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TESI DI LAUREA IN SCIENZE E TECNOLOGIE
PER L'AMBIENTE E IL TERRITORIO

*EFFECTS OF MALEIC HYDRAZIDE AND CHLORPROPHAM
ON BENTHIC DIATOMS OF RIVER ENVIRONMENTS*

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SUMMARY

The study presented in this thesis work was conducted in the context of an Erasmus scholarship in the period from February to July 2008, carried out in France at the INP-ENSAT (Institut National Polytechnique-Ecole Nationale Supérieure d'Agronomie de Toulouse), in the laboratory of Functional Ecology under the supervision of Prof. Eric Pinelli.

The conservation of a good water quality is today a more and more important aspect concerning not only the scientific community, but also the whole population. In this context man plays a negative role, especially with the river contamination by fertilizers and pesticides, because of agricultural activities (Stevenson *et al.*, 1999). Several tools have been originated to assess the ecological status of running waters and, among these, the use of bioindicator organisms has been very successful. Diatoms, for example, are well known bioindicators of trophic pollutions. Their potential use is less evident when other kinds of environmental stress, as pesticide pollution, influence the water quality (Barbour *et al.*, 1999). Diatoms are particular photosynthetic microorganisms, constituted by a siliceous cell wall, called frustule, and high quantities of abnormal frustules have been found in natural diatom communities sampled in streams contaminated by pesticides. With the aim to simulate the exposure of diatoms to pesticides an experiment was carried out by Debenest *et al.* (2008): benthic diatoms of river environments were treated with a genotoxic herbicide, the maleic hydrazide. The induction of frustule abnormalities, associated with the exposure to maleic hydrazide, was also investigated and results showed that abnormal frustules and nucleus alteration abundance (abnormal nucleus location, micronuclei presence, nucleus fragmentation and nucleus membrane breakage) appeared statistically in higher proportions for the maleic hydrazide highest doses (10^{-6} and $5 \cdot 10^{-6}$ M). The link between the formation of frustule and nucleus abnormalities and the genotoxicity remained unclear.

The experiment conducted by Debenest *et al.* (2008) has been repeated and further investigated in this report. In chapter 1 it is introduced the problem of river pollution by pesticides, with particular attention to the French situation, and the main limits of the devices currently used to detect this kind of pollution were analysed. Subsequently, after having described the diatom physiology, the known effects on these organisms resulting from their exposure to pesticides were listed. The aims of the research were: in a first step to validate

adequate biomarkers, obtained through the distinction between nucleus alterations from frustule ones (the genotoxic study), and, in a second step, to understand the freshwater diatom communities change after exposure to pesticides.

As described in chapter 2 (materials and methods), to achieve the first objective benthic diatoms of river environments were exposed to two pesticides, maleic hydrazide (10^{-7} , 10^{-6} and $5 \cdot 10^{-6}$ M) and chlorpropham (10^{-7} and 10^{-6} M). These two products were chosen because they are two pesticides used in France and because of their ability to induce the alterations researched. After a period of 24 hours, using a fluorescent microscope, the observation and study of cellular alterations were carried out. To achieve the second objective other diatom samples were exposed to maleic hydrazide (10^{-7} , 10^{-6} and $5 \cdot 10^{-6}$ M), following the same operating methods used in the first experiment. The change in the composition was investigated after 24 and 48 hours with two different techniques: flow cytometry and identification of species through the study of frustules.

Results of the two works are presented in chapter 3. In the case of the study at a cellular level, after exposure to maleic hydrazide, the nuclear alterations (micronuclei, abnormal location and number of nuclei) increased with the concentration. A similar trend was also found for frustules having an abnormal form. Similarly, after treatment with chlorpropham, a linear increase of nucleus and frustule alterations with the concentration was observed. However, in this case the frustule alterations were more abundant than those concerning the presence of micronuclei. In the study at the community level, from identification of diatoms through the frustule, it was clear that after 24 and 48 hours *Nitzschia Palea* (Kützing) W. Smith species became dominant and could adapt itself to the contaminated environment. The same study, conducted by flow cytometry, showed that after 24 and 48 hours from exposure, the abundance of diatoms in both control and treated samples decreased while that of green algae increased.

In the final part, chapter 4 and 5 (respectively discussion and general conclusions), some conclusions were drawn. About the genotoxic study the hypothesis supported by this report is that maleic hydrazide had two ways of action according to the different doses. With concentrations of 10^{-7} and 10^{-6} M the cytoskeleton seemed to be the first target. This phenomenon, the aneugenic effect, caused abnormal frustules and abnormal number and position of nuclei. On the contrary, micronuclei, were found mainly at the higher

concentration (5×10^{-6} M) and were due to a direct disturbance of DNA, the clastogenic effect. In diatoms exposed to chlorpropham, an aneugenic effect seemed to prevail at all concentrations, causing more cytoskeleton abnormalities and a smaller number of micronuclei. Looking at the community level, in both studies there has been the confirmation that diatom populations suffered the exposure to maleic hydrazide with a consequent decline in their relative abundance and biodiversity. Understanding the precise mechanisms involved in the abnormalities origin, also with future researches, will consent to obtain biomarkers within benthic diatoms of river environments. Biomarkers together with the study of pesticide effects on communities will help actual devices to detect pollution by pesticides into rivers, also after flood episodes where a large number of chemical molecules pass and destroy the natural ecosystem, and to improve the management of the river water quality.

RIASSUNTO

Lo studio presentato in questo lavoro di tesi è stato realizzato in buona parte nell'ambito di una borsa Erasmus nel periodo tra febbraio e luglio 2008, in Francia, presso l'INP-ENSAT (Institut National Polytechnique-Ecole Nationale Supérieure d'Agronomie de Toulouse) nel laboratorio di Ecologia Funzionale, sotto la supervisione del Professor Eric Pinelli.

La conservazione di uno stato di buona qualità dell'acqua è oggi un tema sempre più importante che riguarda non solo la comunità scientifica, ma l'intera popolazione. In questo contesto l'uomo svolge un ruolo negativo, specialmente per quanto riguarda la contaminazione dei fiumi con fertilizzanti e pesticidi derivanti dall'attività agricola (Stevenson *et al.*, 1999). Molti metodi e strumenti sono stati sviluppati per valutare lo stato ecologico dei fiumi e, tra questi, l'uso di organismi bioindicatori si è rivelato molto utile. Un esempio sono le diatomee, utilizzate per valutare lo stato dell'acqua in termini di inquinamento organico. Il loro impiego potenziale per individuare altri tipi di stress ambientale, come l'inquinamento delle acque da pesticidi è, tuttavia, meno noto (Barbour *et al.*, 1999). Le diatomee sono particolari microorganismi fotosintetici, costituite da una parete cellulare silicea (frustulo) la cui morfologia è tipica di ogni specie. Alte quantità di frustuli anormali sono state trovate in comunità campionate in torrenti inquinati da pesticidi. Con lo scopo di simulare l'esposizione delle diatomee a questi prodotti fitotossici è stato condotto un esperimento da Debenest *et al.* (2008) nel quale alcune popolazioni di diatomee bentoniche prelevate in ambienti fluviali sono state trattate con un erbicida genotossico, l'idrazide maleica. L'induzione di frustuli anomali associata con l'esposizione all'idrazide maleica è stata studiata e i risultati hanno mostrato che queste alterazioni, assieme ad altre riguardanti il nucleo (localizzazione e numero anormali del nucleo, presenza di micronuclei, rottura della membrana nucleare), erano statisticamente più abbondanti alle concentrazioni maggiori dell'agente tossico (10^{-6} e $5 \cdot 10^{-6}$ M). Il legame tra i disturbi riguardanti nuclei e frustuli e la genotossicità è rimasto, tuttavia, poco chiaro.

Col presente elaborato si è cercato di riprendere ed approfondire l'esperimento condotto da Debenest *et al.* (2008), di cui sopra. Nel capitolo 1 è stato introdotto il problema relativo all'inquinamento delle acque fluviali da prodotti fitosanitari, ponendo particolare attenzione alla situazione francese, e sono stati analizzati i principali limiti degli strumenti e metodi

attualmente utilizzati per la rilevazione di questo tipo di perturbazione. In seguito, l'attenzione è stata spostata sulle diatomee e, dopo averne presentato la fisiologia, sono stati elencati gli effetti finora noti su questi organismi derivanti dalla loro esposizione a diverse concentrazioni di pesticidi impiegati in agricoltura. Lo studio ha avuto due scopi principali: validare dei biomarcatori, distinguendo le anomalie del nucleo da quelle del frustulo, utilizzabili nella ricerca dell'inquinamento da pesticidi nelle acque superficiali (studio genotossico) e capire come la composizione della comunità di diatomee bentoniche d'acqua dolce cambi in seguito alla sua esposizione a questi agenti tossici.

Come descritto nel capitolo 2, riguardante i materiali e i metodi utilizzati, per conseguire il primo obiettivo alcuni campioni di diatomee prelevate in ambienti fluviali sono state esposte a due diversi pesticidi, idrazide maleica (10^{-7} , 10^{-6} e $5 \cdot 10^{-6}$ M) e chlorpropham (10^{-7} e 10^{-6} M). Questi due prodotti sono stati scelti per due motivi: in primo luogo in quanto appartenenti a delle famiglie di pesticidi utilizzate in Francia e, in secondo luogo, per la loro capacità di indurre le alterazioni ricercate col presente studio. Dopo un periodo di 24 ore, tramite l'utilizzo di un microscopio a fluorescenza, si è provveduto all'osservazione e allo studio delle alterazioni cellulari. Per conseguire il secondo obiettivo altri campioni di diatomee sono stati esposti alla sola idrazide maleica (10^{-7} , 10^{-6} e $5 \cdot 10^{-6}$ M), seguendo le stesse modalità operative del primo esperimento. La variazione della composizione della comunità nel tempo, dopo 24 e 48 ore, è stata studiata tramite due differenti tecniche: citometria di flusso ed identificazione delle specie attraverso lo studio del frustulo.

I risultati relativi ai due lavori sono esposti nel capitolo 3. Nel caso dello studio a livello cellulare, in seguito all'esposizione all'idrazide maleica, si è visto che le alterazioni nucleari (micronuclei, localizzazione e numero anomali dei nuclei) aumentavano con la concentrazione. Un trend simile è stato trovato anche per i frustuli aventi forma anomala. Allo stesso modo, in seguito al trattamento col chlorpropham, si è visto un aumento lineare delle alterazioni di nucleo e frustulo parallelamente alle concentrazioni testate, con la differenza che, in questo caso, le alterazioni della parete esterna erano più abbondanti e quelle riguardanti la presenza di micronuclei erano minori. Nello studio a livello di comunità, dall'identificazione delle diatomee attraverso l'analisi del frustulo, è stato notato che dopo 24 e 48 ore dall'esposizione la specie *Nitzschia palea* (Kützing) W. Smith prendeva il sopravvento sulle altre e si adattava all'ambiente contaminato. Lo stesso studio, svolto attraverso citometria di flusso, ha evidenziato che in seguito a 24 e 48 ore dal trattamento con

l'idrazide maleica, l'abbondanza delle diatomee presenti sia nel controllo che nei campioni trattati diminuiva e quella delle alghe verdi aumentava.

La discussione e le conclusioni generali del lavoro sono presentate rispettivamente nei capitoli 4 e 5. In merito allo studio genotossico si è confermato che il primo pesticida testato, l'idrazide maleica, ha causato due effetti diversi in base alle dosi somministrate: alle concentrazioni 10^{-7} e 10^{-6} M prevalgono i disturbi riguardanti il citoscheletro (effetto aneugenico) con formazione di frustuli anormali e nuclei presenti in numero e posizione erronei, mentre i micronuclei, trovati soprattutto alla concentrazione maggiore ($5 \cdot 10^{-6}$ M), sembrano essere dovuti principalmente ad un disturbo diretto sul DNA (effetto clastogenico). Nelle diatomee esposte al chlorpropham prevale, invece, un effetto aneugenico a tutte le concentrazioni, che causa un maggior numero di alterazioni dovute a disturbi nel citoscheletro e un minor numero di micronuclei. In entrambi gli studi effettuati a livello di comunità è possibile concludere che le popolazioni di diatomee risentono dell'esposizione al prodotto tossico con una conseguente diminuzione della loro abbondanza relativa e della loro diversità specifica. Capendo i meccanismi precisi coinvolti nella formazione delle alterazioni studiate, anche attraverso future verifiche, si potranno ottenere dei biomarcatori validi che insieme allo studio degli effetti dei pesticidi sulle comunità di diatomee bentoniche consentiranno di rilevare con più precisione l'inquinamento da pesticidi nei fiumi, anche in seguito ad eventi di piena dove molte sostanze chimiche vengono rilasciate simultaneamente nelle acque con notevoli effetti negativi sull'ecosistema, e di avere un miglioramento nello studio della qualità delle acque.

1. INTRODUCTION

1.1. A water contamination by agricultural activity

1.1.1. Phytosanitary products and biocides

The main source of water pollution by the agricultural activity concerns the large use of fertilizers and phytosanitary products on cultures. Fertilizers, like nitrates and phosphates, are more and more concentrated in rivers and streams and their effects are visible with the appearance of eutrophication. The supplementary introduction of fertilizers is due to the vegetal production intensification, necessary to the animal and human provisioning. With regard to phytosanitary products, the survey on the cultural practices conducted in France between 1994 and 2001 by Scees (Service central des Enquêtes et Études statistiques of Ministère de l'agriculture et de la pêche) shows that farmers have sensibly reduced the doses of products used, but they have increased the number of treatments and the use of solvent mixtures (IFEN, 2006b).

The European Plant Protection Products Directive 91/414/EEC, of 15 July 1991, defines as phytopharmaceutical products “the active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to: protect plants against all harmful organisms, prevent the action of such organisms, influence the life processes of plants (e.g. growth regulators), preserve plant products, destroy unwanted plants, undesired plants or parts of plants and control or prevent undesired growth of plants” (EUR-Lex, 2008). The Directive 98/8/EC, of 16 February 1998, concerning the placing on the market of biocides products, defined as biocides "the active substances and preparations containing one or more active substances, which are presented in the form in which they are supplied to the user, intended to: destroy, repel or render harmless pests, prevent the action or fight them in any other way, through a chemical or biological action". In this report, the term pesticide, also means a substance employed in the protection and production of cultures that include insecticides, acaricides, herbicides, fungicides and other specialised molecules (e.g. molluscicides, rat-killers etc.).

The use of pesticides in agriculture increased strongly in past decades, making them indispensable to most agricultural practices. As a consequence, pathogen organisms were selected and only the more resistant could survive. Moreover, the decrease of grassland has given place to monocultures, less able to fight illness and parasites (Pointereau *et al.*, 2006). As a chain-reaction more treatments with new products were necessary to limit the phytosanitary risk and to avoid loss of harvest and capitals. Between 1945 and 1985, consumption of pesticides has doubled every ten years. Nevertheless, if pesticides were an enormous progress in the control of food resources and in the improvement of public health (particularly in the fight against insects, disease vectors), the downside has emerged quickly: the incidence of resistance in insects, the disturbance of reproduction in different animals, showed dramatically limits and dangers of these substances not only to the environmental ecosystems, but also to the human beings. Therefore, the market of these toxic agents is very important: Europe and North America alone represent in 2005 more than 50% of pesticide consumers (UIPP, 2008) and, among the different products, herbicides are the most employed, because of their possibility of treat pre-emptively the cultures to avoid the development of pathogens. In the eighties, the French active substance purchase was of about 95 000 tons (ACTA, 2002), but at the beginning of nineties the volumes bought decreased visibly. The growth restarted only in 1997 and reached values of 120 000 tons in 1999. This tendency stopped in the following period, 2001-2004, where the use of phytosanitary substances decreased of 24%, because of the new laws which forbid the sell of many dangerous products for health and environment, the s-triazines (Aubertot *et al.*, 2005). Nevertheless, it remains difficult to assess the real evolution of phytosanitary products in the global market, because of the rapid change in the formulation of active substances and to the different doses used in treatments (Rabaud, 2003).

To have an idea of the Italian market of pesticides, some data collected by ISTAT (National Institute of Statistic) in 2006 showed that the sum of plant protection products distributed for agricultural use amounts to approximately 1 490 000 quintals, -4,7% if compared to 2005. Also the active ingredients contained in preparations released for consumption recorded a decrease down by 851 000 quintals in 2005 to 815 000 quintals in 2006 (-4,3%). With the decline of the active ingredients there has been an increase in their average concentrations contained in phytosanitary products going from 54,4 to 54,7 % in two years (ISTAT, 2007).

1.1.2. State of the water quality in France

In the case of surface raw water the assessment of the water quality in France, for the concentrations in pesticides, refers to a system started in 1999, the SEQ-Eau. It gives the evaluation elements regarding the physical-chemical quality of water and the incidence of this quality on the biology and use of water. At last, the SEQ-Eau gives a list of active substances (pesticides and some of their metabolites), as provided from the Water Framework Directive of 2000, each of them with its own quality threshold, corresponding to five quality classes: very good, good, acceptable, mediocre and bad (Figure 1.1) (IFEN, 2006a; Debenest, 2007). To estimate the water quality, the total sum of the pesticide concentrations is taken into account.

