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Thresholds of contaminants: A synthesis

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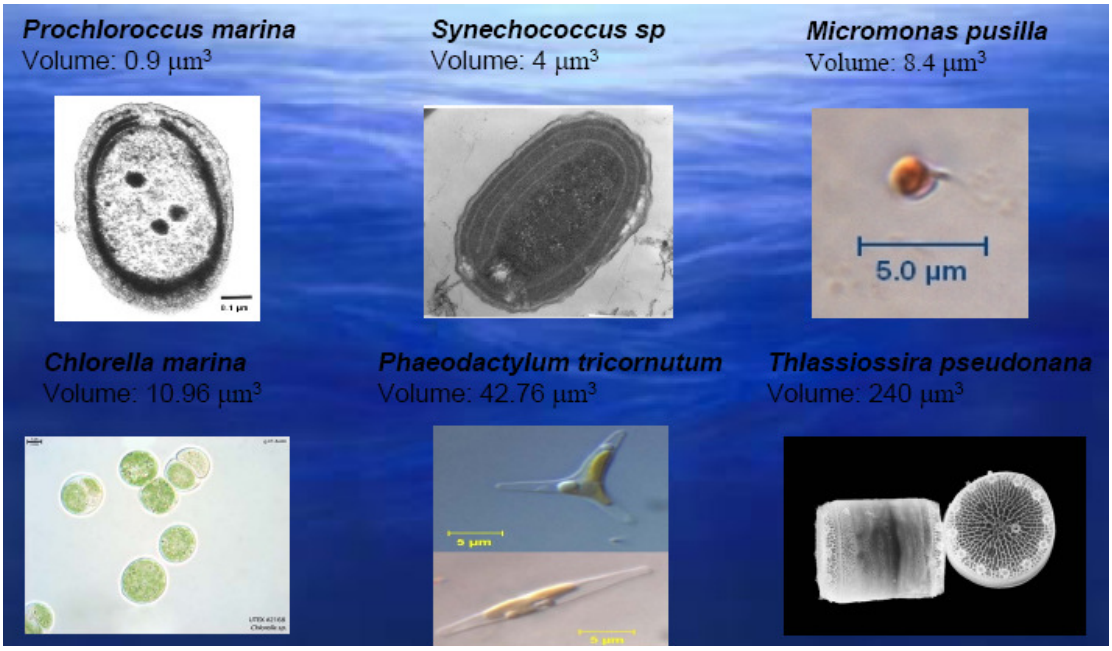

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1. Introduction

A fundamental problem in ecotoxicology is the prediction of population and ecosystem-level effects of contaminant exposure based on dose response data of individuals. Furthermore, long term effects are not assessed when analysing dose-response of few individuals over a short period of time and even no-effect dose concentrations could have a long term effect at population or at ecosystem level. In addition, environmental fluctuations will always affect significantly the population/ecosystem response. Therefore, population/ecosystem resilience to these fluctuations may be affected by a contaminant even though these effects are not observed under dose-response experiments on individuals.

Concerning population-level response a recent study by van Kirk and Hill (2007) suggests that when there are density-dependence relationships, the response to a contaminant may be lower than the one obtained at individual level up to a certain point when toxic effects outweigh compensatory mechanism at this level, after the effects increase sharply. For these reasons, the authors propose a stochastic modelling approach based on individual effects for analysing population response.

At ecosystem level we can find indirect effects (see Fleeger et al., 2003 for a recent survey). These effects even on tolerant species occur by other ecological mechanisms rather than toxic effects, e.g. direct influences of contaminants on predators can lead to cascading indirect effects on resistant species in other trophic levels by altering competitive interactions and therefore modifying substantially its abundance and dynamical behaviour. Such effects are called indirect (or secondary) contaminant effects (Flegger et al., 2003) and sometimes can be as or more significant that the direct (toxic) effects of a contaminant. Ecological models have become effective tools in evaluating indirect effects (Bartell, 1996; Pastorok et al., 2003). In addition, ecological models may be applied to forecast future potential risks or to estimate risks when field experiments cannot be performed, i.e. the release of a new chemical into the environment. They are useful tools for testing alternative hypothesis or to reconstruct past situations where evidence of toxic exposure cannot be demonstrated.

On top of that, the number, diversity and complexity of chemicals produced and released to the environment is overwhelming, and despite this there is only information regarding environmental fate and/or impact on ecosystem and human health for a small fraction (Swoboda-Colberg, 1995).). In fact, of the more than 100,000 synthetic organic chemicals in use (Howard and Muir 2006), the number of chemicals of environmental concern is unknown, and there are environmental fate and transport and ecotoxicological data for less than 1% of the total anthropogenic chemical classes.

Aquatic ecosystems are rarely exposed to only one single contaminant, but typically to mixtures of numerous man-made chemicals with varying constituents in varying concentration ratios (Faust et al., 2003). However, in contrast to this environmental reality, the toxicological reality is that until recently about 95% of the resources were devoted to studies of single chemicals (Groten, 2000). Nevertheless, toxicity data from laboratory tests with single pure chemicals provide essential input to scientific assessments of chemical risks to aquatic ecosystems. On the other hand, the behaviour of chemicals in a mixture may not correspond to that predicted from data on the pure compounds (Altenburger et al., 2003). But the direct testing of all the potential combinations of water contaminants is unfeasible, and thus we are confronted with the task of deriving valid predictions of multiple mixture toxicity from toxicity data on individual compounds (Faust et al., 2003). Therefore, combined exposure is a reality that dictates the necessity to pay a great deal of attention to hazard identification, exposure assessment and risk characterization of mixtures at individual as well as ecosystem level.

In the Thresholds Project we have analyzed some of these questions by using experiments, data analysis tools and modelling approaches. This report is a synthesis of the main findings on these open problems.

2. Toxic effects: Molecular level response[1]

Earlier effects of toxicity of a chemical occur at molecular level. For this reason, the effects of three polycyclic aromatic hydrocarbons (PAHs): pyrene, fluoranthene and benzo[a]pyrene either as single compound or as a mixture were selected to investigate alterations at gene expression level by Real-Time PCR. The marine diatom *Thalassiosira pseudonana* was the selected species because its genome has been recently completely sequenced (Armbrust et al., 2004).

Dose-response curves for growth inhibition were determined (see square symbols in Figs. 2.1 and 2.2.) and four concentrations eliciting from “no effect” up to severe growth inhibition were chosen for further investigation to analyse alterations at gene expression level.

Among the eight selected genes, two were strongly influenced by the PAH treatment: *lacsA*, which is involved in the fatty acid metabolism, was found to be strongly up-regulated by all single PAHs as well as by the mixture (see Fig. 2.1); *sil3*, involved in the formation of the silica shell, was repressed by a factor up to three even at low PAH concentrations not eliciting

[1] Taken from: Bopp, S. K. and Lettieri, T., 2007. Gene regulation in the marine diatom *Thalassiosira pseudonana* upon exposure to polycyclic aromatic hydrocarbons (PAHs). *Gene* **396**, 293-302.

any growth inhibition (see Fig. 2.2). These genes can potentially serve as sensitive and specific biomarkers.

lacsA is involved in biosynthetic pathways of fatty acid derived molecules and it plays an important role in the accumulation of polyunsaturated fatty acids in triacylglycerols (Tonon et al., 2005). Since PAHs are strongly hydrophobic, they tend to accumulate in lipids and it is known that they induce oxidative stress and lipid peroxidation (Kelly et al., 1998). Therefore this gene was a candidate for checking if PAHs could affect the lipid metabolism. It was found that *lacsA* was up-regulated in *T. pseudonana* by PAHs, although stronger induction was found mainly for higher exposure concentrations where also growth was significantly impaired.

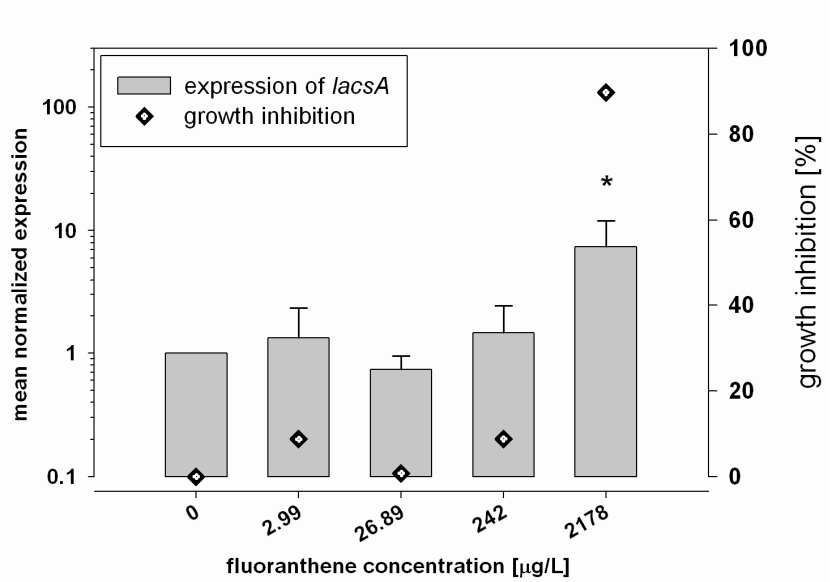

Figure 2.1. Regulation of the *lacsA* gene in *T. pseudonana* due to exposure to fluoranthene. Gray bars represent the relative normalized gene expression of *lacsA* measured in samples from three independent experiments using Real-Time PCR. Vertical lines indicate the standard error of triplicate samples in the Real-Time PCR. Asterisks indicate significant differences from the solvent control sample (One way ANOVA followed by Dunnett test, $p<0.05$). Square symbols represent the growth inhibition (right Y-axis) observed in parallel for the investigated PAH concentrations.

The second clearly regulated gene was *sil3*. Silaffins cause, in combination with long chain polyamines in supramolecular assemblies, the deposition of silicic acid and thus initiate the formation of the silica shell (Poulsen and Kroeger, 2004). According to Frigeri et al. (2006), mRNA levels of *sil1* and *sil3* correspond to distinct stages in cell wall synthesis, i.e. *sil1* levels are enhanced during girdle band formation and *sil3* levels peak during valve formation. Thus, a down-regulation of *sil3* by exposure to PAHs might inhibit the formation of valves and consequently lead to reduced cell division and growth rates. In the current study *sil3* was clearly down-regulated by factors of 2 to 3.5, even at concentrations that did not affect the growth rate. Therefore, it might be an early marker of cell division impairment. *sil1* and *sil2* did not show a clear trend in gene expression after exposure to PAHs. Even if these two genes

are not influenced by PAHs, the formation of the silica shell as a multilevel process might be severely disturbed due to the downregulation of *sil3*.

