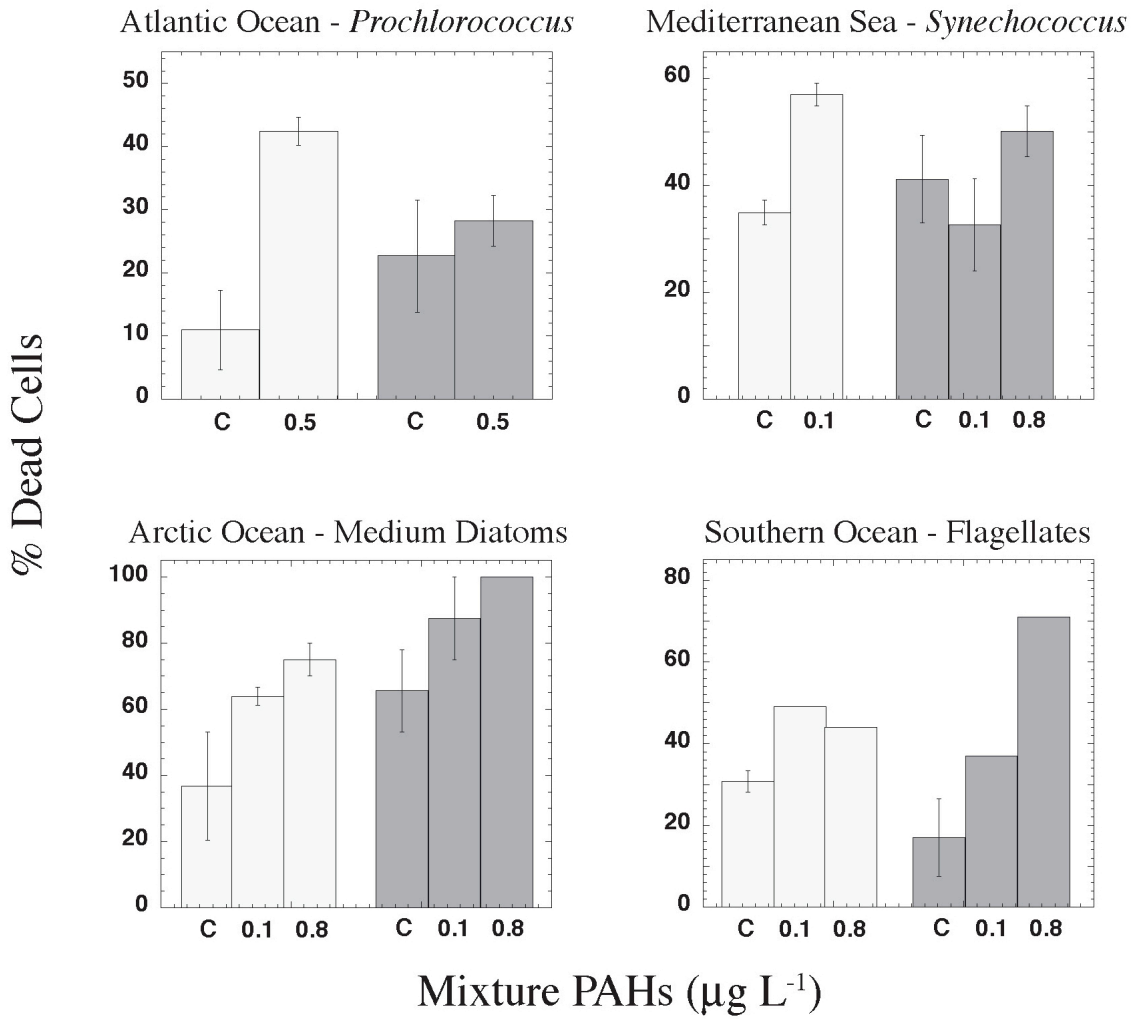

Figure 2.4. Changes in the proportion of dead cells for some of the phytoplankton communities studied, observed in the control (c) and at the lowest concentration of the mixture of PAHs added. Dark columns represent communities exposed to the total solar radiation and clear columns represent the treatments where UVR was removed.

Besides, population decay rates (μ, day^{-1}) were calculated for all the experiments (Table 2.5). At the highest concentration treatments, decay rates varied from 0.09 day^{-1} for the medium sized diatoms of the Arctic Ocean to 4.6 day^{-1} for *Prochlorococcus* of the Atlantic Ocean (Table 2.5). For some of the lowest concentration treatments, decay rates were not measurable, as there was not enough effect of the PAHs on the populations showing in some cases a smooth increase on the populations rather than a decrease of them (represented as a decay rate of 0 in Table 2.5). In many cases, the exposure to total solar radiation increased the decay

rates with respect to the treatments where UVR was removed (Table 2.5). Phytoplankton was exposed to high values of UVR in the experiments performed in oligotrophic waters, where the highest UV index values (7.7 and 9.4 for the Atlantic Ocean and Mediterranean Sea, respectively, Table 2.1) were observed. In contrast, the UVR during the experiments run in polar eutrophic waters were lower (UVI of 4 and 2.4 for the Arctic and Southern Ocean, respectively, (Table 2.1).

Trtmnt (μ**g L^{-1}**)	**UV**	**Atlantic Ocean**				**Arctic Ocean**				**Southern Ocean**				**Mediterranean Sea**				
		P	*S*	P_e	C	F	Sd	M	C	F	Sd	M	C	*S*	P	SE	Npl	C
0.1	**x**	-	-	-	-	0	0	0	0	0	0	0	0.1	0	0.4	0.5	0.3	0.4
	+	-	-	-	-	0	0	0	0	0	0	0	0.2	0	0.8	0.2	0	0.5
0.5	**x**	0.81	0	0.6	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-
	+	0.41	0.07	1.3	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-
0.8	**x**	-	-	-	-	0	0	0	0	0.1	0	0	0.3	0	0.7	0.7	0.5	0.4
	+	-	-	-	-	0.1	0	0	0.1	0	0.1	0.1	0.6	0.3	0.9	0.3	0	0
4	**x**	-	-	-	-	0.1	0.2	0.2	0.4	0.1	0.1	0.1	0.8	0.1	1.0	0.6	0.4	0
	+	-	-	-	-	0.1	0.2	0.1	0.4	0.1	0.1	0.1	1.1	0.2	1.7	0.8	0.6	0.8
10	**x**	2.3	1.6	1.8*	0.4	-	-	-	-	-	-	-	-	0.6	-	0.9	0.7	0.7
	+	4.7*	3.2*	1.8*	0.4	-	-	-	-	-	-	-	-	1.2	-	0.9	0.6	0.6
20	**x**	-	-	-	-	0.5	0.3	0.1	0.5	-	-	-	-	-	-	-	-	-
	+	-	-	-	-	0.5	0.2	0.2	0.5	-	-	-	-	-	-	-	-	-
70	**x**	4.7*	3.2*	1.8*	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-
	+	4.7*	3.2*	1.8*	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2.5. Decay rates (μ, d^{-1}) in the abundance of cells and in Chlorophyll *a* concentration observed in the different experiments. Symbols in the UV column represent treatments in the presence of UVR (+) and when UVR was removed (x). (*P*) *Prochlorococcus*; (*S*) *Synechococcus*; (P_e) Picoeukaryotes; (F) Flagellates; (Sd) Small diatoms; (M) Medium diatoms; (SE) Small eukaryotes; (Npl) Nanophytoplankton. (C) Chlorophyll *a*. (-) no treatments tested. * represents values below the detection limit.

3.3. Additive or synergetic joint action of UVR and PAHs

By using a statistical model (equation 2.3) to analyze the differences in the decay rates, we were able to analyze the phototoxicity of the mixture of PAHs due to the exposure to UVR. In all the cases, independently of the exposure to UVR, the mixture of PAHs significantly ($p<0.0001$) decreased the populations of phytoplankton (Table 2.6), while only for the pico-sized phytoplankton (<2 μm) and the whole

phytoplankton community of the oligotrophic waters (Mediterranean Sea and Atlantic Ocean) there was a significant synergetic effect of the mixture of PAHs and UVR ($p<0.009$ and $p<0.02$, respectively) (Table 2.6).

	Oligotrophic		**Eutrophic**		**<2 μm**		**2-15 μm**		**>15 μm**	
	Est	Prob	Est	Prob	Est	Prob	Est	Prob	Est	Prob
Int a1	0.05	0.7	0.02	0.24	-0.11	0.51	0.34	<0.01*	0.03	0.2
Slope b1	0.15	<0.01*	0.01	<0.01*	0.21	<0.01*	0.06	<0.01*	0.01	<0.01*
Int a2	0.1	0.53	0.01	0.51	0.22	0.28	-0.13	0.12	0.02	0.50
Slope b2	0.1	0.02*	0.00	0.9	0.15	0.009*	0.02	0.26	0.00	0.98

Table 2.6. Values in the statistical parameters obtained after applying the model (equation 2.3) to test the degree of synergy between the mixture of PAH and UVR. The slope b1 is the slope of that linear regression due to the effect of the mixture of PAHs. The Intercept a2 is the variation of the intercept due to UVR. The slope b2 is the variation of the slope due to the effect of both the mixture of PAHs and UVR. The Intercept a1 is the intercept on the Y-axys of the liner regression a1. Tested for the different populations in the experiments and samples grouped by trophic level (Oligotrophic waters = Mediterranean Sea and Atlantic Ocean; Eutrophic waters = Arctic and Southern Ocean) and the phytoplankton cell size (picophytoplankton <2 μm, nanophytoplankton 2-15 μm, microphytoplankton >15 μm). (Int) Intercept; (Est) Estimate; (Prob) Probability.

This effect was clearly observable when comparing decay rates and the concentrations of the mixture of PAHs (Fig. 2.5). The pico-sized phytoplankton (<2 μm) and the whole phytoplankton community of the oligotrophic waters showed a significant higher slope when UVR was present compared to the slope obtained when UVR was absent, indicating the synergetic joint action of UVR and the mixture of PAHs (Fig. 2.5). The nanoplankton (3-15 μm) also showed a larger slope when UVR was present (Fig. 2.5), but it was not significantly synergetic (Table 2.6), suggesting a major synergetic toxic action of UVR and the mixture of PAHs, and showing an intermediate response between the responses observed for picophytoplankton, significantly synergetic, and microphytoplankton, which showed a clear additive effect (Table 2.6, Fig. 2.5).

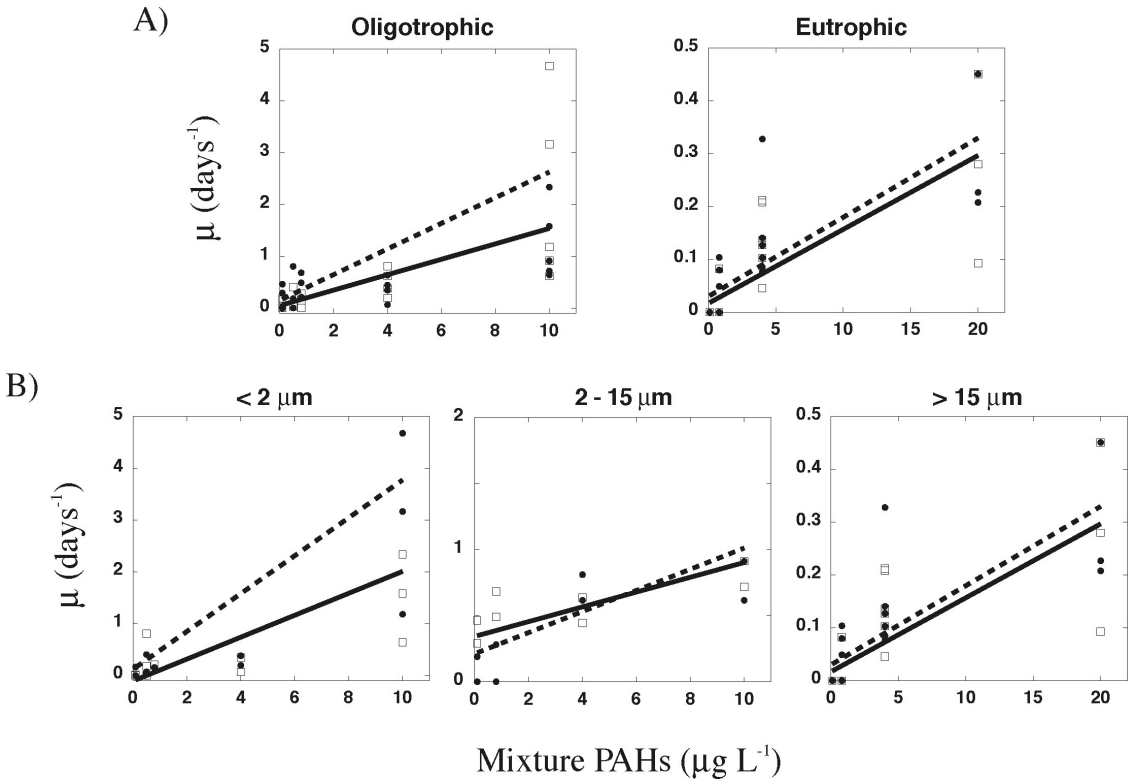

Figure 2.5. Increase in the decay rates (μ, days^{-1}) of phytoplankton as the concentration of the PAHs mixture (μg L^{-1}) increased, under the different light treatments (exposed to solar radiation including or not UVR) considered in the model (equation 2.3). Panel A) Communities classified by the trophic level: oligotrophic (Mediterranean Sea and Subtropical Atlantic Ocean) and eutrophic waters (Arctic and Antarctic waters). Panel B) Phytoplankton communities classified by cell size groups: pico-sized phytoplankton (<2 μm), nanophytoplankton (2-15 μm) and microphytoplankton (>15 μm). The continuous lines represent the fitted linear regressions in the absence of UVR, while the discontinuous lines represent the fitted linear regressions in the presence of UVR. Black dots represent treatments in the presence of UVR, while white squares correspond to the treatments in the absence of UVR.

3.4. Parameters determining phytoplankton sensitivity to the joint action of PAHs and UVR

	Atlantic Ocean		**Mediterranean Sea**		**Arctic Ocean**		**Southern Ocean**	
Int a1	Est	Prob	Est	Prob	Est	Prob	Est	Prob
Slope b1	-0.07	0.66	0.17	0.00*	0.02	0.38	0.00	0.74
Int a2	0.28	<0.01*	0.07	<0.01*	0.01	<0.01*	0.02	<0.01*
Slope b2	0.37	0.09	-0.03	0.63	0.02	0.61	0.01	0.6
Int a1	0.22	0.00*	0.03	0.1	0.00	0.95	-0.00	0.7

Table 2.7. Values in the statistical parameters obtained after applying the model (equation 2.3) to test the degree of synergy between the mixture of PAH and UVR. The slope b1 is the slope of that linear regression due to the effect of the mixture of PAHs. The Intercept a2 is the variation of the intercept due to UVR. The slope b2 is the variation of the slope due to the effect of both the mixture of PAHs and UVR. The Intercept a1 is the intercept on the Y-axys of the liner regression a1. Tested for the different populations in the experiments and samples grouped by location where experiments were performed (Mediterranean Sea and Atlantic, Arctic and Southern Oceans).

When analyzing the variability in b1 and b2 (which are the slopes of the decay rates due to the effect of PAHs in the absence and presence of UVR, respectively) observed (Table 2.7), with respect to Chlorophyll *a* concentration in the water mass, as an indicator of the trophic level, we observed that the slopes decreased as chlorophyll increased, indicating that the effect of PAHs and UVR increased from eutrophic to oligotrophic waters (Fig. 2.6). Moreover, the presence of UVR increased the slope values, especially at the lowest chlorophyll concentration waters (Fig. 2.6).

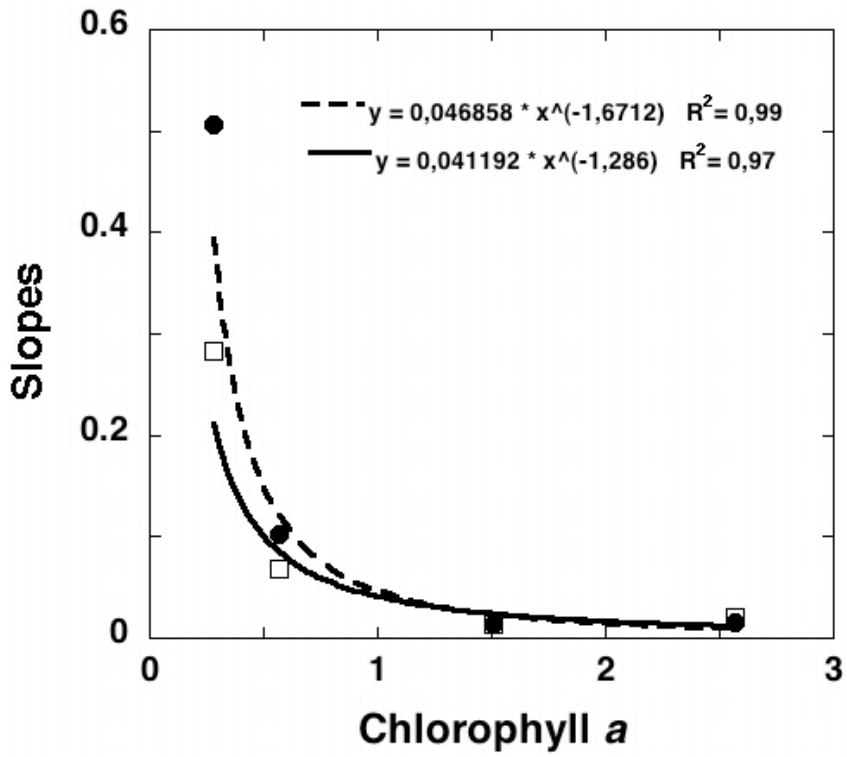

Figure 2.6. Variability in the slopes obtained following the model (Equation 2.3, Fig. 2.2) to analyze the degree of synergy between the mixture of PAHs and UVR, versus the Chlorophyll *a* concentration found in the four areas studied. The discontinuous

line represents the power fit in the presence of UVR (under Total Solar Radiation) and the continuous line represents the power fit for the treatments where UVR was removed. Black dots represent treatments in the presence of UVR, while white squares correspond to the treatments in the absence of UVR.

Although previous studies (Grote et al., 2005; Petersen and Dahllöf, 2007; Petersen et al., 2008) pointed to a joint effect of PAHs and UVR, other experiments did not resolve this question (Sargian et al., 2005; Sargian et al., 2007). In natural phytoplankton communities, Sargian and colleagues (2005) observed non-synergetic effects between the water-soluble fraction (WSF) of crude oil and UVR on subArctic communities, however, they observed synergetic effects between them in subAntarctic communities (2007). Our results showed, however, that different communities could show a different response to the joint action of PAHs and UVR. We observed that picophytoplankton showed a significant synergetic effect of the mixture of PAHs and UVR ($p<0.009$ and $p<0.02$, respectively) (Table 2.6), while the nanophytoplankton and the microphytoplankton showed an additive toxic action of UVR and the mixture of PAHs. Picoplankton was only present in the oligotrophic waters. Nanophytoplankton was found also mostly in oligotrophic waters, but their low abundance in the Arctic and Antarctic waters precluded the analysis. Microphytoplankton was exclusively in eutrophic waters. Cell volume has been identified previously as an important factor determining PAHs lethality to marine phytoplankton (Echeveste et al., 2010a), because small cells have a higher capability to incorporate these contaminants into their cells due to their higher surface to volume ratio, especially due to adsorption at the cell surface (Fan and Reinfelder, 2003). Escher and Hermens (2004) suggested that rather than the nominal concentration, as shown here, the internal concentrations relate exposure to effects. The dependence of LC50 or LC10 values on phytoplankton cell size could depend in fact on the internal

(membrane) concentrations, which will be higher, as per cell, for the small cells at a given nominal concentration. As phytoplankton cell size is associated with trophic level in the oceans (Agawin et al., 2000), oligotrophic regions may be more vulnerable to the joint action of PAHs and UVR.

Sargian and colleagues (2005, 2007) attributed the difference in their two experiments to the possibility that high concentrations of WSF could be strong enough to exceed the potential deleterious effects of UVR, however, others observed phototoxicity of single pollutants even at low concentrations (Petersen and Dahllöf, 2007; Petersen et al., 2008). Our results support the last observations, since our concentrations of PAHs, which were also very high in some of the treatments, did not mask the synergy between UVR and PAHs in the oligotrophic communities.

The levels of UVR received during experiments have not been deeply considered in previous works, but our results suggest that they may have major importance in determining the synergy or additive phototoxicity properties. Thus, experiments performed in the oligotrophic systems were exposed to higher natural UVR than the ones performed in the eutrophic waters. Moreover, oceanic oligotrophic regions receive permanent high UVR levels (e.g. subtropical Atlantic) or are able to receive them seasonally, e.g. summer time in the Mediterranean Sea (Llabrés et al., 2010), receiving also high PAR values that may suffice to induce phototoxicity in some PAHs compounds (Cody et al., 1984; Grote et al., 2005). Our results also suggest that rather than UVR doses, UV intensity may be important in determining the phototoxicity levels of PAHs, suggesting that there may be an irradiance threshold to pass from additive to synergetic effects. UVB doses received were similar for the experiments performed in the polar regions and the subtropical Atlantic Ocean, but the maximum UVI was much higher in the Atlantic. Doses in polar areas reached

values similar to those in the subtropical Atlantic due to the long daylight periods encountered at the polar latitudes sampled (i.e. 19 h and 23 in the Antarctic and Arctic cruises, respectively) although the UVB intensity was low. The maximum UVR values observed in the experiments performed in polar waters represented low values with respect to the maximum values attainable in those areas (e.g. Llabrés and Agustí, 2010) because those experiments were not run during episodes of ozone depletion occurring in spring and early summer in Antarctica and occasionally in the Arctic (Dahlback, 2002; Farman et al., 1985). UVR levels may explain differences in synergetic or the lack of synergy in previous studies (Sargian et al., 2005; Sargian et al., 2007).

4. Conclusions

Our results show that phytoplankton cell size was important in determining the sensitivity of marine phytoplankton to PAHs, been the smallest pico-cyanobacteria, *Prochlorococcus* and *Synechococcus*, the most sensitive. Our results show that synergy between PAHs and UVR, in natural communities, was observed for the smallest phytoplankton, showing as well that the synergetic joint action of UVR and the mixture of PAHs may be specially dramatic in the oligotrophic waters, as they are seawaters dominated by picophytoplankton. In the case of eutrophic regions such as the polar areas studied here, where microphytoplankton cells dominated the communities, an additive effect of the joint action of UVR and the mixture of PAHs rather than a synergy was observed, suggesting that larger cells may be less sensitive to the phototoxicity. The nanophytoplankton also showed higher sensitivity when UVR was present, but the result was not significantly synergetic, suggesting too an additive joint action of UVR and the mixture of PAHs and supporting the importance

of cell size in determining the degree of phototoxicity. The intensity of the joint action may also depend in the UVR levels received, suggesting that there may be an irradiance threshold to pass from additive to synergetic effects. In addition, experiments performed here for simple mixtures of PAH provide valuable information of the general trends of the factors governing the toxicity of organic pollutants to phytoplankton.