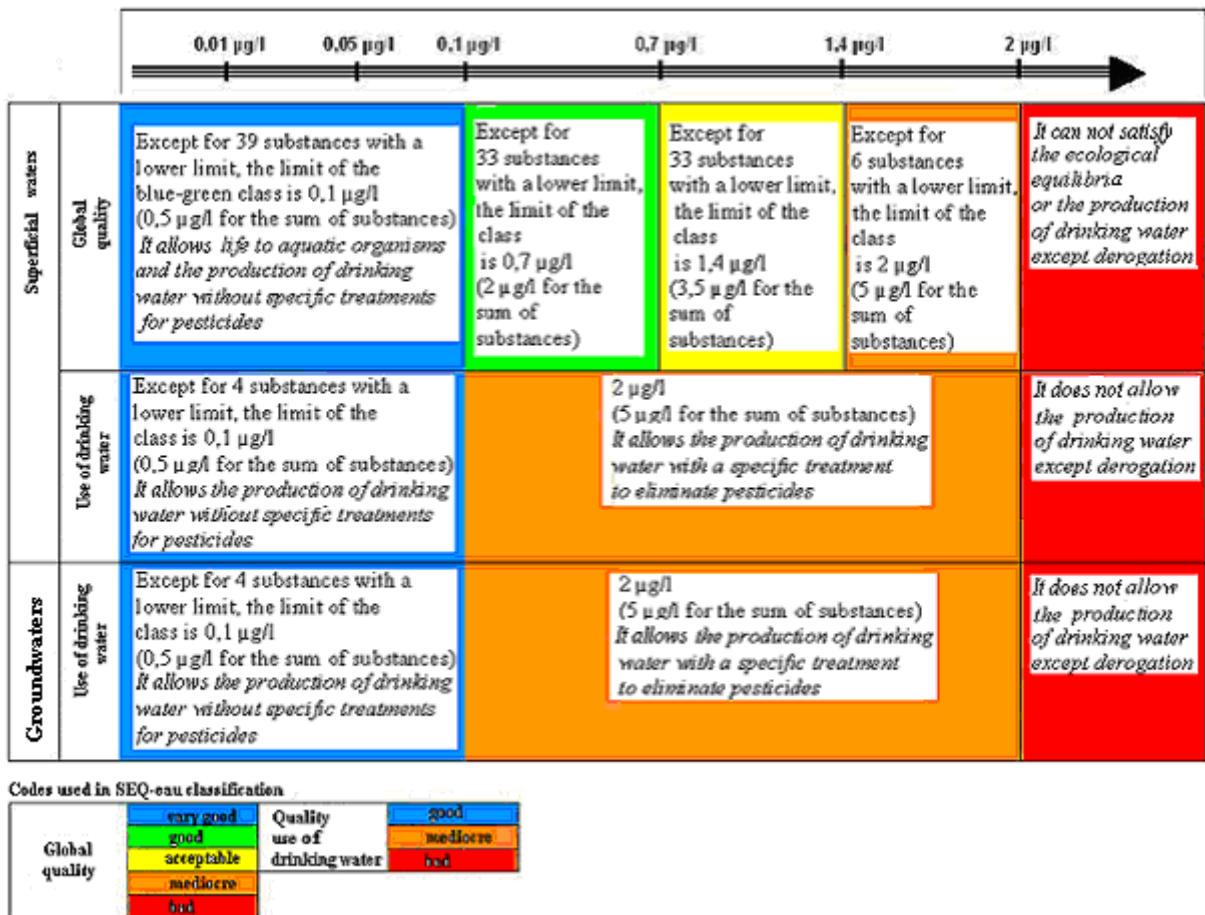


Figure 1.1: The different classes of SEQ-Eau used for pesticides (IFEN, 2006a).

The summary of the latest results, presented by IFEN in 2002 and 2006, highlights the presence of pesticides in all of the measuring points monitored since 1997. For the period 2003-2004, the specific report of IFEN on pesticides in waters, identified the presence of pesticides in 96% of the points of measures for surface water and in 61% for those of groundwater (IFEN, 2006a). During this period, certain thresholds of raw water quality (0,7 µg/l for at least one substance and/or 2 µg/l for the sum of the concentrations of various substances simultaneously) were exceeded at least one time in 50% of the national network stations (Debenest, 2007). The ban of certain molecules has significantly contributed to change the set of the substances found. Thus, the ban of s-triazines is visible in the results of IFEN data for surface waters, between 2002 and 2006, and of Agency of water data, between 2003 and 2004 (IFEN, 2002; Agence de l'eau Adour Garonne, 2005; Agence de l'eau Adour Garonne, 2006; IFEN, 2006a). The low speed of renewal of groundwaters may explain the detection of atrazine in 2003 and 2004 in the national network of groundwater stations. Some molecules appeared after atrazine prohibition: glyphosate is reported in period 2003-2004 in sufficient quantities to lead to the downgrading of many measurement points (IFEN, 2006a). However, it is difficult to distinguish the trends in the short term, at a national scale, of such pollution. Indeed, the heterogeneity of the available data, particularly between large basins (samples date, kind and number of molecules searched), and because of the interannual variability of rainfall regimes during which pesticides are washed and released into rivers, make it difficult to compare the situations across time and space (Aubertot *et al.*, 2005). Moreover, the consequences of the large-scale application of agro-environmental measures (e.g. grassed bands) on the levels of pollution in rivers are not still visible because their application is relatively recent (Debenest, 2007).

1.2. An evolving legislation

Increased agricultural pollution and responsibility (agriculture is the main source of pollutants for surface and groundwater resources) in the continuous decline of the water quality, have helped to raise awareness in society on the environmental risks. The expectation of the society on the reduction of pollution by pesticides is therefore significant. The evolution of the legislation to reduce discards and to restore the already degraded ecosystems falls within this context.

Until 1992 the Common Agricultural Policy (CAP) did not take into account the excessive measure in which pesticides were applied. In fact, the first major reform of CAP has brought aid to agro-environment measures (MAE) in order to promote environmentally-friendly practices. But, these measures did not have the desired effect on the contamination of rivers by agricultural pollution because of their not obligatory character (IFEN, 2002). Countries member have initiated a reform in 2003 with a progressive decoupling of aid in the production and with the introduction of cross-compliance of aid principle (the Agreement of Luxembourg in 2003 established the decoupling of agricultural subsidies from production and the principle of cross-aid, farmers commit themselves to carry out environmental-protection measures in exchange for aid). The aim of this system was to promote the creation of agro-environmental measures by farmers at the expense of their production. Within the framework of the 6th European Action Plan, the European Commission adopted in July 2006 a strategy for the "sustainable use of pesticides". One of the main objectives of this plan is to reduce risks to human and environment health associated with the use of pesticides, defining standards for equipment spray (Ministère de l'écologie, de l'énergie, du développement durable et de l'aménagement du territoire, 2005). The French government established in 2006, for three years, an "inter-ministerial plan of reduction of pesticide risk", which came under the European approach mentioned earlier. The aim of the plan is to reduce the risk posed by these molecules to ecosystems and human health of users and consumers (Ministère de l'écologie, de l'énergie, du développement durable et de l'aménagement du territoire, 2006).

With regard to the water resources context, their deterioration across Europe led European political leaders to implement the Water Framework Directive (2000/60/EC). With this Directive the sectoral approach of the various rules has given way to an integrated administration of the management of water quality. The different key points of this Directive are: the realisation of an inventory of water bodies in terms of quality, quantity and use of this resource (limit 2004), the conquest and conservation of a good ecological status (limit 2015) and the establishment of a comprehensive system for monitoring and recording quality of waters (limit 2006). Nevertheless, in some European countries the requirements set out by this Directive probably will not be reached within the time adopted, especially as regards to the restoration of a good ecological status in 2015 (Aubertot *et al.*, 2005).

Looking at the phytosanitary products, the European Community Directive 91/414/EEC aims to regulate the necessary dispositions to reach a merchandising authorisation for the different

products. This Directive is, in France, the base of the main principles and rules of control of the pesticide commercialisation. In particular, the article 7 of this Directive says that it is possible to obtain a merchandising authorisation, if active principles are declared on a particular list written by the European Community (Annex 1 of Directive 91/414/EEC), which includes only substances whose toxicity has been studied and verified. Since a few years the countries member are overhauling this list with the new information about active products toxicity acquired in these last years. This program is the application of a bigger plan of control and regulation of risk of chemical substance, the Regulation (EC) n. 1907/2006 (REACH), effective from June 2007. It is concerning the total of the chemical substances and by its application all industrials must record their products according with principles and rules similar to that employed in the sector of pesticides (UIPP, 2007).

In Italy the main rules concerning the management of biocides are D.P.R. n. 223 of 1988, D.Lgs. 285 of 1998, D.P.R. n. 392 of 1998 and D.Lgs. n. 174/2000. Instead, concerning phytosanitary products the D.P.R. n. 290 of 2001 is a measure that specifies rules for the production, marketing, sale and use of plant protection products and related coadiuvants. It integrates with D. Lgs. n. 194 of 1995, which transposes the Directive 91/414 EEC and forms with this one a sort of "combined text" on plant protection products.

1.3. Tools used in ecotoxicology to detect pesticide pollution in water and their limits

Ecotoxicology, defined by Truhaut in 1977 as "the branch of toxicology concerning the study of toxic effects on the ecosystem constituents, animals (including human), vegetables and microorganisms, caused by natural or synthetic pollutants in an integral context", is a transversal science that goes across field and laboratory studies, from cells to communities. At the moment, researches in ecotoxicology rarely connect the observed flux and fate of pollutants in the ecosystem with the observed effects on organisms (Forbes *et al.*, 1997; Schulz, 2001; Aubertot *et al.*, 2005; Liess, 2005). With regard to the water quality, actual monitoring programs have many limits in terms of technical analyses, cost, sampling methodology and evaluation of the biological effects of this kind of pollution.

From a methodological point of view, the high number of monitoring networks and sampling frequency are very heterogeneous within and among the different water agencies, depending

on the pollutant sought. In addition, the monitoring of water quality based on discrete samples does not provide an integrated view in the time of pollution levels: a water sample can be pesticides free even if a passenger pollution is appeared in the monitored station. In fact, the release of pesticides into rivers is dependent on significant rainfall events (floods), which in spite of the short duration cause high concentrations of pesticides in rivers and can have a significant biological effect. (Spalding *et al.*, 1989; Forbes *et al.*, 1997; Schulz, 2001; Ferenczi *et al.*, 2002; Neumann *et al.*, 2003; Aubertot *et al.*, 2005). Moreover, the majority of the monitor programs for the water quality, carried on since the nineties, has focused on the contamination of water resources by pesticides without consider the effects of this pollution on aquatic ecosystems.

From an analytical point of view, in the case of chemical analysis the more used tool is chromatography and the sample strategies are different (discrete samples, automated samples, passive samplers etc.). However, the research in water with the only physical-chemical tools of all the pesticide molecules and their metabolites is currently impossible, both in financial and technical terms. In addition, in the natural environments many positive or negative interactions may occur among molecules. Thereby, they can affect the state of ecosystem health, without allow their detection by the physical-chemical analyses.

Looking at the biological tools, examples are the biomarkers, which consist in an observable and/or measurable change at a molecular, bio-chemical, cellular, physiological or behavioural level. The alteration reveals past or present exposure of organisms, at one or more chemical pollutants (Lagadic *et al.*, 1997). However, the adaptation of this technique formulated in the laboratory under controlled conditions to the natural ones, encounters the high abiotic (light, nutrients, pH etc.) or biotic (age, sex, physical conditions etc.) variability. In addition, the specificity of these tools to some molecules prevents the detection of the global pollution, which is a characteristic of the natural environment (Lagadic *et al.*, 1998). Moreover, the extrapolation of results, provided by biomarkers at the cellular level, to estimate the effects of a pollutant to a population or a community level, found difficulties (Lagadic *et al.*, 1998; Aubertot *et al.*, 2005). Other biological tools are the bio-tests, mainly used to evaluate and predict the ecotoxicological potential of substances. Different standardised tests exist, like the Daphnies 24 hours test (EN 1506341), the Algae 72 hours test (NF T90-360) or the Ames' test. However, they provide data which are available only for a very small number of species exposed to a single molecule while, in the natural communities, species interact among

themselves and the answer of one of these can influence the answer of another (De Noyelles *et al.*, 1982). In addition, the natural conditions are extremely variable within a station or among different measuring points. Instead, bioindicators consist in “species or groups of species which, by their presence and/or their abundance, are indicative of one or more properties of the ecosystem where they live” (Lagadic *et al.*, 1997). Combining these various information with environmental preferences (nutrients, acidity, salinity, oxygen etc.) makes it possible to estimate watercourse conditions (Blandin, 1986; Rosenberg *et al.*, 1993; Stevenson *et al.*, 1999). Other devices are the biological key indices. Some examples of French indices are: the Biotic Index and the Global Biological Normalized Index (IBGN) for the benthic macro-invertebrates, the Pollution-Sensibility Index (IPS) and the Biological Diatoms Index (IBD) for the compartment of benthic diatoms, the Biological Macrophyte River Index (IBMR) for superior algae, the River Fish Index (Indice poisson rivière, IPR) and the Oligochaeta Bio-indication Sediment Index (IOBS). Between the two indices regarding diatoms, IPS and IBD, created by Cemagref Bordeaux, only the IBD is normalized (NF T90-354, 2000) (Coste, 1982; Lenoir *et al.*, 1995). Both indices are used in France and Europe (Prygiel *et al.*, 1996) to estimate the overall quality of the water courses. It is necessary to say that biological indices, including that based on the benthic diatoms, do not allow, at the present time, to differentiate the pollution caused by toxic compounds (pesticides and heavy metals) from other pollution (Sabater, 2000; Gold *et al.*, 2002; Dorigo *et al.*, 2004; and Aubertot *et al.*, 2005). They are, in fact, designed to detect the organic pollution. In the case of pollution by pesticides, where a large number of molecules can occur, the adaptation of existing indices is not easy. Moreover, heavy pollution of waterways by fertilizers contributes, as well, to hide the effects of pesticides (Nystrom *et al.*, 1999).

Current strategies, both physical-chemical and biological, do not provide representative information on pollution levels and the use of more complete, automatic, samplers is limited by the cost of such systems. The expectation is, also, high from agencies monitoring the water quality, which wish to origin biological tools sensitive to pollution by pesticides, similar to those concerning benthic diatoms, recognized for detecting organic and trophic pollution (Debenest, 2007).

1.4. Diatoms, a recognised bioindicator

The name diatom comes from the Greek word *diatomos*, which means “cut in half”, because shells of these cells have two overlapping, symmetrical valves, similar to a Petri dish. Diatoms are unicellular organisms that may live as individuals or survive more commonly in colonies. They still thrive in the majority of waters of the Earth, both salt and fresh. Diatoms are perhaps the most abundant aquatic organisms and provide food for a multitude of lake and sea animals. They are especially important in ocean, where they are estimated to contribute up to 45% of the total oceanic primary production (Mann, 1999). Their most distinctive characteristic is the possession of an elaborate, siliceous cell wall, called frustule, feature that is used to define and classify species. The silica that composes the cell wall is synthesized in the cellule by the polymerisation of silicic acid monomers. Thick layers of these diatom shells have become fossilized in rock or packed in shale and clays. Because silica resists to degradation, diatoms are regularly preserved in both freshwater and marine sediments and, in conjunction with knowledge of their ecological specificity, such fossils have been used to infer lake or ocean histories. These algae are non-motile or capable of only limited movement along a substrate by secretion of mucilaginous material by a slit called raphe. They are autotrophic organisms and, therefore, restricted to the photic zone.

1.4.1. Classification

There are more than 200 genders of living diatoms and it is estimated that there are approximately 100 000 species (Round *et al.*, 1990; Canter-Lund, 1995). Although diatoms are usually microscopic their size can vary from 2 μm to 1 mm. Diatoms (Bacillariophyta class) belong to the Heterokontophyta phylum, into the kingdom of Protists (Van Den Hoek *et al.*, 1995; Quéguiner, 2007). They are distinguished from others algae by some of their pigment of the family of xanthophylls (fucoxanthin, diatoxanthin, diadinoxanthin). They also contain chlorophyll “a” and “c” and β -carotene. The systematic is based on the form and ornamentation of valves to identify among the 100 000 existing species (Van Den Hoek *et al.*, 1995).

Diatoms are divided into two orders:

- the Pennales (called Bacillariales), which have valve arranged in relation to a line and tend to appear bilaterally symmetrical. The valves are ornamented with pores, processes, spines and other distinctions;
- the Centrales (called Biddulphiales), which have valves arranged basically in relation to a point, an annulus or a central areola and tend to appear radially symmetrical.