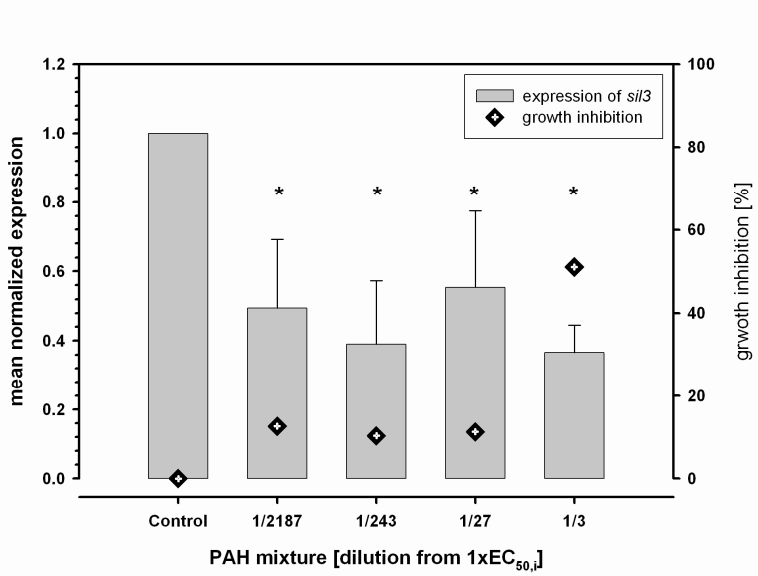

Figure 2.2. Regulation of the *sil3* gene in *T. pseudonana* due to exposure to a mixture of three PAHs. Gray bars represent the relative normalized gene expression of *sil3* measured in samples from three independent experiments using Real-Time PCR. Vertical lines indicate the standard error of triplicate samples in the Real-Time PCR. Asterisks indicate significant differences from the solvent control sample (One way ANOVA followed by Dunnett test, $p<0.05$). Square symbols represent the growth inhibition (right Y-axis) observed in parallel for the investigated PAH concentrations.

Molecular effects were found at concentrations, where no growth inhibition was detected. Our data clearly show that molecular approaches may be useful to develop sensitive biomarkers for detecting environmental exposure in aquatic organisms.

3. Toxic effects: Individual level response

Laboratory experiments were performed with unispecific phytoplankton cultures to analyze the thresholds for the lethal concentration of some PAH's for populations of these organisms. A solution of PAHs dissolved in Acetonitrile, Pyrene dissolved in Methanol and Pyrene and Phenantrene dissolved in DMS were added to exponentially growing cultures of *Prochlorococcus marina* (CCMP1375), *Synechococcus sp* (CCMP833), *Chlorella marina*, *Dunaliella sp*, *Micromonas pusilla*, *Phaeodactyllum tricornutum* and *Thlassiossira pseudonana* (CCMP1335) (see Fig. 3.1).

3.1. CULTURE AND EXPERIMENTAL CONDITIONS

The cultures grew in batch cultures under optimal temperature of 18ºC, but 21ºC for *Synechococcus* and *Prochlorococcus,*and under continuous light conditions, in a nutrient-rich medium (F/2 medium, except *Prochlorococcus marina*, which growth in Pro-99 medium), inside 5 litter glass bottles. Once populations entered in an exponentially growing stage, the

culture was dispensed into different policarbonate bottles of 250 ml volume (2 replicates for each volume) where a gradient of increasing PAHs concentration, including single compounds and mixtures, were added to exponentially growing cultures. The lethality of a solution of a mixture of PAHs dissolved in acetonitrile, pyrene dissolved in methanol, and pyrene and phenantrene dissolved in DMS were tested. Two replicates without adding PAHs were run as controls, and two more replicates with added acetonitrile, methanol or DMSO at the final concentration equivalent to that of the highest PAHs concentration treatment were performed as a control of the effect of the solvents. The evolution of the population was followed from a total of 4 days (*Prochlorococcus marina, Synechococcus sp.*) to a maximum of 28 days (*Dunaliella sp*), depending on the population response.

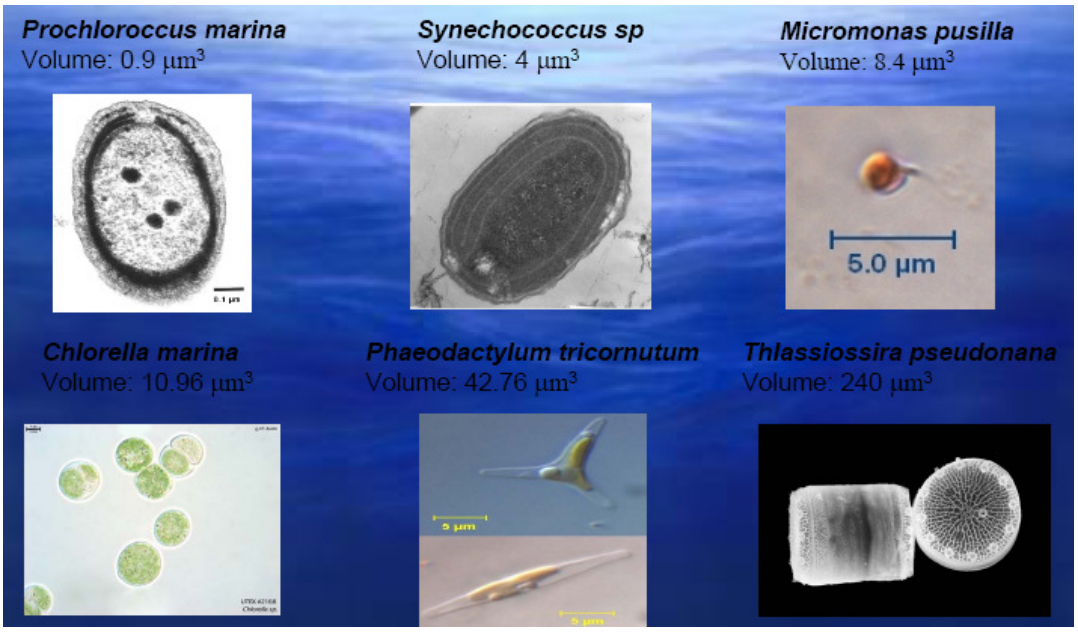

Figure 3.1. Main characteristics of the cell cultures studied.

The changes in the population abundance were followed by sampling the cultures daily or each two days, depending on the species growth rate. Fresh samples were analyzed by flow cytometric techniques using a a FACSCalibur flow cytometer. The proportion of living and death cells in the different populations were followed by applying a cell membrane permeability test, the cell digestion assay (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 Trypsine solution (1% in HBSS) were added, followed by 30 minutes incubation at 35°C.

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

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

where δ_b is the cell death rate (d^{-1}), x is the concentration of living cells, $(x+y)$ is the total concentration and y is the concentration of dead cells. 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.

3.2. RESULTS

The experiments performed with acetonitrile and methanol, used as solvents to prepare the PAHs solutions, indicated that the amounts of the solvents used were toxic for phytoplankton, as indicated by the cell death induced in the solvent controls. However, Pyrene and Phenantrene dissolved in DMSO did not induce lethality and the results presented are restricted to the experiments performed with Pyrene and Phenantrene dissolved in DMSO. The concentrations of Pyrene and Phenantrene analyzed varied from 5 to 1000 $\mu g \cdot L^{-1}$.

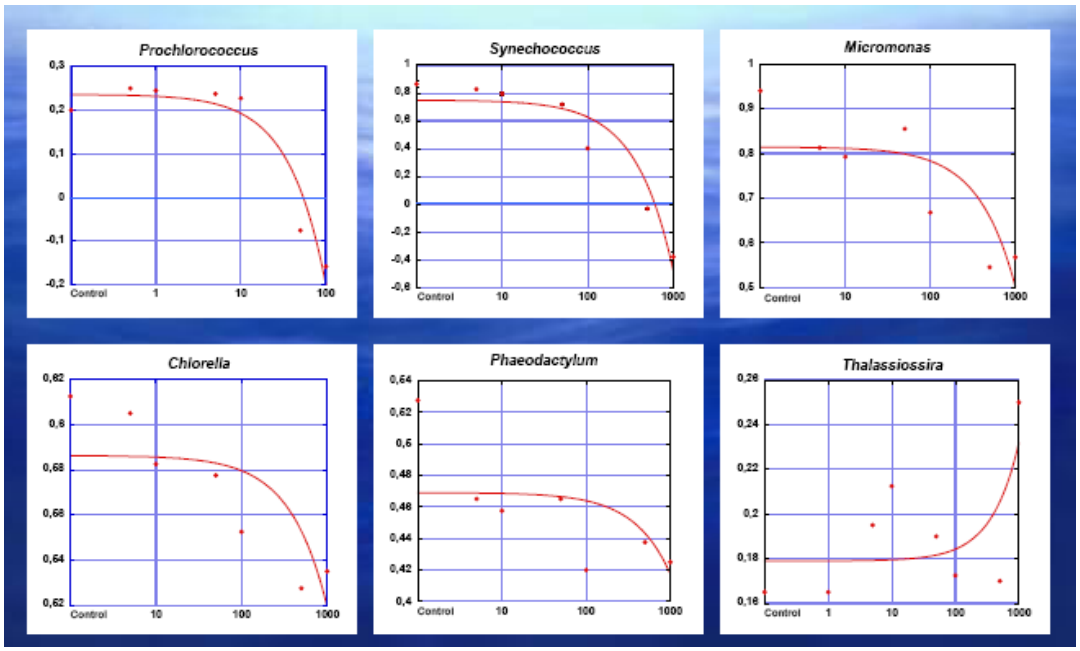

Figure 3.2. Variation of growth rates (d^{-1}) as a function of Pyrene concentrations ($\mu g\ L^{-1}$) (logarithmic scale) dissolved in DMSO.

The results (see figs. 3.2-3.6) indicate that most species were quite resistant to Pyrene and Phenantrene, since there were not population mortality detected, except for the picosized species *Prochlorococcus marina* and *Synechococcus sp.*, which showed strong lethality at high concentrations of Pyrene and Phenantrene.

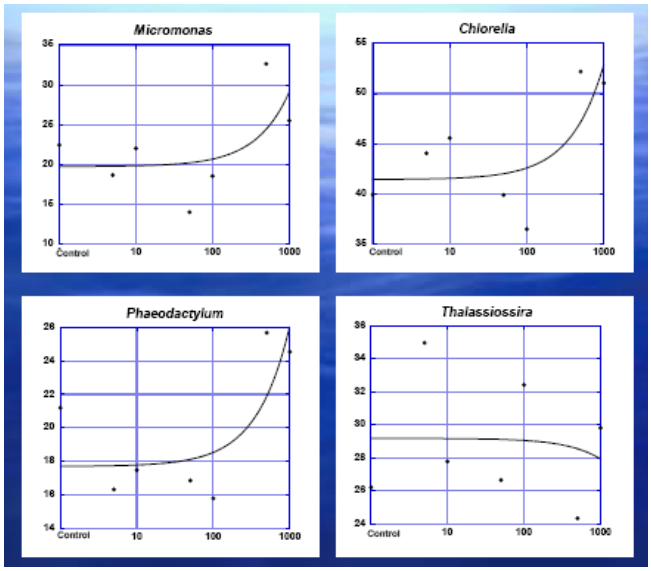

Figure 3.3. Variation of dead cell (%) as a function of Pyrene concentrations (µg L^{-1}) (logarithmic scale) dissolved in DMSO.