5. Acknowledgments

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Chapter 3

Decrease in the abundance and viability of oceanic phytoplankton due to trace levels of organic pollutants

by

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Chemosphere 81, 161–168. 2010

Abstract

Long range atmospheric transport and deposition is a significant introduction pathway of organic pollutants to remote oceanic regions, leading to their subsequent accumulation in marine organisms. Persistent organic pollutants (POPs) bioconcentrate in planktonic food webs and these exert a biogeochemical control on the regional and global cycling of POPs. Therefore, an important issue is to determine whether the anthropogenic chemical perturbation of the biosphere introduced by the myriad of organic pollutants present in seawater influences phytoplankton abundance and productivity. The results reported here from five sets of experiments performed in the NE Atlantic Ocean show that there is a toxic effect induced by trace levels of complex mixtures of organic pollutants on phytoplankton oceanic communities. The levels of single pollutant, such as phenanthrene and pyrene, at which lethality of phytoplankton is observed are high in comparison to field levels. Complex mixtures of organic pollutants, however, have an important toxic effect on phytoplankton abundances, viability and concentrations of Chlorophyll *a* at pollutant concentrations 20-40 folds those found in the open ocean. The toxicity of these complex mixtures of organic pollutants exceeds by 10^3 times the toxicity expected for a single pollutant. Therefore, our results point out the need for a systematic investigation of the influence of complex mixtures of organic hydrophobic pollutants to oceanic phytoplankton communities, a perturbation not accounted for on previous assessments of anthropogenic pressures in the marine environment.

1. Introduction

Persistent organic pollutants (POPs) reach remote oceans by long range atmospheric transport and the subsequent deposition. Once in the water column, hydrophobic organic pollutants will accumulate in planktonic organisms. It has been shown that planktonic food webs influence the oceanic cycle of POPs at regional and global scales (Dachs et al., 2002; Jurado and Dachs, 2008; Lohmann et al., 2007). Phytoplankton is the first step in aquatic food webs and plays an important role in the marine carbon cycle, but the influence of POPs on phytoplankton mediated processes is unknown. Therefore, an important issue that deserves attention is to determine whether the anthropogenic chemical perturbation of the biosphere introduced by the myriad of organic pollutants present in seawater influences phytoplankton abundance and productivity. The synthesis and use of hundreds of thousands of synthetic compounds in agriculture, industry and household applications (Muir and Howard, 2006) contribute to the perturbation of the chemical composition of the biosphere (Dachs and Méjanelle, 2010; Lohmann et al., 2007). Among these, several families of organic compounds have been identified as having an adverse effect on ecosystems and humans, such as persistent organic pollutant (POPs), herbicides, etc. The cocktail of organic pollutants present in oceanic waters is conformed by a large number of compounds (Dachs and Méjanelle, 2010), even though an inventory of these is lacking, there are at least thousands of them (Dachs and Méjanelle, 2010; Lohmann et al., 2007; Muir and Howard, 2006). These chemicals originally entered the environment through emissions to soils, water, air or being the result of degradation processes of other anthropogenic chemicals. Hydrophobic, persistent, and semivolatile organic chemicals have potential for long range atmospheric transport and are eventually deposited to remote oceanic regions after undergoing successive

volatilization and deposition steps around the biosphere (Jurado and Dachs, 2008; Lohmann et al., 2007). The impact of these inputs of pollutants on oceanic phytoplankton is unknown. Indeed, the toxic effect of some herbicides on phytoplankton in coastal environments has been assessed previously (Faust et al., 2003; Magnusson et al., 2008; Petersen et al., 2008), but only recently the influence of single organic pollutants on oceanic phytoplankton populations has received some attention (Echeveste et al., 2010). In spite of this scarcity of studies of the impact of organic pollutants on phytoplankton, it has been recognized as an important issue (Echeveste et al., 2010; Faust et al., 2003; Jamers et al., 2009; Magnusson et al., 2008; Petersen et al., 2008). These previous studies have shown that some individual organic pollutants may interfere with the photosynthetic processes of algae, and that toxicity thresholds are cell-size dependent. However the understanding of the influence of complex mixture of POPs on phytoplankton abundance is lacking. The total inventory of organic pollutants in marine waters is far from complete, in part because we lack appropriate analytical methods to identify most of the myriad of anthropogenic organic compounds (Lohmann et al., 2007; Muir and Howard, 2006), rendering the assessment of their toxicity elusive.

In order to fill in this gap of knowledge, the objective of this study was to elucidate the potential toxic effects of complex mixtures of organic pollutants occurring in seawater to oceanic phytoplankton, by analyzing changes on the abundance and viability of these populations, and by testing the importance of synergetic/additive effects of the myriads of organic pollutants present in seawater as compared with the toxic effects caused by single pollutants or simple mixtures of pollutants. The study of the effects of mixtures of pollutants has been possible by

exposing natural phytoplankton populations to fractions of extracts of seawater.

2. Materials and Methods

2.1. Experimental framework

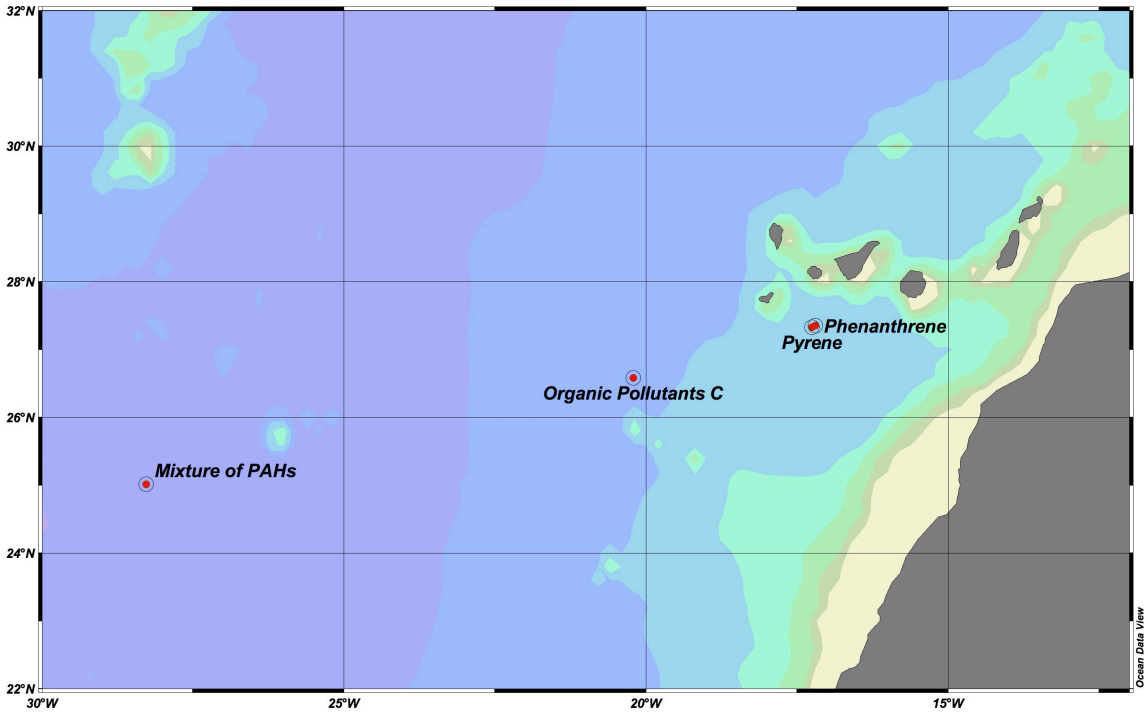

Figure 3.1. Map showing the location where seawater was sampled for the incubation experiments during the RODA cruises in the NE Atlantic Ocean.

A set of experiments to analyze the lethal thresholds of complex mixtures of organic pollutants on natural communities of phytoplankton was performed during the oceanographic cruise RODA-II. The cruise was performed on board the RV Hespérides in the subtropical Atlantic Ocean, from January 31 to March 1, 2007 (Figure 3.1 and Table 3.1).

Contaminant	Campaign	Coordinates	Date	Chlorophyll *a* (mg m^{-3})
Pyrene	RODA-I	27º 21' N - 17º 11' W	08/30/2006	0.44
Phenanthrene		27º 19' N - 15º 25' W	08/31/2006	0.1
Mixture of PAHs	RODA-II	25º 0' N - 28º 27' W	02/21/2007	0.28
Cocktail of Organic Compounds		26º 58' N - 20º 21' W	02/25/2007	0.33

Table 3.1. Position of the stations in the North Atlantic where the natural phytoplankton communities were sampled, the date of sampling, indicating the abundance of phytoplankton (as Chlorophyll *a* concentration) found at each station, and different degree of complexity in the pollutants tested. Experiments with single Pyrene and Phenantrene are described in Echeveste et al. 2010.

The cocktails of organic pollutants in seawater were obtained by concentrating 100 L of seawater on board of the R/V Hespérides using the continuous seawater pumping system during the RODA-I cruise, performed in the same area of the North Atlantic Ocean in August 2006, six months before the RODA-II cruise. The seawater from 5 m depth was filtered on pre-baked GFF filters (Whatmann) and went through a XAD-2 adsorbent for concentration of the hydrophobic organic pollutants as done routinely in analysis of POPs in oceanic conditions (Dachs and Bayona, 1997; Gioia et al., 2008; Nizzetto et al., 2008). The XAD-2 column was kept at 4 ºC until it was extracted in the laboratory following the method described elsewhere (Dachs and Bayona, 1997). The extract was clean up on an alumina 3% deactivated column and three fractions were obtained. The first one was eluted with hexane, the second one with a mixture of hexane-dichloride-methane, and the third one with a mixture of dichloride-methane/ methanol (2:1). After analysis of PCBs and PAHs in the first and second fractions by Gas chromatography coupled to an Electron Capture Detector (GC-ECD) and gas chromatography coupled to mass spectrometry (GC-MS), respectively, the first and second fractions were merged and solvent transferred to

acetone and concentrated to 100 μL. These 2 fractions represented the non-polar fraction, containing most of the chemicals traditionally assumed to be persistent organic pollutants (POP) such as PCBs, PAH, lindane, hexachlorbenzene and others. The third fraction, representing the polar fraction containing organic pollutants with alcohol, ketone and some acid functional groups, was also kept in acetone in the freezer until its use in the RODA-2 campaign for the incubation experiments with phytoplankton. Moreover, field blanks were processed in parallel to the samples, being the field blanks lower than 1% of the measured field concentrations of PAHs and other compounds. In addition, both GC-MS and GC-ECD blank chromatograms (and fragmentograms) were "clean", thus suggesting that the XAD-2 adsorvent used is not a source of contamination in the incubation experiments performed to test the toxicity of organic pollutants to phytoplankton populations. Controls were done with seawater (same depth than experiments) since artificial seawater would contain a higher pollutant load from the MilliQ water (which can be more polluted than open ocean seawater).

In addition, the lethality induced by a "simple" mixture of 16 PAHs was also tested in similar experiments run during the same RODA-2 cruise (Table 3.1). The mixture of PAHs was composed by acenaphtene, acenaphthylene, anthracene, benzo[a]anthracene, benzo[b]fluoranthene, benzo(k)fluoranthene, benzo[ghi]perylene, benzo(a)pyrene, dibenzo[a,h]anthracene, chrysene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, pyrene and phenanthrene in acetone. All PAH in this mixture were present at the same concentration.

Surface seawater (5 m depth) for the experiments carried out in RODA-II campaign was sampled using Niskin bottles attached to a rosette-CTD system and dispensed onto 100 ml acid clean glass bottles. Before the experiments were run,

acetone containing either the mixture of PAHs or the extracts of seawater was let in the bottles to evaporate for 3 hours in order to avoid the presence of the solvent acetone in the treatments. The lethality of both the mixture of PAHs and the cocktail of polar or non-polar organic compounds was tested in samples (seawater) receiving different concentrations. The mixture of PAHs was added at three concentrations; representing 1350, 23400 and 131450 times the concentrations of the PAHs in natural waters from the region where seawater for experiments was collected. These enrichment factors are estimated from the concentrations added in the incubations than the seawater concentrations measured during the RODA-2 cruise, This enrichment factor has a higher influence of the high MW PAHs since all PAH were equally enriched in the incubations (limited by at the more concentrated experiments by the chemical solubility) but high MW PAHs are less abundant in natural seawater. Conversely, the complex mixture (fraction of extract containing non polar and polar organic compounds) was added at concentrations representing 10, 100 and 890 times the concentrations of the organic pollutants present in natural seawater. Besides, for all the experiments duplicated bottles containing sampled seawater without any addition were run as controls. The experimental bottles were incubated on deck under natural solar radiation in a tank with seawater surface running system to keep *in-situ* temperature conditions. Bottles were covered with a neutral net to simulate 5 meters light conditions. Daily sampling was performed in the experiments for 3 days for the experiment with the cocktail of organic pollutants, and 4 days for the experiment with the mixture of PAHs.

2.2. Phytoplankton analysis

The effect of the different pollutant mixtures (simple PAH mixtures versus

concentrated extracts of seawater) on the abundance of the different groups forming the phytoplankton communities (*Prochlorococcus* sp., *Synechococcus* sp. and Eukaryotic picophytoplankton) were analyzed by using flow cytometry counting methods in a FACSCalibur flow Cytometer (Becton Dickinson). An aliquot of a calibrated solution of 1 μm diameter fluorescent beads (Polysciences Inc.) was added to the samples as an internal standard for the quantification of cell concentration. The red, green and orange fluorescence, and forward and side scattering signals of the cells and beads were used to detect different populations and to differentiate them from the fluorescent beads (Marie et al., 1999). The proportion of living and dead cells in the picophytoplankton communities along the experiments were also followed by applying a cell membrane permeability test, the cell digestion assay, which allows the counting and identification of living phytoplankton cells (Agustí and Sánchez, 2002). The cell digestion assay was applied to replicate samples, by adding 200 μl of DNAse I solution (400 μg ml^{-1} in HBSS (Hanks' Balanced Salts)) to 1 ml sample of each treatment, followed by 15 minutes incubation at 35°C in a Digital Dry Bath. After this time, 200 μl of Trypsin solution (1% in HBSS) were added, followed by 30 minutes incubation at 35°C. At the end of this time, samples were kept in cold conditions in order to stop the cell digestion process. Flow cytometric methods were also used for counting living cells using a 1 ml samples in a FACSCalibur Flow Cytometer (Becton Dickinson) installed on board the research vessel Hespérides.

2.3. Chlorophyll *a* estimation

For the estimation of Chlorophyll *a* concentration, 50 ml samples were filtered onto 25 mm diameter Whatmann GF/F filters from each bottle on the day 0 and last day (day 3 or 4 according to the experiment). After filtration, filters were placed in

tubes with 90% acetone for 24 hours for the extraction of the pigment. Then, the fluorescence of the Chlorophyll *a* was measured in a Shimadzu RF-5301 PC spectrofluorimeter and calibrated with pure Chlorophyll *a* as described in Parsons et al. (1984).

2.4. LC50 and LC10 estimation

The 50% lethal thresholds (LC50) and the 10% lethal thresholds (LC10), which are the contaminant concentration at which the different cell populations declined by a 50% or by a 10%, respectively, were calculated applying the equation

$$LC50 = -\ln 0.5 / \Omega \quad \text{(eq. 3.1)}$$

$$LC10 = -\ln 0.9 / \Omega \quad \text{(eq. 3.2)}$$

where Ω is the slope of the relationship between the decay ln of cell abundance and the relative contaminant concentration ($C/C_{Control}$) reached at the end of the experimental treatments.

3. Results and Discussion

3.1. Organic pollutants in the NE subtropical Atlantic

The concentrations of persistent non-polar organic pollutants such as polychlorinated biphenyls (PCB) measured in the Atlantic Ocean during these sampling cruises were on average of 0.5 pg L^{-1} and 0.4 pg L^{-1} for congeners PCB 52 and PCB 180, respectively (Figure 3.2). Concentrations of polycyclic aromatic hydrocarbons (PAH) were higher, and averaged 720 pg L^{-1} and 1100 pg L^{-1} for pyrene and phenanthrene, respectively (Figure 3.2). These levels are comparable to PCB and PAH concentrations reported by Gioia et al. (2008) and Nizzeto et al. (2008) for this

oceanic region. These concentrations can be viewed as representative of oceanic regions, even though it is well known that levels of organic pollutants in this region off-Africa in the NE Atlantic are higher than other more remote oceanic regions. However, the levels in the NE subtropical Atlantic are much lower than those described for coastal regions such as in the NE Mediterranean sea (Garcia-Flor et al., 2009), the NW Atlantic coastal ocean and in estuaries (Dachs and Méjanelle, 2010; Yan et al., 2008).

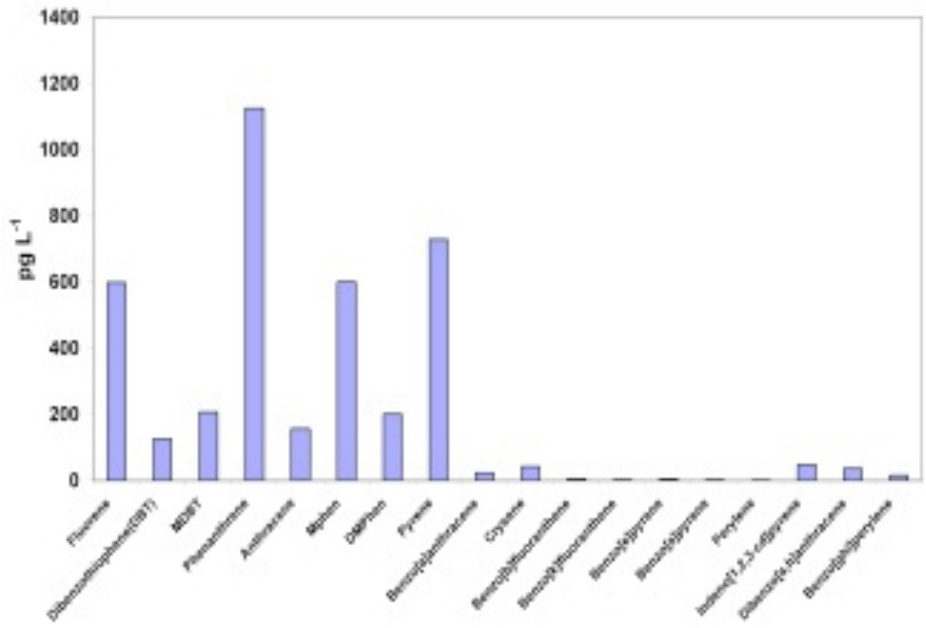

Figure 3.2. Occurrence of persistent organic pollutants (PCBs and PAH) in the NE Atlantic Ocean during the RODA cruises. Upper panel shows the average PCB concentrations and lower panel shows the average PAH concentrations in the dissolved phase at 5 m depth.

3.2. Phytoplankton response to increased concentrations of organic pollutants

Picophytoplankton, represented by *Prochlorococcus* sp., *Synechococcus* sp. and small eukaryotes, dominated the phytoplankton communities in the North East Atlantic Ocean, characterized by low Chlorophyll *a* concentration waters (Table 3.1). The experiments performed with a mix of PAHs at different concentrations, showed that phytoplankton communities were strongly affected by the presence of high

concentrations of PAHs, and all the populations declined as organic pollutants concentrations in the treatments increased. The decline with increasing pollutants concentration was observed in the individual counts of the different populations forming the communities (Fig. 3.3) and when considering total community, quantified as Chlorophyll *a* concentration (Fig. 3.4).

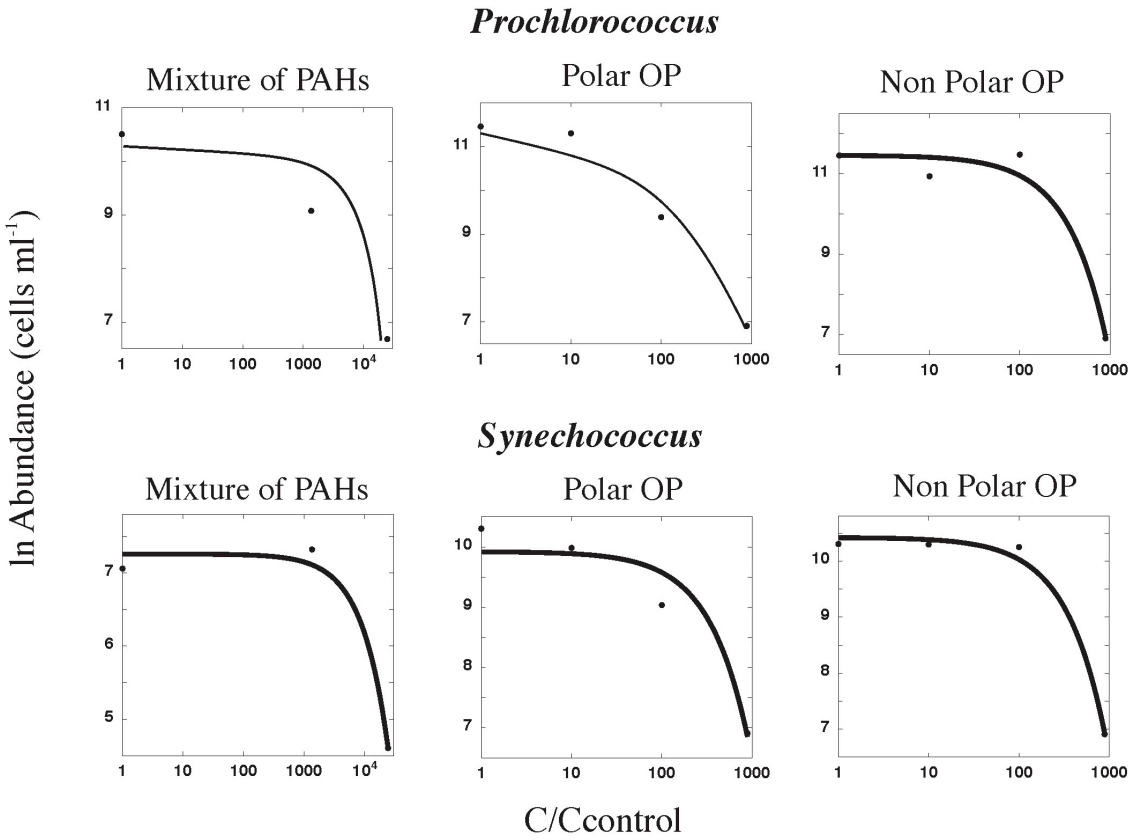

Figure 3.3. Decrease in the cell abundance (cells ml^{-1}) at the end of the experiments of *Prochlorococcus* sp. and *Synechococcus* sp., with the increase in the concentrations of the mixture of 16 PAHs and the cocktail of non-polar organic contaminants and the cocktail of polar organic contaminants, all relative to concentrations present in water. The lines represent the lowess fit. (Non Polar OP) Non Polar Organic Pollutants; (Polar OP) Polar Organic Pollutants.