This classification system, created by Simonsen (1979) and further improved by Round *et al.* (1990), is the most accepted. Pennate diatoms (Figure 1.2) are divided into two suborders: the Fragilariineae, which do not possess a raphe and the Bacillariineae, which possess a raphe (UCL, 2002). Instead, centric diatoms may be divided into three sub-orders based primarily on the shape of the cells, the polarity and the arrangement of the processes: the Coscinodiscineae, with a marginal ring of processes and no polarity to the symmetry, the Rhizosoleniineae, with no marginal ring of processes and unipolar symmetry, and the Biddulphiineae, with no marginal ring of processes and bipolar symmetry (Figure 1.3).

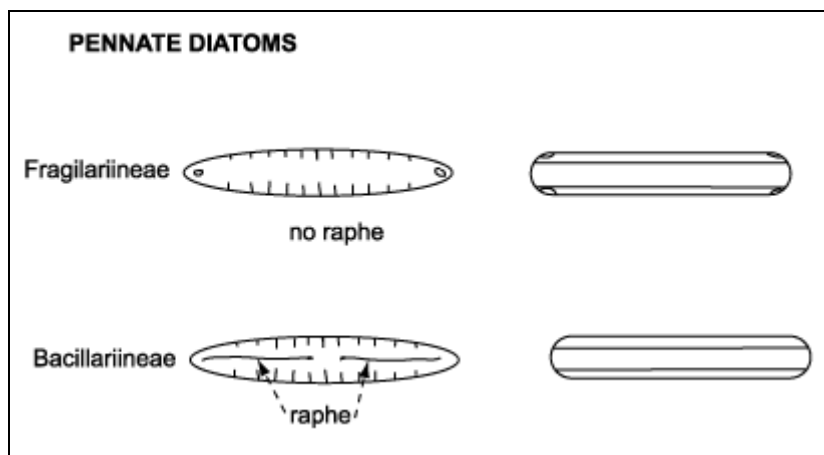


Figure 1.2: Schematic diagram of pennate diatom suborder (UCL, 2002).

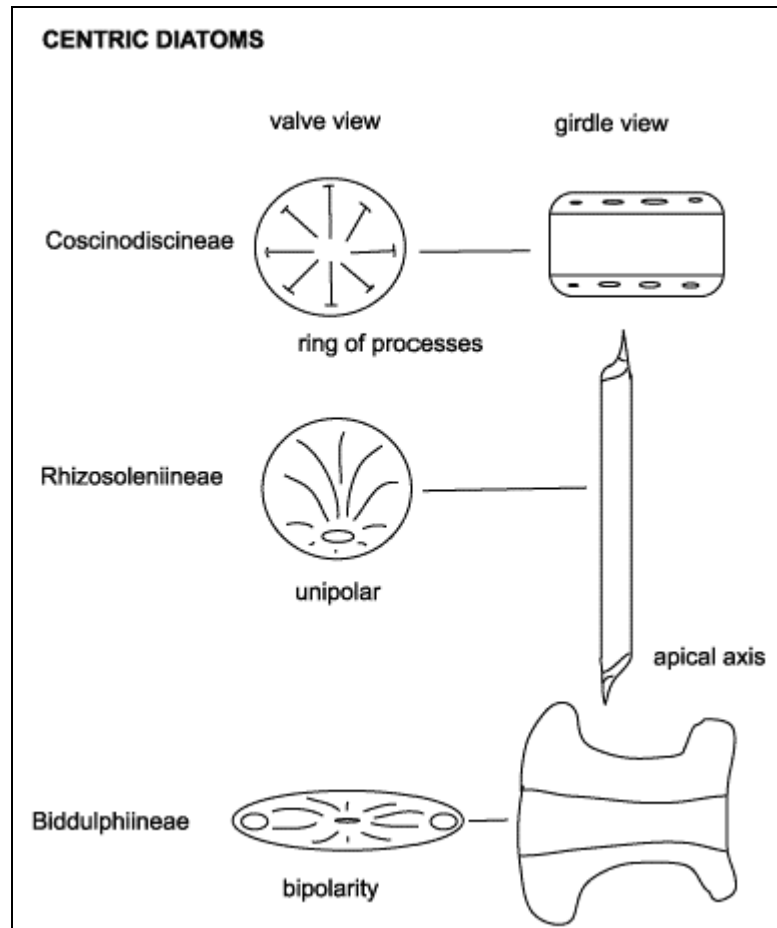


Figure 1.3: Schematic diagram of centric diatom suborder (UCL, 2002).

1.4.2. Ecology

- Environment

The adaptation and survival capacity of these microalgae give them a high ubiquity. Diatoms are found not only in aquatic environments (marine and continental ones), but also in the air (aerophile species) and terrestrial ones (floors, walls of caves) (Prygiel *et al.*, 2000; Berard *et al.*, 2004). When conditions become unfavourable (e.g. abnormal drought, light, nutrient), resting spores are produced by the cell. As soon as optimal conditions are restored, these spores germinate to give the same cells (Quéguiner, 2007). In the aquatic ecosystems can be distinguished: planktonic diatoms, living free suspended in the water column, and benthic diatoms, living attached to the surface of submerged objects. Planktonic diatoms constitute the bulk of phytoplankton in lower parts of watercourses (lentic circles, channel), lake and sea environments. In freshwaters and marine environments they typically exhibit a "bloom and

bust" lifestyle. When conditions in the upper mixed layer (nutrients and light) are favourable (e.g. at the beginning of spring) they can dominate phytoplankton communities (Furnas, 1990). With regard to benthic diatoms, they live attached to the surface of submerged objects, to an equivalent depth to the photic area of rivers, where light is sufficient to ensure photosynthesis. These micro-algae grow in a biological matrix, the biofilm, which includes bacteria, algae and fungi, bound by extracellular polymeric substances (including polysaccharides) secreted by diatoms. It is the nature of this substrate which determines the kind of diatom population found.

- Way of life

On the same substrate, the communities of diatoms change during seasons. With the beginning of spring, pioneer species develop (e.g. *Achnanthes sp.*, *Cocconeis sp.*), mainly pennate of small size, able to adhere very narrowly to the substrate (Korte *et al.*, 1983; Ghosh *et al.*, 1998; Sekar *et al.*, 2004). Thus, diatoms dominate the algal settlements, thanks to the favourable trophic flows at the beginning of spring (Figure 1.4). As soon as these conditions disappear, at the beginning of the summer, diatoms are supplanted by green algae and cyanobacteria. The increase in the grazing of zooplankton and benthic macro-invertebrates also contributes to this decline (Leitão and Couté, 2005). Only about in the middle of the winter, the conditions become too unfavourable to the development of other algal groups and diatoms take again the advantage; strong currents favour the growth of certain species such as *Gomphonema parvulum* (Kützing) and *Achnanthes sp.* (Ghosh *et al.*, 1998). Communities of diatoms, therefore, are subjected to perpetual seasonal reorganizations according to the medium conditions. Their capacity to develop very quickly can explain their importance in the watery ecosystems (Debenest, 2007).

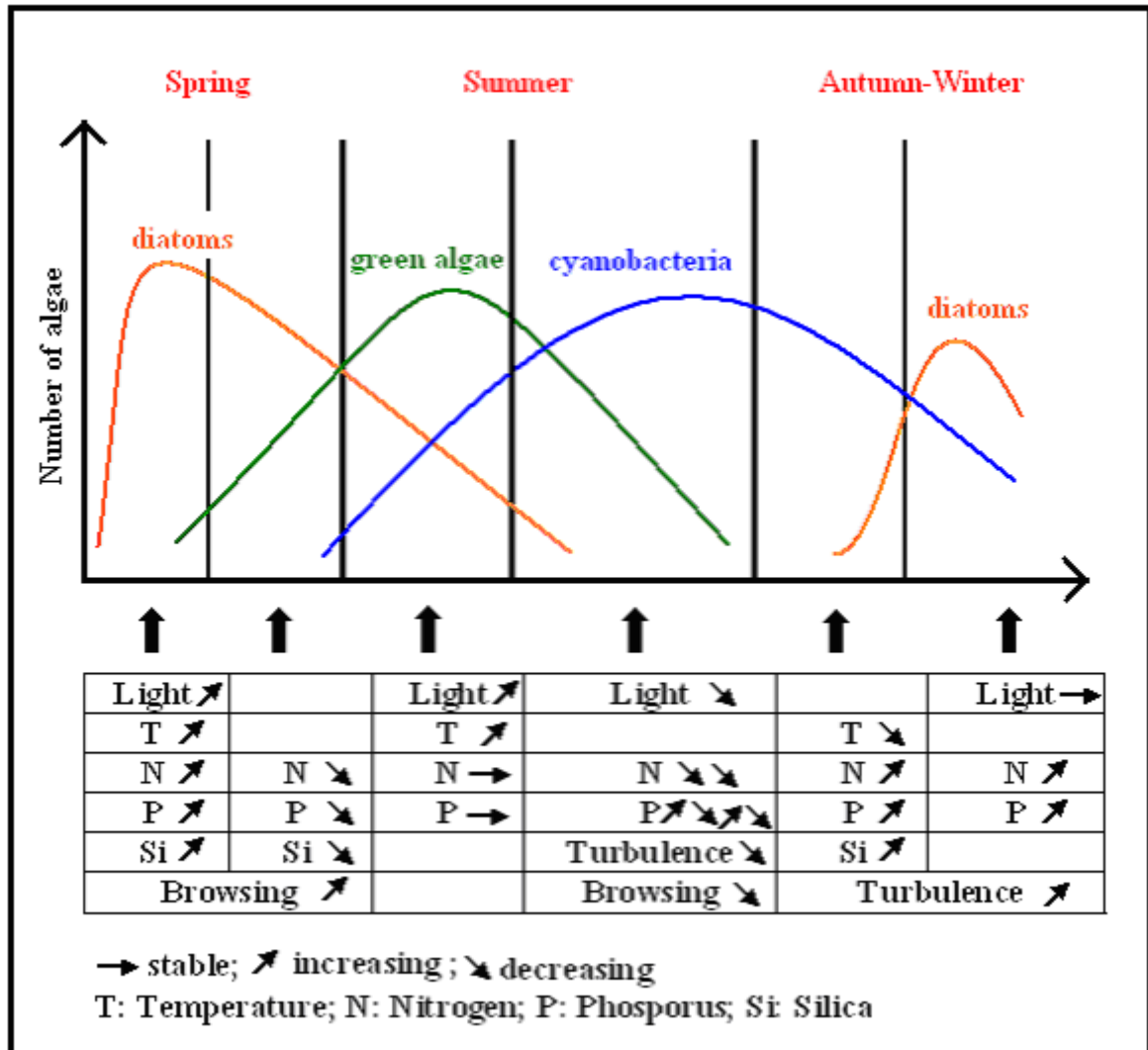


Figure 1.4: Schematic representation of the seasonal patterns of green algae, cyanobacteria and diatom populations (Leitão and Couté, 2005).

- Place in the ecosystem

In all environments, both marine and continental, diatoms are important producers of organic matter. The share of marine diatom primary production, at a planet level over one year, is between 25% and 45%, according to the estimates (Nelson *et al.*, 1995; Van Den Hoek *et al.*, 1995; Quéguiner, 2007). In some cases, where the power of the current limits the development of other algae, primary production is the result of diatoms alone; the algal biomass is then dominated by these micro-algae (Ghosh *et al.*, 1998). As a result, diatoms play a fundamental role as food source for many grazers (e.g. protozoa, macroinvertebrates, fishes) and filter organisms (Round *et al.*, 1990; Stevenson *et al.*, 1999).

- Utility of diatoms: some examples

Because of their ecological importance, diatoms are widely used in aquaculture to feed fishes, crustaceans and molluscs (Avendano-Herrera *et al.*, 2007; Chen, 2007). In fact, diatoms form the basis of some of the most productive food chains and play a fundamental role in the export of carbon to higher trophic levels (Cushing, 1989) and to the deep sea (Goldman, 1993, Buesseler, 1998). Scientific work has, also, tried in recent years to identify diatoms and other microorganisms, which can synthesize lipids used as substitute for fossil fuels (Sudo *et al.*, 1991). Moreover, diatoms in lake sediments have been used to estimate changes in pH and nutrient status, as well as climate change. Finally, these micro-algae are still used by the scientific police in cases of drowning, to determine the cause of death (Ludes *et al.*, 1996).

1.4.3. Ultra-cell structure

Diatoms are unicellular vegetal aquatic eukaryotic organisms and, like all eukaryotic cells, they have a nucleus delimited by a membrane and generally positioned during interphase, in the centre of the cell between chloroplasts (Round *et al.*, 1990; Pickett-Heaps, 1991; Van Den Hoek *et al.*, 1995). In some cases, just before mitosis, the nucleus can be seen against the wall, and more specifically, against the connective bands (Figure 1.5), but always in the central plan of the cell perpendicular to the transapical plan (Edgar *et al.*, 1984). The shape of the nucleus varies a little bit for the same species. The differences among species are linked to the size and the shape of the cell, factors that also affect other organelles (vacuoles, cytoplasm, filaments etc.) (Duke and Reimann, 1977). Regarding the chloroplasts, their number and shape vary between centric and pennate diatoms: centric diatoms, usually, have a large number of small discoid chloroplasts, while pennate limit themselves to two major plastids (Van Den Hoek *et al.*, 1995; Raven *et al.*, 2000).

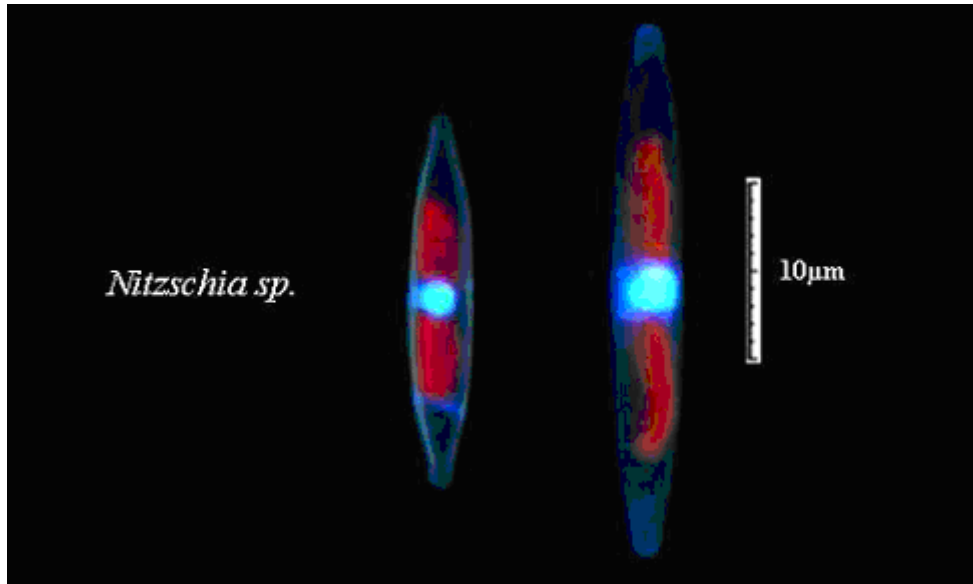


Figure 1.5: Sight by fluorescence microscope of the pennate *Nitzschia sp.* whose nucleus (blue) marked by Hoechst 33342 is surrounded by two chloroplasts (red) (Debenest, 2007).

The main peculiarity of diatoms lies in the composition of the cell wall structure, own of all plant cells. In contrast to other vegetal organisms, this structure consists of a siliceous hull (opaline silica polymerized hydrated, $\text{SiO}_2 \cdot n\text{H}_2\text{O}$) embedded in an organic matrix (Campbell, 1995; Raven *et al.*, 2000). This shell, or frustule, consists of two valves (epivalve and hypovalve), that fit one in the other and the suture between these valves are provided by a set of interlayer siliceous tapes, forming a connective belt (Figure 1.6).

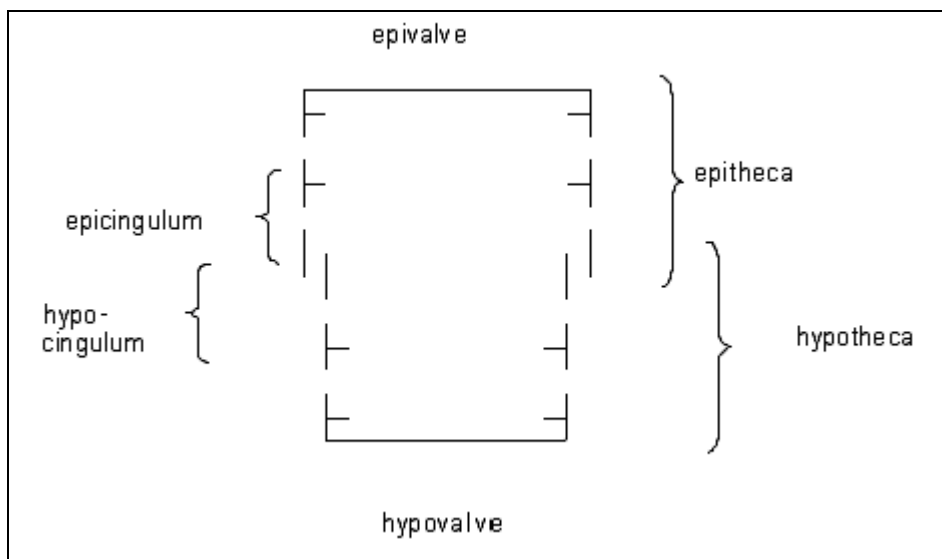


Figure 1.6: Diagrammatic section showing the frustule terminology (UCL, 2002).