The lethal threshold concentration (50% abundance) for Pyrene was 80 µg·L^{-1} for *Prochlorococcus marina*, whereas for Phenantrene it was 175 µg·L^{-1}. *Synechococcus sp* appeared to be more resistant than *Prochclorococcus marina* for Pyrene (350 µg·L^{-1}) and less for Phenantrene (150 µg·L^{-1}).*Chlorella marina*, *Dunaliella sp*, *Micromonas pusilla*, *Phaeodactyllum tricornutum* and *Thlassiossira pseudonana* (CCMP1335), did not show catastrophic mortality of their populations when growing at the concentrations of pyrene and phenantrene tested. However, we could detect some effect of Pyrene and Phenantrene toxicity as, for example, *Chlorella marina*, showed an important decrease in growth rate as the Pyrene concentration increased (Fig. 3.2). Expected lethal dosis (50% abundance) for *Micromonas pusilla* *Chlorella marina*, *Phaeodactyllum tricornutum* and *Thlassiossira pseudonana* are 1550, 4700, 7300 and 35800 µg L^{-1}, respectively; whereas considering (50% living cells) the values are 75, 60, 80 and 940 µg L^{-1}, respectively.

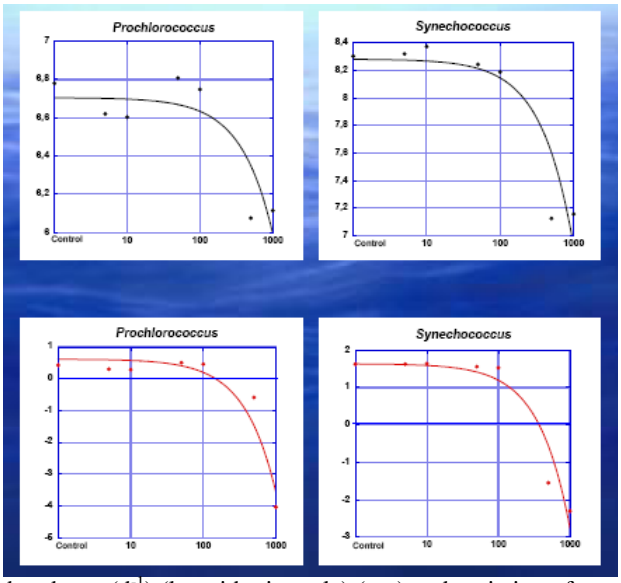

Figure 3.4. Variation of Abundance (d^{-1}) (logarithmic scale) (top) and variation of growth rates (d^{-1}) (bottom) as a function of Phenantrene concentrations (µg L^{-1}) (logarithmic scale) dissolved in DMSO for *Prochlorococcus marina* and *Synechococcus sp*.

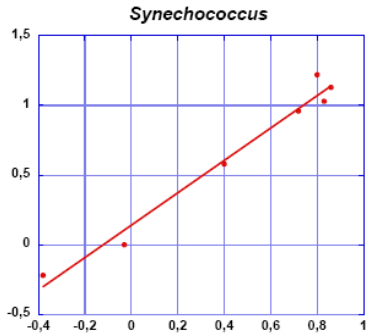

Figure 3.5. Dead rate (d^{-1}) as a function of growth rate (d^{-1})for *Synechococcus sp* (CCMP833).

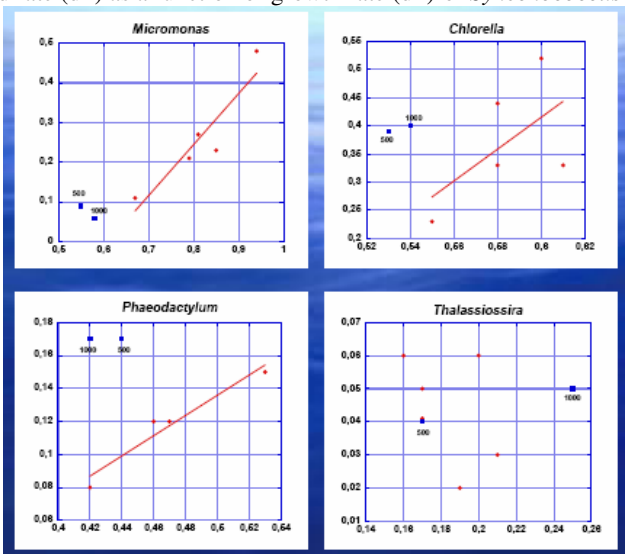

Figure 3.6. Dead rate (d^{-1}) as a function of growth rate (d^{-1}) for *Micromonas pusilla, Chlorella marina, Phaeodactyllum tricornutum and Thlassiossira pseudonana* (CCMP1335).

4. Toxic effects: Population level response

Plankton abundance and community composition were studied during the two oceanographic cruises THRESHOLD-1 (2006) and THRSHOLD-2 (2007), on board the RV Garcia del Cid, along the Mediterranean and Black Seas.

Experiments to analyze the lethal threshold of PAH's and metals on natural populations of phytoplankton were performed with Mediterranean and Black Sea plankton, sampled during the oceanographic cruises.

4.1. PLANKTON ABUNDANCE

The abundance of bacteria and phytoplankton was quantified during the cruises in all the stations and depths sampled. Niskin bottles attached to a rosette-CTD system were used for sampling. Samples for bacteria and picophytoplankton were taken and analyzer on board using flow cytometric techniques using a Becton & Dickinson FACScalibur bench machine with a laser emitting at 488 nm. Samples for bacteria were stained for a few minutes with Syto13 (Molecular Probes) at 2.5 µM, and run through the fow cytometer at low speed, and data were acquired in log mode until around 10,000 events had been acquired. We added a small volume (10-20µL) per sample of a calibrated solution of yellow-green 1 µm Polysciences latex beads as an internal standard.

Bacteria were detected by their signature in a plot of side scatter (SSC) vs. green fluorescence (FL1) as suggested by del Giorgio et al. (1996). Picophytoplankton communities composed by *Synechoccoccus*, *Prochlrococcus* and eukaryotic phytoplankton was quantified in fresh samples by using the same flow cytometer. An aliquot of a calibrated solution of 1 µm diameter high-green fluorescent beads (Polysciences) was added to the samples as an internal standard for the quantification of cell concentration. Bead concentration in the standard solution was calculated by filtering replicated aliquots onto black nuclepore filters and counting the beads under an epifluorescence microscope. The red, green and orange fluorescence emissions, and the forward and side scattering of the cells and beads, were used to detect different cell populations and to differentiate them from the fluorescent beads.

Chlorophyll *a* concentration was measured in all the stations and depths sampled as a quantification of total phytoplankton abundance. 50-100 ml of water was filtered through Wahtman GF/F filters and the pigment extracted in 90% acetone for 24 hours. After extraction, filters were centrifuged and the fluorescence of the supernatant reader ina Shimadzu RF2400 spectrofluorimeter calibrated following Parsons et al. (1984).

4.2. DESCRIPTION OF EXPERIMENTS

The experiments consisted on incubate replicated bottles on deck incubators, simulating seawater and air conditions (light, temperature, etc.). The experiments, 8 altogether, were carried out with PAH's (pyrene and phenantrene), metals (Cadmium and Lead) and methanol. For the PAH's and metal experiments we run duplicated bottles of 5-6 treatments plus duplicated controls. Experiments began with surface water collection from sea in day 0 and inoculation of the contaminant, following daily the effects of these contaminants in the communities of picoplankton and microplankton during 4 days. The concentrations used in the treatments varied for Cadmium and Lead from 0.01 ppb to 112 ppb-1000 ppb, and for Pyrene and Phenantrene from 5 $mg \cdot L^{-1}$ to 500 $mg \cdot L^{-1}$. Changes in total phytoplankton abundance during the experiments were followed by analyzing Chlorophyll *a* concentration. The effect of PAH's and metals on the picophytoplankton community was followed by analyzing changes in the abundance of *Prochlorococcus* sp, *Synechococcus* sp and Eukaryote picoplankton by using a flow cytometer.

4.3. RESULTS

Synechococcus showed lethality to high lead and cadmium concentrations although total phytoplankton community was resistant, see figs. 4.1 and 4.2. In Lead experiment, *Synechococcus* lethality was independent of population size; whereas thresholds for Lead and Cadmium were similar for Mediterranean phytoplankton.

Table.4.1. Lethal doses (50% abundance) of Cadmium and Lead in ppm.

	Cadmium		**Lead**	
	Synechococcus	**Chlorophyll-a**	**Synechococcus**	**Chlorophyll-a**
Mediterranean Sea	2	3	16	30
Black Sea	17	550	16	3000

Table.4.2. Lethal doses (50% abundance) of Pyrene and Phenantrene in $\mu g\ L^{-1}$.

	Pyrene		**Phenantrene**	
	Synechococcus	**Chlorophyll-a**	**Synechococcus**	**Chlorophyll-a**
Mediterranean Sea	85	145	80	240
Atlantic Ocean	40		45	
Cell cultures	350		150	

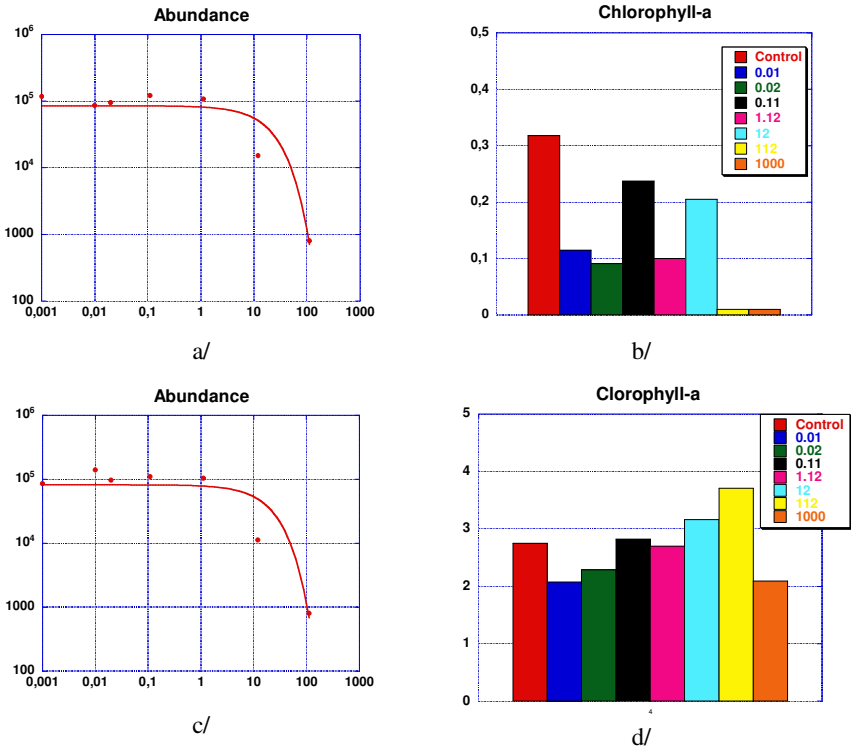

Figure 4.1. Lead effects as a function of its concentration ppm: a/ in Synechococcus (Mediterranean Sea); b/ total phytoplankton community (Mediterranean Sea); c/ in Synechococcus (Black Sea); d/ total phytoplankton community (Black Sea).