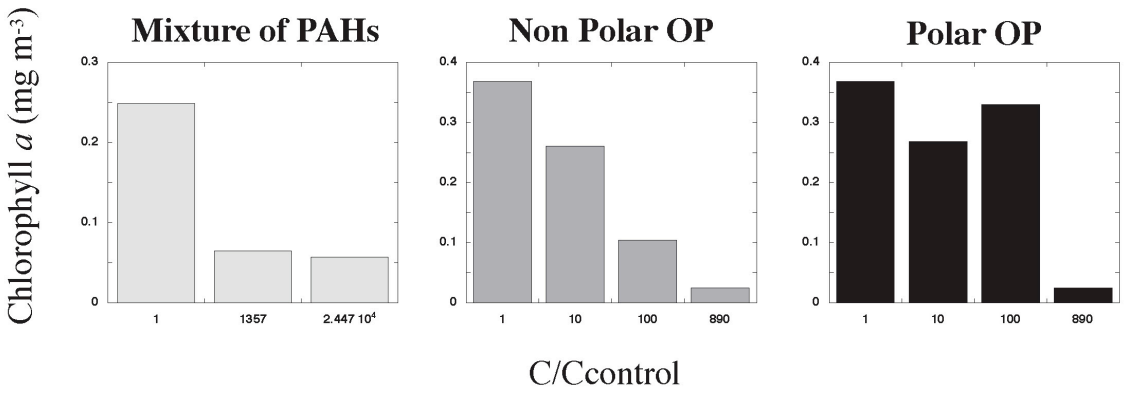

Figure 3.4. Chlorophyll *a* concentrations at the end of the incubations for treatments with different concentrations of the mixture of 16 PAHs and the cocktail of non-polar organic contaminants and the cocktail of polar organic contaminants. Concentrations of pollutants in treatments (x-axys) are shown relative to the concentration in the natural seawater what was used as control. (Non Polar OP) Non Polar Organic Pollutants; (Polar OP) Polar Organic Pollutants.

The same trends were observed with the seawater extract fractions containing the complex mixtures of non-polar and polar organic pollutants. The abundance of *Prochlorococcus* sp. and *Synechococcus* sp decreased when increasing the concentration of the complex mixture of chemicals, for both the polar and non-polar fractions (Fig. 3.5). The same trend is observed for Chlorophyll *a*, a surrogate of total phytoplankton community. Indeed, results show a significant negative correlation between Chlorophyll *a* concentration and the enrichment of non-polar (Chl = -0.052 Ln(C/CControl) + 0.37, $r^2 = 0.99$) and polar (Chl = -0.042Ln(C/CControl) + 0.39, $r^2 = 0.65$) organic compounds.

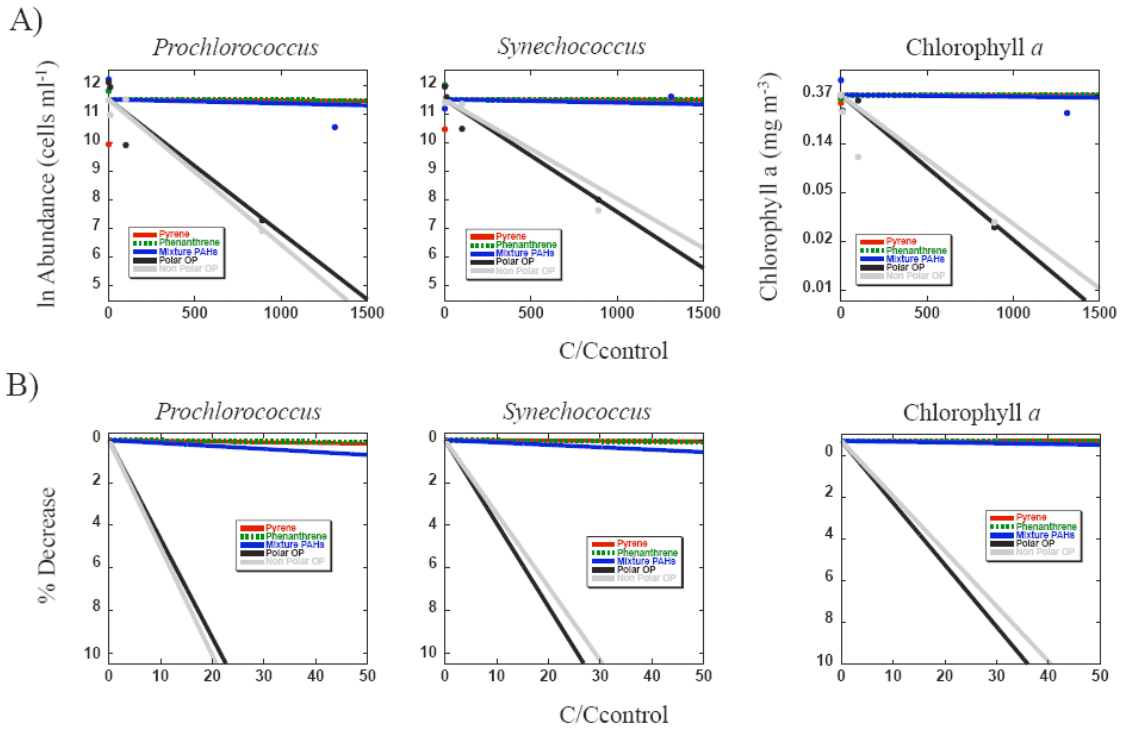

Figure 3.5. Decrease in the phytoplankton biomass with increasing pollutants concentrations (C/Ccontrol). Panel A) Decrease of *Prochlorococcus* sp. and *Synechococcus* sp. abundance (cells ml^{-1}) and Chlorophyll *a* concentration with increasing pollutants concentrations (C/Ccontrol). Panel B) Percentage decrease of *Prochlorococcus* sp., *Synechococcus* sp. and Chlorophyll *a* concentration with increasing pollutants concentrations (C/Ccontrol). (Proc_Pyr) *Prochlorococcus*_Pyrene; (Proc_Phen) *Prochlorococcus*_Phenanthrene; (Proc_Mix) *Prochlorococcus*_Mixture of PAHs; (Proc_Non P) *Prochlorococcus*_Non Polar Organic Pollutants; (Proc_Pol) *Prochlorococcus*_Polar Organic Pollutants; (Syn_Pyr) *Synechococcus*_Pyrene; (Syn_Phen) *Synechococcus*_Phenanthrene; (Syn_Mix) *Synechococcus*_Mixture of PAHs; (Syn_Non P) *Synechococcus*_Non Polar Organic Pollutants; (Syn_Pol) *Synechococcus*_Polar Organic Pollutants; (Chlor_Pyr) Chlorophyll *a*_Pyrene; (Chlor _Phen) Chlorophyll *a*_Phenanthrene; (Chlor _Mix) Chlorophyll *a*_Mixture of PAHs; (Chlor _Non P) Chlorophyll *a*_Non Polar Organic Pollutants; (Chlor _Pol) Chlorophyll *a*_Polar Organic Pollutants. Cell abundance and Chlorophyll *a* decreases of phenanthrene and pyrene are taken from Echeveste et al. 2010.

The observed decay rates showed a higher decay when the complexity of the contaminant added increased (Table 3.2). The addition of single pollutants resulted in the lowest decay rates ($3x10^{-6}$ and $4.94\ x10^{-6}$ for the Chlorophyll *a* for pyrene and phenanthrene, respectively). Higher decay rates were measured for the mixture of

PAHs ($3.60x10^{-5}$) and the highest decay rates were observed for the non-polar and the polar mixtures of organic pollutants ($2.64x10^{-3}$ and $2.97x10^{-3}$, respectively) (Fig. 3.5).

	Prochlorococcus	***Synechococcus***	**Chlorophyll *a***
Pyrene	$3.34\ 10^{-05}$	$1.22\ 10^{-05}$	$3.00\ 10^{-06}$
Phenanthrene	$2.10\ 10^{-05}$	$1.80\ 10^{-05}$	$4.94\ 10^{-06}$
Mix PAHs	$1.39\ 10^{-04}$	$1.13\ 10^{-04}$	$3.60\ 10^{-05}$
Polar Organic Pollutants	$4.65\ 10^{-03}$	$3.45\ 10^{-03}$	$2.97\ 10^{-03}$
Non Polar Organic Pollutants	$5.06\ 10^{-03}$	$3.92\ 10^{-03}$	$2.64\ 10^{-03}$

Table 3.2. Decay rates obtained for Atlantic *Synechococcus* sp., *Prochlorococcus* sp. and total phytoplankton, as Chlorophyll *a*, for the treatments with the cocktail of concentrated non-polar and polar organic compounds, and for simple mixtures of polycyclic aromatic hydrocarbons (PAHs). The decay rates of phenanthrene and pyrene are taken from Echeveste et al. 2010.

Indeed, there is a toxic impact on phytoplankton at only few times the natural concentrations of mixtures of organic pollutants (Fig. 3.3-3.5). The relative concentration ($C/C_{Control}$) at which the Chlorophyll *a* is reduced by 10% (here indicated as LC10), which would represent a significant impact in terms of oceanic carbon fluxes mediated by phytoplankton, is of 36±8 and 40±22 for the non-polar and polar organic compounds, respectively. As far *Prochlorococcus* sp. and *Synechococcus* sp. populations are concerned, the 10% decrease of their abundance was obtained at concentrations of 21±6 and 27±3 folds the control (oceanic) levels of non-polar organic compounds and at concentrations of 23±13 and 31±12 fold the control levels of polar organic compounds (Table 3.3, Figure 3.5).

	LC50 (C/Ccontrol)			LC10 (C/Ccontrol)		
	***P.* sp**	***S.* sp**	**Chloro-phyll *a***	***P.* sp**	***S.* sp**	**Chloro-phyll *a***
Non-polar organic compounds	137 ± 37	177 ± 23	263 ± 148	21 ± 6	27 ± 3	40 ± 22
Polar organic compounds	149 ± 84	201 ± 77	234 ± 52	23 ± 13	31 ± 12	36 ± 8
Mixtures of PAHs	4987 ± 3866	6134 ± 1723	19254 ± 53233	660 ± 580	810 ± 260	2450 ± 8090
Phenan-threne	33007 ± 8044	38508 ± 9404	140427 ± 46366	5017 ± 1223	5853 ± 1429	21345 ± 7090
Pyrene	20759 ± 9927	56629 ± 14072	231049 ± 108083	3155 ± 1509	8608 ± 2139	35120 ± 16429

Table 3.3. Thresholds levels for 50% and 10% reduction in abundance (LC50 and LC10, respectively) expressed as relative enrichment to the field oceanic values used as control ($C/C_{Control}$), obtained for Atlantic *Synechococcus* sp. (*S.* sp), *Prochlorococcus* sp (*P.* sp). and total phytoplankton, as Chlorophyll *a*, for the treatments with the cocktail of concentrated non-polar and polar organic compounds, and for simple mixtures of polycyclic aromatic hydrocarbons (PAH). Phenanthrene and pyrene field oceanic dissolved concentrations were 1100 and 720 pg L^{-1}, respectively. The total concentration of non-polar and polar compounds in oceanic waters is obviously unknown. The data of thresholds levels calculated for additions of single compounds as phenanthrene and pyrene are taken from Echeveste et al. 2010.

Most part of toxicity studies of organic pollutants mixtures on marine phytoplankton have been focused on pesticides and herbicides (i.e. Arrhenius et al., 2004; DeLorenzo et al., 2003; Faust et al., 2003; Fernández-Alba et al., 2002; Magnusson et al., 2008; Walter et al., 2002), but few studies have been performed with natural communities (Arrhenius et al., 2004; Echeveste et al., 2010), and besides, none of them have analyzed lethal levels of PAH mixtures or real complex mixtures of organic pollutants to phytoplankton. Our study showed, for the first time, the lethal effects of mixtures of organic pollutants on field marine phytoplankton, including the natural mixture found in the Atlantic Ocean, indicating that mixtures of organic

pollutants presently found in the ocean are influencing and impacting the marine carbon cycle in open waters of the Atlantic Ocean, by reducing phytoplankton biomass and production, and by inducing cell mortality i.e. influencing the fate of oceanic primary production.

Experiments done during the same sampling cruises inoculating natural seawater with varying amounts of pyrene or phenanthrene (Echeveste et al., 2010) indicated that individual pollutants induced a 10% reduction to *Prochlorococcus* sp. and *Synechococcus* sp. at the very high concentrations of 2 μg L^{-1} and 6 μg L^{-1} of pyrene, respectively, much larger than the measured dissolved phase concentrations in the NE Atlantic (Figure 3.2). However, these pyrene concentrations are consistent with those described by Petersen (2008) at which a decrease in Chlorophyll *a* of coastal benthic microalgae is observed. In the NE Atlantic, the 10% reduction due to phenanthrene was observed at a concentration of 6 μg L^{-1}. Total phytoplankton community (measured as Chlorophyll *a* concentration), needed as well large concentrations of phenanthrene and pyrene to decrease (Table 3.3). These phenanthrene and pyrene concentrations exceeded 21000 and 35000 times the oceanic measured concentrations (Figure 3.3, Table 3.3). The experiments performed with the mixture of PAHs showed an effect on phytoplankton abundance (Table 3.3) at 660 and 810 fold the natural levels of these mixtures in natural waters, thus at a concentration higher than for complex mixtures but lower than single compounds (Table 3.3).

Therefore, the toxic effect of individual organic pollutants, such as phenanthrene or pyrene, to oceanic phytoplankton is not significant at natural levels. Conversely, the complex mixtures of organic pollutants, especially those with chemical properties similar to those of POPs, have a measurable impact on

phytoplankton communities at concentrations similar (factor of 20-40) to those found in the oceanic environment. Furthermore, this work shows, for the first time, that the levels of pollution reaching oceanic waters are becoming close to the levels required to significantly affect oceanic phytoplankton, due to the complex cocktail formed by a mixture of thousands of organic anthropogenic compounds. There are a limited number of modes of action of pollutants on phytoplankton (Cedergreen et al., 2008; Faust et al., 2003; Nizzeto et al., 2008) while there are thousands of different pollutants in oceanic waters. Therefore, the joint effect of these complex mixtures follow primarily the joint phytoplankton toxicity of concentration addition instead of independent action (Cedergreen et al., 2008; Nizzeto et al., 2008), where a large number of chemicals at low concentrations induce an effect equivalent to a single chemical at high concentration. This is reasonable since the cell abundance and viability, parameters assessed here, are integral toxicity endpoints and there are presumably many pollutants in oceanic waters with the potential to induce this toxicological response independently of the mechanism of action.

Echeveste et al. (2010) has shown that the toxicity thresholds decrease for smaller phytoplankton species, as sensitivity increase with decreasing cell size. Therefore, the higher concentrations needed to decrease the Chlorophyll *a* concentrations in comparison to *Prochlorococcus* sp. and *Synechococcus* sp. is due to the higher resistance of larger cells that are also forming the community and contributing to the overall phytoplankton Chlorophyll *a*. Escher and Hermens (2002, 2004) have reviewed the issue of internal concentrations driving toxicological effects. The concentrations of hydrophobic organic chemicals at the cell membrane per cell or phytoplankton biomass increase for smaller cells due to higher surface/volume ratios (Del Vento and Dachs, 2002), then for a given dissolved concentrations, the internal

membrane exposure concentration, specially due to surface adsorption, can be higher for smaller cells. If this is so, then the higher sensitivity of *Prochlorococcus* sp. and *Synechococcus* sp could be due to the fact than the internal exposure concentration inducing lethal effects is observed at lower dissolved phase concentrations.

According to the funnel hypothesis (Warne and Hawker, 1995), the simple mixture of PAHs may have an additive behavior, as equitoxic mixtures of narcotic toxicants tend to approach toxic additivity (i.e., they become less synergistic or antagonistic) as the number of components increases. In the case of the simple mixtures of PAHs tested here, even though pyrene and phenanthrene show similar toxicities, high MW PAHs (BaPyrene and others) may be more toxic to phytoplankton. If this was true, then the overall toxicity of the mixture of PAHs would have a higher weight from the high MW PAHs, but this will need of experimental evidence, not provided here, since Del Vento & Dachs (2002) have shown that phytoplankton cell membrane permeability is a limiting factor for the uptake of high MW organic compounds. In any case, the issue that deserves further attention is whether complex natural organic pollutants have an additive, a synergetic or an antagonistic behavior on phytoplankton. As observed in our results, the presence of more contaminants on the mixture would imply that each contaminant may not need to be as concentrated as when acting alone, suggesting an additive effect, even though synergetic and antagonistic effects will be happening for such a large set of chemicals, even though the overall effect can cancel resulting in a simple additive effect. To resolve this, we would require to know the composition and toxicity of pollutants presently found in nature, which is not available. From Table 3.2, we estimated that it would be necessary between 250 to 1000 compounds with similar toxicities of pyrene and phenanthrene to reach the observed effect of complex

mixtures to phytoplankton population (LC10) due to additive effect. Presumably, there are organic pollutants high higher equivalent toxicities than these two PAHs, this would result in a total number of organic compounds in seawater lower than that indicated above. However, considering that pyrene and phenanthrene are two pollutants at considerably high concentrations in the environment in comparison to other pollutants, such as PCBs and other POPs, then the number of pollutants found in the oceanic environment could also be of several thousands. Indeed, in coastal waters, thousands of organic pollutants have been reported (Dachs and Méjanelle, 2010), and many more are potentially present in the marine environment. It is also possible that there is, to some extend, some antagonistic and synergistic effect among chemicals found in the environment. This assumption would be in consonance to the funnel hypothesis (Warne and Hawker, 1995), which concludes that the toxicity of mixtures measured using biological endpoints that require high toxicant concentrations (as in this case the LC50s), deviates more from toxic additivity than endpoints that require low concentrations. However, in this case, this would be compensated by the large number of chemicals thus with a potential scenario of direct addition (Warne and Hawker, 1995). As the exact number and concentration of organic pollutants in nature cannot be measured due to the lack of appropriate analytical methods, we only can speculate about whether all organic pollutants present in nature are acting additively, synergistically or antagonistically. The inventory of marine, and environmental, pollutants is now a research priority together with the understanding of their cycling and impacts.

In addition to changes in the abundance of the populations of *Prochlorococcus* sp. and *Synechococcus* sp., we also observed that the number of alive and dead cells counted following the methodology described elsewhere (Agustí and Sánchez, 2002),

has been altered when the populations were exposed to the natural cocktail of organic pollutants. The results showed that the percentage of dead cells of both *Prochlorococcus* sp. and *Synechococcus* sp. increased when the concentrations of cocktails of polar and nonpolar organic compounds increased (Figure 3.6). The induction of cell death was significant, representing and increase up to 15% in dead cells (Figure 3.6), and was detected at concentrations of organic pollutants below those resulting in a significant decrease (10%) in the total population abundance.

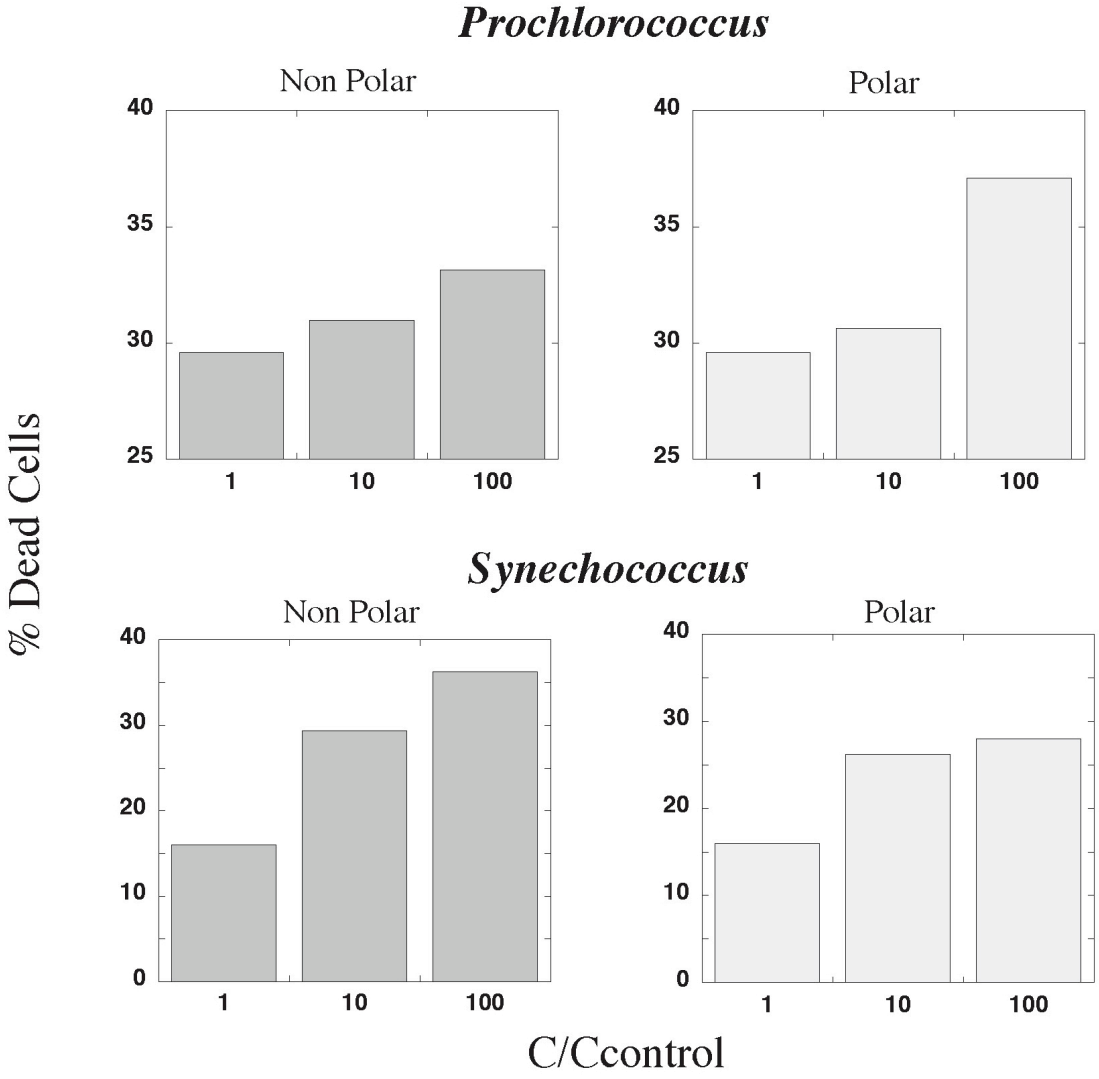

Figure 3.6. Number of dead cells, given as percentage of total cell abundance, for Atlantic *Prochlorococcus* sp. and *Synechococcus* sp. after three days of experiment with complex mixtures of non-polar and polar organic compounds. The percentage of dead cells increased during the treatments for both species. (Non Polar) Non Polar Organic Pollutants; (Polar) Polar Organic Pollutants.