Valves present many pores and, in some pennate diatoms, also a longitudinal cleft called raphe, which allows interaction with the external environment (Figure 1.7). Diatoms secrete the mucilage through these openings in the form of polysaccharides, which are involved in adherence to substrates, in the cell mobility and in the colony formation (Bourrelly, 1981; Van Den Hoek *et al.*, 1995; Bertrand, 1999).

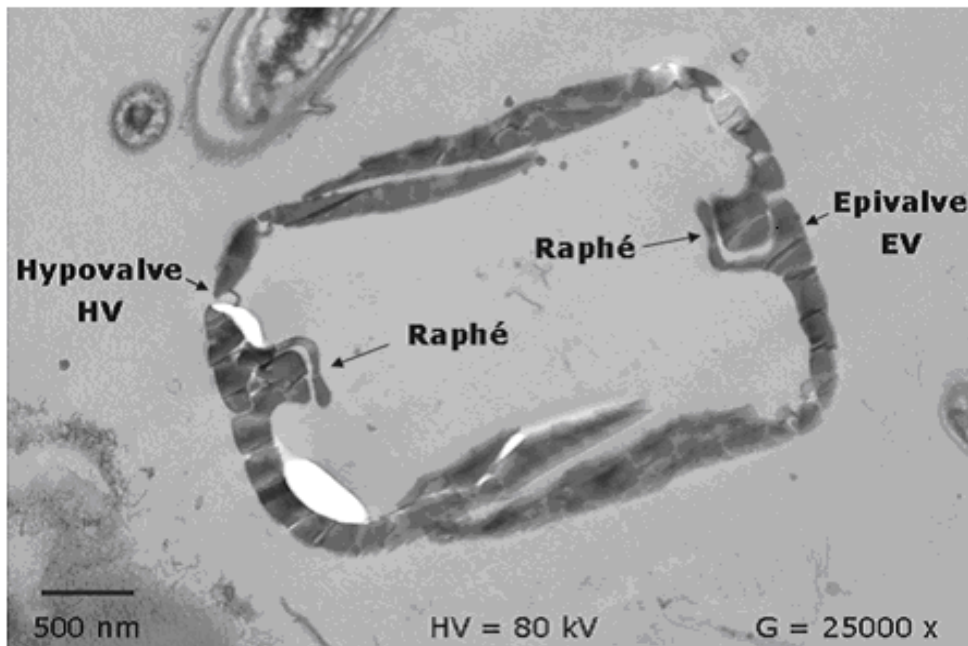


Figure 1.7: Transversal cut of a pennate diatom with two raphes, seen by transmission electronic microscope (Debenest *et al.*, 2008).

The mechanisms responsible for the formation of this mineral structure are subject to discussion in the scientific community. Silica absorbed by the cell, would be accumulated in the form of silicic acid in a storage vesicle, through the merger of microvesicles, whose origin has not yet been established. Some writers quote the Golgi apparatus, while others the granular endoplasmic reticulum (Lee *et al.*, 1992). Transport of microvesicles would be controlled by the microfilaments, while, transport of the storage vesicle, would be the responsibility of microtubules (Duke and Reimann, 1977; Pickett-Heaps *et al.*, 1979; Lee *et al.*, 1992; Van Den Hoek *et al.*, 1995). When arrived on the surface of the cell membrane, siliceous material condenses and polymerizes on the hydroxyl groups of two amino acids, serine and threonine, to form a new valve (Round *et al.*, 1990). For some writers polymerization of silica should take place directly in the storage vesicle (Wilt, 2005).

Membrane and organic material present in the storage vesicle form an ephemeral envelope around the valve in formation. This structure consists of primary sulphated polysaccharides, including fucose, mannose, and galactose, synthesized in the early stages of the valve development. (Round *et al.*, 1990; Van Den Hoek *et al.*, 1995). A second envelope, called diatopetic layer, is then synthesized. It is composed of polypeptides, acid polysaccharides and, in some diatoms (including *Navicula pelliculosa*), a sulphated polysaccharide, the glucuronomannan (Round *et al.*, 1990; Van Den Hoek *et al.*, 1995; Quéguiner, 2007). This organic matrix protects frustule against any form of dissolution conditions linked to physical and chemical environments (Debenest, 2007).

1.4.4. Multiplication and reproduction

Vegetative cell multiplication is the predominant method of multiplication in diatoms. When conditions are favourable for their proliferation, the mother cell turns into two daughter cells. The mitosis of benthic diatoms fundamentally does not differ from that of other plant organisms. Starting with two centromeres, the mitotic spindles, along which chromosomes migrate during the anaphase, deploy (Pickett-Heaps *et al.*, 1979; Round *et al.*, 1990; Pickett-Heaps, 1991). The main feature of cell division in diatoms is the synthesis, from each daughter cell, of a new valve within each valve inherited from the mother cell (Figures 1.8 and 1.9). The two valves do not have the same size and also diatoms daughter are of different size: the diatom that comes from the big valve has the same size of the diatom mother, while the diatom that comes from the small valve is slightly smaller. This process is repeated with each cycle, thus, the average diatom size gets progressively smaller with each round of replication (Becker, 1996). When a threshold equal to the 30% of the initial size is reached, sexual reproduction is triggered and restores the original size of the cell. The rate of reproduction is extremely variable among species. Some writers believe that small pioneer species develop faster than large cell, but data are reduced to a few species (Round *et al.*, 1990; Leitão and Couté, 2005).

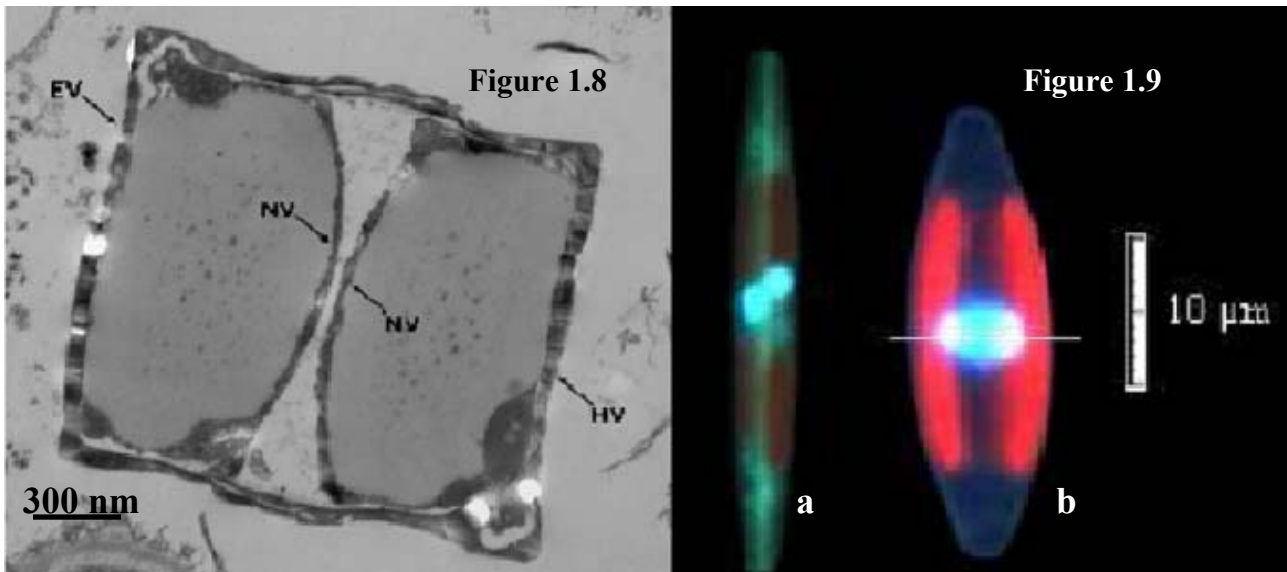


Figure 1.8: Transversal cut seen by transmission electronic microscope of a diatom in the final division. Two new valves (NV) have been secreted into epivalve (EV) and hypovalve (HV) (Debenest *et al.*, 2008).

Figure 1.9: View by the fluorescence microscope of diatoms in division. “a”: anaphase or telophase, the walls are being synthesized; “b”: metaphase, the nucleus is divided into two along a transapical white axis (Debenest *et al.*, 2008).

As already said, vegetative reproduction progressively reduces the average size of diatom frustule and sexual reproduction restores the original size. Diatoms have a monogenetic diplontic life cycle: meiosis takes place at the formation of gametes (Figures 1.10). In diatoms, sexual reproduction is characterized by the origin of one auxospore, which gives birth to a new diatom (Gayral, 1975). Centric and pennate diatoms have two different ways of sexual reproduction. In centric diatoms vegetative cells act as gametocysts, some of them produce one or two motionless oospheres and other small uniflagellate cells (Round *et al.*, 1990; Quéguiner, 2007). In this case, sexual reproduction is like an oogamic reproduction, which leads to auxospore formation, whose division produces a diatom of maximum size (Reviere, 1953; Raven *et al.*, 2000). For pennate diatoms, haploid gametes form after an uneven cell division: the smallest haploid mass gives male gametes and the other oospheres. The fusion of gametes of two adjacent cells, thanks to a separation between the two valves, produces two auxospores. Each auxospore gives birth to a zygote, which becomes a cell morphologically slightly different to clones it has engendered by vegetative propagation (Round *et al.*, 1990; Quéguiner, 2007). The frustule formation starts after maximal size is reached. Gamete formation to appearance of initial cells (auxospores) takes 2-4 days (entire process of sexual reproduction).

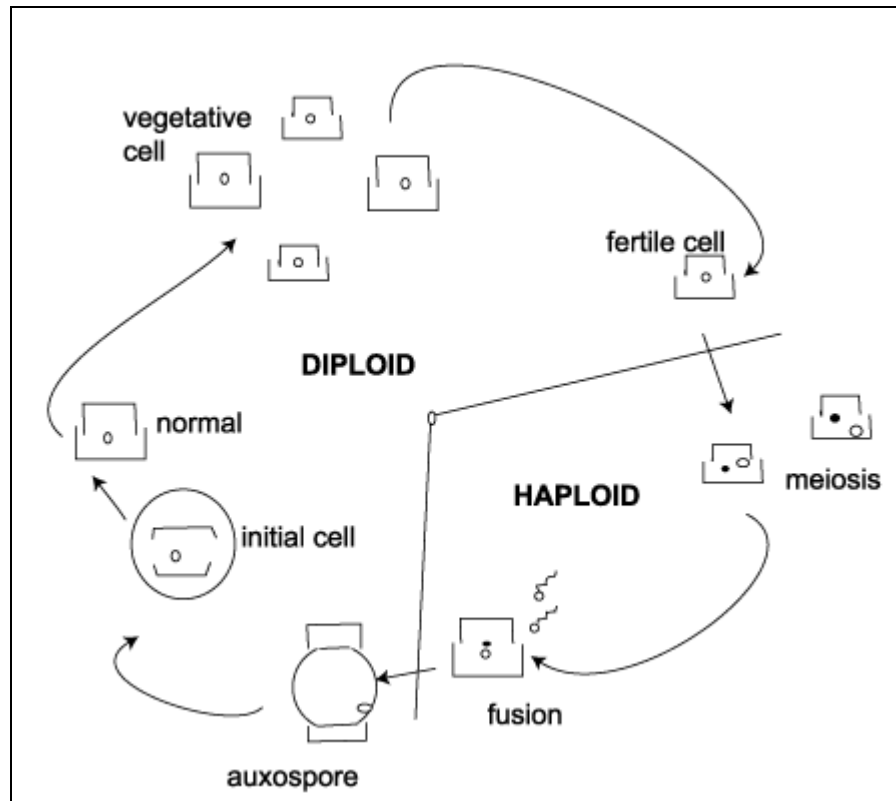


Figure 1.10: Diagram showing simplified life cycle of a typical diatom (UCL, 2002).

1.5. Effects of pesticides on diatoms

The effects of pesticides, particularly herbicides, on algae and diatoms have been subjects of a lot of researches. In this paragraph some of the known effects of pesticides on diatoms are presented.

1.5.1. Cytology and ultra-structure

Diatoms have an internal network of filaments (microtubules, actin filaments, microfilaments), which is involved in the phases of proliferation and plays an important role in the maintenance of the internal cell layout. These filaments are essential to the cell survival. Therefore, many herbicide molecules have been created around this target and algae can be exposed to this type of active substances in waters (Debenest, 2007). Coss and Pickett-Heaps (1974) have showed that an herbicide of the carbamate family (Isopropyl N-Phenyl Carbamate, IPC) leads to distortions of mitotic spindles in a green alga (*Oedogonium*

cardiacum). Moreover, effects of colchicine, a mitosis inhibitor acting like IPC on microtubules, were studied (Coombs *et al.*, 1968; Edgar *et al.*, 1984; Puiseux-Dao, 1989). Exposure to this compound disrupts tubulin polymerization and leads to a disorganisation of mitotic spindles (Coombs *et al.*, 1968; Puiseux-Dao, 1989). Similarly, Spurck and Pickett-Heaps (1994) have showed that the ordered arrangement of chromosomes in metaphase could be altered by the effect of the active ingredient of a drug, diazepam, on mitotic spindles in two diatom species, *Surirella robusta* and *Hantzschia amphioxys*. Regarding the involved cellular mechanism, the effects on microtubules have been mainly studied. For example, the colchicine blocks the aggregation of tubulin “ α ” and “ β ” binding these molecules (Puiseux-Dao, 1989). According to Coss and Pickett-Heaps (1974), the action mechanisms of colchicine and IPC are similar. Herbicides could, therefore, also disrupt the synthesis of microtubules. The IPC belongs to a family that includes both fungicides and herbicides. Therefore, the internal skeleton of diatoms can also be affected by fungicides (Debenest, 2007).

Few studies have been conducted on the impact of toxic chemicals and pesticides on the nucleus. Cassoti *et al.* (2005) have observed a dispersion of DNA in cells among a marine diatom (*Thalassiosira weissflogii*) exposed to an aldehyde (2-trans,4-trans-decadienal). This alteration appears linked to a form of apoptosis, a kind of programmed cell death. Another chemical, the colchicine, would lead to a fragmentation of the nucleus in diatoms; multinuclear cells have been observed in a species of diatom (*Navicula pelliculosa*) exposed to this molecule (Coombs *et al.*, 1968; Duke and Reimann, 1977). The responsible mechanisms for such nuclear deterioration can be extremely varied. Rijstenbil (2001) reported that oxidative stress induced by solar radiation may alter the membranes and DNA in diatoms. It is to consider that toxics, including pesticides, origin in diatoms similar mechanisms already observed in higher plants exposed to these compounds (Debenest, 2007). The effects of toxic substances on the nucleus can also be related to alterations in microtubules. As previously seen, some compounds (herbicide IPC and colchicine) can disrupt microtubules, which play an essential role in the separation of the genetic equipment during mitosis. This is known as aneugenic effect. At the same time, Coombs *et al.* (1968) and Duke and Reimann (1977) have shown a disruption of mitotic spindles (consisting of microtubules) and an origin of multi-nuclear cell under the effects of colchicine. Therefore, the disruption of microtubules, under the action of toxic, lead to a fragmentation of nucleus in more than two parts during mitosis (Debenest, 2007).

Several hypotheses have been suggested to the origin of frustule distortions (Figure 1.11). Of all the possible causes that could lead to the induction of frustule abnormalities, heavy metals are most often cited in the scientific literature. Studies *in situ* have, in fact, identified abnormal forms in environments contaminated by heavy metals pollution (Feldt *et al.*, 1973; McFarland *et al.*, 1997; Dickman, 1998). In the case of more specific experiments, abnormal forms were observed in communities exposed to cadmium, copper, mercury and zinc (Thomas *et al.*, 1980; Adshead-Simonsen *et al.*, 1981; Fisher *et al.*, 1981; Rijstenbil *et al.*, 1994; Ruggiu *et al.*, 1998; Gold *et al.*, 2003; Gomez *et al.*, 2003; Cattaneo *et al.*, 2004). Instead, the responsibility of chemicals, especially pesticides, in the appearance of abnormalities in the siliceous wall, has been little studied. Schmitt-Jansen and Altenburger (2005) have observed abnormal forms on diatoms exposed to high concentrations of isoproturon (maximum 312 µg/l), a widely used agricultural herbicide. Several authors reported that molecules, such as colchicine or antimicrotubule drugs, could disrupt the general shape of frustule (Duke and Reimann, 1977; Edgar *et al.*, 1984; Van Den Hoek *et al.*, 1995). Also free radicals (e.g. HO[•]) are dangerous compounds. They are produced in natural conditions, for example by light, or under the influence of abnormal exposure to toxic chemicals or radiation. When defence mechanisms are no longer enough, the high oxidizing ability of these compounds can alter the membrane integrity of the cell. For Rijstenbil *et al.* (1994 and 2001) oxidative stress, induced by these compounds, can also lead to the formation of abnormal frustules, without the cellular mechanisms have been yet identified. Other environmental factors, such as nutrient deficiencies and pH, play probably an important role in the origin of abnormal forms of diatoms (Dickman, 1998). According to Thomas *et al.* (1980) and McFarland *et al.* (1997) strong silica deficiencies would give appearance of this kind of alteration. The appearance of deformed frustules could also be the result of a mechanical pressure of the various individuals in the event of overcrowding (Andresen *et al.*, 1991). In addition, for Stoermer (1998), the presence of abnormal forms in a community could be due to the development of a clonal population started from an individual, which has suffered a genetic mutation, responsible for the deformation of its cell wall. Cellular mechanisms involved in the genesis of the abnormal forms are still poorly understood. Despite of that, the change in the internal layout of the cell seems to have an impact on the frustule secretion (Edgar *et al.*, 1984; Round *et al.*, 1990).