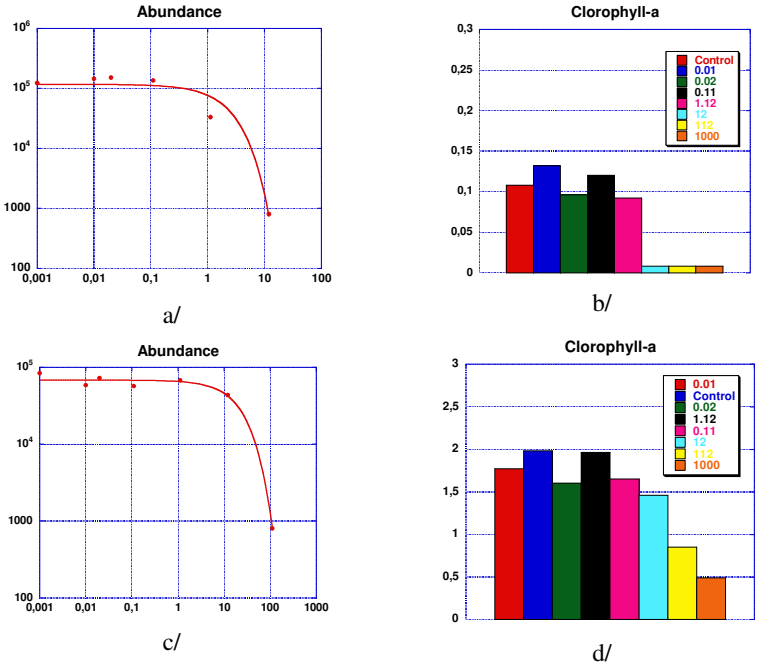

Figure 4.2. Cadmium effects as a function of its concentration ppm: a/ in Synechococcus (Mediterranean Sea); b/ total phytoplankton community (Mediterranean Sea); c/ in Synechococcus (Black Sea); d/ total phytoplankton community (Black Sea).

Results for Pyrene and Phenantrene are summarised in Table 4.2 and Figs. 4.3 and 4.4. Concerning the lethality thresholds obtained, these correspond to higher concentrations than those found at Mediterranean or Black Seas by one to two orders of magnitude. However, these values are lower, at least for PAHs, than those found for cell cultures by a factor of 2 or

3, indicating than controlled dose-response experiments can give inadequate results. In addition, there are also differences between different natural communities, i.e. Mediterranean and Black Seas and Atlantic Ocean, as it can be observed in Tables 4.1 and 4.2. The reasons for these differences between laboratory experiments and field conditions can be due to a number of processes. Under natural conditions, planktonic populations will be under a number of environmental pressures, such as nutrient availability, light and synergy of added PAHs with many of the thousands of trace level pollutants present in natural waters. Unfortunately, these interactions can not be elucidated here.

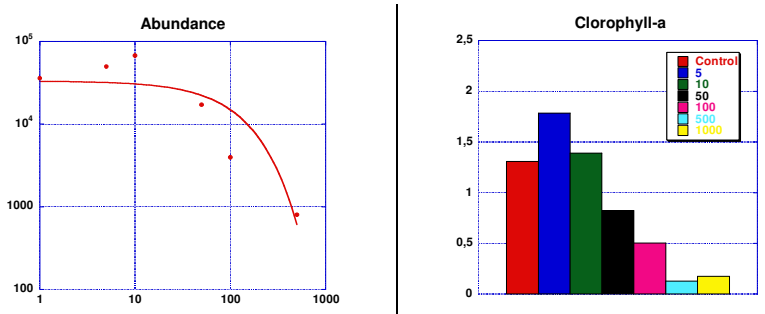

Figure 4.3. Pyrene effects as a function of its concentration (µg L^{-1}): a/ in Synechococcus (Mediterranean Sea); b/ total phytoplankton community (Mediterranean Sea).

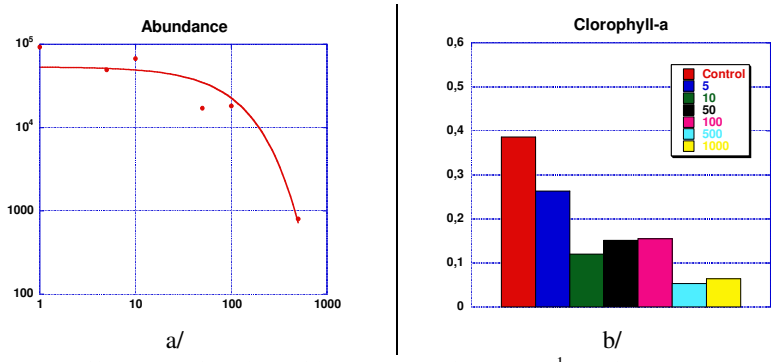

Figure 4.4. Phenantrene effects as a function of its concentration (µg L^{-1}): a/ in Synechococcus (Mediterranean Sea); b/ total phytoplankton community (Mediterranean Sea).

As it can be seen in Fig. 4.5, there are no toxic effects of Methanol in Bacteria, *Synechococcus* or total phytoplankton community at the studied concentrations.

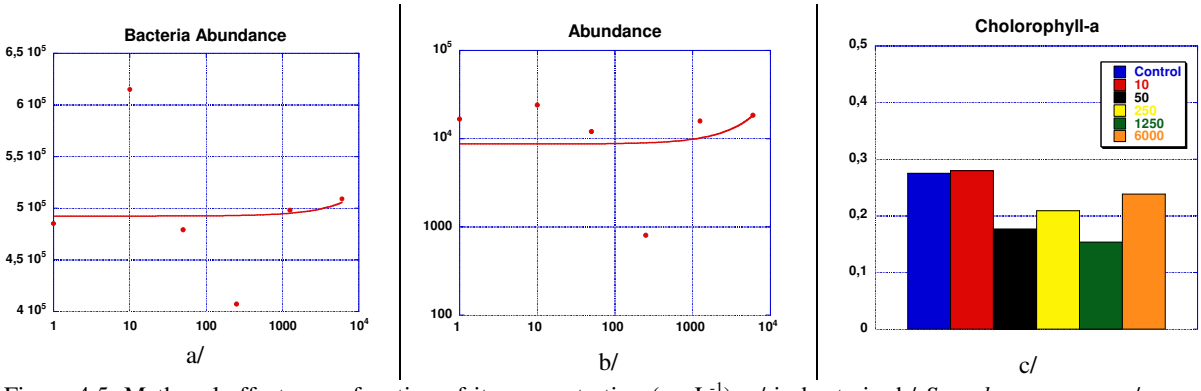

Figure 4.5. Methanol effects as a function of its concentration (µg L^{-1}): a/ in bacteria; b/ *Synechococcus* sp; c/ total phytoplankton community.

5. Toxic effects: Ecosystem level response

An integrated model including fate of and effects of contaminants on an ecological model was developed and presented in D4.3.4. The model was used to simulate the dynamic behaviour of the mesocosm experiments carried out at NERI in the Isefjord, Denmark (see D4.3.1-D4.3.3) for different conditions of nutrient and contaminant concentrations, to elucidate the combined effects of these two drivers at ecosystem level.

The model is composed of 6 main compartments: two phytoplankton groups, diatoms and flagellates (*Pd, Pf*), two zooplankton groups representing microzooplankton (< 200 μm) and mesozooplankton (0.2-2 mm) (*Zs, Zl*), bacteria (*B*) and detritus (*D*) to account for the mineralization of dead organic matter performed by the bacteria. Nitrate and ammonium concentration in the water column were considered as forcings. The model also requires meteorological data for wind (speed and direction), humidity, cloud coverage, temperature and rainfall. The meteorological forcing is very important for the food web model, especially for simulating primary production.

The comparison of the outcome of the simulations with the experimental results of the mesocosm showed that it was possible to represent the main dynamics observed in the mesocosm experiments over a relatively short time (11 days) with a rather simplified food-web model. This confirmed that the model contains the features necessary to represent the system correctly even on a small scale.

Therefore, we applied the model to study the ecosystem level response and specifically the variability of the response depending on the time of the release of a contaminant pulse and on the environmental conditions.

5.1. METHODOLOGY

Numerical experiments were carried out by running the model for a 3-months period (92 days) during spring, between 1st March and 31st May, using meteorological data of two different years, 2001 and 2002. The nutrient level was kept constant during the simulation at a concentration of 17.6 μmol/L for NO_3+NO_2 and 1.13 μmol/L for NH_3. The spring period was chosen in order to see the phytoplankton and zooplankton blooms and to check the sensitivity of the ecosystem response for a release of contaminant before, during and at the end of the bloom. The water temperature conditions obtained from the simulation using the forcing meteorological conditions are shown in Fig. 5.1. We observe that in 2002 the mean temperature of the water was slightly higher for the entire period of the simulations and this will affect the primary production of the system.

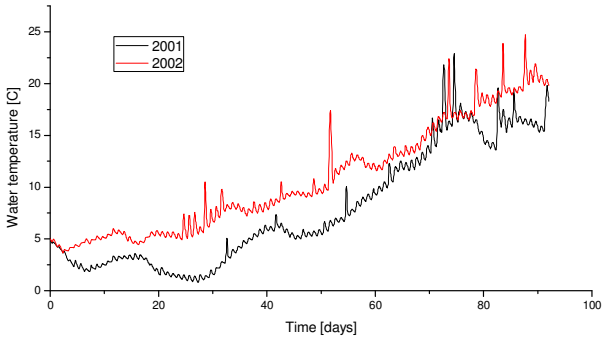

Figure 5.1. Water temperature obtained for 2001 and 2002

The result of the basis-run without contaminant addition for the year 2001 shows a phytoplankton bloom around day 55 followed by a zooplankton bloom and a bloom of the bacteria population (Fig. 5.2). Similarly, in 2002 there is first a phytoplankton bloom followed by a zooplankton bloom, but the phytoplankton bloom is anticipated to day 40 and has smaller amplitude (Fig. 5.3). The basis scenario of 2002 shows that at the end of the simulation phytoplankton population (flagellates) is growing towards a second bloom.

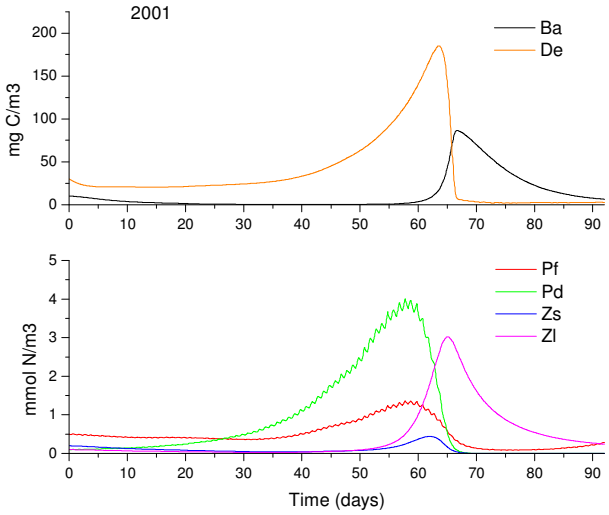

Figure 5.2. Basis scenario of the food-web for meteorological condition of 2001.

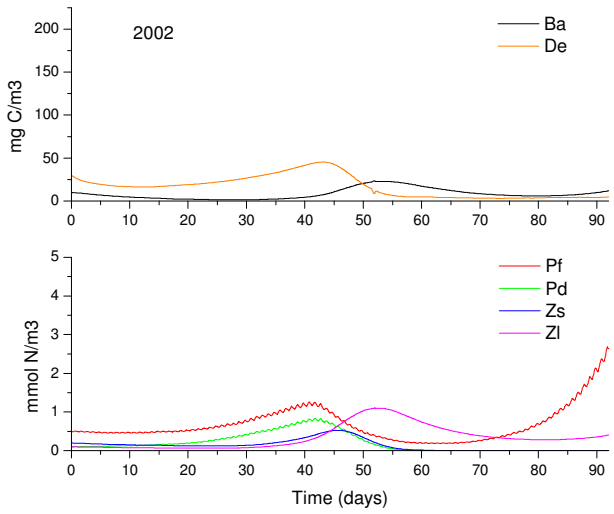

Figure 5.3. Basis scenario of the food-web for meteorological condition of 2002.