3.3 Implications of the influence of organic pollutants on oceanic phytoplankton and research needs

So far, the marine carbon cycle has been studied without accounting for the influence and impact of the regional and global dynamics of organic pollutants (Sarmiento and Gruber, 2006). Even though the results from the *in-vitro* experiments can not be extrapolated directly in terms of ecological implications, this work shows that a coupling between the anthropogenic perturbations of the biosphere by the chemical anthropogenic "universe", and the carbon fluxes mediated by phytoplankton is feasible, even though much research will be needed on the influence of pollutants on phytoplankton productivity and bacterial respiration before its influence on carbon fluxes can be delimitated. The estimated oceanic gross primary productivity carbon flux (del Giorgio and Duarte, 2002) is of the order of 59 Gt C y^{-1}. Any factor affecting this flux by 10% would imply a perturbation of the order of the anthropogenic inputs of carbon, while the atmosphere ocean exchange of CO_2 is lower and in the order of 2.2 Gt C y^{-1} (Takahashi et al., 2002). Moreover, by contributing to phytoplankton losses by cell death, organic pollutants are influencing the fate of the carbon incorporated by phytoplankton in the photosynthesis, and reducing the capacity of the oceanic biological pump (Kirchman 1999). Therefore, even if complex mixtures of organic pollutants induce a perturbation on phytoplankton mediated carbon fluxes of only 1%, a value ten fold lower than the thresholds limits discussed in this study, the impact of organic pollutants on oceanic phytoplankton would be very important for understanding the regional and global carbon cycle and how humans are affecting it.

The oceanic high productivity regions are those receiving higher inputs of pollutants from atmospheric deposition (Dachs et al., 2002, Jurado et al., 2005), thus with presumable a close coupling between the carbon and pollutant cycles. In coastal

regions, where pollutant concentrations can be orders of magnitude higher than in open ocean, a significant coupling of the biogeochemical cycle of pollutants and the marine carbon cycle may be occurring. Further research is needed to delimit the consequences of organic pollutants on the marine carbon fluxes and global change, as mediated by phytoplankton and bacteria, and characterize the wide range of anthropogenic chemical perturbations in the biosphere.

4. Conclusions

The results reported here from five sets of experiments performed in the NE Atlantic show that there is a toxic effect induced by trace levels of complex mixtures of organic pollutants on phytoplankton oceanic communities. The levels of single pollutant, such as phenanthrene and pyrene, at which lethality of phytoplankton is observed are high in comparison to field levels. Complex mixtures of organic pollutants, however, have an important toxic effect on phytoplankton abundances, viability and concentrations of Chlorophyll *a* at pollutant concentrations 20-40 folds those found in the open ocean. The toxicity of these complex mixtures of organic pollutants exceeds by 10^3 times the toxicity expected for a single pollutant. Therefore, our results point out the need for a systematic investigation of the influence of complex mixtures of organic hydrophobic pollutants to oceanic phytoplankton communities, a perturbation not accounted for on previous assessments of anthropogenic pressures in the marine environment and that will need a interdisciplinary research effort during the next decade.

5. Acknowledgments

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Chapter 4

Toxic thresholds of cadmium and lead to oceanic phytoplankton: cell size and ocean basins dependent effects

by

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Abstract

Toxic and nutritive thresholds of cadmium and lead to natural oceanic phytoplankton were examined in experiments with communities from Mediterranean and Black Seas and North East Atlantic Ocean. At concentrations of Cd added below 0.11 μg L^{-1}, picocyanobacteria showed some positive responses, as observed by decreases in the percentage of death cells in the populations. At higher concentrations, for all phytoplankton populations, cell abundances and growth rates decreased with increasing addition of Cd and Pb. The lethal concentrations at which populations decreased by a half (LC50s), ranged from 0.23 to 498.7 μg L^{-1} Cd for Atlantic *Prochlorococcus* and Black Sea picoeukaryotes, respectively, and from 20 to 465.2 μg L^{-1} Pb for Mediterranean *Synechococcus* and Black Sea nanoplankton, respectively. This lethal concentrations were significantly lower than those previously reported for phytoplankton cultures. LC50s were strongly related to population cell size, increasing as cell size increased, indicating that oceanic *Prochlorococcus* and *Synechococcus* populations were the most sensitive, and the largest phytoplankton groups the most resistant. Based on this relationship, differences in the sensitivity to Cd across systems were detected, with Black Sea phytoplankton community been more resistant (up to 100 times) than similar sized phytoplankton of the Mediterranean Sea and Atlantic Ocean. Despite the similar metal concentrations across basins, the recurrent inputs of Cd and other metals may have selected more resistant populations in the Black Sea.

1. Introduction

Trace metals in the oceans can act as nutrients (e. g. Fe, Zn, Co, Mo and Ni), been essential for the growth of cells, and as toxicants, mainly due to their ability to denature protein molecules (e.g. Hg, Ag, Pb, Sn, Cr). Toxicity is often related to their capacity to displace nutrient metals from their metabolic sites, as they can enter into cells through the same transport systems of nutrient metals (Sunda and Huntsman 1998; Bruland et al. 1991). Moreover, some of the trace metals that act as nutrients can be toxic at high concentrations, such as Zn, Cu and Ni (Wong and Chang 1991). In oceanic areas, besides of the introduction of metals by biogenic agents (Cattell and Scott 1978), the submarine volcanic or hydrothermal activities (e.g. Klinkhammer et al. 2001) and mainly the deposition of aerially transported elements may represent the way of entrance of trace metals (Duce et al. 1991; Guieu et al. 1991; Paytan et al. 2009).

Among all the trace metals in oceans, two of the most toxic are Cd and Pb (e.g. Sorentino 1979; Hollibaugh et al. 1980; Rai et al. 1981). Despite of Cd is, together with Hg, one of the most toxic metals (e.g. Sorentino 1979; Rai et al. 1981), at very low concentrations it can act too as nutrient, due to its physical and chemical similarities with other metals like Zinc (Price and Morel 1990). However, at high concentrations it may block passive or active entrance of other nutrients essential for growth of phytoplankton (Gipps and Coller 1980; Brand et al. 1986). Combustion of fuels, particularly coal, represents the main source of anthropogenic emissions of metals such as Cd, which arrives to open oceans by atmospheric deposition (e.g. Duce et al. 1991). On the other hand, the main source of Pb is the combustion of gasoline, arriving to oceans by direct spillage from ships or by continuous atmospheric deposition (e.g. Duce et al. 1991). Lead has only been described as toxic for

microalgae, with no description of been acting as nutrient (e.g. Hessler 1974).

Shaw (1990) made a complete review describing the toxic effects of metals on phytoplankton such as reduction of photosynthetic electron transport and photosynthetic carbon fixation, inhibition of respiratory oxygen consumption, disruption of nutrient uptake processes, inhibition of enzyme reactions or protein synthesis, production of abnormal morphological development and ultrastructural changes, impairment of motility (i.e. lost of flagella), degradation of photosynthetic pigments, etc. All these effects may inhibit primary production in marine ecosystems (Davies 1978; Thomas et al. 1980) but most of these studies have been performed in laboratory cultures and have been focused on changes at the sublethal level. However, fewer studies have been performed in natural communities and less have analyzed the effect of metals at the lethal level, i.e. the levels of contaminant overpassing the adaptive capacity of cells (e.g. Jennings 1979; Devi Prasad and Devi Prasad 1982; Romero et al. 2002). The analysis of lethal levels of POP's in phytoplankton had revealed very clear patterns between species, suggesting that lethal thresholds are able to express the different sensitivity and resistance capacity of phytoplankton species (Echeveste et al. 2010*a*, 2010*b*).

The goal of this study was to quantify the lethal thresholds of two metals, Cd and Pb to field populations of marine photosynthetic plankton. On-board experiments analyzing the effect of different metal concentrations on the population abundance and cell death of phytoplankton in the Mediterranean Sea, Black Sea and the subtropical North Atlantic Ocean, which are mostly oligotrophic waters dominated by pico-sized phytoplankton. The resulting cell death-metal relationships observed will be used to define the thresholds and points of no return of metals concentration to marine phytoplankton. We tested the relation of these thresholds with cell volume.

We also determined the sensitivity of phytoplankton from the different communities to low levels of increased Cd and Pb to identify, especially in the case of Cd, the metal concentrations at which natural phytoplankton may show a positive response.

2. Methods

The experiments to analyze the lethal-nutritional thresholds of Cd and Pb on natural communities of phytoplankton were performed with Mediterranean, Black Sea and North East Atlantic Ocean phytoplankton, sampled during the oceanographic cruises THRESHOLDS-I and RODA-I. The cruise THRESHOLDS-I was performed on board the RV Garcia del Cid, along the Mediterranean and Black Seas from June 3 to July 5, 2006, while the cruise RODA-I was performed on board the RV Hespérides, in the subtropical Atlantic Ocean from August 17 to September 5, 2006 (Table 4.1).

	Experiment	**Coordinates**	**Date**	**Chlorophyll *a* (mg m^{-3})**	**Surface Water Temperature (ºC)**
Mediterranean Sea (THRES)	Cd	36º 42' N - 12º 04' E	06/30/2006	0.23	23.5
	Pb	36º 47' N - 14º 14' E	06/07/2006	0.25	20
Black Sea (THRES)	Cd	41º 52' N - 30º 04' E	06/19/2006	1.90	20.3
	Pb	41º 56' N - 29º 06' E	06/20/2006	1.70	18.8
Atlantic Ocean (RODA)	Cd	28º 27' N - 18º 10' W	08/26/2006	0.23	23.3
	Pb	30º 00' N - 23º 02' W	08/21/2006	0.12	24.7

Table 4.1. Position of the stations where the natural phytoplankton communities were sampled, and the date of sampling, indicating the abundance of phytoplankton (as Chlorophyll *a* concentration) found at each station, the surface water temperature, and whether the experiments were performed with Cd or Pb. THRESHOLDS campaign (THRES) was carried out in the open Mediterranean Sea and Black Sea, while RODA campaign was carried out in the subtropical North Atlantic Ocean.

Cadmium and Pb concentrations in the field stations were studied by sampling surface seawater (1 m depth) collected from a Zodiac deployed from the research vessel. Seawater was pumped through acid-cleaned Teflon tubing coupled to a C-flex tubing (for the Cole-Parmer peristaltic pump head), filtered through an acid-cleaned

polypropylene cartridge filter (0.22 μm, MSI, Calyx®), and collected in a 0.5 L LDPE bottle. Samples were acidified on board to pH <2 with Ultrapure-grade HCl (Merck) in a class-100 HEPA laminar flow hood, and stored for at least one month before extraction. Metals (Cd and Pb) were pre-concentrated by the APDC/DDDC organic extraction method of Bruland et al. (1979), and analyzed by ICP-AES (Perkin Elmer Optima 5300 DV).

Surface waters (5 m) used in the experiments were sampled by using Niskin bottles attached to a rosette-CTD system. Experiments began with the distribution of sampled water into 250 ml acid cleaned Pyrex bottles. After this gathering, Cd and Pb were inoculated at different concentrations, in duplicated bottles, and were incubated on deck under natural solar radiation in a tank with seawater surface running system to keep "in situ" temperature conditions. Metal solutions for experiment bottles were prepared with seawater, from Pb and Cd standard solutions of 1000 mg/L (Scharlau Chermie S.A). Bottles were covered with a neutral net to simulate 5 meters light conditions. After addition of a gradient of concentrations of Cd and Pb, we got final concentrations in the incubation bottles of 0.01, 0.02, 0.1, 1.12, 11.2, 112 and 1000 μg L^{-1}. Duplicated bottles without metal additions were also run as controls. Daily sampling was performed in the experiments for as long as 4 days. A total of 6 experiments were performed, with one experiment of Pb and Cd, respectively, done in each water mass (Table 4.1).

Changes in total phytoplankton abundance during the experiments were tested by analyzing Chlorophyll *a* concentrations. For this estimation, 50 ml samples were filtered onto 25 mm diameter Whatmann GF/F filters from each bottle on the day 0, day 2 and last day (day 4). After filtration, filters were placed in tubes with 90% acetone for 24 hours for the extraction of the pigment. Then the fluorescence of the

Chlorophyll *a* was measured in a Shimadzu RF-5301 PC spectrofluorometer and calibrated with pure Chlorophyll *a*, as described in Parsons et al. (1984).

The effect of Cd and Pb on the different groups forming the phytoplankton communities was analyzed. For picophytoplankton communities (*Prochlorococcus* sp., *Synechococcus* sp. and eukaryotic picophytoplankton) changes in the abundance and viability of cells along the experiment were quantified by using duplicated 1 mL fresh samples counted in a FACSCalibur Flow Cytometer (Becton Dickinson), as described before (Echeveste et al. 2010*a*, 2010*b*). An aliquot of a calibrated solution of 1 μm diameter fluorescent beads (Polysciences Inc.) was added to the samples as an internal standard for the quantification of cell concentration. The red, green and orange fluorescence, and forward and side scattering signals of the cells and beads were used to detect different populations and to differentiate them from the fluorescent beads (Marie et al. 2000).

The proportion of living and death cells in the picophytoplankton communities along the experiments were also followed by applying a cell membrane permeability test, the cell digestion assay (Agustí and Sánchez 2002), which allows the counting and identification of living phytoplankton cells. This test consists on the exposure of samples to an enzymatic cocktail, DNAse and Trypsin, which penetrates inside of the cells with permeable membranes (i.e. dead cells, Darzynkiewicz et al. 1994) resulting in the elimination of the death cells in the sample by enzymatic digestion, resting only the living cells of the population. The cell digestion assay was applied to replicate samples, by adding 200 μL of DNAse I solution (400 μg ml^{-1} in HBSS (Hanks' Balanced Salts)) to 1 mL sample of each treatment, followed by 15 minutes incubation at 35°C in a Digital Dry Bath. After this time, 200 μL of Trypsin solution (1% in HBSS) were added, followed by 30 minutes incubation at 35°C. At the end of

this time, samples were kept in cold conditions in order to stop the cell digestion process as described before. After the incubation, samples were counted using the flow cytometer as described above.

The cells counted after the cell digestion assay represent the living cells in the population, whereas the cells counted in untreated samples represented the total population (living and dying cells). The percentage of living (or viable) cells was calculated as the ratio between the concentration of cells after the enzyme digestion, and the cell concentration of untreated samples, which represent the total (dead plus living) cell population.

The changes in the abundance of nano and microphytoplankton communities were analyzed at the beginning and the end of the experiment by using epifluorescence microscopy. In order to have a representative number of cells, 50 ml of water were sampled from each replicated treatment bottle and filtered onto polycarbonate 2 μm pore diameter black filters. During the filtration, with the last mL to be filtered, 2 mL of gluteraldehyde (25%) were added to the sample in order to fix it. Once filtered, filters were freezed until their analysis in order to preserve them. Cells were then counted in a Zeiss Axioplan Imaging epifluorescence microscope and classified into 3 groups: small eukaryotes (3-4 μm size cells), nanophytoplankton (5-20 μm size cells) and microphytoplankton (larger than 20 μm). The cell volume of the nano and microplankton in each sample was calculated by approximation to the nearest simple geometric shape, from the dimensions (at x1000) of the different cells within the community. The cell diameters of *Synechococcus* and *Prochlorococcus* populations were assumed to be 0.6 and 1.2 μm, respectively, as measured in laboratory strains (Echeveste et al. 2010*a*).

The evolution of the populations in the different metal treatments were followed by analyzing the changes in the population abundance and Chlorophyll *a* concentration with time, and calculating the growth and decay rates of the populations and total community (Chlorophyll *a* concentration values). From the decay rates, the half lives ($t_{1/2}$) of the different species and size groups in each treatment were calculated by applying the formula

$$t_{1/2} = \ln 2 / \mu \qquad \text{(eq. 4.1)}$$

where μ is the slope of the natural logarithm (ln) of the decay of cell abundance with time in days.

The 50% Lethal Thresholds (LC50) of Cd and Pb for each species tested was calculated as the metal concentration at which the cell population will be decreased by a half, applying the equation

$$LC50 = \ln 2 / \Omega \qquad \text{(eq. 4.2)}$$

where Ω is the slope of the relationship between the decay ln of cell abundance and the metal concentration ($\mu g\ L^{-1}$) reached at the end of the experimental treatments.

The significance difference observed between treatments was analyzed by using the t-student test.

3. Results

Picophytoplankton (represented by *Prochlorococcus* sp., *Synechococcus* sp. and small eukaryotes), and nanophytoplankton dominated the phytoplankton communities found in the stations sampled in the Mediterranean and Black Seas and in waters of the North East Atlantic Ocean. The waters studied were in general

oligotrophic but the Black Sea, as indicated by the higher Chlorophyll *a* concentration (Table 4.1). Phytoplankton abundance was low in the Mediterranean and Atlantic waters, although the phytoplankton biomass in the Black Sea was higher. There were some differences in the community composition of phytoplankton between the areas studied, with *Prochlorococcus* been more abundant in Atlantic waters than in the Mediterranean Sea, where was often absent, as was for the Black Sea, where nano-sized dinoflagellates dominated the biomass, been *Synechococcus* sp. highly abundant.

Surface dissolved (<0.22 μm) Cd concentrations were 9.15 ng L^{-1}, 8,97 ngL^{-1} and 4.0 ng L^{-1}, in the Black Sea, Mediterranean Sea and North East Atlantic Ocean, respectively. On the other hand, dissolved Pb concentrations were below the detection limit (< 0.01 μg L^{-1}) in all the samples. The addition of the lowest Cd concentrations supposed an increase of Cd of about 2 times (0.01 μg L^{-1}) or 3 times (0.02 μg L^{-1}) with respect to the natural concentrations of Cd observed in the Mediterranean and Black Seas, and an increase of about 3 times (0.01 μg L^{-1}) and 5 times (0.02 μg L^{-1}) to respect the natural concentrations of Cd in the Atlantic Ocean.

Cd (μg L^{-1})	**Mediterranean Sea**			**Black Sea**				**Atlantic Ocean**				
	Proc	*Syn*	Chl *a*	*Syn*	P_euk	Npl	Chl *a*	*Proc*	*Syn*	P_euk	Npl	Chl *a*
C	0.39	0.47	-0.19	0.57	0.14	0.18	0.01	-0.19	0.3	nd	nd	-0.05
0.01	0.42	0.53	-0.14	0.54	-0.10	-0.14	-0.02	-0.38	0.23	-0.01	-0.04	0
0.02	0.34	0.54	-0.22	0.61	-0.01	-0.05	-0.04	-0.16	0.32	-0.03	0.071	0.01
0.11	0.2	0.48	-0.17	0.61	0.14	0.12	-0.04	-0.35	0	0.16	-0.02	-0.07
1.12	-0.42	0.03	-0.23	-0.7	0.12	0.06	0.01	-1.2	-0.6	-0.12	-0.04	-0.26
12	-3.04	-1.4	-0.84	-0.9	-0.29	-0.5	-0.07	-	-	-	-	-
112	*	*	*	*	0.05	-0.07	-0.2	-	-	-	-	-
1000	*	*	*	*	-0.34	-0.57	-0.34	-	-	-	-	-

Table 4.2. Growth and decay rates (d^{-1}) of the different groups forming the natural phytoplankton communities examined in the experiments with Cd. (*Proc*) *Prochlorococcus*; (*Syn*) *Synechococcus*; (P_euk) Picoeukaryotes; (Npl) Nanophytoplankton; (Ch *a*) Chlorophyll *a*; (nd) not detectable. (-) no treatments. (*) above measurable limits.

A slight positive response of phytoplankton in some of the experiments was observed in the treatment with the lowest Cd concentrations added (0.01 and 0.02 μg L^{-1}), (Table 4.2). Thus, when Cd was added at concentration of 0.01 μg L^{-1} to the Mediterranean community, an increase in the growth rates was observed for *Prochlorococcus* sp. (0.42 d^{-1}) and *Synechococcus* sp. (0.53 d^{-1}), although this increase was not significant (P>0.05) when compared with the control bottles where no Cd was added (0.39 d^{-1} and 0.47 d^{-1}, respectively, Table 2). When 0.02 μg L^{-1} of Cd was added an increase (P>0.05) in the growth rate was also observed for Mediterranean *Synechococcus* sp. (0.54 d^{-1}, Table 4.2). Not significant (P>0.05) increases in growth were detected in the populations of the Black Sea at the low Cd treatments (Table 4.2). The whole phytoplankton community (expressed as Chlorophyll *a*) of the Atlantic Ocean showed also a small, not significant (P>0.05), increase in growth rate to respect to the control community (Table 4.2). In the case of Pb no positive effects were observed, although Atlantic *Synechococcus* sp. population showed a slight increase in the growth rates at the lowest Pb concentrations (P>0.05).

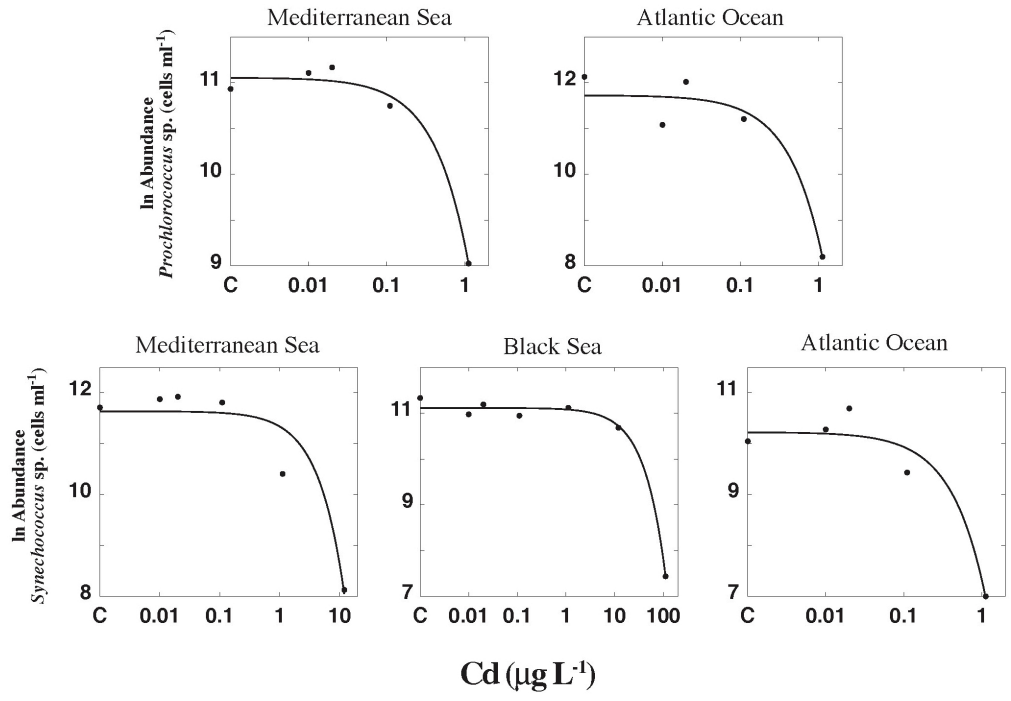

Figure 4.1. Decrease in the cell abundance of *Prochlorococcus* sp. and *Synechococcus* sp. (cells ml^{-1}) exposed to different levels of Cd observed in the experiments carried out with natural communities. The lines represent the lowess fit.

In the presence of high concentrations of Cd and Pb, the results of the experiments showed that the natural communities were strongly affected, as most of the populations declined as Cd and Pb concentrations in the treatments increased (Figs. 4.1 and 4.2, respectively). The decline in the phytoplankton biomass was more important when the communities were exposed to Cd than when exposed to Pb (Figs. 4.1 and 4.2, respectively).