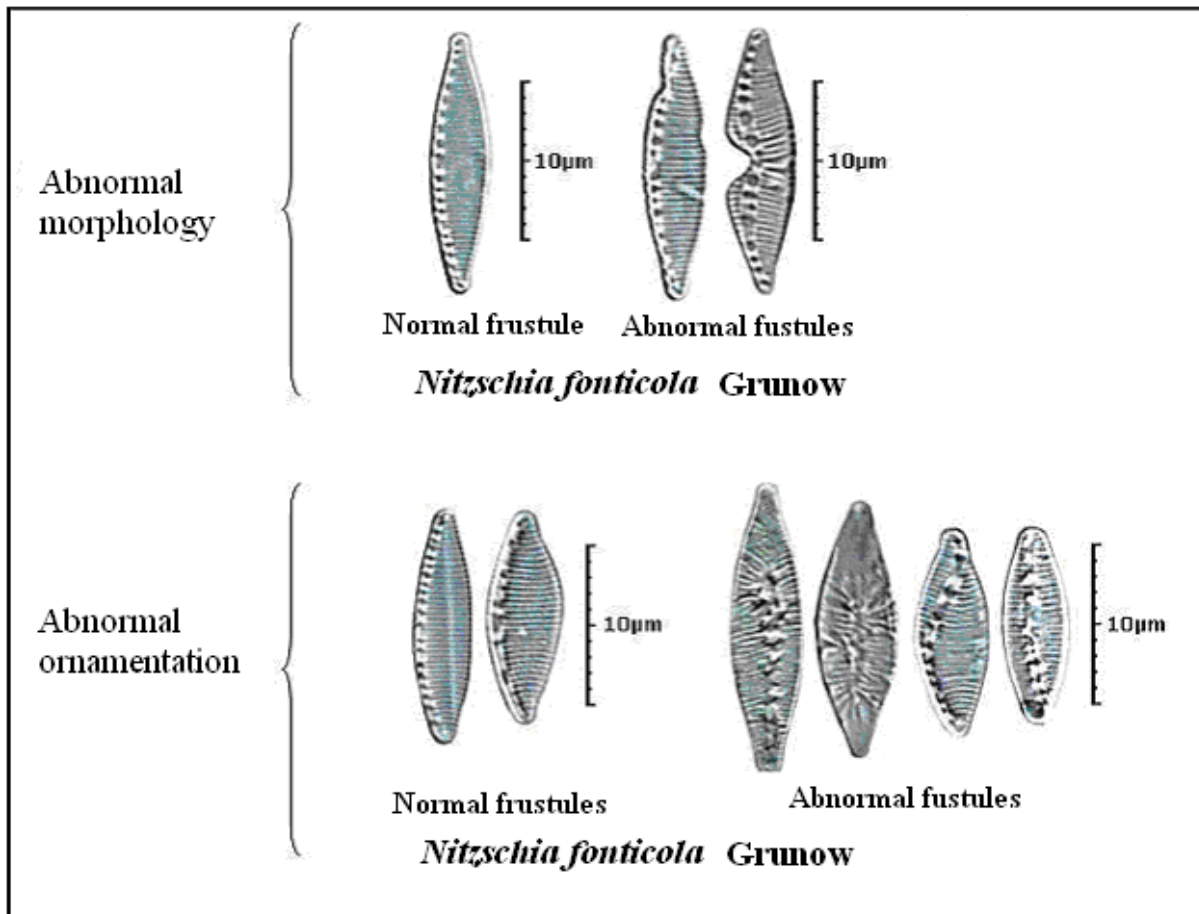


Figure 1.11: Photos by optical microscope of normal and abnormal frustules in different benthic diatom species (Debenest, 2007).

1.5.2. Cell metabolism

The photosynthetic activity is the target of a great range of herbicides used in agriculture, as the s-triazines and the substituted ureas (phenylurea and sulphonylurea). These molecules inhibit the operating entities of photosynthetic complex, the photo-system II. They bind themselves with the protein D1 and block the transfer of electrons necessary for a redox reaction (Hill reaction), consisting in the splitting of water to give two electrons to the reaction centre P680 (Berard and Pelte, 1996; Peres *et al.*, 1996; Dorigo *et al.*, 2001; Leboulanger *et al.*, 2001; Berard *et al.*, 2003; Dorigo *et al.*, 2004). It has been observed that concentrations of atrazine in the range from 20 and 500 µg/l could significantly affect photosynthesis of phytoplanktonic algae (De Noyelles *et al.*, 1982). Different short term (some days) studies, showed the great potential of irgarol, s-triazines family, used to eliminate fixed algae and diatoms on the ships hulls, inhibitor of photosynthesis in algae (Dahl *et al.*,

1996; Nystrom *et al.*, 2002; Berard *et al.*, 2003). In the case of long term studies (a few weeks), effects even appeared at relatively low concentrations, 0,063-0,25 µg/l (Dahl *et al.*, 1996). With this same type of experimental system, in the long term, Kasai *et al.* (1995) tested an herbicide from the same family, simetryne, which reduced the photosynthetic activity of phytoplankton, but only at high concentrations (0,1 and 1 mg/l). Other authors obtained similar results with the exposure to other pesticides: diquat, hexazinone (Peterson *et al.*, 1997), several substances of the sulphonylureas family (Nystrom *et al.*, 2002) and glyphosate (Goldsborough *et al.*, 1988).

Few data concerning the impact of herbicides on the cellular respiration are available. Hamala and Kollig (1985) observed, on periphytic communities, that atrazine treatments increased their heterotrophic activity. The ratio photosynthesis/respiration was less than 1 after the addition of atrazine and the net primary productivity decreased. These authors observed that the respiratory demand gained as the photosynthetic activity declined.

For several species of diatoms, it was shown that exposure to atrazine could significantly reduce the production of proteins, including D1 and D2, which play an important role in the cellular mechanisms of photosynthesis (Weiner *et al.*, 2007). Other active substances, as nicosulfuron, which inhibits the synthesis of amino acids (valine and isoleucine), can also disrupt the proteins production in algae (Rimet *et al.*, 1999). Lipid accumulation was also observed in diatoms exposed to atrazine: in fact, increasing lipid can be a sign of nutrient deficiencies. Other molecules of the chloroacetamide family, as alachlor, may, at the opposite, inhibit fatty acid synthesis (Weiner *et al.*, 2007).

Herbicide molecules also affect the absorption of nutrients (NO₃, NO₂, Si) by algae. Krieger *et al.* (1988) have observed in microalgae exposed to a continuously high concentration of atrazine (134 µg/l) a decrease in the absorption of nitrate, nitrite and silica. Atrazine, therefore, disrupts nutrient absorption in cells. Many authors obtained similar results in experimental systems with other herbicides (Goldsborough *et al.*, 1986; Herman *et al.*, 1986; Gurney *et al.*, 1989). The increase in environmental nutrients concentrations is linked to the inhibition of periphyton, which does not retain more minerals of the upper layers of the sediment (Peres *et al.*, 1996). Temperature plays a significant role in this cellular mechanism. Krieger *et al.* (1988) noted some effects at 10°C, but not at 25°C, in the absorption of

nutrients from several periphytons exposed to peaks of four herbicides (alachlor, atrazine, metolachlore, metribuzin).

1.5.3. Reproduction and biomass growth

Some carbamates and toluidines have been formulated to inhibit cell division. Coss *et al.* (1974) have shown that Isopropyl N-Phenyl Carbamate (IPC) originated abnormal division of centrosomes. Pesticides affect, therefore, microtubules involved in the segregation of chromosomes during mitosis. Such mechanisms were highlighted among higher plants as *Vicia faba* L., treated with a genotoxic herbicide, maleic hydrazide (MH) (Grant *et al.*, 1992; Marcano *et al.*, 2004). In the pennate diatom *Navicula pelliculosa* exposed to an inhibitor of mitosis, colchicine, Coombs *et al.* (1968) have also observed the formation of multinuclear cells, sign of a disorganisation of mitotic spindles. The cellular mechanisms that are responsible for these changes are not still well known.

Diatoms, in particularly benthic ones, live in a community where species interact with each other. The impact of pesticides on one or more species affects, therefore, the whole community. Regarding the total biomass of biofilm, which includes amorphous (extracellular polysaccharides) and alive organic matter, a chronic exposure from medium to high concentrations of atrazine (from 2 µg/l to 500 µg/l) led to a significant decrease in total biomass (De Noyelles *et al.*, 1982; Krieger *et al.*, 1988; Jurgensen *et al.*, 1990; Carder *et al.*, 1998). The algal biomass is assessed by conventional dosage, with spectrometry, of some pigments, including chlorophylls “a”, “b”, “c” or by liquid chromatography. The concentration of chlorophyll “a” is one of the most important parameters for assessing the effects of pesticides on algal biomass. Several authors have shown that, among diatom communities, exposure to concentrations in the range of 10-1000 µg/l of atrazine led to a reduction of the quantity of chlorophyll “a” (De Noyelles *et al.*, 1982; Kosinski *et al.*, 1984; Krieger *et al.*, 1988; Jurgensen *et al.*, 1990; Berard and Pelte, 1996; Guasch *et al.*, 1997; Tang *et al.*, 1997; Carder *et al.*, 1998; Guasch *et al.*, 1998a). It was also found that a molecule of the family of substituted-ureas, the isotroturon, disturbed at high concentrations (40 and 312 µg/l) the development of algal biomass (Schmitt-Jansen *et al.*, 2005). Regarding the cell density, many authors have shown that atrazine could induce a decline of cell density in some

species of diatoms, overall centric, or at the opposite, an increase in other species, mostly pennate (Tang *et al.*, 1997; Berard *et al.*, 2001; Berard *et al.*, 2004). Regarding isoproturon, even at lower concentrations, Peres *et al.* (1996) have shown that this herbicide had a pronounced effect on cell density in benthic diatoms.

1.5.4. Community composition

Effects of pesticides on algae development are not necessarily direct. They can express themselves through the specific competition in a community: some tolerant species, multiplying more rapidly, take advantage of the most sensitive species (Berard *et al.* 2003). Berard and Pelte (1996) indicate that atrazine can alter the physiology of certain species by reducing their competitive capacity in the communities. Exposure to toxics may, therefore, lead to a selection of the most suitable species, breaking the existing balance within communities (Debenest, 2007). From the various studies conducted, it appears in the majority of cases that exposure to herbicides alters the diversity of the communities of diatoms. Thus, it was shown that the diversity of aquatic, but also land, communities of diatoms decreased as a result of exposure to atrazine (10-90 µg/l) (Hamala *et al.*, 1985; Berard and Pelte, 1996; Berard *et al.*, 2004). Isoproturon, also, induced a decline in the diversity of diatom communities exposed to high concentrations in the range from 40 to 160 µg/l (Peres *et al.*, 1996; Schmitt-Jansen *et al.*, 2005). On the contrary, at lower concentrations (2 µg/l), it was observed an increase of the diversity with the development of some minority species at the expense of other more sensitive, but still present at low levels of exposure (Schmitt-Jansen *et al.*, 2005).

Within diatom communities, benthic species preferring eutrophic environments seem to be more tolerant to herbicides exposure. In Appendix A it is possible to see the list of abbreviations used to identify diatom species. Characteristic species of environments polluted by trophic elements are: *Gomphonema parvulum* Kützing (GPAR), *Nitzschia palea* (Kützing) W. Smith (NPAL), *Asterionella formosa* Hassall (AFOR), *Navicula lanceolata* (Agardh) Ehrenberg (NLAN), *Fragilaria capucina* Desmazieres var. *vaucheriae* (FCVA), *Synedra acus* Kützing (SACU), *Achnanthes lanceolata* (Brébisson) (ALFR), *Achnanthes minutissima* Hustedt (AMIN) and *Cocconeis placentula* (Ehrenberg) (CPLA). These species are favoured

in the communities of benthic diatoms after exposure to herbicides of the family of s-triazines (atrazine and irgarol), under controlled conditions (microcosms, cell cultures) (Hamala *et al.*, 1985; Goldsborough *et al.*, 1986; Berard and Pelte, 1996; Muñoz *et al.*, 2001; Berard *et al.*, 2003) or natural conditions (Guasch *et al.*, 1998a; Berard *et al.*, 2003). ALFR and NPAL species are known for their resistance to atrazine (Kosinski *et al.*, 1984; Kasai, 1999; Dorigo *et al.*, 2004). Similar observations were performed among communities of diatoms treated with isoproturon. Some species tolerant to eutrophic conditions, mainly navicules, *Navicula minima* Grunow (NMIN), *Navicula halophila* (Grunow) Cleve (NHAL), *Navicula cryptocephala* Kützing (NCRY), but also *Gomphonema parvulum* Kützing (GPAR), have seen their health increase in communities exposed to this herbicide (Peres *et al.*, 1996; Schmitt-Jansen *et al.*, 2005).

Several studies in semi-controlled conditions (microcosms) have demonstrated that an exposure to herbicides, like atrazine or isoproturon, promoted the development of small species, *Navicula minima* (NMIN) in particular, *Sellaphora seminulum* (SSEM) and the achnanthes, *Achnantheidium minutissimum* (ADMI), ALFR, AMIN, CPLA (Goldsborough *et al.*, 1986; Peres *et al.*, 1996; Muñoz *et al.*, 2001; Schmitt-Jansen *et al.*, 2005). The scientific literature mentions that small species are pioneer and the first to colonize the substrate (Korte *et al.*, 1983; Sekar *et al.*, 2004). The strategy to survive of these "opportunistic" species is based on a high multiplication rate, allowing them to grow faster and colonize early substrates, even in unfavourable conditions (Goldsborough *et al.*, 1986; Peres *et al.*, 1996). In environments of high toxic pollution, these species are the only to sustain higher rates of reproduction. However, the primary colonization of substrates in unpolluted environments by small species, rather than by bigger ones, is discussed by the scientific community (Acs *et al.*, 1993). Another explanation, given by Rijstenbil *et al.* (2001), is that smaller species present concentrations of gammaglutamylcysteinyl-glycine, an antioxidant molecule important in the detoxification activity of cells, from 5 to 10 times higher than in bigger species. However, according to Tang *et al.* (1998), the sensitivity of algae to herbicides, such as atrazine, is linked to their ability to bioconcentration. The accumulation of herbicide increases with the biovolume. For Lockert *et al.* (2006), the relationship between cell volume and sensitivity to atrazine is not significant.

Some authors have noted that the N-heterotrophic species, as *Cocconeis placentula* Ehrenberg (CPLA), *Fragilaria crotonensis* Kitton (FCRO), NHAL, AFOR or NMIN, were tolerant of

exposure by inhibitors of photosynthesis (simazine, terbutryne and isoproturon) (Goldsborough *et al.*, 1986; Peres *et al.*, 1996). Diatoms are therefore able to maintain, in parallel, two metabolic pathways and moving from one to another, depending on the environmental conditions. The scientific literature explains the survival of certain pennate diatoms in adverse conditions (e.g. darkness, organic pollution) through such mechanisms (Hellebust *et al.*, 1977). In case of photosynthesis inhibition by an herbicide, certain species would be able to survive in the same way as in a medium with a low light. This fact explains their relative tolerance to herbicides which inhibit photo-system II (Hamilton *et al.*, 1988).

1.6. Factors interfering with response of diatoms to pesticides and with detection of effects

Response of algae and diatoms in particular to pesticide is not uniform. In fact, many environmental or ecological parameters can interfere with the degree of reaction of these microorganisms or even hide their responses (Debenest, 2007).