In the model the dose-response effects of pyrene have been simulated using the Weibull equation: $f(x) = 1 - \exp[-\exp(\theta_1 + \theta_2 \log_{10} x)]$. Therefore, the toxicity of pyrene for each compartment of the food web model is described by the parameters θ_1 and θ_2, which define the shape of the dose-response curve (Fig. 5.4). For diatoms and flagellates the dose-response curve was fitted to data from the mesocosm experiment (D 4.3.3) and data from a study on phytoplankton communities in Greenland (Hjorth 2005), while for zooplankton they were taken from an internet database http://www.pesticideinfo.org/ and other studies (Barata et al. 2005, Bellas and Thor 2007). No data has been found for bacteria, therefore the toxicity data have been assumed low and comparable with unicellular species in the above mentioned database.

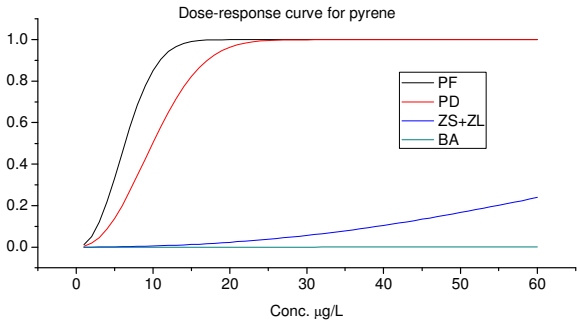

Figure 5.4. Dose-response curves used in the model.

5.2. RESULTS: ECOSYSTEM LEVEL RESPONSE

The ecosystem level response was tested for different concentrations of pyrene (from 0 to 50 µg/L) and different times of contaminant release. In fact the model was run three times, considering a pulse of contaminant before the phytoplankton bloom (day 21), during the increase (day 41) and at the end of the bloom (day 61).

To evaluate the ecosystem level response we had to define a criterion that could be compared between different simulations. For this purpose, we integrated the biomass, expressed in mmolN m^{-3}, of the 2 phytoplankton and 2 zooplankton populations over the simulation period. Thereafter, we calculated the sum, and normalized the value, meaning that the simulation without contaminant has a value of 1 (maximal total biomass) and the simulation with the minimum total biomass has a value of zero.

The graph representing the decrease of total biomass associated to each contaminant addition shows a similar trend for both years, 2001 and 2002, and for all times of contaminant inputs (Fig. 5.5). In all the simulations there is a decrease of the total biomass between 0 and 30 µg/L, corresponding to the shape of the dose-response curve for phytoplankton. Even though the basis scenarios are different, both years show the steepest decrease of total biomass for the addition of contaminant after the blooms (d61). On the other hand, we observe that the ecosystem is less able to recover when the pulse of contaminant is released before the blooms (d21).

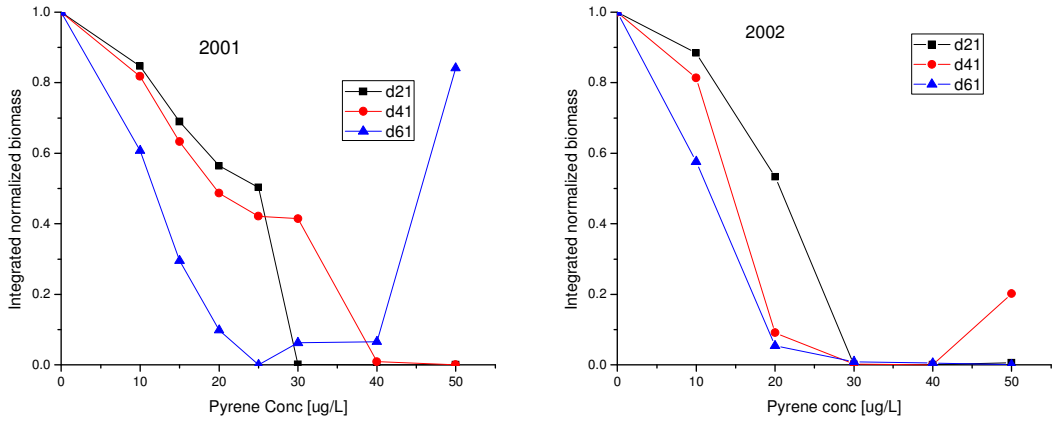

Figure 5.5. Integrated normalized biomass of the ecosystem for the different scenarios of pyrene input.

The results also show that in some cases the zero value does not correspond to the maximal contaminant addition, meaning that the minimum total biomass was reached before reaching the maximal concentration of contaminant. For example, the simulation of year 2001 for a contaminant addition at day 61 shows a final value of the biomass equal to 0.85. Moreover the simulation of 2002 shows a final increase of the biomass subjected to a contaminant pulse at

day 41. In both cases, even though the phytoplankton community was affected very strongly by the contaminant addition, the zooplankton populations still grows, because microzooplankton feeds on bacteria, which are increasing due to the increase of detritus in the system, and the large zooplankton feeds on the small zooplankton. However, this increase of the zooplankton population cannot persist without the primary producers.

From the results it is also possible to evaluate the variability of the ecosystem level response for a given contaminant input. The difference between the maximum and the minimum effect varies from 20% to 50%.

This example illustrates the difficulties faced when assessing ecosystem health or ecological risk due to the presence of a toxic pollutant based only on dose response data without taking into account the interactions within the ecosystem neither the environmental variability present in natural systems.

6. The regulatory approach

Article 16 of the Water Framework Directive (EC 2000) mandates the European Commission (EC) to select priority substances (PS) and identify priority hazardous substances (PHS) with a provision to review the list of PS at the latest four years after the entry into force of the WFD. Furthermore, the EC has to propose measures to control and reduce point and diffuse sources for the progressive reduction of PS and the cessation of PHS within 20 years after the adoption of the EC proposal. In addition quality standards applicable to the PS concentrations in surface water, sediments and biota have to be developed.

In 2001, a list of PS was adopted (Decision 2455/2001/EC) identifying 33 substances of priority concern at EU level, including a subset of PHS (11 and 14 as 'PS under review' for possible classification as PHS) that require the cessation or phase-out of discharges, emissions and losses within 20 years of adopting measures for that purpose.

A proposed Daughter Directive (COM (2006) 397) was developed by the EC and published in July 2006 in order to comply with the requirements of Article 16 of the WFD. The Directive has two objectives:

1 to ensure a high level of protection of the aquatic environment and human health by setting environmental quality standards (EQS) for the PS and certain other pollutants;
2 to define good chemical status in surface water bodies through compliance with EQS.

Environmental Quality Standards (EQS) are concentrations of pollutants or groups of pollutants which should not be exceeded in order to protect human health and the environment (COM (2006) 397). Some EQS were already defined at community level and most Member States had their own EQS. However, all these EQS varied considerably across

the EU. Therefore the EC decided to harmonise the EQS values based on toxicity and ecotoxicity data as well as bioaccumulation potential, environmental contamination and human risk.

EQS derivation is based on effects assessment methodology. This methodology based on a European consensus is described in the Technical Guidance Document (TGD) in support of European regulation on new notified, existing substances and biocidal products (European Commission, 2003). According to this methodology, Predicted No Effect Concentration (PNEC) can be determined based on NOECs (No Observed Effect Concentrations). PNEC is considered as the thresholds above which an unacceptable effect is most likely to occur in the ecosystem considered. EQS for one substance is then derived from aquatic PNEC.

EQS were derived by European Commission experts according to this methodology described by Lepper (2005) for the 33 substances of Annex X of WFD and those substances were classified as follows:

1 Priority substances (PS): Alachlor, atrazine, benzene, chlorofenvinphos, chloropyrifos, 1,2-dichloroethane, dichloromethane, di(2-ethylhexyl)phthalate (DEHP), diuron, fluoranthene, isoproturon, lead and compounds, naphthalene, nickel and compounds, octylphenol, pentachlorophenol, simazine, trichlorobenzenes, trichloromethane and trifluralin.

2 Priority Hazardous Substances (PHS): Anthracene, brominated diphenylether (PentaDBE), cadmium and compounds, chloroalkanes (C_{10-13}), endosulfan, hexachlorobenzene, hexachlorobutadiene, hexachlorocyclohexane, mercury and compounds, nonylphenols, pentachlorobenzene, polyaromatic hydrocarbons (PAHs) and tributylin (TBT) compounds.

EQS have also been derived for 8 substances of List I of Annex of Directive 76/464/EEC named as “other pollutants” in the daughter directive proposal.

6.1. ENVIRONMENTAL QUALITY STANDARDS FOR PRIORITY SUBSTANCES IN SURFACE WATER

The established EQS for the priority substances list taken from the Directive proposal have been summarized bellow. The units are in µg/l and refer to the total concentrations in the whole water sample; AA refers to Annual average and MAC to maximum allowable concentration. In the case of metals the EQS refers to the dissolved concentration, i.e. the dissolved phase of a water sample obtained by filtration through a 0.45 µm filter or any equivalent pre-treatment.

Name of substance	AA-EQS Inland surface waters	AA-EQS Other surface waters	MAC- EQS Inland surface waters	MAC-EQS Other surface waters
Alachlor	0.3	0.3	0.7	0.7
Anthracene	0.1	0.1	0.4	0.4
Atrazine	0.6	0.6	2.0	2.0
Benzene	10	8	50	50
Pentabromodiphenylether	0.0005	0.0002	*not applicable*	*not applicable*
Cadmium and its compounds *(depending on water hardness classes)*	≤ 0.08 (Class 1) 0.08 (Class 2) 0.09 (Class 3) 0.15 (Class 4) 0.25 (Class 5)	0.2	≤ 0.45 (Class 1) 0.45 (Class 2) 0.6 (Class 3) 0.9 (Class 4) 1.5 (Class 5)	
C10-13 Chloroalkanes	0.4	0.4	1.4	1.4
Chlorfenvinphos	0.1	0.1	0.3	0.3
Chlorpyrifos	0.03	0.03	0.1	0.1
1,2-Dichloroethane	10	10	*not applicable*	*not applicable*
Dichloromethane	20	20	*not applicable*	*not applicable*
Di(2-ethylhexyl)phthalate (DEHP)	1.3	1.3	*not applicable*	*not applicable*
Diuron	0.2	0.2	1.8	1.8
Endosulfan	0.005	0.0005	0.01	0.004
Fluoranthene	0.1	0.1	1	1
Hexachlorobenzene	0.01	0.01	0.05	0.05
Hexachlorobutadiene	0.1	0.1	0.6	0.6
Hexachlorocyclohexane	0.02	0.002	0.04	0.02
Isoproturon	0.3	0.3	1.0	1.0
Lead and its compounds	7.2	7.2	*not applicable*	*not applicable*
Mercury and its compounds	0.05	0.05	0.07	0.07
Naphthalene	2.4	1.2	*not applicable*	*not applicable*
Nickel and its compounds	20	20	*not applicable*	*not applicable*
Nonylphenol	0.3	0.3	2.0	2.0
Octylphenol	0.1	0.01	*not applicable*	*not applicable*
Pentachlorobenzene	0.007	0.0007	*not applicable*	*not applicable*
Pentachlorophenol	0.4	0.4	1	1
Polyaromatic hydrocarbons (PAH)	*not applicable*	*not applicable*	*not applicable*	*not applicable*
Benzo(a)pyrene	0.05	0.05	0.1	0.1
Benzo(b)fluoranthene	Σ=0.03	Σ=0.03	*not applicable*	*not applicable*
Benzo(k)fluoranthene				
Benzo(g,h,i)perylene	Σ=0.002	Σ=0.002	*not applicable*	*not applicable*
Indeno(1,2,3-cd)pyrene				
Simazine	1	1	4	4
Tributyltin compounds	0.0002	0.0002	0.0015	0.0015
Trichlorobenzenes (all isomers)	0.4	0.4	*not applicable*	*not applicable*
Trichloromethane	2.5	2.5	*not applicable*	*not applicable*
Trifluralin	0.03	0.03	*not applicable*	*not applicable*

Moreover, Member States shall ensure that the following concentrations of hexachlorobenzene, hexachlorobutadiene and mercury are not exceeded in prey tissue (wet weight) of fish, molluscs, crustaceans and other biota:

a) 10 µg kg^{-1} for hexachlorobenzene,

b) 55 µg kg^{-1} for hexachlorobutadiene,

c) 20 μg kg^{-1} for methyl-mercury.