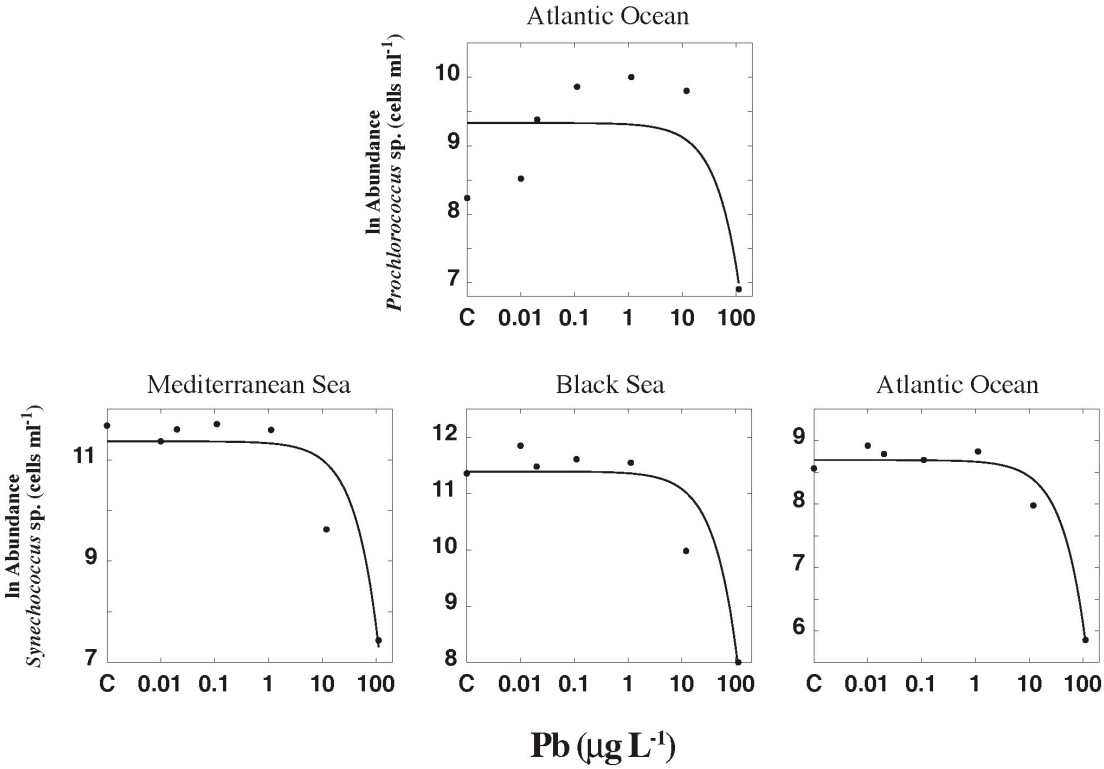

Figure 4.2. Decrease in the cell abundance of *Prochlorococcus* sp. and *Synechococcus* sp. (cells ml^{-1}) exposed to different levels of Pb observed in the experiments carried out with natural communities. The lines represent the lowess fit.

When analysing the total communities, by following the decline in Chlorophyll *a* concentration, important differences in the sensitivity to Cd and Pb were observed (Fig. 4.3), with Atlantic and Black Sea communities been highly

resistant to Pb, showing a minor decrease in the biomass at the high concentration treatments (Fig. 4.3).

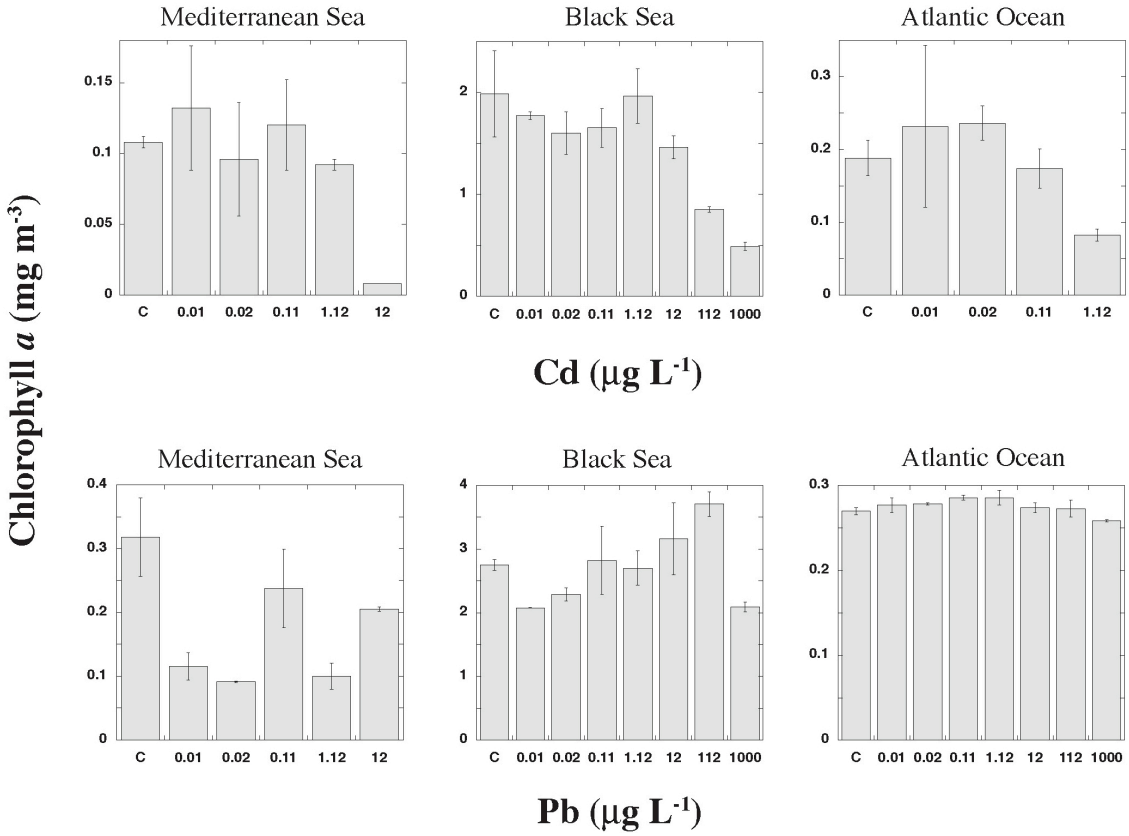

Figure 4.3. Decrease in the Chlorophyll *a* concentration with increasing trace metals concentrations (Cd and Pb, μg L^{-1}) observed in the experiments with phytoplankton communities from the Mediterranean Sea, Black Sea and Atlantic Ocean.

The LC50s varied from 0.23 μg L^{-1} for Atlantic *Prochlorococcus* sp. to 498.7 μg L^{-1} for Black Sea picoeukaryotes in the case of Cd and from 20.02 μg L^{-1} for Mediterranean *Synechococcus* sp. to 465.2 μg L^{-1} for Black Sea nanoplankton in the case of Pb (Table 4.3). The 10% Lethal Thresholds (LC10) were also calculated, representing the metal concentration required to reduce the 10% of the population, and were 6.5 times lower, on average, than those of the LC50 (Table 4.3). The LC50s of Pb obtained using the Chlorophyll *a* concentration values of the experiments from the Black Sea and the Atlantic Ocean were very high, 3080 and 9240 μg L^{-1}, since the decrease in the Chlorophyll *a* observed was very soft (Fig. 4.3). These data should be

considered only orientational since the error of this calculation was very high (Fig. 4.3). The Pb LC50s of *Prochlorococcus* sp. and *Synechococcus* sp. were always lower than those obtained for pico-eukaryotes and nanophytoplankton (Table 4.3), indicating the larger sensitivity of the smallest phytoplankton to Pb.

			Volume (μm^3)	**LC50 ($\mu g\ L^{-1}$)**	**LC10 ($\mu g\ L^{-1}$)**
Cd	Mediterranean Sea (THRES)	*Prochlorococcus*	0.11	0.38 ± 0.07	0.06 ± 0.01
		Synechococcus	0.9	2.34 ± 0.6	0.35 ± 0.09
		Chlorophyll *a*		3.18 ± 0.59	0.48 ± 0.09
	Black Sea (THRES)	*Synechococcus*	0.9	21.25 ± 2.25	3.23 ± 0.34
		Picoeukaryotes	22.98	498.7 ± 660	75.8 ± 100.6
		Nanoplankton	157.96	332.1 ± 395	50.5 ± 60.15
		Chlorophyll *a*		560 ± 180	85 ± 27
	Atlantic Ocean (RODA)	*Prochlorococcus*	0.11	0.23 ± 0.07	0.03 ± 0.01
		Synechococcus	0.9	0.24 ± 0.07	0.04 ± 0.01
		Picoeukaryotes	20.53	1.28 ± 9.91	0.19 ± 1.51
		Nanoplankton	152.52	3.9 ± 9.8	0.59 ± 1.49
		Chlorophyll *a*		0.81 ± 0.26	0.12 ± 0.04
Pb	Mediterranean Sea (THRES)	*Synechococcus*	0.9	20.02 ± 6.79	3.04 ± 1.03
	Black Sea (THRES)	*Synechococcus*	0.9	22.19 ± 4.45	3.37 ± 0.68
		Picoeukaryotes	24.37	268.5 ± 126.5	40.8 ± 19.23
		Nanoplankton	188.57	465.2 ± 290	70.7 ± 44.28
		Chlorophyll *a*		3080 ± 9580	468 ± 1450
	Atlantic Ocean (RODA)	*Prochlorococcus*	0.11	33.28 ± 17.2	5.1 ± 2.6
		Synechococcus	0.9	27.1 ± 4	4.1 ± 0.6
		Chlorophyll *a*		9240 ± 6250	1405 ± 950

Table 4.3. Cell volumes (μm^3) of the different groups forming the natural phytoplankton communities examined, and the Cd and Pb LC50 and LC10 ($\mu g\ L^{-1}$) values obtained for each phytoplankton group in all the experiments carried out. THRESHOLDS campaign (THRES) was carried out in the open Mediterranean Sea and Black Sea, while RODA campaign was carried out in the subtropical North Atlantic Ocean.

There was a significant ($p<0.05$) linear relationship between the LC50, for both Cd and Pb, and phytoplankton cell volume, with the LC50s increasing as the cell volume increased (Fig. 4.4, panel A), as described in the equations:

ln LC50 (μg L^{-1} Cd) = 0.64 + 0.68 x ln cell volume (μm^3), $R^2 = 0.44$ (eq. 4.3)

ln LC50 (μg L^{-1} Pb) = 3.64 + 0.47 x ln cell volume (μm^3), $R^2 = 0.82$ (eq. 4.4)

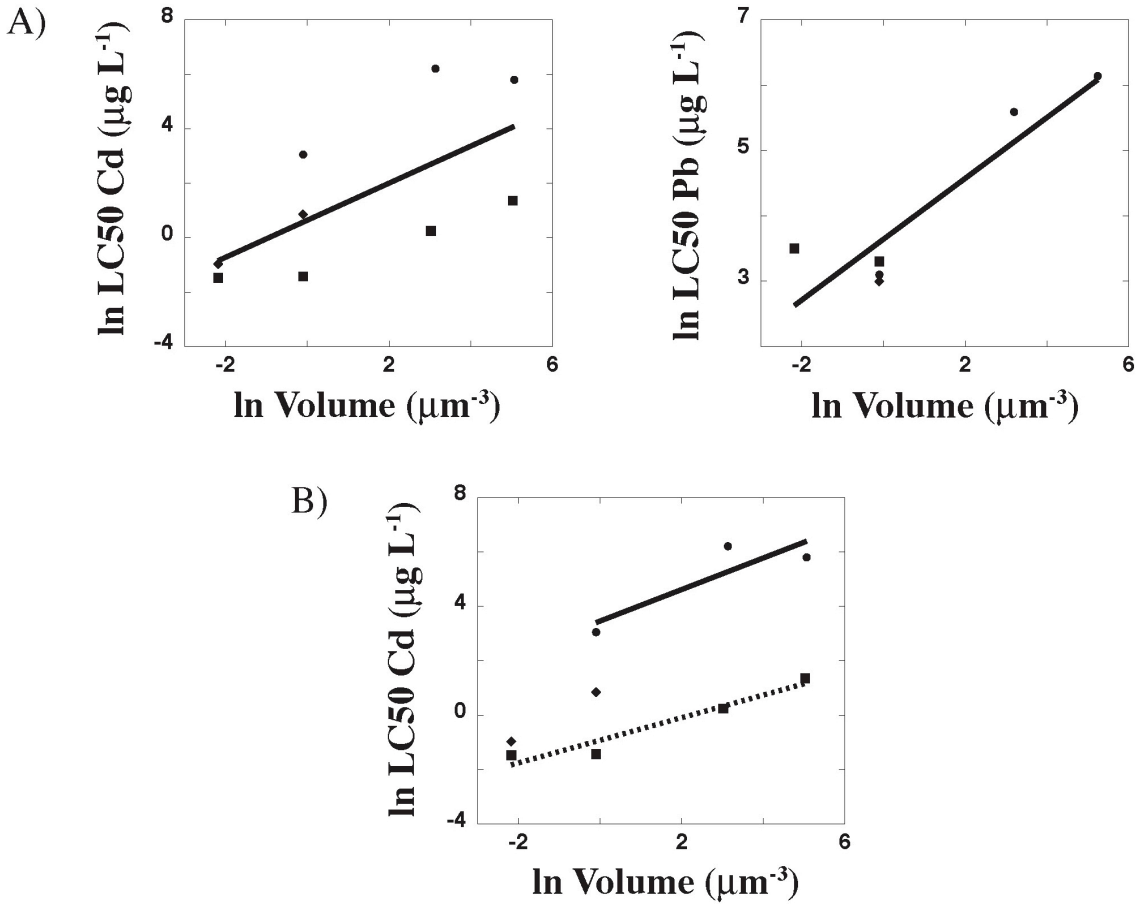

Figure 4.4. The relationship between Cd and Pb LC50s and phytoplankton cell volume obtained for the natural phytoplankton communities of the Mediterranean and Black Seas and the Atlantic Ocean. Panel A: The continuous lines represent the fitted linear regressions for the phytoplankton communities exposed to Cd (eq. 4.4) or Pb (eq. 4.5). Panel B: Relationship between Cd LC50 and phytoplankton cell volume. The continuous line represents the fitted linear regression for the phytoplankton community of the Black Sea (eq. 4.6), while the discontinuous line represents the fitted linear regression for the phytoplankton community of the Atlantic Ocean (eq. 4.7). Dots represent populations from the Black Sea, diamonds represent populations from the Mediterranean Sea and squares represent populations from the Atlantic Ocean.

The relationship between Pb LC50 and cell size was strongest than that observed for Cd (eq. 4.3). However, for Cd, the relationship with cell size was

stronger when considering the results from the different water masses independently (Fig. 4.4, panel B), indicating that the sensitivity of the populations of the Black Sea was lower for the three different size categories forming the community (eq. 4.5; Fig. 4.4, panel B). By contrary, the Atlantic populations (AO) were more sensitive than populations of similar cell size from the Black Sea (BS) (eq. 4.6; Fig. 4.4, panel B).

$$\ln LC50\ BS\ (\mu g\ L^{-1}\ Cd) = 3.46 + 0.57 \times \ln \text{cell volume}\ (\mu m^3),\ R^2 = 0.77 \qquad \text{(eq. 4.5)}$$

$$\ln LC50\ AO\ (\mu g\ L^{-1} Cd) = 0.92 + 0.41 \times \ln \text{cell volume}\ (\mu m^3),\ R^2 = 0.93 \qquad \text{(eq. 4.6)}$$

The half lives obtained under the different treatments, calculated for the two species showing catastrophic cell death in the natural communities examined (*Prochlorococcus* sp. and *Synechococcus* sp., Table 4.4), varied from 3 days to 0.1 days, showing shorter half lives at the highest metal concentrations (Table 4.4). Other phytoplankton populations showed half lives values between 0.2 and 1.4 days for picoeukaryotes, and between 0.3 and 2.4 days, for nanophytoplankton.

			Half Live (d)			
			0.11 ($\mu g\ L^{-1}$)	**1.12** ($\mu g\ L^{-1}$)	**12** ($\mu g\ L^{-1}$)	**112** ($\mu g\ L^{-1}$)
Cd	Mediterranean Sea (THRESHOLDS)	*Prochlorococcus*	-	1.7	0.23	-
		Synechococcus	-	-	0.5	0.24
	Black Sea (THRESHOLDS)	*Synechococcus*	-	0.95	0.82	0.16
	Atlantic Ocean (RODA)	*Prochlorococcus*	2.15	0.58	-	-
		Synechococcus	-	1.11	-	-
Pb	Mediterranean Sea (THRESHOLDS)	*Synechococcus*	-	-	0.82	0.22
	Black Sea (THRESHOLDS)	*Synechococcus*	-	2.27	0.69	0.38
	Atlantic Ocean (RODA)	*Prochlorococcus*	-	1.43	1.24	0.23
		Synechococcus	-	3.17	1.08	0.51

Table 4.4. Values of half lives (d) calculated for *Prochlorococcus* sp. and *Synechococcus* sp. for the highest concentrations of Cd and Pb (μg L^{-1}) tested in the experiments performed with natural communities. (-) no decay of the populations.

The induction of cell death was also analyzed by measuring the increase in the proportion of dead cells within the populations. In the treatments where the concentration of metals was below the LC50 values, and where the decline in the populations were not catastrophic, we were able to detect the induction of cell death in both *Prochlorococcus* sp. and *Synechococcus* sp. populations. Mediterranean *Prochlorococcus* sp. increased cell death at the lowest concentrations of Cd (Fig. 4.5), but Atlantic *Prochlorococcus* sp. showed a decrease in the percentage of dead cells at the lowest concentrations of Cd indicating a positive effect (Fig. 4.5). For *Synechococcus* sp., consistent changes in the percentages of dead cells were observed in some of the experiments (Fig. 4.5). *Synechococcus* sp. population of the Black Sea, increased cell viability with respect to the control (i.e. a reduction in the proportion of dead cells) at the lowest Cd concentration treatments (Fig. 4.5), parallel to the smooth increases in the growth rates observed at the lowest concentration treatments with respect to the control, as pointed out before. For Mediterranean and Atlantic *Synechococcus* sp. populations, the percentage of dead cells increased at the lowest concentrations of Cd added (Fig. 4.5), although there was not a clear effect on cell death on Mediterranean *Synechococcus* sp. at the low Cd concentrations (Fig. 4.5). For the experiments with Pb, Atlantic *Synechococcus* sp. population increased its viability (i.e. a reduction in the proportion of dead cells) at the lowest Pb concentrations (Fig. 4.5), in agreement with the slightly increase in growth rates observed. Above 0.11 μg L^{-1} of Cd and Pb, the percentages of dead *Synechococcus* sp. cells tended to increase in the Black Sea and Atlantic Ocean populations, respectively (Fig. 4.5), as well as observed for Atlantic *Prochlorococcus* sp. at the Pb experiment

(Fig. 4.5). For the rest of Cd experiments the treatment of 0.11 μg L^{-1} appeared to be a threshold for picophytoplankton cell mortality, since above this concentration, catastrophic decline of the populations were observed for both *Prochlorococcus* sp. and *Synechococcus* sp.

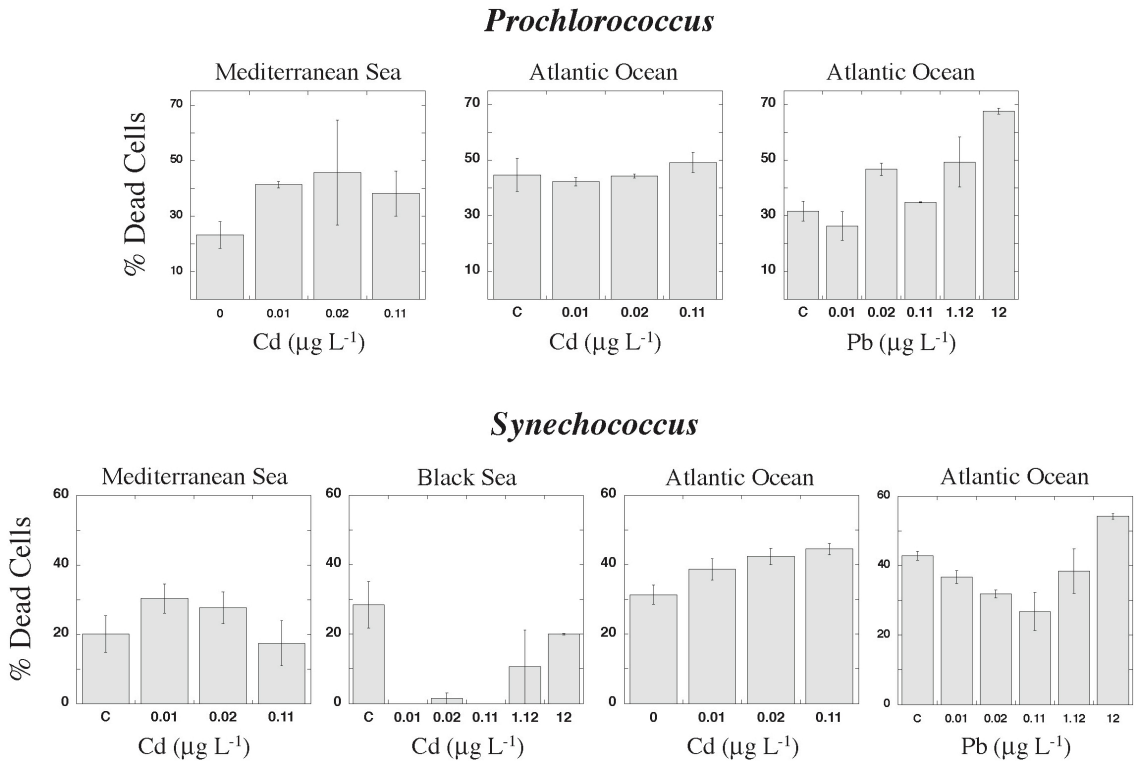

Figure 4.5. Changes in the proportion of dead cells of *Synechococcus* sp. and *Prochlorococcus* sp. of the Mediterranean and Black Seas and the Atlantic Ocean, observed in the control (C) and at the lowest concentrations of Cd and Pb added. The ranges of concentrations are below the LC50s.

4. Discussion

The results presented here identify the lethal thresholds for Cd and Pb of natural communities of marine phytoplankton, and point to the picophytoplankton communities as the most sensitive to those metals. The comparison of the results obtained with the present literature on the lethality of Cd and Pb to marine phytoplankton, indicated that the LC50s obtained in this work were 2 or 3 orders of magnitude lower than those reported previously (Jennings 1979; Devi Prasad and

Devi Prasad 1982; Romero et al. 2002). One of the factors that could be responsible for the different values obtained could be that, while previous studies were performed with larger species growing in cultures (Jennings 1979; Devi Prasad and Devi Prasad 1982; Romero et al. 2002), our experiments were held in oligotrophic waters dominated by pico-sized phytoplankton. Thus, our results show that phytoplankton cell size was important determining the sensitivity to high concentrations of metals, with the smallest pico-cyanobacteria, *Prochlorococcus* sp. and *Synechococcus* sp., been the most sensitive, and the largest phytoplankton populations the most resistant. The results also show that the positive effect of low values of Cd were also associated to phytoplankton cell size, showing pico-cyanobacteria a positive response when Cd was added at low concentrations.