Ecological parameters can affect the response of these microalgae to pesticides (Debenest, 2007). Regarding the specific competition, in uncontaminated environments communities live in equilibrium among the different species. In the case of diatoms, cells are able to secrete chemical mediators, as the 2-trans,4-trans-decadienal aldehyde, which enable them to regulate their own growth (Casotti *et al.*, 2005). Therefore the species studied, isolated or in communities, do not have the same reaction to pesticides (De Noyelles *et al.*, 1982; Badr *et al.*, 1997; Peterson *et al.*, 1997). The competition and the interdependence among the different species affect, therefore, largely the response of algae. Most opportunistic competitors, as CPLA, are widely advantaged in adverse environment (Goldsborough *et al.*, 1986). The overall structural parameters (biomass, population density) are less sensitive to pesticides than the specific structural parameters (density by species, specific composition). Looking at the biofilm, among diatoms some species live in various kinds of substrates. Part of the biofilm organic matter is secreted by these organisms in the form of extracellular polysaccharides. These substances protect algae from the effects of pesticides (Peres *et al.*, 1996). The dynamic of substrate colonisation and the degree of biofilm development influence benthic diatom communities exposed to toxic compounds as atrazine (Guasch *et al.*, 1997). Moreover, biofilm represents an important source of food for many grazer organisms. The work of

benthophages modifies deeply the structure of the biofilm, altering the protective layer for cells. Grazing also affects the structure of cells, encouraging the effect of toxic elements (Muñoz *et al.*, 2001). However, the role of grazers in algae exposed by pesticides is difficult to evaluate under natural conditions (Debenest, 2007).

Environmental factors determine deeply the effects of pesticides on algal communities (Guasch *et al.*, 1997; Berard *et al.*, 2001; Navarro *et al.*, 2002). Several scientific studies have shown that diatoms are more susceptible to atrazine in case of high exposure to light (Guasch *et al.*, 1997; Guasch *et al.*, 1998a; Guasch *et al.*, 1998b). In natural conditions, where brightness varies greatly from one point to another, it is therefore difficult to compare effects on communities of different stations exposed to pesticides. At the end of spring and at the beginning of summer, the development of river bank vegetation can also potentially reduce the effects of pesticides on diatoms (Debenest, 2007). Navarro *et al.* (2002) have shown that tolerance to atrazine in periphytic communities was lower in spring than in summer. Also the availability of nutrients has a deep impact on the algae development. Indeed, it impacts on the physical conditions of individuals, on the level of development of biofilm in the case of periphytic species and on the species composition of communities. Therefore, the effects of pesticides on algae communities depend on the levels of nutrient concentration in the medium. Thus, it has been shown that nitrogen and phosphorus deficiencies could increase the sensitivity of algae (Lin *et al.*, 2005). Under natural conditions, spatial and temporal variations in the concentrations of nutrients must affect the sensitivity of algae to toxics, especially in case of rivers draining agricultural watersheds (Berard *et al.*, 2001; Navarro *et al.*, 2002). Consequently the effects of pesticides are more difficult to discover in this type of environment, because pollution by pesticides is concomitant with pollution from nitrates and phosphates. Development of benthic diatoms largely depends on hydraulic conditions too (Ghosh *et al.*, 1998). The abrasive effect of high currents induces a species selection encouraging the most tolerant to lotic environments. Hydraulic conditions influence, thus, responses of diatoms to pesticides shaping the structure of communities and restricting the creation of the protective biofilm. With floods episodes a part of diatoms is lost, species are selected and pesticide pollution increases. Response of diatom communities does not depend only on concentration, but also on the duration of exposure and on the species present (Jurgensen *et al.*, 1990). Moreover, interactions among toxic molecules present in waters can induce different effects like synergies, antagonisms or combined effects (Hoagland *et al.*, 1993; Carder *et al.*, 1998; Lagadic *et al.*, 1998). Effects of global pollution by pesticides are also difficult to assess.

1.7. Objectives of the research

The potential impact of pollution by pesticides on freshwater diatom communities has not been demonstrated yet. In this context, the study of the frustule and nucleus abnormalities can be particularly interesting to improve the detection in water of phytosanitary products.

With the aim to simulate the exposure of diatoms to pesticides, in a study carried out by Debenest *et al.* (2008) under controlled conditions, diatoms were treated with three concentrations of a genotoxic herbicide, maleic hydrazide ($5 \cdot 10^{-6}$, 10^{-6} , 10^{-7} M). Results showed the appearance of abnormal frustules and nucleus alterations, which were statistically more abundant for maleic hydrazide highest doses (10^{-6} and $5 \cdot 10^{-6}$ M). The main hypothesis suggested to explicate the mechanisms involved in the anomaly formation, concerns an indirect genotoxic effect: the toxic agent would cause interferences within the diatom cytoskeleton (microtubular system and microfilaments). This phenomenon is known like aneugenic effect. The microtubular system plays a central role in nucleus division during mitosis. It would also manage the migration of certain components for the cell wall formation (Pickett-Heaps *et al.*, 1979; Round *et al.*, 1990; Pickett-Heaps, 1991; Lee and Li, 1992; Van Den Hoek *et al.*, 1995). Edgar and Pickett-Heaps (1984) reported that nucleus position and microtubules integrity are important for the development of valves. In diatom cells treated with microtubule inhibitors, different authors have recorded multinuclear cells and also abnormal frustules (Coombs *et al.*, 1968; Duke and Reimann, 1977; Lee and Li, 1992).

The first aim of this study was to validate the hypothesis supported by Debenest *et al.* (2008), according to which multinuclear cells and abnormal frustules are due to a disturb in diatom cytoskeleton structure during cell division. With this purpose freshwater benthic diatom communities were exposed to different concentrations of two pesticides (maleic hydrazide and chlorpropham) and the nature of alterations regarding the abnormal number of nuclei, the micronuclei (nuclei ten times smaller than a normal one) and the abnormal frustules presence was investigated. Maleic hydrazide was chosen as positive control to reconfirm data obtained by Debenest *et al.* (2008), while chlorpropham was preferred for its disrupting effect on the cytoskeleton integrity. A second aim of this study was to understand how maleic hydrazide could change freshwater benthic diatom community, taken as a whole, in order to ameliorate the detection of pesticide pollution in rivers. Different diatom cultures were exposed to three concentrations of this pesticide and the change of community composition was followed after

24 and 48 hours from the exposure. One diatom culture was analysed through frustules identification and one other through flow cytometry technique.

The structure adopted in this report tries to separate in chapters (“Materials and methods”, “Results” and “Discussion”) the two different studies: the first one, the genotoxic study, concerning the validation of biomarkers in diatoms and the second one, concerning the change of diatom populations exposed to maleic hydrazide.

2. MATERIALS AND METHODS

2.1. Biological material employed

2.1.1. Sites of sampling

To carry out this study it has been necessary to collect numerous samples of diatoms to obtain a final community with a number of cells high enough to allow pesticide exposures and microscope observations. Two places situated in the South-West of France have been used to collect diatoms. A community of diatoms was collected in the Garonne River (Pinsaguel village, Midi-Pyrénées region). The Garonne is the most important river of Midi-Pyrénées region, rising in the Spanish central Pyrenees and flowing into the Atlantic by way of the estuary, called the Gironde. It is 575 km long and its basin is 56 000 square km in area (Figure 2.1 and 2.2). The reason why this place has been selected is that there was the necessity to have a river with accessible conditions, sufficient clear water and a developed diatom biofilm attached to the stones. The nearest place to ENSAT laboratories that satisfied these requests was Pinsaguel. However, the period of this study, from March to June 2008, has been characterized by numerous rain events and, as a consequences, by a situation of flood in the Garonne River and in all the rivers of its basin (Adour-Garonne basin). Therefore, another site with accessible conditions, clear water and a developed diatom biofilm has been found. The second river of sampling was the Sor, at Durfort (a village belonging to the Tarn department, Figure 2.1), in a tract characterised by a water level of about 30-60 cm. The full extent of the Sor stream is of 60 km and the drained surface covers 470 square km. The Sor basin (Figure 2.3) belongs to a major basin, the Adour-Garonne, which drains surface water over 118 000 square km. Moreover, Durfort is one of the few villages in the Midi-Pyrénées region, which is placed in an area relatively away from the cultivated field. This means that, in case of flood, especially in spring when the main treatments on cultures are done, concentrations of pesticides and fertilizers in stream are low, which is a good condition in our study, where diatoms possibly without alterations induced by chemicals and toxics were required. A research conducted by Agence de l'eau Haute Garonne (2007) confirmed the general state of good quality of the Sor water, with a value of IBD (Diatoms Biological Index)

equal to 19,3 in year 2007, which corresponds to a very good quality of water in the SEQ-Water classification.



Figure 2.1: Sites of diatom collection. Durfort and Pinsaguel, Midi-Pyrénées region.

The reason why the period of sampling March-June has been chosen for this study is because diatoms dominate the algal settlements at the beginning of spring, thank to favourable trophic conditions. In fact, as soon as these conditions disappear, at the beginning of the summer, diatoms are supplanted by green algae and cyanobacteria, making their collection difficult. Only in one occasion, for the flow cytometry study, diatoms were collected after the recommended period, in the month of July.



Figure 2.2: The Garonne River and its basin (UNEP/DEWA/GRID-Europe, 2008).

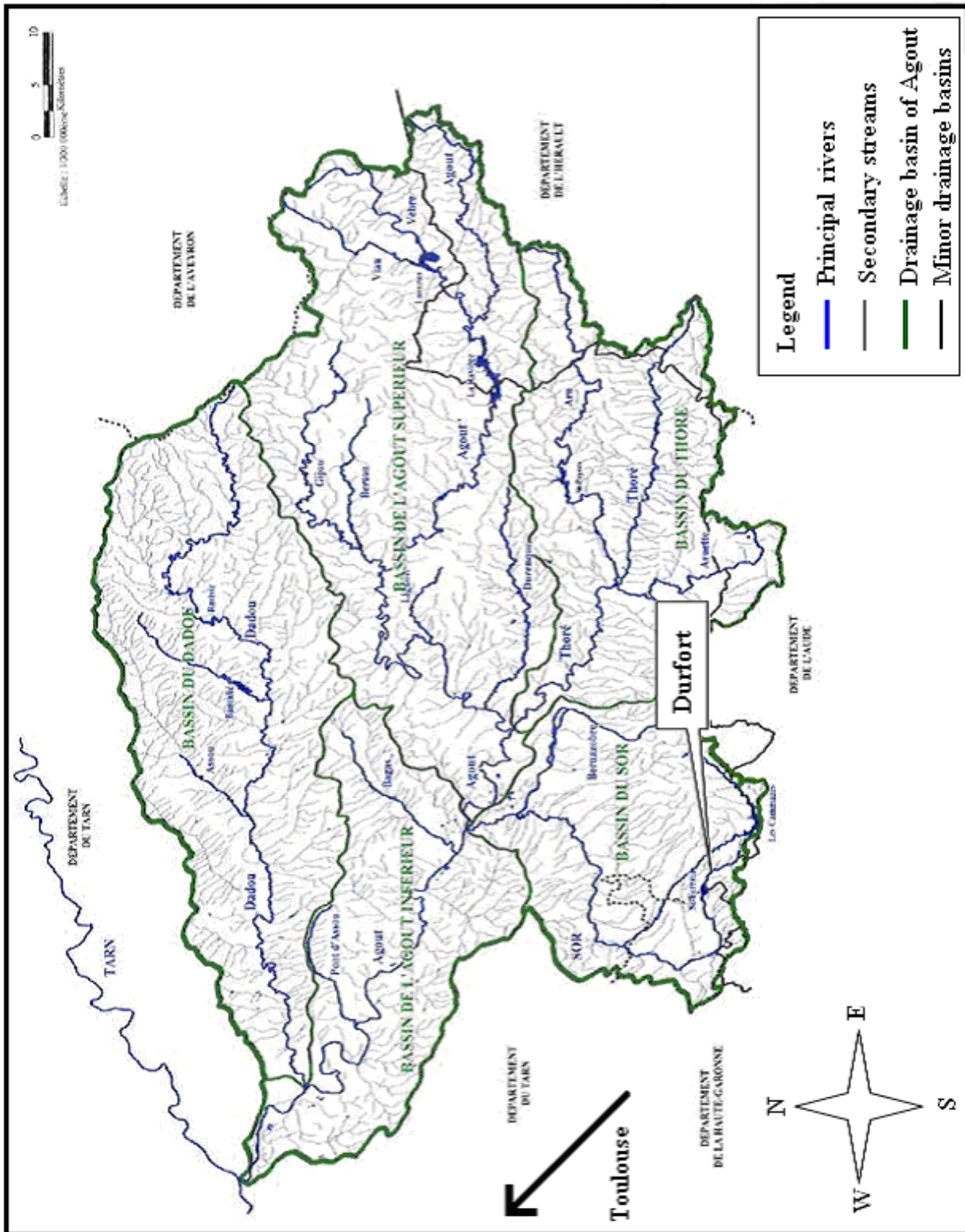


Figure 2.3: The Sor River and its basin in the Tarn department (SAGE, 2008).

2.1.2. Sampling and isolation

In the sampling operation, stones have been selected amongst those which had an evident biofilm, according to Kelly *et al.* (1998). Generally pebbles were preferred over boulders, for a reason of manoeuvrability. Pebbles were picked up mostly on bank, where the current was not so strong, and were scraped with a knife, focusing on biofilm with rust colour that indicated the presence of diatoms generally attached to the upper surface of stones (Figure 2.4 and 2.5). After having sampled about 20-30 cobbles, the biofilm obtained was then placed at the bottom of a clear plastic tray, of about 50 cm X 30 cm, with some river water and then transferred to the laboratory.



Figure 2.4: The diatom scraping from the stones in the Sor River.



Figure 2.5: The presence of diatoms on the upper surface of a stone.

A quick examination of the sample was done on the return to the laboratory and a note was made of any unusual features (e.g. large number of empty frustules). In the box containing the collected biofilm were introduced two pieces of dense polystyrene where microscope slides were set vertically through specific slits: the use of microscope slides to collect diatoms has proven efficiently by different authors (Dorigo *et al.*, 2001; Navarro *et al.*, 2002; Berard *et al.*, 2003; Guasch *et al.*, 2003). Slowly nutritious solution Chu No. 10 was added until the level of slides (Figure 2.6). It is important not to deposit pieces of biofilm on slides to avoid the attachment of unwanted microorganisms like green algae. Slides were necessary to isolate benthic diatoms, which tend to attach to a substrate. Then, the box was closed with a lid not hermetically to consent the entrance of the air and placed in a phytotron, where it remained under controlled conditions for three days (temperature $24 \pm 0.5^\circ\text{C}$ / $18 \pm 0.5^\circ\text{C}$ day/night cycles; photoperiod 16 hours under daylight fluorescent lamps providing $400 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ [Philips 600W, Eindhoven, Netherlands]; relative humidity 70%). The time of submersion, three days, was chosen to collect a maximum of benthic diatoms as well as to limit the presence of other microorganisms (especially other algae) on slides (Debenest *et al.*, 2008).



Figure 2.6: Plastic box with vertical slides to isolate benthic diatoms.

2.1.3. Cell culture

After three days of incubation in phytotron, microscope slides were scraped with a knife and washed with nutritive solution (Figure 2.7). In this operation it was necessary to avoid the use of too much nutritive solution and to scratch biofilm piles, where probably high quantity of green algae were present. The inoculum obtained was introduced with the nutritious solution until reaching 40 ml of content in apposite flasks of culture (Corning[®] Flask, 150 cm² Cell Culture Flask, Canted Neck), provided with a perforated cap to allow respiration. The number of used flasks depended on the quantity of biofilm collected: generally for 20-30 pebbles sampled one or two flasks were necessary. Flasks were than placed in the phytotron on a platform in rotatory motion to facilitate the adhesion of diatoms to walls (Figure 2.8). To optimise the culture conditions a protection against excessive light has been made over the culture flasks, through the use of box cups in polypropylene: this kind of opaque screen had the aim to attenuate the stark of the internal light, simulating the effect of vegetation near the river banks.



Figure 2.7: The operation of slide scraping.



Figure 2.8: The flasks of culture with the light protection structure placed in the phytotron.

The development of cultures was checked every day with an optical microscope (20X magnification, Micro Mecanique, IM, Olympus, Japan) to observe the growth of diatom populations. The culture technique has been changed during the study, with the aim to individuate an optimal strategy to cultivate a dense community of diatoms, limiting the development of other undesired microorganisms. The evolution of the technique is represented in Figure 2.9.

Following the first protocol used at the beginning of the study, diatoms collected from the plastic box were simply left in the culture flask and, three days after the transfer, treated with an antibiotic to limit the development of green algae, protozoa, bacteria or fungi. The antibiotic used was Amoxicillin sodium (CAS: 34642-77-8) 10 mg/l: in a culture flask were introduced 45 ml of nutritious solution with diatoms and 5 ml of antibiotic. The antibiotic was removed after one day and substituted by 50 ml of nutritious solution to avoid any negative effect on diatom development. In the next days, if no other antibiotic treatments were required, the nutritive solution was changed every 7 days to avoid any nutrient deficiency situations. An example of diatom culture where an antibiotic treatment was necessary because of the extensive presence of green algae is presented in Figure 2.11. Instead, an example of a good diatom culture with numerous cells in division is presented in Figure 2.12.

However, a simple antibiotic treatment was not sufficient to limit the development of undesired microorganisms, which may compromise the experiment. Therefore, a filtration was added. A second protocol was created according to which flasks were scrapped (Figure 2.10) with an apposite cell scraper (BD, Falcon TM, REF 353086) and the cell suspension was than filtered in a 100 µm nylon filter (BD, Falcon TM, REF 352360). This operation retained the unwanted algae, bigger than diatoms.