The discussions on lead, nickel and their compounds on the risk assessment have not yet been finalized. Therefore the proposed values are provisional.

6.2. ENVIRONMENTAL QUALITY STANDARDS FOR OTHER POLLUTANTS

Name of substance	AA-EQS Inland surface waters	AA-EQS Other surface waters
DDT total	0.025	0.025
para-para-DDT	0.01	0.01
Aldrin	Σ=0.01	Σ=0.005
Dieldrin		
Endrin		
Isodrin		
Carbontetrachloride	12	12
Tetrachloroethylene	10	10
Trichloroethylene	10	10

6.3. EQS FOR THRESHOLDS SUBSTANCES

The same approach was applied to the selected substances in the Thresholds project in order to compare with experimental results (D4.1.2, James, 2006). Given the low availability of data it was not possible to define a robust dose-response relationship for none of the substances studied except for cadmium. For the same reason it was not possible to derive thresholds of no effect for most substances. Thresholds could be derived for pelagic organisms for benzo[a]pyrene, benzo[k]fluoranthene, benzo[g,h,i]perylene and cadmium; as well as thresholds of no effect for sediment dwelling organisms for benzo[k]fluoranthene. Pentabromodiphenyl ethers and fluoranthene were the only substances for which thresholds of no effect were derived for the three media: water (pelagic organisms), sediment (benthic organisms) and top predators (secondary poisoning). No thresholds were derived for pyrene, benzo[b]fluoranthene and indeno[1,2,3-cd]pyrene. The most complex substance studied was mercury since it is necessary to consider also secondary poisoning effects and the role of speciation. Given the knowledge on bioaccumulation and toxicity of organic mercury and considering the considerable uncertainties encountered, no reliable overall threshold of no effect of mercury could be derived for the marine environment. It was deemed more appropriate to wait until a decision is taken at the European Commission level in the context of the Water Framework Directive. Table 6.1 summarizes the main findings during the application of the common methodology. For more details the reader is referred to D1.4.2 (James, 2006).

Table 6.1. Overview of thresholds of no effect reported in the present study. AFM: Assessment Factor Method; SEM: Statistical Extrapolation Method; EqPM: Equilibrium

Chemicals	Thresholds of no effect for			Overall threshold of no effect for the marine environment
	Pelagic organisms	benthic organisms	marine top predators	
	$ng.l^{-1}$	$\mu g.kg^{-1}$ dw	$\mu g.kg^{-1}$ ww	$ng.l^{-1}$
PCB 105	-	-	-	-
PCB 118	-	-	1.1 (low reliability)	-
PCB 156	-	-	-	-
PCB 180	-	-	-	-
Pentabromodiphenyl Ethers	53	310	333	12.2 (←top predators)
Pyrene	-	-	-	-
Fluoranthene	100	155	11,500	32 (←sediment)
Benzo[a]pyrene	5	-	-	0.005 (←seawater)
Benzo[b]fluoranthene	-	-	-	-
Benzo[k]fluoranthene	3.6	60 (AFM) 38-537 (EqPM)	-	0.4 (←sediment)
Benzo[g,h,i]perylene	0.8	-	-	0.8 (←seawater)
Indeno[1,2,3-cd]pyrene	-	-	-	-
Cadmium	1.6 (AFM) 210 (SEM)	-	-	210 (←seawater)
Mercury	1 (not relevant)	-	-	-

7. The role of mixtures in aquatic environments

Mixtures can be of different types depending on the number and groups of chemicals present. Identifying and quantifying these chemicals is never an easy task even for simple mixtures. Furthermore, another problem arises: how will the chemicals behave in the mixture? In this case, there is a need to know the chemicals mechanism of action, which can constitute a major problem in complex mixtures where components of the mixture are not known beforehand.

In a mixture, chemicals may basically behave in two ways from a toxicological point of view: they can have a joint action or they can interact. In the first case they may act through independent action (IA), when the toxicity of the individual chemical is independent of the other compounds in the mixture, or by concentration addition (CA) when the overall toxicity equal the sum of the toxicity of the mixture. In the second case, the effects of the interaction may be antagonistic or synergistic. There is a general consensus that, in most of the chemical mixtures in aquatic environments, the toxicity acts according to concentration addition (CA)

and, even for mixtures that have dissimilar modes of action (IA), at low concentrations, they still might behave according to CA toxicity approach (Maciel and Zaldivar, 2005).

7.1. MODELLING TOXICITY OF SINGLE COMPOUNDS

One of the most important concepts used in toxicology to determine risk assessment and regulation is the dose-response relationship for which several models have been used. In the past, the most used approach was to consider a linear function with or without threshold, i.e. at increasing concentrations there is an increase in the response and nonlinear with saturation at 100%. Actually, dose-response curves of single chemicals are fitted to sigmoidal shape curves with values between 0-1 (0-100%). Several models have been proposed in literature (Backhaus *et al*., 2004), between them:

- Weibull (W):

$$f(x) = 1 - \exp[-\exp(\theta_1 + \theta_2 \log_{10} x)] \quad (1)$$

- Box-Cox transformed Weibull (BCW):

$$f(x) = 1 - \exp\left[-\exp\left(\theta_1 + \theta_2 \frac{x^{\theta_3} - 1}{\theta_3}\right)\right] \quad (2)$$

- Morgan-Mercier Flodin:

$$f(x) = 1 - \frac{1}{1 + \theta_1 \cdot x^{\theta_2}} \quad (3)$$

- Logit (L)

$$f(x) = \frac{1}{[1 + \exp(-\theta_1 - \theta_2 \log_{10} x)]} \quad (4)$$

- Generalized Logit (GL):

$$f(x) = \frac{1}{[1 + \exp(-\theta_1 - \theta_2 \log_{10} x)]^{\theta_3}} \quad (5)$$

- Box-Cox-Probit (BCP):

$$f(x) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\theta_1 + \theta_2 \left[\frac{x^{\theta_3} - 1}{\theta_3}\right]} \exp\left(\frac{-u^2}{2}\right) du \quad (6)$$

where θ_1,θ_2,and θ_3 are parameters of the equations. As said before, normally the functions have a lower (L) and upper (U) asymptotes with values of 0 and 1. However, in some cases, at low concentrations chemicals shown stimulating effects (hormesis effect) having an U-type shape in the lower part of the concentration-response relationship (Calabrese and Baldwin, 2003). In this case, it is possible to move along the y-axis the function using the following expression:

$$F(x) = L + (U - L) f(x) \quad (7)$$

However, the U-type shape form cannot be reproduced with this approach (Backhaus *et al*., 2004).

Figure 7.1 summarizes the different functions that have been considered for dose-response, whereas in fig. 7.2, the individual concentration response curves for algal toxicity obtained by Faust *et al* (2003) are shown.

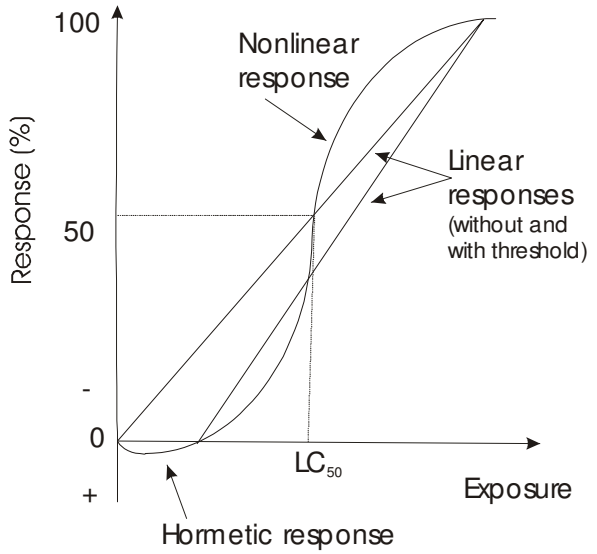

Figure 7.1. General dose-response functions: a/ linear with and without thresholds and nonlinear with hormesis.

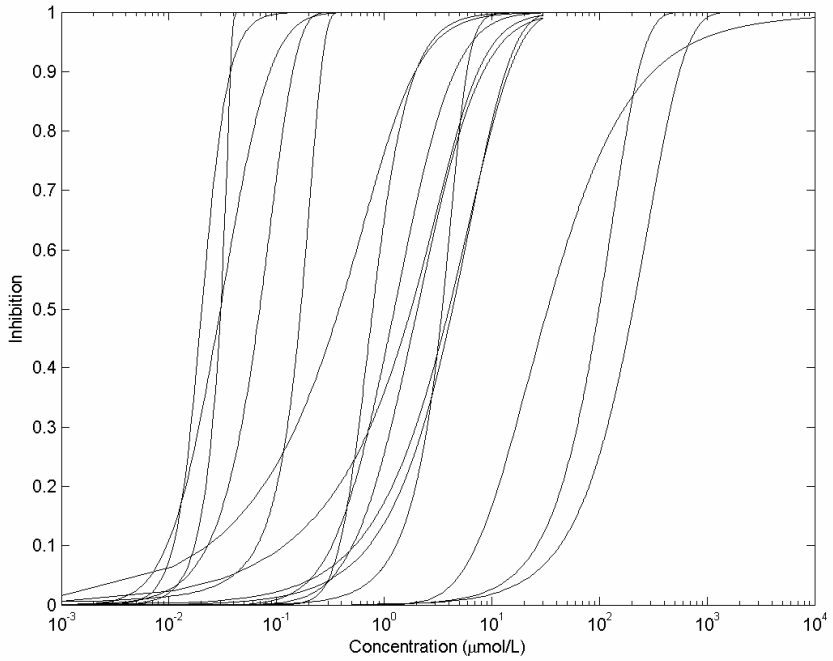

Figure 7.2. Individual concentration response curves for the algal toxicity of 16 dissimilarly acting chemicals (Norflurazon, Aclonifen, DTMAC, Terbuthylazine, Metazachlor, 8-Azaguanine, Paraquat dichloride, CCCP, Azaserine, Kresoxim-methyl, Triadimenol, Metsulfuron-methyl, Fenfuram, Chloramphenicol, Nalidixic acid, Metalaxyl. Fitting functions from Table 4 in Faust et al. (2003).