For both Cd and Pb, pico-sized phytoplankton showed lower LC50s than nanophytoplankton with LC50s up to three orders of magnitude higher. As a consequence, a strong relationship between LC50 and cell volume was found, confirming that the variability in the lethal levels of Cd and Pb appeared to be strongly related to phytoplankton cell size. Our results agree with those of Brand and colleagues (1986) which showed that the cyanobacteria were most sensitive to Cd toxicity, been largest diatoms the more resistant, and with other studies identifying phytoplankton cell size as an important property determining the level of toxicity of other contaminants (Fan and Reinfelder 2003; Ruangsomboon and Wongrat 2006; Echeveste et al. 2010*a*). The higher surface to volume ratio associated with small cells has been identified as the factor increasing their capacity to incorporate contaminants (Fan and Reinfelder 2003; Ruangsomboon and Wongrat 2006; Echeveste et al. 2010*a*).

The lower resistance of the phytoplankton populations studied here with respect to those from previous studies could be due to the fact that most of the literature data was obtained from phytoplankton species growing in laboratory cultures. Although a previous study observed similar sensitivities of cultured and natural phytoplankton to the exposition of trace metals (Hollibaugh et al. 1980), Echeveste et al. (2010*a*) found significant differences in the sensitivity of phytoplankton to organic pollutants between natural communities and laboratory cultures, been the cultures orders of magnitude more resistant. As argued by Echeveste et al. (2010*a*), this difference may be due to the optimal growth conditions in the laboratory compared to natural sea conditions; and by the possibility that more resistant genotypes may have been selected at the laboratory. Anyway, specific studies should be done to assess these assumptions.

The results revealed a high variability in the sensitivity of phytoplankton to the two metals tested. We found significant differences in the degree of toxicity for phytoplankton between Cd and Pb, being more toxic Cd, as previously described (Sorentino 1979; Rai et al. 1981). Despite of its toxic condition, previous studies have shown that Cd can act as nutrient too (e.g., Tkachenko et al. 1974; Kuiper 1981). Cd may be used by phytoplankton populations substituting Zn, a nutritive metal that is a co-factor for the enzyme carbonic anhydrase, which catalyses a critical rate-limiting step for carbon use in photosynthesis (Goldman and Horne 1983; Price and Morel 1990). In this work, we have tested low concentrations of Cd within the ranges reported to act as nutrient in marine phytoplankton, 1-50 μg L^{-1} (Kuiper 1981) and 1-10 μg L^{-1} (Tkachenko et al. 1974), but no growth enhancement was detected at such high levels with natural communities in this study. However, at lower concentrations of Cd added in our treatments (0.01-0.02 μg L^{-1}), we were able to observe a smooth,

but not significant (P>0.05), growth enhancement of *Prochlorococcus* sp. and *Synechococcus* sp. in most cases. At concentrations above 0.11 μg L^{-1} of Cd added, only negative effects were observed in the phytoplankton community, determining a threshold below which Cd could have beneficial effects for phytoplankton substituting nutritive metals.

Moreover, at the lowest Cd concentrations added (0.01 to 1.12 μg L^{-1}), significant ($p<0.05$) decreases in the percentage of dead cells of the Black Sea *Synechococcus* sp. were detectable, with viability increasing to values between 85-100%, although no enhancement of the net growth rate was observable. The quantification of the proportion of living and dead cells within the natural populations tested, allowed to detect the induction of cell death at the treatments where the metals concentration analyzed did not result in a substantial decline of the population. For *Prochlorococcus* sp., we observed a minor positive effect at low concentrations of Cd, since the percentages of dead cells did not increase as observed when higher Cd concentrations were added (e.g., Atlantic *Prochlorococcus* sp.).

For a more extensive analysis of the nutritive or toxic activity of these metals, a more accurate gradient of concentrations should be used in one sense (low concentrations for nutritive studies) or another (high concentrations for lethality studies). This is an important issue to consider when carrying out experiments with natural communities in open seawater, as those performed for this work, since the number of treatments are usually limited and the techniques used for these analysis, like the cell digestion assay, cannot be preserved and, therefore, need to be performed in situ.

Our metals LC50 values of phytoplankton allow the comparison of the sensitivity of phytoplankton with that reported for other organisms. Thus, the reported

Cd LC50s in these works ranged from 180 to 55 000 μg L^{-1} (Eisler 1971; Madoni et al. 1992), while the Pb LC50s ranged from 600 to 5880 μg L^{-1} (Baudouin and Scoppa 1974; Bat et al. 1999), showing generally higher values than those reported here for the different phytoplankton populations, and showing also that the LC50s increased with the increase in the size of the organism. When comparing the half lives observed in this work for phytoplankton with those obtained for larger organisms in these previous works, we observed that the picophytoplankton showed, in almost all the cases, lower half lives values, 0.1 to 2 days when exposed to high concentrations of Cd and from 0.2 to 3 days when exposed to high concentrations of Pb, than those reported for larger marine organisms, which were on average of 4 days for both Cd or Pb.

Although Pb concentrations were not detectable during the cruises, previous studies reported concentrations ranging from 8.8 to 30.4 ng L^{-1} in the Atlantic Ocean (Helmers et al. 1991), from 13.9 to 63.4 ng L^{-1} in the Mediterranean Sea (Yoon et al. 1999) and 35.8 ng L^{-1} in the Black Sea (Lewis and Landing, 1992). These levels of Pb, together with those obtained in this work of Cd, suggest that the environmental level concentrations of Cd and Pb in the Mediterranean and Black Seas and in the Atlantic Ocean are highly below the LC50s obtained in this work. For example, the LC50 of Cd for *Prochlorococcus* sp. ranged from 0.23 to 0.38 μg L^{-1}, while the Pb LC50 was 33.28 μg L^{-1}, which represented more than 10 and 1000 times higher concentrations than those measured in nature, respectively, suggesting that these species may be strongly resistant to natural levels of individual trace metals.

Thus, and besides the general dependence of sensitivity to metals related to the populations cell size found here, our results also show that growth in different regions may have also influenced in the sensitivity degree of phytoplankton to Cd. The

phytoplankton populations from the Black Sea were the most resistant to high Cd concentrations, while Atlantic Ocean populations were the most sensitive. These different sensitivities were of orders of magnitude different, been Black Sea populations an order of magnitude more resistant to Cd than Mediterranean Sea populations (e.g. *Synechococcus* sp.), and been the later an order of magnitude more resistant than Atlantic Ocean populations.

These significant different sensitivities to Cd were detected despite Cd concentrations in seawater were not significantly different among the water masses analyzed (9.15 ng L^{-1}, 8,97 ngL^{-1} and 4.0 ng L^{-1}, in the Black Sea, Mediterranean Sea and North East Atlantic Ocean, respectively). The Mediterranean and Black Seas receive higher amounts of contamination directly from land by rivers and are seawaters with limited water exchange, especially in the Black Sea, where there is a predominant role of atmospheric fallout and the transport of trace elements from nearshore regions (Lewis and Landing, 1992; Zeri et al., 2000). Therefore, these seawaters may be more influenced to recurrent higher inputs of pollutants, including metals such as Cd. Moreover, the anoxic conditions of the Black Sea may decrease dissolved Cd concentrations due to the formation of insoluble metal sulphides (Lewis and Landing, 1992; Zeri et al., 2000). The introduction of such temporally higher inputs of metals in the environment may have developed adaptation processes and the selection of more resistant populations. Besides, the presence of high concentrations of other metals than Cd and Pb, due to antagonistic interactions, may also result in increased tolerance of the organisms to Cd and Pb, the so-called co-tolerance. Bariaud and Mestre (1984) observed that a Cd-resistant strain of *Euglena gracilis* was more resistant to the addition of other metals than a non-Cd resistant strain. Other works

also showed this process of co-tolerance in phytoplankton (Visviki and Rachlin 1991; Abd-el-Monem et al. 1998).

The higher resistance to Cd observed in the Black Sea community with respect to its concentration in nature, suggest the idea that more resistant populations may have been selected, as an adaptation to higher levels of contamination. It has been observed that phytoplankton is able to genetically adapt to crude oil exposition as a result of rare spontaneous mutations (Carrera-Martínez et al. 2010), so we tried to resolve this question analyzing the populations of *Synechococcus* sp., present in all the systems studied, by 18S rRNA genes amplification through the polymerase chain reaction (PCR) technique. However, the low abundance of *Synechococcus* sp. populations in the seawaters examined precluded the proper DNA extraction (Arrieta, personal communication). Therefore, future studies may consider genetic adaptation processes of oceanic phytoplankton to increasing pollutants concentrations.

5. Conclusions

In summary, our results show that cell size determined phytoplankton sensitivity to Cd and Pb, with the smallest pico-cyanobacteria, *Prochlorococcus* sp. and *Synechococcus* sp., being the most sensitive, and the largest phytoplankton populations the most resistant. The induction of cell death was already detectable at the lowest concentration treatments (0.01 and 0.02 μg L^{-1}), but some populations showed higher growth rates or lower decay rates at these concentrations ($P>0.05$), or even a decrease in the percentage of dead cells (e.g. Black Sea *Synechococcus* sp.), suggesting a positive effect. The LC50s obtained in this work were significantly lower than those reported previously with laboratory cultures, showing the higher sensitivity of natural phytoplankton communities. Moreover, the results showed that the Black

Sea phytoplankton community was more resistant than similar sized phytoplankton of the Mediterranean Sea and Atlantic Ocean, suggesting that the recurrent inputs of Cd and other metals in the environment may have induced the selection of resistant populations.

6. Acknowledgments

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Chapter 5

Dissimilar sensitivities of Arctic and Southern Ocean phytoplankton to Cd, Pb and Hg

by

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Abstract

Despite their remoteness from contaminant source areas, polar regions are no more the pristine areas they used to be. Among the pollutants reaching these regions, Hg, Cd and Pb are three of the most toxic trace metals. The goal of this work was to analyze the toxicity of Hg, Cd and Pb to natural phytoplankton communities of the Arctic and Southern Oceans. Mercury was the most toxic metal for both communities, being Cd and Pb toxic too for Antarctic communities but not for those of the Arctic. These Arctic communities were resistant to high concentrations of Cd and Pb, probably due to a reduction in the grazing. Cell size played a key role in determining phytoplankton sensitivity to the metals studied, being the lowest cells the most sensitive due to their higher surface to volume ratio. Through changes in the average cell size of the groups forming the phytoplankton community, we were able to observe changes in the community structure, where more resistant species might have been selected due to higher resistances to the metals added. However, unclear trends were obtained, precluding the determination whether phytoplankton may enlarge or reduce its size to resist pollutants presence.

1. Introduction

Marine phytoplankton is responsible for about half the photosynthetic fixation of carbon on Earth (Field et al., 1998) and represents the base of the marine ecosystems' food web. Among these ecosystems, polar ecosystems are one of the most sensitive ecosystems and are exposed to many threatens such as anthropogenic contamination. In the past, polar ecosystems were considered pristine areas far from industrial activities and other sources of anthropogenic pollutants, but since the Industrial Revolution, contaminants have reached both Arctic and Antarctic regions via atmospheric long-range transport and local mining activities (Barrie et al., 1992; Djupström et al., 1993; Macdonald et al., 2000; Muir et al., 1992), deriving in accumulation of pollutants in these marine ecosystems (Waldichuk, 1989; Daelemans et al., 1992).

Among these contaminants, 4 major groups are of special interest: organochlorines, polycyclic aromatic hydrocarbons, radionucleotides and trace metals (MacDonald et al., 2000). Metals are natural components of seawater and sediments (Cattell and Scott, 1978; Klinkhammer et al., 2001; Mason et al., 1994). Among them, the most toxic and present in polar regions are Hg, Cd and Pb (de Moreno et al., 1997; Honda et al., 1997; Macdonald et al., 2000; Muir et al., 1992). They displace nutrient metals from their metabolic sites, entering into cells through the same transport systems of nutrient metals (Sunda and Huntsman, 1988; Bruland et al., 1991), denaturing protein molecules. At very low concentrations Cd can act too as nutrient due to its physical and chemical similarities with other metals belonging the group two, like Zinc (Price and Morel, 1990).

Although these 3 trace metals occur naturally, anthropogenic activities have increased the rate at which are mobilized to these remote areas (Bargagli, 2000; Muir,

1992; Pacyna and Keeler, 1995). Combustion of fuels, particularly coal and gasoline, represents the main source of anthropogenic emissions of Cd, Pb and Hg (Muir et al., 1992). Approximately 60-80 T of human mobilized Hg (from Eurasia and North America) is deposited in the Arctic annually (Pacyna and Keeler, 1995) accumulating in Arctic biota (Muir, 1999), but there is no change in Hg concentration since the late 1980's in the Antarctic biota (Dalla Riva et al., 2003). In the case of Pb, anthropogenic emissions have significantly altered the biogeochemical cycle of Pb despite recent emission controls (Bargagli, 2000; MacDonald et al., 2000). Cd emissions have increased too during this century (Shen et al., 1987), and despite the difficulty to discriminate anthropogenic or natural origin of Cd contamination in these regions due to its biogeochemical activity, high concentrations in water and biota have been reported (Bargagli, 2000; MacDonald et al., 2000).

Many biological parameters have been used to define the sublethal levels of trace metals in cells (Shaw et al., 1990), such as reduction of photosynthetic electron transport, inhibition of respiratory oxygen consumption or disruption of nutrient uptake processes, which may inhibit primary production in marine ecosystems (Davies, 1978; Thomas et al., 1980). Most part of the studies on behalf the toxicity of trace metals to natural marine phytoplankton have been focused on the phytoplankton community of temperate and cold areas of the Northern hemisphere exposed to Cu (Goering et al., 1977; Thomas and Seibert, 1977; Kuiper, 1981a; Mann et al., 2002; de la Broise and Palenik, 2007; Debelius et al., 2009;), Hg (Knauer and Martin, 1972; Davies and Gamble, 1979) or Cd (Kuiper, 1981b; Echeveste et al., submitted). The present work contributes with new data to the few (or almost absent to our knowledge) data concerning the acute and chronic levels of Cd, Pb and Hg to natural polar phytoplankton communities.

The goal of this study was to quantify the lethality of three toxic trace metals, Hg, Cd and Pb, to natural marine photosynthetic plankton of the Arctic and Southern Oceans, by analyzing the effect of different concentrations on the population abundance and cell death of natural phytoplankton communities. The resulting cell death-metal relationships observed will be used to define the thresholds and points of no return of trace metals to marine phytoplankton. We tested the relation of these thresholds with cell volume since it has been observed that contaminant accumulation in phytoplankton depends on cell size (Fan and Reinfelder, 2003; Ruangsomboon and Wongrat, 2006; Echeveste et al., 2010a; Echeveste et al., submitted).

2. Methods

The experiments to analyze the lethal thresholds of Cd, Pb and Hg on natural communities of phytoplankton were performed with Arctic and Southern Oceans plankton communities, sampled during the oceanographic cruises ATOS-I and ATOS-II, on board the RV Hespérides. The cruise ATOS-I was performed along the Arctic Ocean from June 27 to July 27, 2007, while the cruise ATOS-II was performed along the Southern Ocean from January 24 to March 2, 2009 (Table 5.1).

	Experiment	**Coordinates**	**Date**	**Chlorophyll *a* (mg m^{-3})**
Arctic Ocean	Cadmium	80º 46' N - 13º 26' E	07/18/2007	1.13
	Lead	72º 58' N - 12º 39' W	07/03/2007	4.02
	Mercury	78º 44' N - 2º 58' E	07/07/2007	2.83
Southern Ocean	Cadmium	69º 02' S - 75º 06' W	02/14/2009	0.57
	Lead	62º 39' S - 59º 0' W	01/28/2009	2.72
	Mercury	62º 10' S - 57º 14' W	02/06/2009	3.44

Table 5.1. Position of the stations where the natural phytoplankton communities were sampled, and the date of sampling, indicating the abundance of phytoplankton (as Chlorophyll *a* concentration) found at each station and whether the experiments were performed with cadmium, lead or mercury.

Surface water (5 m) used in the experiments was sampled by using Niskin bottles attached to a rosette-CTD system. Experiments began with the distribution of sampled water into 2 L acid clean polycarbonate bottles. After this gathering, Hg, Cd and Pb were inoculated at different concentrations in duplicated bottles, and were incubated on deck under natural solar radiation in a tank with seawater surface running system to keep "in situ" temperature conditions. Metal solutions for experiment bottles were prepared with seawater, from Pb, Cd and Hg standard solutions of 1000 mg/L (Scharlau Chermie S.A). Bottles were covered with a neutral net to simulate 5 meters light conditions. The gradient of concentrations of Cd, Pb and Hg used represented final concentrations of 0.05, 0.5, 5, 50 and 500 ppb. Duplicated bottles without chemical additions were also run as controls. Daily sampling was performed in the experiments for as long as 6-8 days. A total of 6 experiments were performed, with one experiment of Cd, Pb and Hg performed in each water mass (Table 5.1).

Changes in total phytoplankton abundance during the experiments were followed by analyzing Chlorophyll *a* concentration. For this estimation, 50 ml samples were filtered onto 25 mm diameter Whatmann GF/F filters from each bottle on the day 0, day 2 and last day (day 4). After filtration, filters were placed in tubes with 90% acetone for 24 hours for the extraction of the pigment. Then the fluorescence of the Chlorophyll *a* was measured in a Shimadzu RF-5301 PC spectrofluorometer and calibrated with pure chlorophyll a as described in Parsons et al. (1984).

The effect of the trace metals on the different groups forming the phytoplankton communities was also analyzed. The changes in the abundance of nano and microphytoplankton communities were analyzed at the beginning and the end of

the experiment by using epifluorescence microscopy, in the case of the experiment with Hg in the Arctic Ocean, and by using a FlowCam, a submersible flow cytometer and microscope (FlowCAM, Fluid Imaging, Inc., Edgecomb, ME, USA), at the beginning, at the middle and at the end of each of the rest of the experiments. The FlowCam has been recently developed for the monitoring of phytoplankton communities in freshwater and marine environments (Sieracki et al. 1998, Sterling et al. 2004; See at al., 2005), allowing us to count, image and analyze cells in situ, measuring the size and parameters of the fluorescence waveform generated, and storing a digital image of each passing particle.

Regarding epifluorescence microscopy analysis, and in order to have a representative number of cells, 50 ml of water were sampled from each treatment bottle and filtered onto policarbonate 1 μm pore diameter black filters. During the filtration, with the last ml to be filtered, 2 ml of glutaraldehyde (25%) were added to the sample in order to fix it. Once filtered, filters were frozen until their analysis in order to preserve them. Cells were then counted in a Zeiss Axioplan Imaging epifluorescence microscope and classified into 3 groups: small eukaryotes (3-4 μm size cells), nanophytoplankton (5-20 μm size cells) and microphytoplankton (larger than 20 μm). The cell volume of the nano and microplankton in each sample was calculated by approximation to the nearest simple geometric shape, from the dimensions (at x1000) of the different cells within the community.

Regarding FlowCam analysis, duplicate water samples were pumped through a glass flow chamber (2x0.1mm) at a rate of approximately 0.3 ml min^{-1} for 3-4 min from each treatment bottle and then cells monitored. Data were collected in "trigger" mode and then classified in 4 different groups: Large diatoms with equivalent

Spherical Diameter (ESD) larger than 65 μm, medium diatoms with ESD between 35 and 65 μm, and small diatoms and flagellates with ESD lower than 35 μm.

The proportion of living and dead cells in the picophytoplankton communities along the experiments were also followed by applying a cell membrane permeability test, the cell digestion assay (Agustí and Sánchez, 2002), which allows the counting and identification of living phytoplankton cells. This test consists on the exposure of samples to an enzymatic cocktail, DNAse and Trypsin, which penetrates inside of the cells with permeable membranes (i.e. dead cells, Darzynkiewicz et al., 1994) resulting in the elimination of the death cells in the sample by enzymatic digestion, resting only the living cells of the population. The cell digestion assay was applied to replicate samples, by adding 200 μl of DNAse I solution (400 μg ml^{-1} in HBSS (Hanks' Balanced Salts)) to 1 ml sample of each treatment, followed by 15 minutes incubation at 25°C in a Digital Dry Bath. After this time, 200 μl of Trypsin solution (1% in HBSS) were added, followed by 30 minutes incubation at 25°C. At the end of this time, samples were kept in cold conditions in order to stop the cell digestion process as described before. After the incubation, samples were counted using the flow cytometer as described above.

The cells counted after the cell digestion assay represented the living cells in the population, whereas the cells counted in untreated samples represented the total population (living and dying cells). The percentage of living (or viable) cells was calculated as the ratio between the concentration of cells after the enzyme digestion, and the cell concentration of untreated samples, which represent the total (dead plus living) cell population.

Eukaryotic picophytoplankton community was also analyzed for the experiments performed in the Southern Ocean. The changes in the abundance and

viability of cells along the experiments were quantified by using duplicated 1 ml fresh samples counted in a FACSCalibur Flow Cytometer (Becton Dickinson), as described before (Echeveste et al., 2010a). An aliquot of a calibrated solution of 1 μm diameter fluorescent beads (Polysciences Inc.) was added to the samples as an internal standard for the quantification of cell concentration. The red, green and orange fluorescence, and forward and side scattering signals of the cells and beads were used to detect different populations and to differentiate them from the fluorescent beads (Marie et al., 2000).

For Pb cellular metal content, a volume of 50 ml of samples were collected at a depth of ~10 meters using an acid-cleaned all-plastic 50 micron mesh plankton net deployed from the zodiac. The samples were filtered through 0.22 μm Polycarbonate filters under a class-100 laminar flow hood, and stored frozen in Teflon microcentrifuge vials and transported back to the laboratory for acid digestion and analysis. Metal concentrations were determined by ICP-AES (Perkin Elmer Optima 5300 DV) after an acid-digestion (Tovar-Sánchez and Sañudo-Wilhelmy, 2011).

2.1. Statistical analysis and calculations

The evolution of the populations in the different metal treatments were followed by analyzing the changes in the population abundance, and when observed the decay of the population the half lives ($t_{1/2}$) of the different species and size groups in each treatment were calculated by applying the formula:

$$t_{1/2} = \ln 2 / \mu \qquad \text{(eq. 5.1)}$$

where μ is the slope of the ln of the decay of cell abundance with time in days.

The 50% and 10% lethal thresholds of Cd, Hg and Pb for each species tested was calculated as the trace metal concentration at which the cell population will be decreased by a half or 10%, respectively, applying the equations:

$$LC50 = -\ln 0.5 / \Omega \quad \text{(eq. 5.2)}$$

$$LC10 = -\ln 0.9 / \Omega \quad \text{(eq. 5.3)}$$

where Ω is the slope of the relationship between the decay ln of cell abundance and the trace metal concentration (μg L^{-1}) reached at the end of the experimental treatments.

The significant differences observed between treatments and the changes in the average cell size of the populations between treatments were analyzed using the T-student test.

3. Results

Chlorophyll *a* concentrations tended to be high in both Arctic and Southern Ocean waters sampled (Table 6.1), where phytoplankton communities were dominated by large nanophytoplankton and microphytoplankton groups, such as diatoms and flagellates. In the Arctic Ocean *Phaeocystis pouchetii* was also found, while in the Southern Ocean picoeukaryotes and small nanoplankton were also present in the water masses analyzed. The addition of high concentrations of trace metals had a different effect on the phytoplankton communities depending on the metal and on the community studied, as observed by the changes in Chlorophyll *a* concentration among the experiments (Fig. 5.1).