After having seen that also with filtration competitors continued to develop, a third protocol was formulated. In addition to the antibiotic treatment and to the filtration, more attention was paid to the scraping operation. Flasks were scraped avoiding areas with high density of green algae and the obtained solution was placed in new flasks to allow to filtered diatoms a new colonisation of the clean site. Moreover, a daily agitation was done to detach diatoms from walls, stimulating their development and, meanwhile, inhibiting the one of different algae.

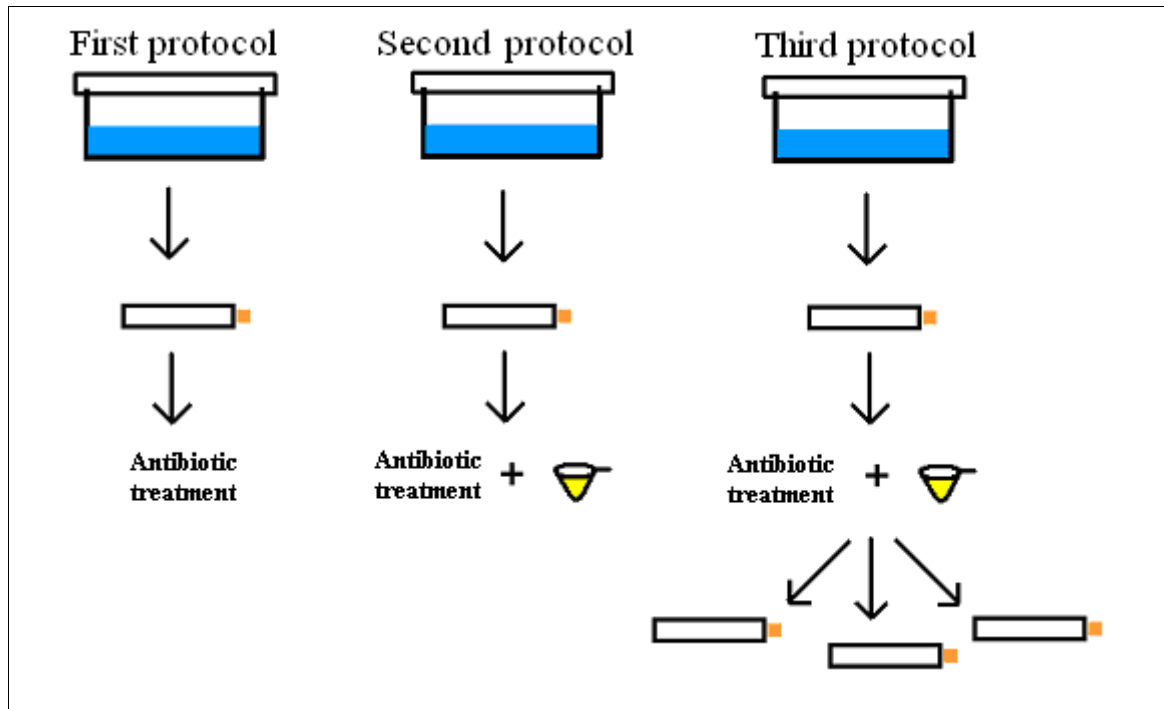


Figure 2.9: Evolution of culture technique. In the first protocol collected diatoms were treated with antibiotic; in the second protocol diatoms were treated with antibiotic and then filtered; in the third protocol diatoms were treated with antibiotic, filtered and placed in new culture flasks.

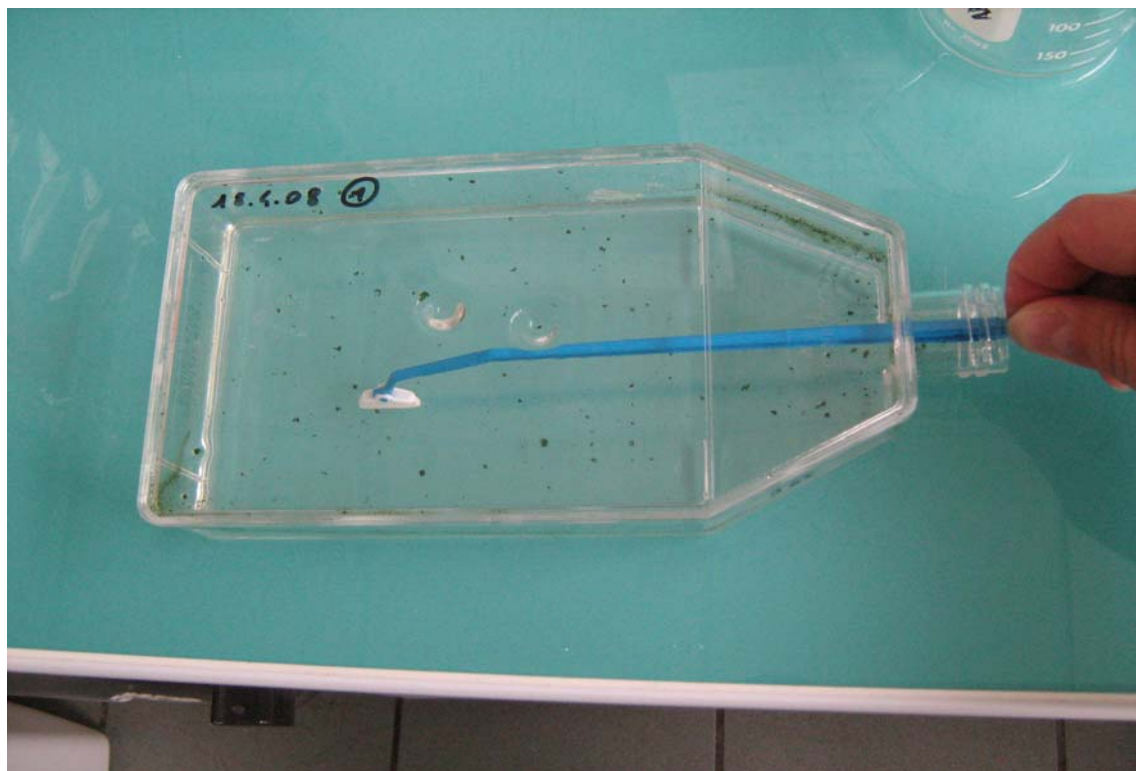


Figure 2.10: The operation of flask scraping.

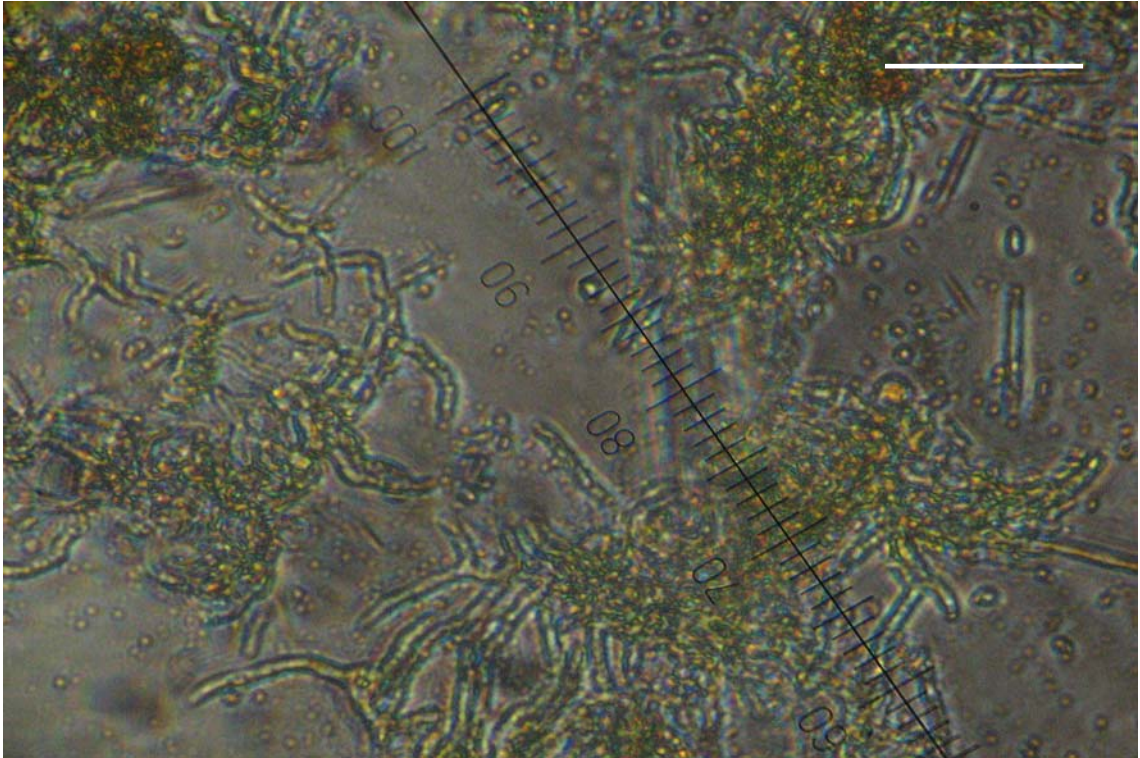


Figure 2.11: Diatom culture, seen by optical microscope, where an antibiotic treatment was necessary. The white axis corresponds to 50 μm .

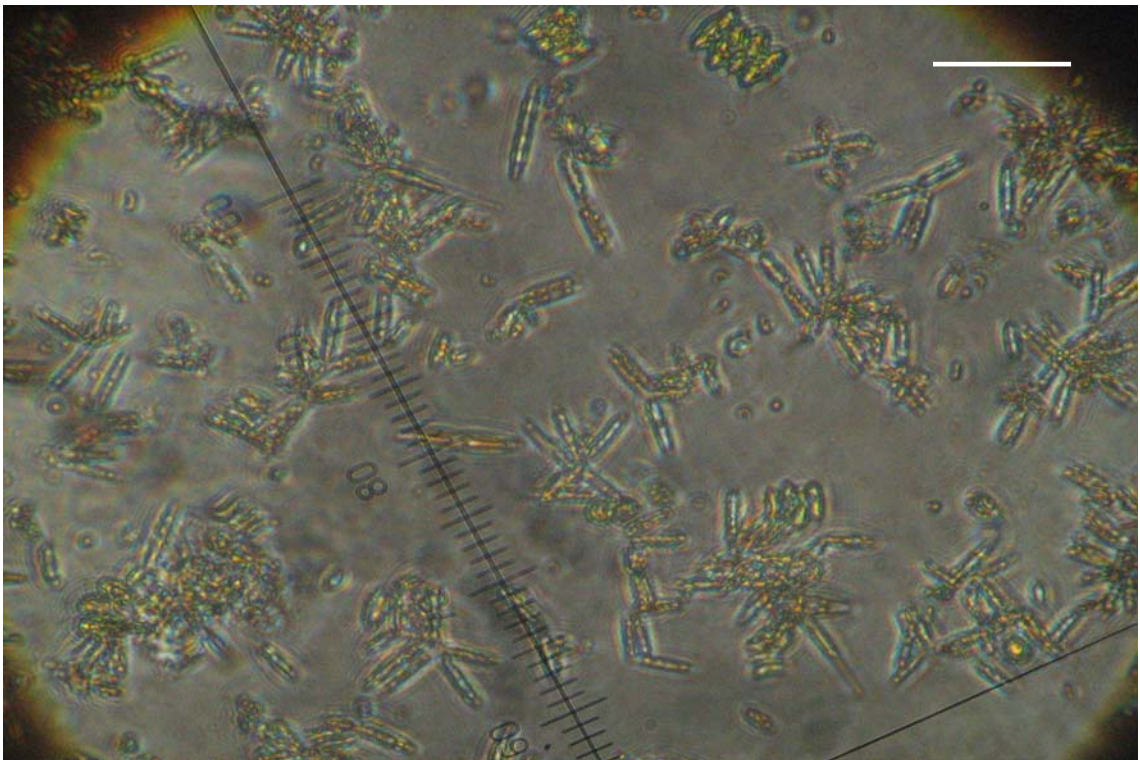


Figure 2.12: An example of correct diatom culture, seen by optical microscope, free from undesired microorganisms. The white axis corresponds to 50 μm .

The nutritive solution used in this study has been the Chu No. 10 medium (Nichols, 1973), because of some reasons exposed in a study of Debenest *et al.*, (2008): these authors studied two nutrient media for the diatom culture, the Chu No. 10 medium (Nichols, 1973), modified by Hughes and Lund, and a nutrient solution formulated in the laboratory of ENSAT, similar to the Freshwater “WC” medium (Guillard *et al.*, 1972). Cell density was assessed during the diatom culture in the two different nutritious solutions to study the growth of biomass. Results showed that it was higher for the diatom community cultivated in the Freshwater “WC” modified medium than in the Chu No. 10. However, comparison between the Freshwater “WC” modified medium and the Chu No. 10 one indicated that the first was rapidly colonized by cyanobacteria and green algae. This contamination could be due to the absence of inhibitor elements in the micronutrient solution. Even if with the Freshwater “WC” modified solution the diatom biomass growth was higher, proliferation in the culture of other microorganisms was a negative factor for further ecotoxicological experiments. These microorganisms produce extracellular polymeric substances which agglomerate diatoms and other algae disturbing microscope observations. In contrast Chu No. 10 solution allowed a sufficient production of diatom biomass and it was very selective for other microorganisms and algae (Debenest *et al.*, 2008). For these reasons, the Chu No. 10 medium was considered the better nutritious solution to use in the study (see Appendix B).

2.2. Genotoxic study

2.2.1. Pesticides employed

The optimum time of culture, to obtain a community close to the original one and to produce enough biomass for experiments, was studied by Debenest *et al.* (2008). To this aim the best duration for the culture was of about 72-96 hours. However, in this study after 72-96 hours the cell density was not high enough to consent exposure to pesticides. Only with two weeks of culture it was possible to start experiments. Diatoms were cultivated no more than fourteen days because after this period the increasing abundance in green algae did not consent microscope observations. Any kind of density measurement was made to decide the beginning of pesticide treatments; this choice was based on the daily culture observations.

With the aim to understand mechanisms involved in origin of nucleus and frustule alterations, two pesticides belonging to some important families of active phytosanitary substances used on big cultures in France (EUROSTAT, 2007) were selected. The phytopharmaceutical products employed are:

- chlorpropham (isopropyl 3-chlorocarbanilate, CIPC): a selective systemic plant growth regulator belonging to the carbamate family; it is used for pre-emergence control of different vegetables and cultures (carrots, garlic, seed grass, onions etc.). Sumida *et al.* (1977) showed that chlorpropham inhibited the growth of an alga, *Chlorella*, by 50% at 1,3 μM , under non-photosynthetic conditions, and by 50% at 4 μM , under photosynthetic conditions. It has been chosen for its capacity for inhibiting mitosis, through a disruption of the organiser centre of microtubules and the achromatic spindle (ACTA, 2007) (Figure 2.13).
- Maleic hydrazide (6-hydroxy-2H-pyridazin-3-one, MH): a well known genotoxic herbicide, the only one in this family; it is used in France as growth regulator for potatoes, onions, tobacco and as herbicide in agriculture or in green spaces. This chemical is used as a positive control in the micronucleus tests (Cotelle *et al.*, 1999). In higher plants, maleic hydrazide induces micronuclei formation, DNA strand breaks or chromosome bridges (Cotelle *et al.*, 1999; Grant, 1999; Kong and Ma, 1999; Marcano *et al.*, 2004; Hajjouji *et al.*, 2007). In a study carried out by Debenest *et al.* (2008) benthic diatoms were exposed to different concentrations of maleic hydrazide and a lot of nuclear alterations appeared at maleic hydrazide highest concentrations (10^{-6} and $5 \cdot 10^{-6}$ M). It has been chosen because its effects were already known thanks to Debenest *et al.* (2008) (Figure 2.14).

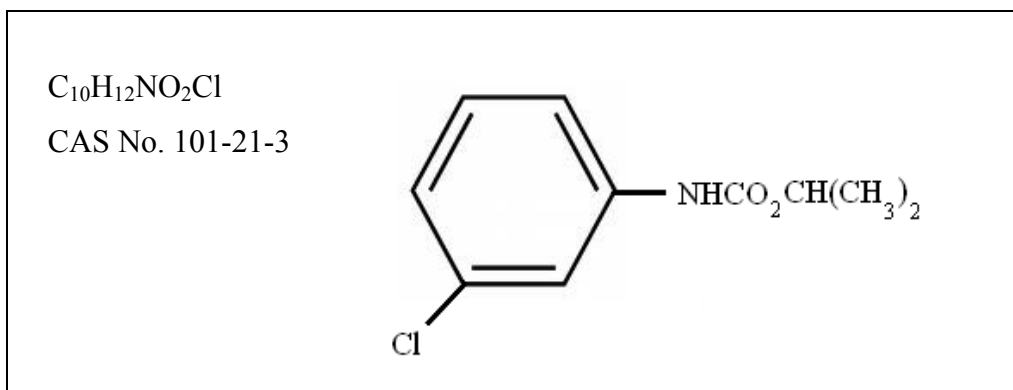


Figure 2.13: Molecule of chlorpropham (CIPC).