7.2. JOINT ACTION (NON-INTERACTIVE) AND INTERACTION MODELS

Even though early toxicological studies were devoted to the characterization on single chemicals, Bliss defined in 1939 several categories of chemical action, which are still relevant (Dybing *et al.*, 2002). These are: Concentration Addition (CA), Independent Action (IA) and interactions.

a/ Concentration Addition (CA): Assumes that the components in the mixture have a similar action but differ only with respect to their individual potency. Introduced by Loewe and Muischnek (1926), it is also know as Loewe additivity, simple joint action or dose addition. This may be expressed in terms of toxic units (TUs) which are the ratio of the concentration *i–th* substance in the mixture to the concentration needed to provoke a certain effect (Backhaus *et al.*, 2004):

$$TU_i = \frac{C_i}{ECx_i} \tag{8}$$

whereas C_i is the concentration of toxicant i in the mixture producing $x\%$ effect (e.g. EC_{50}). Therefore the overall toxic unit, for a mixture with n components, is equal to:

$$TU_{mix} = \sum_{i=1}^{n} TU_i = \sum_{i=1}^{n} \frac{C_i}{ECx_i} = \frac{C_{mix}}{ECx_{mix}} = 1 \tag{9}$$

Individual concentrations can be expressed as constant proportions p_i of the total concentration C_{mix}, with $p_i = C_i / C_{mix}$. In order to calculate the ECx_{mix}, this equation can be re-written as:

$$ECx_{mix} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{ECx_i}} \tag{10}$$

The concentration addition is the most common approach to risk assessment of mixtures and it is applicable over the whole range of exposure levels from low non-toxic levels when all chemicals in the mixture act in a similar way (Feron and Groten, 2002).

In concentration addition the components of the mixture exerting their effect via membrane perturbation as narcotic toxicants only or if the concentrations of specifically acting compounds are so low that only these baseline toxicities contribute to an overall effect (Escher and Hermens, 2002). This is the case of the studies on s-triazine mixtures on algal toxicity reported by Faust et al. (2001) and (2003) or for the application of toxic equivalency factors (TEF) used to describe the combined toxicity of isomers or structural analogues such as dioxins or PCBs (Dybing et al., 2002) where the total potency of the combined occurrence is calculated as the sum of the concentration of each individual congener multiplied by its specific. Also toxicity of PAHs (Fent and Batscher, 2000; Ankly et al., 1996; Birnbaum and

DeVito, 1995; Calamari and Vighi, 1992; Könemann, 1981), also confirmed by Swartz et al. (1997) and Erickson et al. (1999) for phototoxic PAHs. Furthermore, a number of models have been proposed to predict the toxicity of mixtures to organisms, all of which are generally based on the concept of additivity (Konemann, 1981, Ribo and Rogers, 1990; Stratton, 1988 and Stratton, 1989).

However, it is important to considerer that the mode of action of a certain group of chemicals may only be the same for a particular species and therefore it may be not possible to generalize to other organisms.

b/ Independent Action (IA): IA, also known as Bliss independence (Bliss, 1939) and response addition (Greco et al., 1995), is based on a the concept of statistically independent distribution of the sensitivities of the individuals towards the toxicants. In this case, it is assumed that the joint probability, p^s_{mix}, that an individual survives a concentration, $C_{mix} = \sum_{i=1}^{n} C_i$, is given by:

$$p^s_{mix} = 1 - \prod_{i=1}^{n} [1 - p^d(C_i)] \qquad (11)$$

whereas the probability of dying p^d is the complementary of the survival probability, i.e. $p^d = 1 - p^s$. Although, originally it was formulated for mortality/survival analysis, it can be applied in dose-response analysis as:

$$ECx_{mix} = 1 - \prod_{i=1}^{n} (1 - ECx_i) \qquad (12)$$

IA predicts that a mixture of chemicals will not exert an adverse effect when individual chemicals in that mixture are present below their individual No Observable Adverse Effect Level (NOAEL). According to USEPA, 2000, IA should be used for mixtures of chemicals that produce the same toxic effect in the same target organ, but which do so by dissimilar mechanisms of action (Borgert et al., 2004).

Both approaches have shown their validity (Faust et al., 2001; Faust et al., 2003;Vighi et al., 2003, a.o.), CA when used for chemical mixtures with similar action and IA when used for chemical mixtures with dissimilar action. Combination of both approaches has been also attempted (Altenburger et al., 2004). Although both models (CA, IA) involve summing, either the component doses or their toxic effects, differences between models may produce large differences in the risks estimated for a particular mixture. However, with a regulatory perspective, i.e. worst case, CA may be defendable as a pragmatic assumption by default since normally high mixture toxicity is predicted. Alternatively, the use of QSAR criteria was proposed by Vighi et al. (2003) to classify the substances as supposedly similarly or dissimilarly acting when no information is available.

c/ Interactions: In any case, both proposed approaches (CA, IA) to evaluate joint toxicity are "non-interaction" approaches, that is, they assume that chemicals are simply additive, and neither synergistic nor antagonistic, when combined in mixtures (Borgert et al., 2004). Several approaches have been proposed to take into account the interactions between chemicals to describe their combined effect that may result in a stronger effect (synergism, potentiation) or weaker effect (antagonism, inhibition) than expected on the basis of either CA or IA.

Antagonistic effects were explained by Escher *et al*. (1996), at the molecular level, by competition for sites in the membrane that may decrease toxicity. Synergistic effects can be explained by damage in the cell membrane. Organic solvents, in particular, will affect the membrane permeability and cause proton leak leading to uncoupling (Escher et al., 1999; Lewis et al., 1994). In order to study these effects mechanistic studies have shown (Andersen and Jennison, 2004) that interactions should be described at the level of target tissue dose and are best categorized as either pharmacokinetic (PK) or pharmacodynamic (PD). PK interactions occur when the presence of other chemical alter the relationship between the applied dose and the target tissue dose of a compound.

PD interactions occur when the presence of a second chemical alters the relationship between target tissue dose and tissue response.

Joint or interactive effects of a mixture observed at a clearly toxic-effect-levels of the individual chemicals in the mixture do not predict the joint or interactive effects of the mixture that might occur at exposure levels of the mixture similar to or lower than the highest no-toxic-effect-levels of the individual chemicals. This conclusion is highly relevant for designing further toxicity studies of mixtures as well as for low dose extrapolation of mixture toxicity data (Feron and Groten, 2002).

All three basic principles of joint action and interaction are theoretical. In reality, however, it is likely to have to deal with these concepts at the same time, especially when mixtures consist of more than two compounds and when the targets (individuals rather than cells) are more complex (Groten, 2000).

A frequent goal in mixture toxicology is primarily to determine situations where the effects of combinations of chemicals differ from the additive effects of the chemicals given individually. A great deal of effort has focused on creating various statistical methods for assessing when differences from additivity become significant and on identifying potentially important interactions that would change perceptions of the risks of mixtures of chemicals (Andersen and Dennison, 2004).

7.3. CALCULATING MIXTURE'S TOXICITY FROM INDIVIDUAL COMPONENTS

Concentration response curves for single substances describe the intensity of a defined effect as a function of the toxicant concentration, see Fig. 7.2. Similar curves can be obtained for mixtures when the ratio of the concentrations of the individual components is kept constant and only the total concentration is varied.

For the case the assumed action mechanism is CA and we are interested in calculating the total effect caused by a mixture there is an iterative procedure where the function:

$$error = \left(1 - \sum_{i=1}^{n} \frac{C_i}{f_i^{-1}(E(C_{mix}))}\right)^2 \tag{13}$$

has to be minimised. The procedure consists on defining an effect (E) and a mixture concentration C_{mix}, then calculate the individual concentrations that will produce this effect using the inverse of Eqs. (1-6). For example for the Box-Cox-Weibull (BCW), we will have:

$$f_i^{-1}(E(C_{mix})) = \left[1 + \frac{\theta_3}{\theta_2}(\ln[-\ln(1-E)] - \theta_1)\right]^{1/\theta_3} \tag{14}$$

Then the Eq. (12) is calculated and the procedure repeated by changing the mixture concentration until the error is minimized. Figures 7.3-7.4 show two examples for two mixtures of dissimilarly toxicants selected by Faust *et al.* (2003).

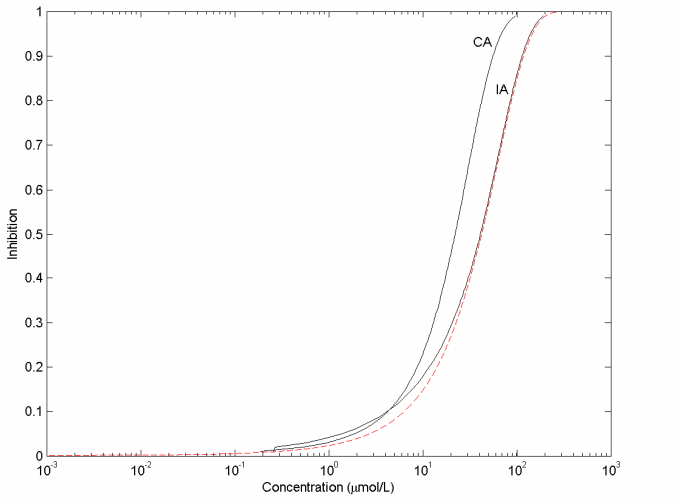

Figure 7.3. Observed and predicted (CA and IA) algal toxicity of the mixture of 16 dissimilarly acting substances with components mixed in the ratio of their EC50 values (Faust *et al.*, 2003; Table 5). Discontinuous red line: fitted experimental values (Faust *et al.*, 2003; Table 6).

The procedure in the case of IA also requires iteration. In this case the error to minimize is:

$$error = \left[x\% - 1 + \prod_{i=1}^{n}(1 - f_i(p_i(ECx_{mix})))\right]^2 \tag{15}$$

whereas the total effect is x%. In this case one defines a total effect and a mixture concentration, then calculates the individual effects of each component in the mixture at their specific concentration and evaluates Eq. (15). The procedure is repeated until the appropriate mixture concentration is obtained. Figures 7.3-7.4 show two examples for mixtures of dissimilarly acting compounds. In these two cases IA gives better results, since the chemical mixture was specifically chosen from dissimilarly acting substances.

Ii is generally accepted that for dissimilarly acting toxicants, IA will produce a better fit of the mixture toxicity (Backhaus et al., 2000; Faust et al., 2003; a.o.), whereas in the case of similarly acting chemicals CA will adjust more accurately the experimental results (Könemann, 1981; Calamari and Vighi,1992; Altenburger et al. 2000; Faust et al., 2001; a.o.). However, with a regulatory perspective, i.e. worst case, CA by predicting higher toxicity (see figs. 7.3-7.4) seems a more pragmatic option (Vighi et al., 2003). In any case, no-interactions have been assumed to occur in these two approaches so interactive aquatic toxicity is not taken into account (Gunatilleka and Poole, 1999). Thus although the additivity models are mathematically simple, they require assumptions about the mechanisms of action (only similar or dissimilar) and the high to low dose extrapolation. Therefore theoretical considerations in risk assessment of chemical mixtures should be verified by simple case studies (Groten, 2000).