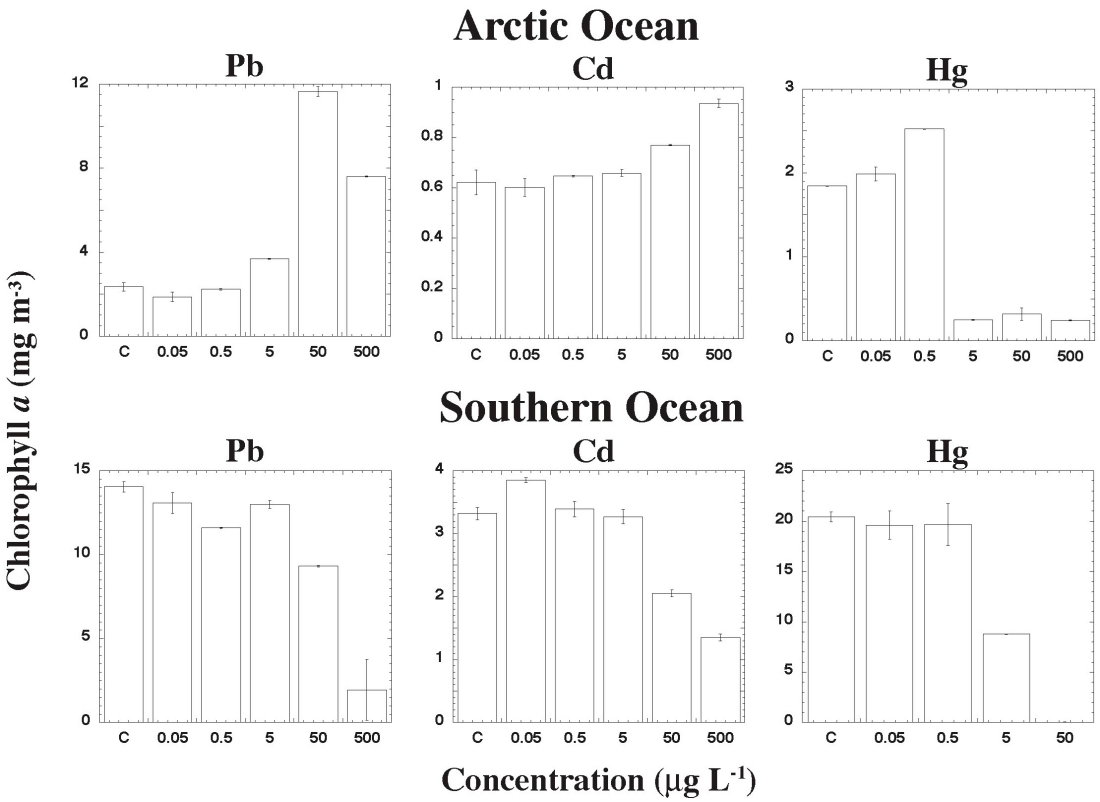

Figure 5.1. Chlorophyll *a* concentrations (mg m^{-3}) with increasing trace metals concentrations (Cd, Pb and Hg, μg L^{-1}) observed in phytoplankton communities from the Arctic and Southern Oceans.

A decline in the phytoplankton Chlorophyll *a* of both the Arctic and Southern Oceans experiments was observed at high concentrations of Hg. While phytoplankton of the Southern Ocean decreased at high concentrations of Cd or Pb (Fig. 5.1), the Arctic phytoplankton community increased however at high concentrations of Cd and Pb (Fig. 5.1). The Pb incorporated in phytoplankton cells, calculated as the Pb content per unit Chlorophyll *a*, in both Arctic and Southern Oceans increased as Pb concentration in the treatments increased, in a magnitude relative to the concentration of Pb added, i.e., one fold increase in the Pb added increased one fold the Pb content in the phytoplankton cells (Table 5.2). However, this result contrasts with the differences in Pb toxicity observed in the Arctic and Antarctic communities (Fig. 5.1).

	Arctic Ocean	Southern Ocean
C	nd	0.003
0.05	nd	0.012
0.5	nd	0.007
5	0.051	0.031
50	0.263	0.213
500	2.552	1.213

Table 5.2. Pb content (μg) per unit Chlorophyll *a* (μg). (nd) not detectable.

Growth and decay rates calculated for the different populations forming the communities indicated high variability in the sensitivity of the different phytoplankton groups. In the case of Hg, the decay rates observed increased as the concentration of Hg increased in the treatments, especially for the smallest phytoplankton cells, *Phaeocystis pouchetii* of the Arctic Ocean and picoeukaryotes from the Southern Ocean, that showed maximum decay rates of 0.72 and 3.17 day^{-1}, respectively (Table 5.3).

	ARCTIC OCEAN				
CADMIUM (μg L^{-1})	Flagellates	Small diatoms	Medium diatoms	Large diatoms	Chloro-phyll *a*
C	-0.11	-0.14	-0.06	0.07	-0.09
0.05	-0.16	-0.22	0.06	0.27	-0.1
0.5	-0.17	-0.18	0.15	0.35	-0.08
5	-0.25	-0.21	0.18	0.38	-0.08
50	-0.19	-0.19	0.13	0.25	-0.05
500	-0.16	-0.12	0.1	0.23	-0.02
LEAD (μg L^{-1})	Flagellates	Small diatoms	Medium diatoms	Large diatoms	Chloro-phyll *a*
C	0.24	0.33	-0.09	-0.17	-0.07
0.05	0.17	0.29	-0.32	-0.05	-0.1
0.5	0.26	0.32	-0.26	-0.17	-0.08
5	0.22	0.29	-0.19	-0.04	-0.01
50	0.26	0.39	0.03	-0.07	0.16
500	0.25	0.33	0.01	-0.07	0.1
MERCURY (μg L^{-1})	*Phaeocystis pouchetii*		Nano-plankton		Chloro-phyll *a*
C	-0.15		-0.01		-0.06
0.1	-0.2		-0.07		-0.05
1	-0.23		-0.11		-0.02
50	-0.37		-0.4		-0.59
500	-0.39		-0.4		-0.59
1000	-0.72		-0.53		-0.58

Table 5.3. Growth and decay rates (day^{-1}) of the different groups forming the natural phytoplankton communities examined in the Arctic Ocean.

However, while in the case of the Arctic Ocean the populations always decayed when exposed to the all treatments tested (Table 5.3), in the case of the Southern Ocean, at the lowest concentrations of Hg, growth rates increased for all the groups forming the phytoplankton community (Table 5.4).

	SOUTHERN OCEAN					
CADMIUM (μg L^{-1})	Picoeu-karyotes	Flage-llates	Small diatoms	Medium diatoms	Large diatoms	Chloro-phyll *a*
C	0.29	0.1	0.06	0.15	-0.09	0.23
0.05	0.24	0.08	0.01	0.14	0.19	0.25
0.5	0.3	0.06	0.04	0.16	0.2	0.23
5	0.27	0.1	-0.02	0.17	0.2	0.23
50	0.23	0.07	-0.03	0.03	0.09	0.17
500	0.18	-0.03	-0.03	0.03	0.16	0.12
LEAD (μg L^{-1})	Picoeu-karyotes	Flage-llates	Small diatoms	Medium diatoms	Large diatoms	Chloro-phyll *a*
C	0.09	0.13	0.41	0.54	0.42	0.27
0.05	0.02	0.11	0.39	0.4	0.28	0.26
0.5	0	0.19	0.42	0.49	0.41	0.24
5	0.04	0.17	0.45	0.48	0.39	0.26
50	-0.02	0.14	0.4	0.57	0.46	0.15
500	-0.13	-0.04	0.16	0.16	0.04	-0.06
MERCURY (μg L^{-1})	Picoeu-karyotes	Flage-llates	Small diatoms	Medium diatoms	Large diatoms	Chloro-phyll *a*
C	0.33	0.16	0.16	0.11	0.1	0.31
0.05	0.29	0.16	0.13	0.1	0.03	0.3
0.5	0.3	0.16	0.18	0.11	0.11	0.3
5	0.12	-0.04	0.11	0.02	-0.3	0.16
50	-3.17	-0.3	-0.12	-0.21	-0.28	-0.62
500	-	-	-	-	-	-0.54

Table 5.4. Growth and decay rates (day^{-1}) of the different groups forming the natural phytoplankton communities examined in the Southern Ocean. (-) not observed.

When Cd toxicity was tested, we observed that for the Arctic Ocean at the highest concentrations the growth rates of the medium diatoms (0.152 and 0.179 day^{-1}, for 50 and 500 $\mu g\ L^{-1}$ respectively), and large diatoms (0.346 and 0.375 day^{-1}, for 50 and 500 $\mu g\ L^{-1}$ respectively) increased, but the populations of the smallest diatoms and flagellates decreased showing decay rates in all the treatments (Table 5.3). In the Southern Ocean, at the lowest Cd concentrations growth was observed, with a smoothing of the growth rates as Cd concentrations increased, but showing decay rates for the smallest flagellates and diatoms at the highest Cd concentrations tested (Table 5.4). Similarly, at the lowest Pb concentrations, the Southern Ocean populations showed growth rates, with values smoothing as Pb concentrations increased and showing decay rates at the highest Pb concentration tested, 500 $\mu g\ L^{-1}$, for the picoeukaryotes and the small flagellates, -0.13 and -0.04 day^{-1}, respectively (Table 5.4). In the Arctic Ocean, at all the Pb concentration treatments tested, Chlorophyll *a* showed decay rates, which values smoothed as Pb concentrations increased but showing growth rates at the highest Pb concentrations, 50 and 500 $\mu g\ L^{-1}$, respectively (Table 5.3). However, these changes in the growth and decay rates varied strongly among the different phytoplankton groups, showing growth when exposed to Pb the smallest flagellates and diatoms, although the population of larger diatoms decayed (Table 5.3), with the medium sized diatoms growing at the highest Pb concentrations, 50 and 500 $\mu g\ L^{-1}$, respectively (Table 5.3).

These differences in the sensitivity to the metals added between the different Arctic phytoplankton populations varied greatly (Fig. 5.2). While Hg toxicity was very high, resulting in the decrease of all the populations present, the sensitivity to Cd and Pb showed opposite responses of the phytoplankton groups forming the community. The addition of Cd decreased the abundance of the smallest flagellates

and diatoms (Fig. 5.2), but increased their abundances when Pb was added. By the contrary, the opposite trend was observed for the medium and large sized diatom populations, decreasing when exposed to Pb and increasing when exposed to Cd (Fig. 5.2).

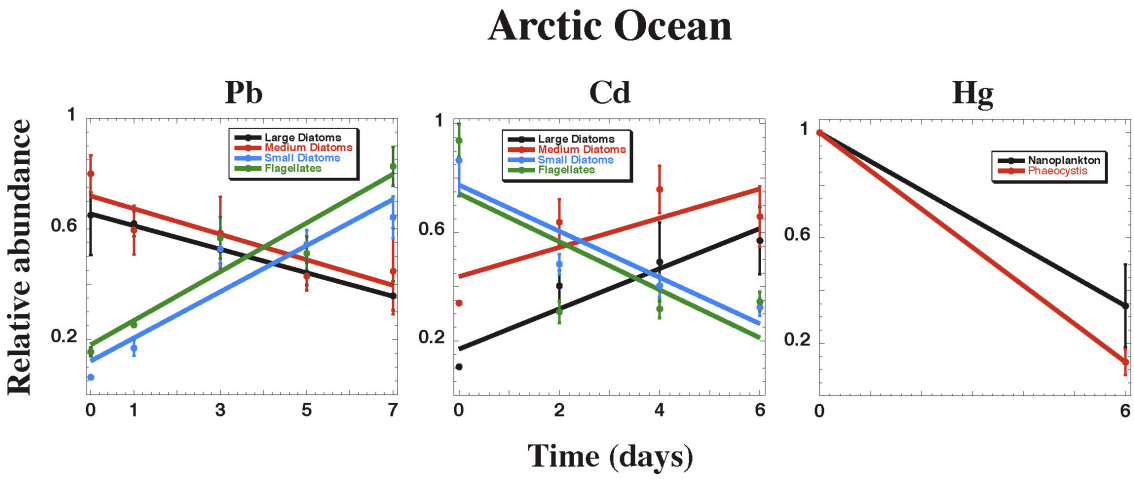

Figure 5.2. Relative abundances of the phytoplankton populations analyzed during the experiments with Cd, Pb and Hg (μg L^{-1}) in the Arctic Ocean.

When the decrease of the phytoplankton populations was observed, the 50% Lethal Thresholds (LC50), the metal concentrations required to reduce the 50% of the population, were calculated (Table 5.5). In the case of Hg, LC50s varied from 1.2 μg L^{-1} for the nanoplankton of the Arctic Ocean to 18.47 μg L^{-1} for the small diatoms of the Southern Ocean (Table 5.5), while in the case of Cd and Pb, it was only possible to calculate the LC50s for the Sothern Ocean populations. Thus, in the case of Cd, LC50s varied from 407.7 μg L^{-1} for the medium size diatoms to 1188.9 μg L^{-1} for the picoeukaryotes (Table 5.5), while in the case of Pb, the LC50s varied from 146.23 μg L^{-1} for the medium diatoms to 362.5 μg L^{-1} for the picoeukaryotes (Table 5.5). Some of these LC50s are only approximated values, since high errors were obtained due to smooth decreases of the population with the increase of the trace metals concentrations. The 10% Lethal Thresholds (LC10), representing the metal

concentrations required to reduce the 10% of the population, were lower than those of the LC50 but still high (Table 5.5), been only on average, 6.5 times lower. The consideration of LC10 is relevant because a decrease of 10% in population would already have a dramatic effect on the ecosystem and marine carbon cycle.

		CADMIUM		**LEAD**		**MERCURY**	
		LC50 (μg L^{-1})	**LC10 (μg L^{-1})**	**LC50 (μg L^{-1})**	**LC10 (μg L^{-1})**	**LC50 (μg L^{-1})**	**LC10 (μg L^{-1})**
Arctic Ocean	Picoeukaryotes	-	-	-	-	1.7 ± 0.9	0.3 ± 0.1
	P. pouchetii	-	-	-	-	2.3 ± 1.1	0.4 ± 0.2
	Nanoplankton	-	-	-	-	1.2 ± 0.3	0.2 ± 0
	Chlorophyll *a*	X	X	X	X	1.6 ± 0.3	0.2 ± 0
Southern Ocean	Picoeukaryotes	1189 ± 839	181 ± 128	280 ± 141	43 ± 21	2 ± 0.1	0.3 ± 0
	Small nanoplk	-	-	363 ± 55	55 ± 8.3	-	-
	Flagellates	412 ± 153	63 ± 23	304 ± 96	46 ± 15	13 ± 4.2	1.9 ± 0.6
	Small diatoms	1038 ± 4444	181 ± 128	169 ± 69	26 ± 11	18.5 ± 7	2.8 ± 1.1
	Med diatoms	408 ± 501	62 ± 76	146 ± 81	22 ± 12	17 ± 3.4	2.6 ± 0.5
	Large diatoms	X	X	175 ± 107	27 ± 16	15 ± 13	2.2 ± 1.9
	Chlorophyll *a*	387 ± 130	59 ± 20	116 ± 50	18 ± 7.6	7.6 ± 0.9	1.2 ± 0.1

Table 5.5. Cadmium, lead and mercury LC50 and LC10 (μg L^{-1}) values obtained for each phytoplankton group in all the experiments carried out. (X) not measurable. (-) not observed. (*P. pouchetii*)*Phaeocystis pouchetii*; (Small nanoplk) Small nanoplankton; (Med diat) Medium diatoms.

The induction of cell death was also analyzed in the treatments where the concentrations of metals were below the LC50 values. In these treatments, despite not observing a catastrophic decline in the cell abundance, induction of cell death was detectable by measuring an increase in the proportion of dead cells. In some cases, this increase was moderate or even not detectable, but in others, increases were extremely high, representing increases of 60-75% of dead cells at the highest metal concentrations tested with respect to the lowest treatments (Fig. 5.3). However, often these increases were not directly related to decreases in the cell abundance. In the Arctic Ocean, the addition of the highest concentrations of Pb or Cd (500 μg L^{-1}) did not result in catastrophic cell deaths, but instead, induced the growth of some groups:

the smallest diatoms and flagellates in the case of Pb and the medium and large diatoms in the case of Cd. However, as the concentration of Pb in the water increased, the percentage of dead cells in the smallest diatoms and flagellates increased (Fig. 5.3), as happened when Cd was added, which also induced cell death of the medium diatoms (Fig. 5.3). In the Southern Ocean, the highest concentrations of the metals added derived in dramatic decreases in the population abundances, but lower concentrations also induced cell death (Fig. 5.3), as observed with the medium diatoms when Pb was added (Fig. 5.3), with the flagellates when Cd was added (Fig. 5.3) or with the small diatoms when Hg was added (Fig. 5.3). In all these populations, despite cell growths were observed, the percentage of dead cells increased as the metal concentrations in the treatments increased. Increases in cell abundances due to increases in metal concentrations may not be necessarily related to enhanced growth, since cell death can be induced too (Fig. 5.3), but be related to decreases in other cell losses, such as grazing of the populations.

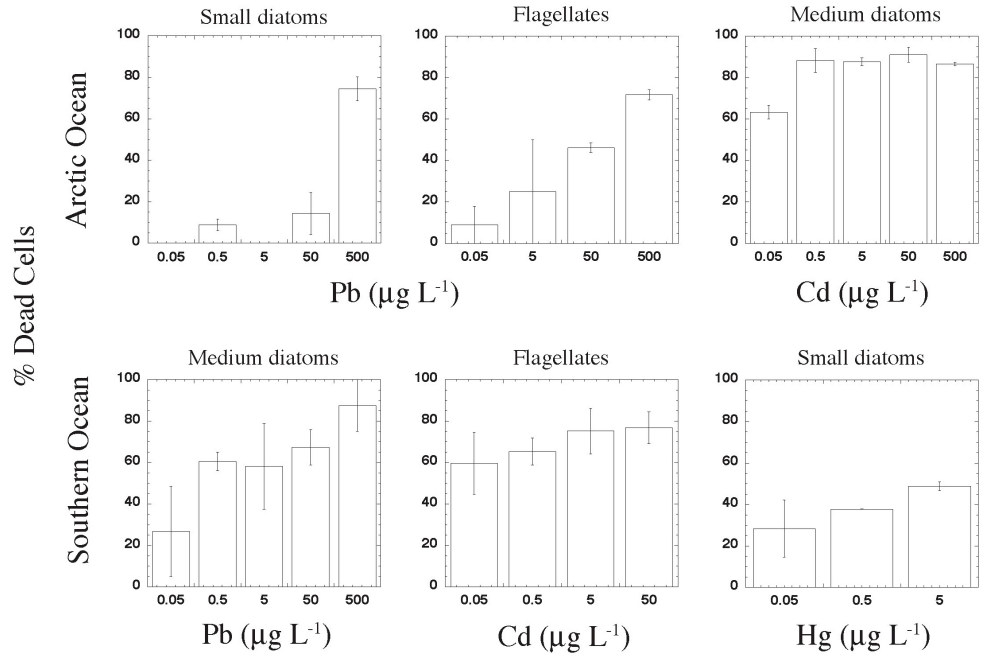

Figure 5.3. Changes in the proportion of dead cells of different phytoplankton populations of the Arctic and Southern Oceans at low and high concentrations of Cd, Pb and Hg (μg L^{-1}).

At the end of the experiments, the differences in cell size and populations diversity induced by metals were analyzed too. In many cases, the exposure to high concentrations of metals significantly ($p<0.05$) changed the average cell size of the populations (Table 5.6), as well as some changes in the community composition. In the Arctic, centric diatoms such as *Chaetoceros* sp. were the dominant groups, except in the experiment with Hg, where *Phaeocystis pouchetii* was the dominant species. Here changes in the average cell size of the populations were only observed when the large diatoms were exposed to high concentrations of Pb, showing significant higher sizes with the increasing concentrations of Pb (Table 5.6). In the Southern Ocean, where pennate diatoms were the predominant populations, Cd did not significantly force changes in the average size of the different populations (Table 5.6), although the proportion of small pennate diatoms increased. Hg only induced changes in the smallest diatoms and flagellates, significantly decreasing their body sizes (Table 5.6), despite the disappearance of the medium and large sized centric diatoms (mostly *Thalassiosira sp.*). Pb also induced significant changes in the Antarctic populations, increasing the average size of the largest diatoms but decreasing that of the small sized diatoms (Table 5.6) and that of the medium sized diatoms (Table 5.6), where centric diatoms disappeared.

	Arctic Ocean						**Southern Ocean**			
	Flag	Sd	Md	Ld	*P. p.*	Npl	Flag	Sd	Md	Ld
Cd	0	0	-0.01	-0.03	-	-	0	0	-0.01	0.02
Pb	0	0	0	0.15*	-	-	0	-0.01*	-0.01*	0.06*
Hg	-	-	-	-	0.01	-0.02	-0.02*	-0.09*	-0.03	-0.12

Table 5.6. Differences in cell size induced by cadmium, lead and mercury for each phytoplankton group in the Arctic and Southern Oceans. Values represent the slope of the relationship between the average ESD (μm) and the metal concentration ($\mu g\ L^{-1}$). Positive slope values imply increases in the average size, although Negative values imply a decrease. (-) absent population. (Flag) Flagellates; (Sd) Small diatoms; (Md) Medium diatoms; (Ld) Large diatoms; (*P. p.*) *Phaeocystis pouchetii*; (Npl) Nanoplankton;.

4. Discussion

Most part of the studies on behalf the toxicity of trace metals to natural marine phytoplankton have been focused on the phytoplankton community of temperate and cold areas of the Northern hemisphere exposed to Cu (Goering et al., 1977; Thomas and Seibert, 1977; Kuiper, 1981a; Mann et al., 2002; de la Broise and Palenik, 2007; Debelius et al., 2009;), Hg (Knauer and Martin, 1972; Davies and Gamble, 1979) or Cd (Kuiper, 1981b; Echeveste et al., submitted). The present work contributes with new data to the few (or almost absent to our knowledge) data concerning the acute and chronic levels of Cd, Pb and Hg to natural polar phytoplankton communities.

The results of cell death induction obtained revealed a high variability in the sensitivity of phytoplankton to the three trace metals tested. Hg has been identified as the most toxic trace metal, followed by Cu or Cd, and then Ag and Pb (Sorentino, 1979; Rai et al., 1981), and our results also showed this trend as phytoplankton was more sensitive to Hg than to Cd or Pb. Although previously Cd was found to be more toxic to oceanic phytoplankton than Pb (Echeveste et al., submitted), no significant differences were found in the degree of toxicity of Cd and Pb, as indicated by the similar LC50s obtained for each metal. Even though, Pb induced more changes at the community composition level, which will be explored later.

The most surprising results of this work were concerned to the high resistance of some Arctic populations to the toxicity of Cd and Pb, especially for the latter. Although previously the proliferation of insensitive species of temperate areas exposed to Cu were reported (Thomas and Seibert, 1977), no previous records with Cd or Pb were found in polar waters.

Despite at high concentrations Cd acts as toxicant, it has been observed that it can act too as nutrient at low (<0.11 μg L^{-1}, Echeveste et al., submitted) or medium

concentrations (1-50 μg L^{-1}, Tkachenko et al., 1974; Kuiper, 1981b). Cd may be used by phytoplankton populations substituting Zn, a nutritive metal that is a co-factor for the enzyme carbonic anhydrase, which catalyses a critical rate-limiting step for carbon use in photosynthesis (Goldman and Horne, 1983; Price and Morel, 1990). However, even at the highest concentrations (500 μg L^{-1}) tested here, Cd had a positive rather than a toxic effect for the medium and large diatoms. It is therefore hypothesized that, probably as a result of the development of different resistant mechanisms against metals toxicity (González-Dávila, 1995), Arctic diatoms may be using Cd as nutrient. The results observed in this work and in previous works (Echeveste et al., submitted) may be agree with Finkel and colleagues' findings (2007), as they observed that while cyanobacteria presented a metabolic sensitivity to Cd, diatoms instead presented a high metabolic demand to Cd.

However, it was even more surprising the response of Arctic phytoplankton populations to Pb, as they showed the highest cell abundances at the highest concentrations tested. Although a previous work performed by Malanchuk and Gruendling (1973) observed an increase in the CO_2 fixation of the cultured species *Ochromonas malhamensis* in the presence of Pb, it is quite improbable that Pb could have positive effects on phytoplankton as any physiological use of Pb has been yet described. To our knowledge, and based on previous hypothesis (Kuiper, 1981a; Thomas and Seibert, 1977), probably Pb induced the death of the populations of cilliates and protists, avoiding the graze of the smallest phytoplankton groups as Pb concentrations increased. These authors hypothesized that trace metal additions may have decreased the number of zooplankton organisms, and thereby the grazing pressure on the larger phytoplankton, probably as a consequence of zooplankton preference of large species over small microflagellates (Gamble et al., 1977).

Although the Arctic and the Antarctic phytoplankton communities assimilated Pb in a same degree, significant differences were observed between these two communities, being the former resistant to Pb and the later sensitive.

It has been observed that the exposition to trace metals such as Cu (Thomas and Seibert, 1977) or Hg (Kuiper, 1981a) can vary the phytoplankton community structure. Thomas and Seibert (1977) observed that Cu addition favored the presence of microflagellates and pennate diatoms, probably due to a lower grazing pressure as previously noticed. By instance, Kuiper (1981a) observed that Hg favored larger species and argued that the lower surface to volume ratio of larger species may be the reason explaining their higher resistance to metal stress. Although no remarkable changes in the community composition were observed among the treatments in all our experiments, in some cases changes in the average size of the different groups were observed, specially in the Southern Ocean community. Although Pb increased the average size of the largest Arctic and Antarctic diatoms, it decreased that of the smallest and medium sized Antarctic diatoms, as did Hg with the smallest Antarctic flagellates and diatoms. These increases or decreases in the average size of the population did not necessary mean that the size of the species was changing, but rather that a selection to more resistant species was occurring. Even though, more experiments should be perform to determine the general trend of marine phytoplankton exposed to metals towards larger or smaller sized cells.

The LC50s obtained in this work are agree with previous works that found cell size as an important parameter explaining phytoplankton sensitivity to trace metals (Goering et al., 1977; Thomas and Seibert, 1977; Kuiper, 1981a; Echeveste et al., submitted). Polar populations, of larger size, showed in general higher resistance to the trace metals studied, as observed by their higher LC50s observed in comparison of

those obtained for smaller sized groups (Echeveste et al., submitted). This is due to their lower surface to volume ratio, which derives in a slower metal uptake and a higher resistance. However, Arctic and Antarctic communities showed different sensitivities to the metals tested. Arctic communities were more resistant to Cd and Pb than those from Antarctica, which showed lower LC50s and changes in the community structure. Due to the closeness to contaminant sources, which may regularly increase Cd and Pb concentrations in seawater, Arctic communities may have developed adapting mechanisms, a process already observed with Cd in temperate areas (Echeveste et al., submitted). In the case of Hg, despite the higher concentrations in the Arctic, Antarctic communities were more resistant, but this was probably due to the higher size of the populations of Antarctica in comparison of those found in the Arctic (mostly *Phaeocystis pouchetii*).

The LC50s obtained in this work represented in overall 3-5 orders of magnitude higher concentrations than those observed in these polar waters (Honda et al., 1987; Muir et al., 1992; Tovar-Sánchez, personal communication). For example, Hg concentrations in the Arctic Ocean (Muir et al., 1992) were on average of 0.037 μg L^{-1}, while the LC50 observed for *Phaeocystis* sp. was 2.3 μg L^{-1}. In the Arctic, Cd and Pb concentrations were 0.034 and 0.006 μg L^{-1}, respectively, while in the Southern Ocean Cd and Pb concentrations were 0.058 and 0.016 μg L^{-1}, respectively (Tovar-Sánchez, personal communication), representing lower values than those previously reported (Honda et al., 1987; Muir et al., 1992). These concentrations were much lower than the LC50s reported here, as observed for example for the Antarctic flagellates, 303.5 μg L^{-1} Pb and 412 μg L^{-1} Cd, respectively. However, and as a decrease by a half of the phytoplankton community would have dramatic consequences for the ecosystem, the LC10s were also calculated, as a 10% reduction

of the phytoplankton community would already have a significant effect on the ecosystem. These LC10s would still represent higher concentrations than those observed in the polar seawaters by 2-4 orders of magnitude, but as trace metals do not impact individually and, besides, there other environmental factors such as UVR or temperature that enhance their impact, they may already be impacting phytoplankton communities.

Moreover, we were able to calculate the half lives for those populations suffering catastrophic cell death, varying from 0.2 days to more than 30 days. Half lives depended on the trace metal tested, the phytoplankton population considered and the trace metal concentration. Thus, shorter half lives were observed as the trace metal concentrations increased, specially when Hg was added, as it induced shorter half lives due to its higher toxicity. Moreover, generally for the same metal and concentration tested, the lowest sized phytoplankton groups showed the lowest half lives.

When the trace metals concentration treatments analyzed here did not result in a substantial decline of the population, the induction of cell death was revealed from our direct estimates of the proportion of living cells within the populations. By doing this analysis, we detected an increase in the percentage of dead cells even at the treatments where a decline in the total population was not detected. Thus, as the concentrations of Cd, Pb or Hg increased, the proportion of dead cells increased, resulting in increases of more than 20% of the dead cells in the treatments with the concentrations close to the LC50s found. These results are agree with previous works (Echeveste et al., 2010a; Echeveste et al., submitted), which showed that the increase of the contaminants derived in an increase of the percentage of dead cells below the LC50s.

As pointed by Chapman and Riddle (2003, 2005), there is a need of more research on behalf the biogeochemical cycles occurring in polar ecosystems and how they influence marine organisms, including phytoplankton. Polar regions are increasingly subject to anthropogenic chemical contamination, and although it is assumed their impact on marine organisms, it is yet not clear in which degree they affect them, as observed in the case of arctic phytoplankton, where the mortality induced by Cd or Pb was absent for some phytoplankton populations.

5. Conclusion

The results of cell death induction obtained in this work revealed a high variability in the sensitivity of polar phytoplankton to Hg, Cd and Pb between Arctic and Southern Ocean communities. Mercury was the most toxic metal for both communities, being Cd and Pb toxic too for Antarctic communities but not for those of the Arctic. Arctic communities were resistant to high concentrations of Cd and Pb, probably due to a reduction in the grazing. Cell size played a key role in determining phytoplankton sensitivity to the metals studied, being the lowest cells the most sensitive due to their higher surface to volume ratio. Through changes in the average cell size of the groups forming the phytoplankton community, we were able to observe changes in the community structure, where more resistant species might have been selected due to higher resistances to the metals added. However, unclear trends were obtained, precluding the determination whether phytoplankton may enlarge or reduce its size to resist pollutants presence.

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General Discussion

Oceanic phytoplankton plays a crucial role in the marine food web and the global biogeochemical processes (Berger et al., 1989; Falkowski and Woodhead, 1992; Field et al., 1998). In the oceans, phytoplankton is often exposed to many environmental stressors (Suttle, 1992; Berges and Falkowski, 1998; Llabrés and Agustí, 2006) that can induce phytoplankton cell death, including exposition to toxic pollutants (i.e., Mann et al., 2002). This doctoral thesis was born with the idea of providing new data on behalf the toxicity of pollutants to marine phytoplankton, an effect clearly defined… but poorly analyzed (Kennish, 1997). With this aim, many experiments with pollutants were performed in different oceanic regions to define the thresholds and points of no return of the oceanic phytoplankton exposed to pollution. Moreover, the parameters determining phytoplankton sensitivity to pollutants were also examined.

The results obtained showed that cell size was the principal parameter determining phytoplankton sensitivity to pollutants. As previously noticed, many cell processes such as growth or maximal abundance depend on the species cell size (i.e., Banse, 1976; Agustí et al., 1987) and phytoplankton sensitivity to pollutants does as well, being the smallest phytoplankton cells more sensitive to pollutants than larger cells. Uptake of light and nutrients and its release as waste products and heat is influenced by the surface/volume ratio, being higher for the smallest cells (Lewis, 1976). The introduction of pollutants into the cell also depends in this ratio (Del Vento and Dachs, 2002; Fan and Reinfelder, 2003). Thus, we observed that *Prochlorococcus* and *Synechococcus*, the smallest picocyanobacteria, were the most sensitive to PAHs such as pyrene or phenanthrene (Echeveste et al., 2010a) or metals

such as Cd or Pb (Echeveste et al., submitted). This fact may force the sensitivity of phytoplankton in oligotrophic seawaters such as the Mediterranean Sea or the Atlantic Ocean, where picophytoplankton populations dominate the phytoplankton community and are, therefore, the main primary producers of these ecosystems (e.g. Agawin et al., 2000; Alonso-Laita and Agustí, 2006). Looses of these populations due to contamination could derive in the collapse of the marine food chain, as the picophytoplankton communities are those dominating in these oligotrophic conditions (Chisholm, 1992; Raven, 1998; Agawin et al., 2000; Irigoien et al., 2004).

However, cell size is not the only parameter determining phytoplankton sensitivity to pollutants, since we observed that similar sized phytoplankton may show different sensitivities according to the growing conditions. This was observed when cultured species exposed to pyrene and phenanthrene were compared to similar sized natural communities exposed to both PAHs (Echeveste et al., 2010a). Due to optimal growing conditions and continuous exposition to higher concentrations of contaminants, cultured species appeared to be more resistant than natural communities (Echeveste et al., 2010a). Organisms have developed a wide range of behavioural, morphological, physiological and life-history strategies to face external environmental factors, both at large and short timescales, also called macro and microevolution, respectively, leading to the selection of resistant species (Bijlsma and Loeschcke 1997, 2005). Similarly, and through an intense search in the literature of almost 300 articles, we were able to observe this difference between cultured species and natural phytoplankton communities exposed to various metals. This search, centred in the effects of Hg, Cd or Pb to both freshwater and marine phytoplankton, also showed a significant ($p<0.05$) higher resistance of cultured phytoplankton species with respect to natural communities (Fig. GD.1).

It is remarkable that most of the experiments were performed in laboratory conditions with cultured species (92%), in contrast with those performed with natural communities (8%), showing the important contribution of this doctoral thesis to increase the knowledge of the effects of these metals in natural phytoplankton communities. Moreover, it is astonishing that almost any of these studies was performed with pico-sized phytoplankton, although they are the dominant species in oligotrophic oceans. The results of the experiments performed during this doctoral thesis provide some thresholds to this lack of data.

Thus, when we compared the slopes for the different metals tested, we did not observe significant ($p>0.05$) increases in the toxicity to Hg related to decreases in cell size (Fig. GD.1), however, for the two other metals analyzed we observed that the toxicity of Pb significantly ($p<0.05$) varied with cell size (Fig. GD.1), while in the case of Cd, this toxicity significantly ($p<0.05$) varied for the cultured species and greatly ($p>0.05$) for the natural communities. These results pointed that low variations in the cell size determine big differences in the toxicity of metals to phytoplankton (Fig. GD.1). When we analyzed the intercepts of the graphs, we observed that Hg was the most toxic metal, followed by Cd, and being Pb the less toxic, which confirms the toxic degree of those metals to phytoplankton included in the literature (Sorentino, 1979; Rai et al., 1981). However, in the case of metals, in contrast with the results observed with PAHs (Echeveste et al., 2010a), the slopes did not significantly vary between cultured species and natural communities, meaning that in both cases cell size, and not the growing conditions, strongly explained the sensitivity to these metals.

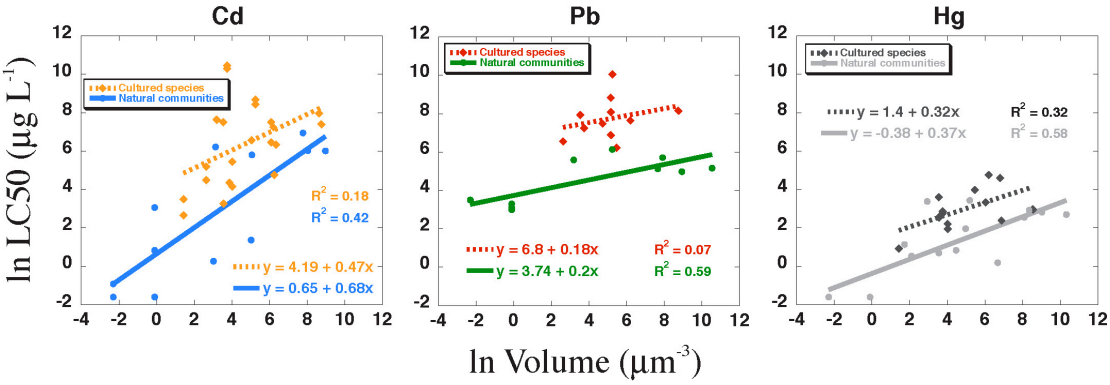

Figure GD.1. Relationship between Cd, Pb and Hg LC50s (μg L^{-1}) and phytoplankton cell volume (μm^3). The continuous lines represent the fitted linear regressions for the natural communities examined during this thesis, while the discontinuous lines represent the fitted linear regressions for cultured phytoplankton data from the literature. (Cd_Cult) $p<0.05$*; (Cd_Nat) $p>0.05$; (Pb_Cult) $p<0.05$*; (Pb_Nat) $p<0.05$*; (Hg_Cult) $p>0.05$; (Hg_Nat) $p>0.05$.

Many power rules have been described controlling phytoplankton metabolic processes. Maximal cell abundance was assumed to decrease as a reason of -3/2 log cell size (Agustí et al., 1987), while others found that growth decreased with size as power rule of -3/4 (Banse, 1976). When analyzed the allometric relationships of each of the individual pollutants used during this thesis (Cd, Pb, Hg, pyrene and phenanthrene), it was observed that generally, except in the case of Cd, the sensitivity to pollutants decreased with cell size in a power rule between -1/5 and -1/3, while Cd sensitivity decreased by -2/3. This lower power rule of the sensitivity to pollutants compared to other metabolic processes implies larger cells to be less sensitive than smaller, indicating that the largest cells may have developed higher adapting capacities probably due to their higher complexity, which may give them the capacity to face pollutants toxicity (López-Rodas et al., 2006; Ipatova et al., 2008). Moreover, it would point that the smallest cells would be proportionally more sensitive, as the toxic slope would be lower than those of the maximum abundance and growth.

As pointed before, cell size is not the only parameter explaining phytoplankton sensitivity to pollutants, since the environmental factors, through evolving behaviours, morphologies, etc., also determine different sensitivities/resistances in organisms (Bijlsma and Loeschcke, 1997, 2005), This fact was also observed among the different natural communities tested. For example, Black Sea phytoplankton exposed to Cd was more resistant than Mediterranean and Atlantic phytoplankton (Echeveste et al., submitted), probably due to a recurrent exposition for several generations to higher concentrations of contaminants in these areas, including metals such as Cd. Thus, this exposition may select more resistant communities in polluted areas compared to those of unpolluted areas, which was previously observed (Say et al., 1977; Gavis et al., 1981; Murphy et al., 1982; Foster, 1982). This may be one of the reasons why Arctic phytoplankton was more resistant to Cd and Pb than similar sized phytoplankton of the Southern Ocean, as these communities are more exposed to pollution from the near industrial zones (Echeveste et al., submitted).

Organic pollutants may induce too structural changes in phytoplankton communities, including genetic adaptations to grow in polluted areas with crude oil (Carrera-Martínez et al., 2010, 2011). To test whether organic pollutants may already been inducing changes in natural phytoplankton communities, we analyzed the effects of natural cocktails of organic pollutants in natural phytoplankton communities of the Mediterranean Sea, the Atlantic Ocean and the Southern Ocean. As picophytoplankton dominates the Mediterranean and Atlantic communities, these communities may be more vulnerable to these pollutants due to their lower cell size. However, we observed that Antarctic communities, of larger cell size, were more sensitive than those of the oligotrophic seawaters (Echeveste et al., in preparation),

showing that the proximity to the contaminant sources (i.e. Mediterranean communities) could force the selection of resistant strains (Echeveste et al., in preparation). Moreover, these results would point that Antarctic communities, due to the remoteness of the main contaminant sources (i.e. Northern Hemisphere), may have not yet been exposed to enough pollution to induce genetic adaptation (Echeveste et al., in preparation). Pollutants can induce rapid genetic changes or small-scale evolutionary processes, the so-called microevolution due to pollution (Klerks, 1989; Medina et al., 2007), and the results of this thesis point to this assumption.

However, it is necessary to remark that mainly all the points discussed until now have been achieved with experiments developed with single pollutants. Single pollutants exert a significant negative effect in natural communities at concentrations highly above those found in seawater (Echeveste et al., 2010a; Echeveste et al., submitted). However, in nature phytoplankton communities are always exposed to numerous pollutants acting altogether in seawater. By analyzing the effects of single (pyrene and phenanthrene), simple mixtures (of 16 PAHs) and complex mixtures of organic pollutants (those occurring in seawater), it was observed that increasing the pollutants complexity increased some orders of magnitude the toxicity observed for single pollutants (Echeveste et al., 2010b), and showed that the natural cocktails of organic pollutants may already be affecting phytoplankton communities at concentrations close to those found in seawater (Echeveste et al., 2010b). This fact would show that not only cell size, but also contaminants complexity, may determine the sensitivity of oceanic phytoplankton to pollutants.

Moreover, in nature phytoplankton communities are not only affected by the presence of myriads of contaminants, but also by variations in the environmental

conditions, such as nutrient limitation and light deprivation (Berges and Falkowski, 1998) or ultraviolet radiation (UVR) (Llabrés and Agustí, 2006), that also affect phytoplankton communities. In our experiments, UVR significantly increased PAHs toxicity, showing the phototoxicity of organic pollutants (Echeveste et al., 2011), and showed that phytoplankton sensitivity to PAHs and UVR depended on cell size and UVR levels received. In the future, the combination of pollutants' toxicity and the incoming environmental changes will have dramatic consequences to organisms, not only through alterations of the organisms' homeostasis, which may exacerbate the adverse effects of contaminants (Heugens et al., 2001; Gordon, 2003), but also through indirect effects. For example, the bioavailability and toxicity of pollutants in nature may increase in response to rising temperatures and salinity (Cairns et al., 1975; Sokolova and Lanning, 2008; Heugens et al., 2001; Schiedek et al., 2007).

It is estimated that there are 80 000 to 100 000 pollutants on Earth (USEPA, 1998), and each year about 1700 new chemicals are released to the environment (Cairns, personal communication). This is why global pollution, together with climate change, the loss of biological diversity and other six drivers (Rockström et al., 2009), has become one of the main threats to the Earth system. Although measurable, the effects of many pollutants have not been properly analyzed in the environment. Many of these contaminants are not quantifiable as there have not yet been developed appropriate techniques to measure them, avoiding, therefore, the quantification of the impacts of each contaminant in the ecosystem.

This doctoral thesis has provided new tools to understand the toxicity of pollutants to oceanic phytoplankton. Since most of the knowledge is based on the results with cultured tests, it is remarkable that probably the scientific community may have underestimated the impact of pollutants to oceanic phytoplankton, as they

may be more sensitive to them than we thought. This doctoral thesis provides valuable data concerning the effects of pollutants to natural phytoplankton communities, especially in the case of picophytoplankton, which data were almost absent until now. Moreover, the parameters determining phytoplankton sensitivity to pollutants have been addressed, and cell size was found to be the principal parameter determining phytoplankton sensitivity to pollutants, but not the unique. Other aspects such as growth in different ocean basins or the joint action of multiple pollutants and UVR were also found to significantly determine the sensitivity or resistance of the phytoplankton communities. Since some of the experiments were performed considering future environmental scenarios, the present thesis also contributed to the understanding of the effects of pollutants to oceanic phytoplankton in a changing world.

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Conclusions: Research Highlights

1. During this thesis several thresholds and points of no return of different POPs and trace metals to marine phytoplankton have been defined and quantified.

2. Cell size has been identified as the major parameter determining phytoplankton sensitivity to pollutants, being the smallest species the most sensitive due to its higher surface to volume ratio, which favours pollutants uptake.

3. The induction of cell death by pollutants, as observed by increases in the percentage of dead cells, has been also detectable at concentrations lower than the thresholds values observed to cause catastrophic cell death.

4. Cultured species are less sensitive to pollutants exposure than natural communities, as a result of the optimization of the growth conditions or to the selection of more resistant strains in the laboratory.

5. Despite high concentrations of trace metals are highly toxic for oceanic phytoplankton, the Arctic phytoplankton community was found to be highly resistant.

6. Low concentrations of toxic trace metals (i.e. Cd and Pb) were beneficial for some oceanic phytoplankton communities as observed by smooth increases in cell abundances or decreases in the percentage of dead cells.

7. The concentrations of individual pollutants found in the ocean are significantly below the lethal thresholds observed for natural phytoplankton communities.

8. The mixture of 16 PAHs increased an order of magnitude the induced toxicity to respect that of single PAHs (pyrene and phenanthrene).

9. The natural complex mixture of POPs increased by an order of magnitude the toxicity observed for the mixture of PAHs, indicating that POPs in the oceans may impact phytoplankton communities at concentrations close to those presently found in seawater.

10. UVR enhances PAHs toxicity, showing synergetic effects for the pico-sized phytoplankton and additive effects for nano and micro-sized phytoplankton. Moreover, this synergy was also observed for the whole communities of the Mediterranean Sean and the Atlantic Ocean, which are oligotrophic waters dominated by picophytoplankton.

11. Different sensitivities were found among phytoplankton communities growing in different ocean basins exposed to different contamination levels, probably as a result of the selection of resistant strains.

12. More experiments with natural communities are needed to assess the real impacts of global pollution to oceanic phytoplankton.

13. Although cell size was found to be an important parameter explaining phytoplankton sensitivity to pollutants, it is not the unique, and it is therefore necessary to consider other aspects to understand the variability observed in the phytoplankton community, such as adaptation processes by growing in different ocean basins or the joint action of multiple pollutants and environmental stressors.

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