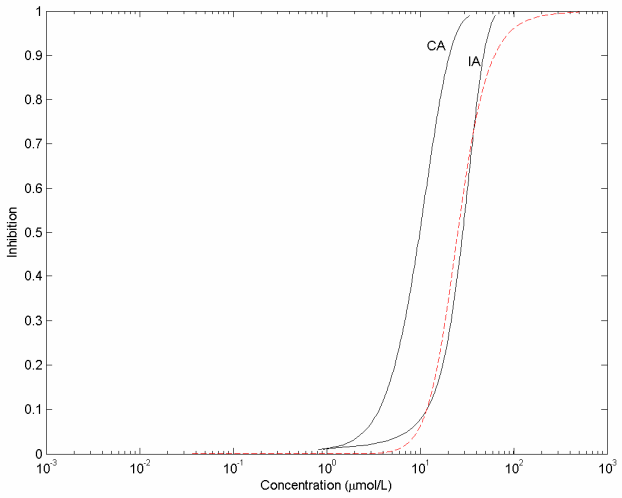

Figure 7.4. Observed and predicted (CA and IA) algal toxicity of the mixture of 16 dissimilarly acting substances with components mixed in the ratio of their EC1 values (Faust et al., 2003; Table 5). Discontinuous red line: fitted experimental values (Faust *et al.*, 2003; Table 6).

General toxicity refers to narcosis that acts by non-specific disruption of the proper functioning of the cell membrane (generally thought of as the site of action). Compounds exhibiting narcotic toxicity are not reactive and do not interact with specific receptors in an organism (Verhaar et al., 1992). Specific toxicity refers to reactive toxicity that is realized

through disruption of the function of a defined receptor site in the cell (Gunatilleka and Poole, 1999).

Effects of mixtures usually exceed those of the most active constituents alone. As a consequence, risk assessment procedures for contaminants in aquatic systems may no longer be restricted to single pure contaminants, but have to be considered combined effect resulting from multiple chemical exposures.

Typically aquatic environmental concentrations are lower than the concentrations that cause statistically significant effects in laboratory toxicity tests, they are below NOEC. Whether such low concentrations are relevant for a predictive mixture toxicity assessment is a controversial issue (Faust et al., 2003). Hence the relevance of low concentrations for the predictive assessment of mixture toxicity is a critical point (Könemann and Pieters, 1996). Under the assumption of concentration addition any concentration of any mixture component is expected to contribute to the overall toxicity of a mixture; there would be no threshold concentration other than zero. Under the Independent action the situation is different. Only those concentrations of individual toxicants that cause individual effects greater than zero are expected to contribute the overall toxicity.

However, in all these toxicity assessments there is the limitation of our knowledge of the anthropogenic chemical pressures, since only a small fraction of man-made chemicals introduced in the environment have been studied and thus the assessment of the complete chemical perturbation of environmental media remains undone and unknown.

7.4. CASE STUDY: NORTHERN ADRIATIC

In this example, we will assess the combined effects of plant protection products on algal species. The measured environmental concentrations refer to a station in the Adriatic Sea in front of a coastal lagoon (Sacca di Goro) (Viaroli et al., 2006) and were obtained during one year experimental campaign (Carafa et al., 2007). The results for the Adriatic Sea station are summarized in Table 7.1. For a more detailed information on material, methods and concentrations in the watershed, inside the lagoon (water and sediments), and biota (*Ulva* and clams) the reader is referred to Carafa et al. (2007). Modelling results for s-triazides (atrazine, simazine and terbuthylazine) can be found at Carafa et al. (2006).

Unfortunately data on algal toxicity was not found for molinate which is one on the compounds at higher concentrations at this site. However, as it can be observed, all the concentrations are well bellow the EC_{50} values. In this case the resulting algal toxicity for the Adriatic Sea station is low independently of the mode of action, i.e. CA or IA with values around $0.6\ 10^{-4}$ in percentage of inhibition.

Table 7.1. Average and highest plant protection product concentration in the water column at the Adriatic Sea station (carafa et al., 2007) and concentration response function for algal toxicity (Faust et al., 2001; Faust et al., 2003; Arrhenius et al., 2004; Junghans et al., 2006).

Substance	Average conc. (ng/L)	Highest conc. (ng/L)	Concentration response function				EC_{50} (µg/L)
			Regression Model	θ_1	θ_2	θ_3	
Atrazine	3.75	5.93	GL	6.765	17.391	0.1118	38.83
Simazine	6.18	25.96	W	0.83	2.18	-	56.88
Cyanazine	0.10	0.28	BCP	8.64	7.40	0.6482	27.20
Chloridazon	9.55	40.59	W	-2.375	2.777	-	1172.53
Metamitron	1.09	2.55	W	-0.995	1.912	-	430.73
2-ethyl-6methylaniline	1.81	3.40	-	-	-	-	-
Tribenuron-methyl	1.85	4.68	W	0.670	1.735	-	100.03
Isoproturon	0.26	0.53	BCW	1.246	1.073	-0.0235	47.03
Diuron	6.65	25.32	W	2.847	2.349	-	10.02
Terbuthylazine	52.11	234.50	W	4.165	3.908	-	15.92
Molinate	30.65	175.51	-	-	-	-	-
Prometryn	0.04	0.23	GL	2.57	2.51	0.0105	12.50
Linuron	0.42	1.08	W	1.769	2.020	-	21.85
Terbutryn	0.21	0.31	GL	26.28	20.00	0.1948	7.80
Metolachlor	12.96	59.29	BCW	0.239	3.156	0.4930	232.15
Alachlor	2.38	12.20	W	4.009	5.127	-	37.77
Chlorvenfinvos	0.0	0.0	-	-	-	-	-
Pendimethalin	0.15	0.72	W	5.752	2.957	-	2.40
Chlorpyrifos	0.30	1.18	-	-	-	-	-
Diethylanilline	2.30	5.66	-	-	-	-	-

8. Managing under thresholds: Contaminants

The WFD defines five categories: High, Good, Moderate, Poor and Bad. However, the most important boundary is between moderate and good. In this case restoration measures have to be taken into account which implies monetary considerations. Assuming that our system respond in a non-linear fashion, then the assessing of the five categories should be done in a manner of avoiding the excessive restoration measures that would be necessary when the threshold point has been reached and taking into account the uncertainty associated with the threshold's determination.

Under an ecosystem with thresholds one should define the boundaries between the classification accordingly with the type of response (see Fig. 8.1). Under these circumstances, the common practice to divide the system equally does not hold. In addition, in case of a sharp threshold, the intermediate categories: Moderate and Poor could be difficult to distinguish. Furthermore, the point of non-return should also be assessed taking into account also socio-economic considerations.

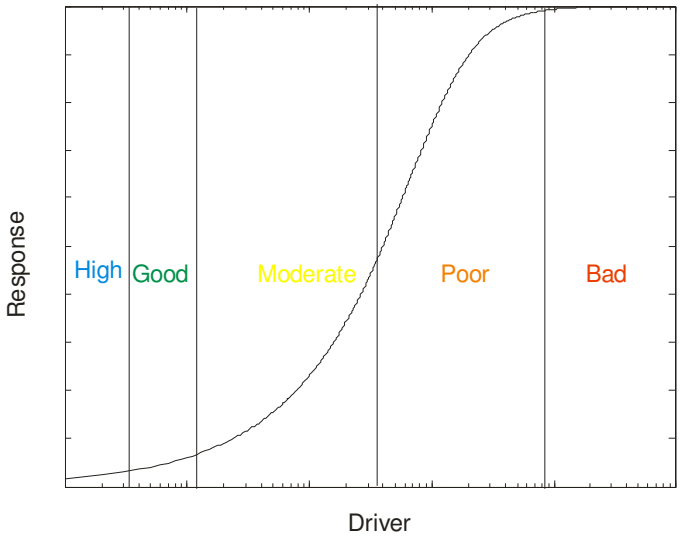

Figure 8.1. WFD classification under non-linear ecosystem response.

However, these considerations would only apply to biological quality elements. Concerning chemicals, in particular, priority substances (PS), the Directive (COM (2006) 397) actually under discussion at the European Parliament establishes a set of Environmental Quality Standards that refers to the concentration of the substance in the water column (see Section 6). If any of these concentrations is exceed then the water body should be defined in bad status. Since these concentrations have been chosen in basis of ecotoxicological studies which are based on the results of acute and chronic toxicity test in which mortality, reproductive effects or other end points have been measured for a relatively small number of organisms exposed, under controlled conditions, to varying contaminant concentrations, then it is not clear to assess under which extent we are overprotecting the environment. On the other hand, if we consider that organism are exposed to a myriad of chemical products and that regulations apply to few of them it is also questionable if these measures are really protecting ecosystems.

9. Conclusions

In the Thresholds project we have analyzed several open questions concerning the role and effects of contaminants in coastal ecosystem through experiments, data analysis tools and modelling. Even though there is still a long route to follow before been able to calculate the

effects of contaminants at ecosystem level, we have found several important points that may be summarized as follows:

1. Molecular level effects are detected even at concentrations that did not affect the macroscopic end point studied, i.e. growth rate.
2. Natural populations are more sensitive that populations in cultures.
3. There are differences for the same species at different environments, e.g. Mediterranean, Black Seas and Atlantic Ocean.
4. The environmental conditions and the time of release of the contaminant cause a variability of the response at ecosystem level that can reach 50% effects.
5. At the actual level of knowledge it is difficult to assess if the legal approach, based on the precautionary principle, is over or under conservative, when considering molecular and its long term effects, the combined effects of mixtures and the environmental fluctuations that affect all ecosystems.
6. A similar colour code to the one adopted for biological quality elements should be adopted for the definition of EQS, with values higher than the EQS as orange (poor). This will allow assessing contamination trends and an early detection of a chemical contamination problems.
7. In aquatic environments ecosystem experience the combined effects of mixtures. Ecotoxicological risk assessment should be performed taking this aspect into account. However, with the amount of new chemicals being produced and the detection limits required it is clear that new integrated indicators are necessary. Limiting the levels of certain chemicals in the environment is one step to improve ecosystem health but alone it will not prevent further deterioration.
8. Due to practical limitations, knowledge on ecotoxicology is only available for a small fraction of the anthropogenic chemical pressure. The importance of this simplification has not been comprehensibly assessed and introduces uncertainty in the appropriate outcome of current legislation and managing practices.

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Abstract
A fundamental problem in ecotoxicology is the prediction of long term population and ecosystem-level effects of contaminant exposure based on dose response data of few individuals obtained over a short time period. In addition, environmental fluctuations will always affect significantly the population/ecosystem resilience. However, these fluctuations are not taken into account under dose-response experiments on individuals.
In the Thresholds project we have analyzed some of these questions by using experiments, data analysis tools and modelling approaches. Several important finding may be summarized as follows:
1 Molecular level effects are detected even at concentrations that did not affect the macroscopic end point studied, i.e. growth rate.
2 Natural populations are more sensitive that populations in cultures.
3 There are differences for the same species at different environments, e.g. Mediterranean, Black Seas and Atlantic Ocean.
4 The environmental conditions and the time of release of the contaminant cause a variability of the response at ecosystem level that can reach 50%.
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8 Due to practical limitations, knowledge on ecotoxicology is only available for a small fraction of the anthropogenic chemical pressure. The importance of this simplification has not been comprehensibly assessed and introduces uncertainty in the appropriate outcome of current legislation and managing practices.

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