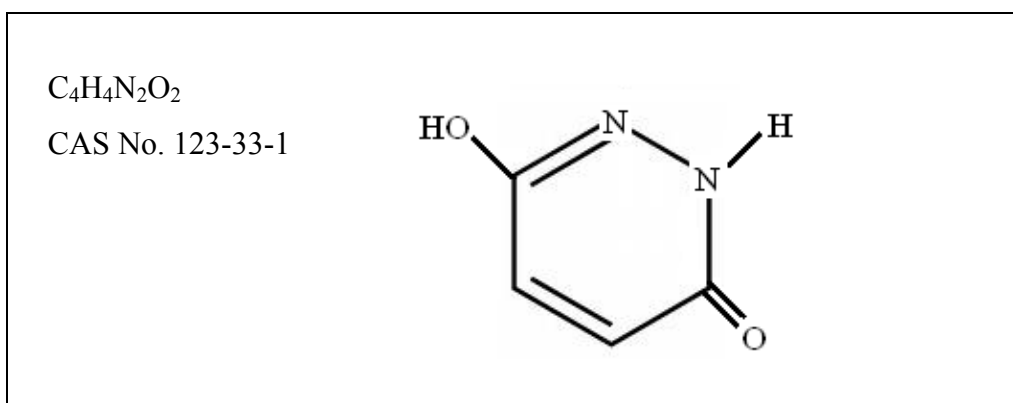


Figure 2.14: Molecule of maleic hydrazide (MH).

2.2.2. Selected concentrations

After the culture period samples were exposed to different doses of phytosanitary products, which best simulated the concentration of pesticides during flooding events in rivers reflecting a polluted situation. In fact, a study conducted by Debenest, in year 2007, showed that after leaching events, rivers in flood state presented the higher pesticide concentrations (between 10^{-6} and 10^{-9} M) which were the more dangerous for diatoms health. Also other authors noticed a relation between river pollution by toxic products and raining episodes (Brockway *et al.*, 1984; Hamala *et al.*, 1985; Williams *et al.*, 1995; Lockert *et al.*, 2006). For chlorpropham doses used have been 10^{-6} and 10^{-7} M. No concentrations superior to 10^{-6} M have been chosen to avoid the inhibition of cell division. For maleic hydrazide the doses

chosen have been 10^{-7} , 10^{-6} and $5 \cdot 10^{-6}$ M, whose effects on benthic diatoms were already known thanks to the study of Debenest *et al.* (2008).

2.2.3. Exposure methodology

The benthic diatom community, isolated from the natural biofilm and cultured under controlled conditions, was scraped from the culture flasks and placed in centrifuge tubes from 15 ml. The selected pesticides were then added. Three replicates were prepared for each concentration and, also, three tubes for the control (containing only the nutritive solution with diatom cells) (Table 2.1). Every tube was initially filled with scrapped diatoms and aliquots of nutritive solution until the desired volume. Only afterwards, to avoid a shock for cells, pesticide quantities were added until the desired concentrations in a total volume of 10 ml. Tubes were held horizontally in a small box (Figure 2.15) and slowly shaken.

Table 2.1: Concentrations and volumes of chlorpropham (CIPC) and maleic hydrazide (MH). MS means mother solution of pesticide and NS nutritious solution with diatom cells.

Treatment	MS (mol/l)	Volume of MS (ml)	Volume of NS (ml)	Total volume (ml)
CIPC (10^{-7} M)	CIPC 10^{-5}	0,101	9,899	10
CIPC (10^{-6} M)	CIPC 10^{-5}	1,111	8,889	10
MH (10^{-7} M)	MH 10^{-5}	0,101	9,899	10
MH (10^{-6} M)	MH 10^{-5}	1,111	8,889	10
MH ($5 \cdot 10^{-6}$ M)	MH 10^{-5}	5,555	4,445	10
Control	-	-	10	10

Diatoms were exposed to toxic agents for 6 hours. The duration of this exposure was chosen in comparison with bibliographic data (Cotelle *et al.*, 1999; Casotti *et al.*, 2005). At the end of exposure cells were centrifuged a first time (2700 revolutions per minute, during 10 minutes at 19°C) to eliminate the pesticide and, a second time, after addition of 10 ml of nutritious

solution, to eliminate any possible pesticide residues. Finally, tubes were completed with 10 ml of fresh medium and placed horizontally in the phytotron. Diatom cells were kept for a recovery time of 24 hours in these conditions of culture, with the tubes disposed on the rotatory desk to simulate a natural current. The recovery time was fixed in relation to growth rates assessed previously during some cell cultures prepared by Debenest *et al.* (2008).

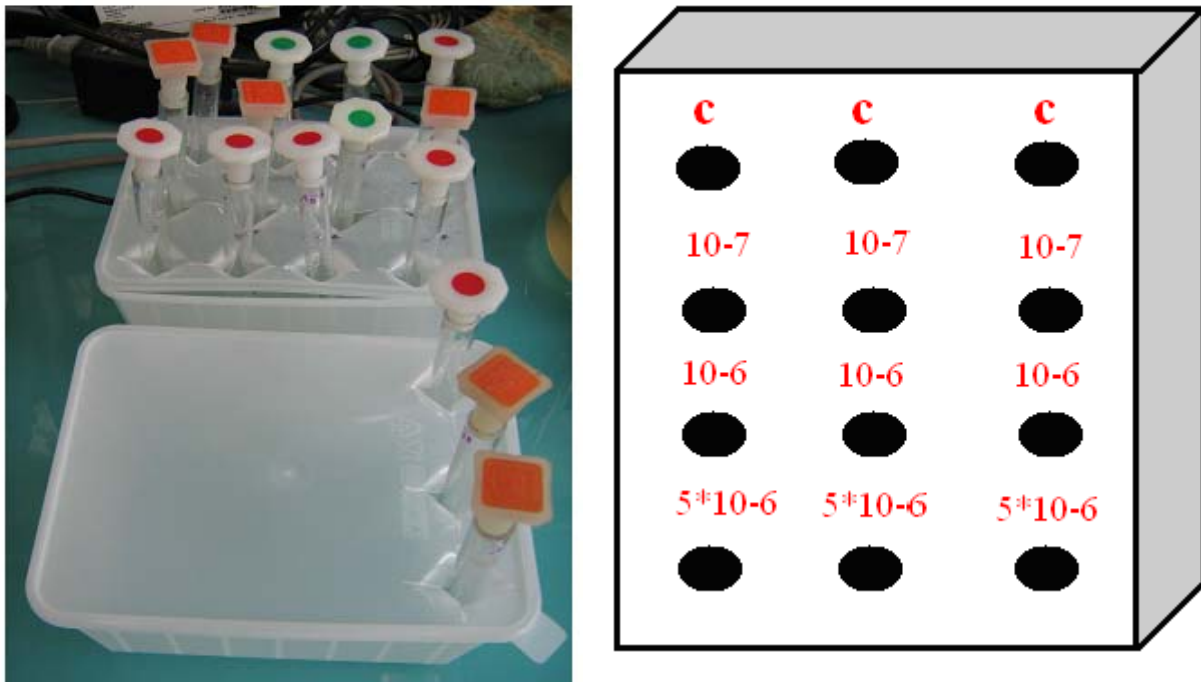


Figure 2.15: An example of diatom exposure with maleic hydrazide. Three repetitions for every treatment were done: “C” indicates the control; “10⁻⁷”, “10⁻⁶” and “5*10⁻⁶” indicate respectively the exposure to pesticide concentrations of 10⁻⁷, 10⁻⁶ and 5*10⁻⁶ M.

2.2.4. Staining and microscopic observations

The marker used in fluorescence microscopy to stain diatom nucleus has been Hoechst 33342 (CAS No. 23491-52-3) 2%. This marker has been selected comparatively to one other fluorochrome, Propidium Iodide (IP, CAS No. 25535-16-4), which was tested in a study of Debenest *et al.* (2008) and did not make possible to discern nucleus from chloroplasts. Hoechst 33342 marks DNA and it is also used to stain cells. Cells are illuminated with an argon laser (ultraviolet light at around 353-365 nm) and the resulting violet/blue fluorescence light is detected at 480 nm (KuKuruga, 1997). This fluorochrome emits to a minor wave

length (450-500 nm) than that of chlorophyll (640-670 nm). If fluorochromes with the same spectra of chlorophyll are used, as in the case of IP, the natural autofluorescence of the chlorophyllian pigments forbids the distinction between nuclei and chloroplasts (Debenest, 2007). Hoechst 33342 is able to pass through membranes and can, therefore, visualize nuclei in living cells as well as in fixed cells (University of Western Australia, 2008). Moreover, its fluorescence is very sensitive to DNA conformation and chromatin state in cells, consequently, it can detect nuclear damages (Molecular ProbesTM, 2005). In the observations, nuclei labelled by Hoechst 33342 appeared blue coloured, while chloroplasts, not marked by Hoechst 33342 but identifiable for the autofluorescence of chlorophyll, appeared as red (Figure 2.16).

It is also necessary to say that Hoechst 33342 can disrupt DNA replication during cell division: it is potentially mutagenic and carcinogenic. However, this effect is not influent on the nuclear integrity of diatoms because to see its effect it is necessary to wait a whole reproduction cycle, which requires at least 6 hours. In this study diatom exposure to this stain was carried out only some minutes before fluorescent microscope observations.

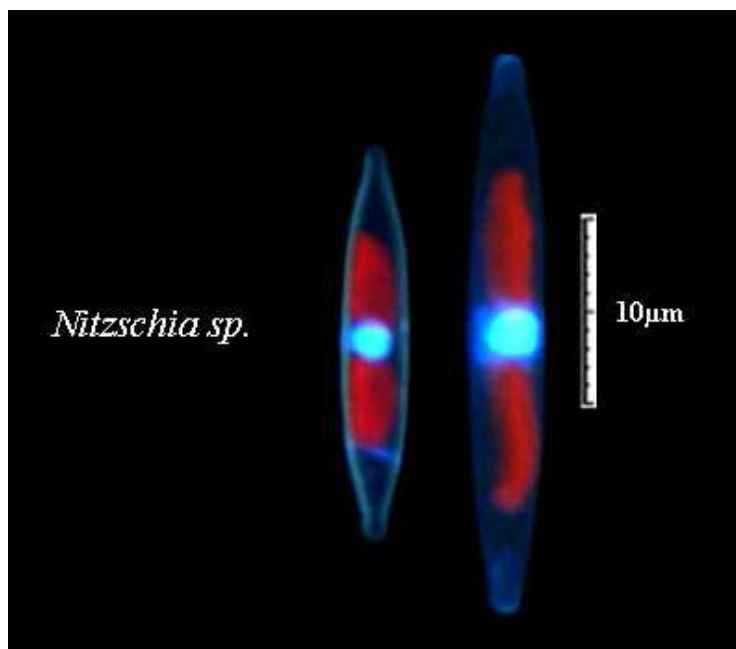


Figure 2.16: Cells of *Nitzschia sp.* with nuclei stained in blue (Hoechst 33342). Chloroplasts appear in red (Debenest *et al.*, 2008).

Another aim of this research was to ameliorate the protocol for marking diatoms, to facilitate the study and the identification of these algae with the use of fluorescence microscope. To avoid the problem given in many study by the use of formaldehyde, which alters the structure of cells damaging the observations, paraformaldehyde 4% alone and paraformaldehyde 4% mixed to glutaraldehyde 25% have been tested in two different steps. In a first step, the marker has been added after fixation, while, in a second one, the marker has been added before the fixation. In any case two different solutions, presented in Table 2.2, have been prepared. With fixation before marking, solutions A and B were taken and added in an Eppendorf where 20 µl of liquid Hoechst 33342 (2 g/l) were added. Differently, in the case of marking before fixation, the first product introduced in the Eppendorf was the marker, 20 µl of liquid Hoechst 33342 (2 g/l), followed by the nutritious solution with diatoms and, finally, by the fixing agents (solutions C and D). However, to have a control also a solution composed only of nutritious solution and diatoms was marked by Hoechst 33342. For each sample one slide was prepared.

Table 2.2: The four solutions tested and the order of addition of their components (NS means nutritious solution with diatoms).

Solutions	Composition	Sequence
A	500 µl of paraformaldehyde 4% 480 µl of NS	fixation before marking
B	500 µl of paraformaldehyde 4% 20 µl glutaraldehyde 25% 460 µl of NS	fixation before marking
C	500 µl of paraformaldehyde 4% 480 µl of NS	marking before fixation
D	500 µl of paraformaldehyde 4% 20 µl glutaraldehyde 25% 460 µl of NS	marking before fixation
Control	980 µl of NS	no fixation

Nuclear and frustule alterations were counted under a 60X objective with a fluorescence microscope (BX41 Olympus America Inc. Center Valley, PA USA) with a specific DAPI

filter (U-MWU2, Ex. Filter: 330-385 λ , Em. Filter: 420 λ , Dichromatic filter: 400 λ) (Figure 2.17). The software used to visualize and organize diatom images and files has been XnView for Windows (Pierre-E Gougelet).

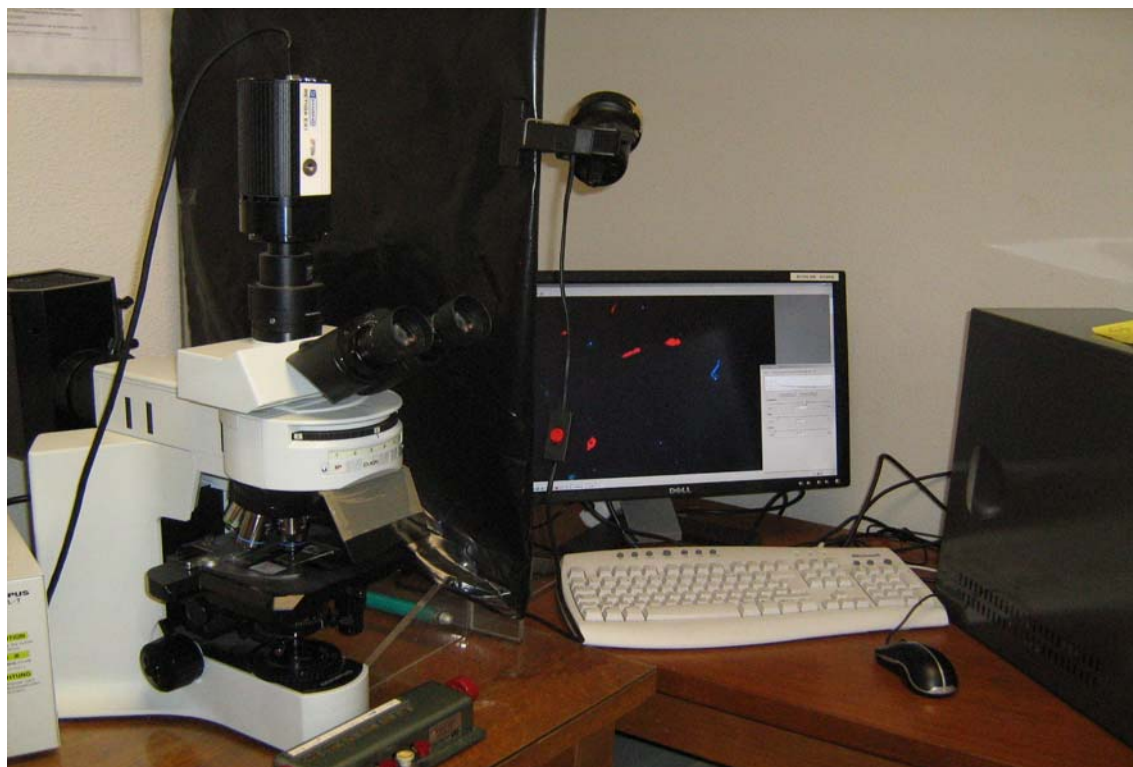


Figure 2.17: The fluorescence microscope (BX41 Olympus America Inc. Center Valley, PA USA) used to count diatom alterations.

A total of at least 1000 cells (about 350 cells for every slide) for each treatment were counted to determine the mitotic index and the frequency of alterations. Based on the methodology formulated for the micronucleus tests in higher plants (Cotelle *et al.*, 1999) the mitotic index (number of cells in division divided by the total number of cells counted and multiplied by 100) was calculated. The calculation of this index was necessary to confirm the presence of at least 2% of cells in division, ensuring that the pesticides did not inhibit the cell division. It becomes impossible to see the genotoxic effect on cells if pesticides cause their immediate death. Observations of diatom cross sections with a transmission electronic microscope showed that during mitosis new valves were secreted inside each valve (epivalve and hypovalve) of the mother cell (Debenest *et al.*, 2008). Therefore two cells were regarded as

divided if they were stuck side by side. Likewise, cells were considered in mitosis if two similar nuclei were observed in the transapical plane (white axis in Figure 2.18).

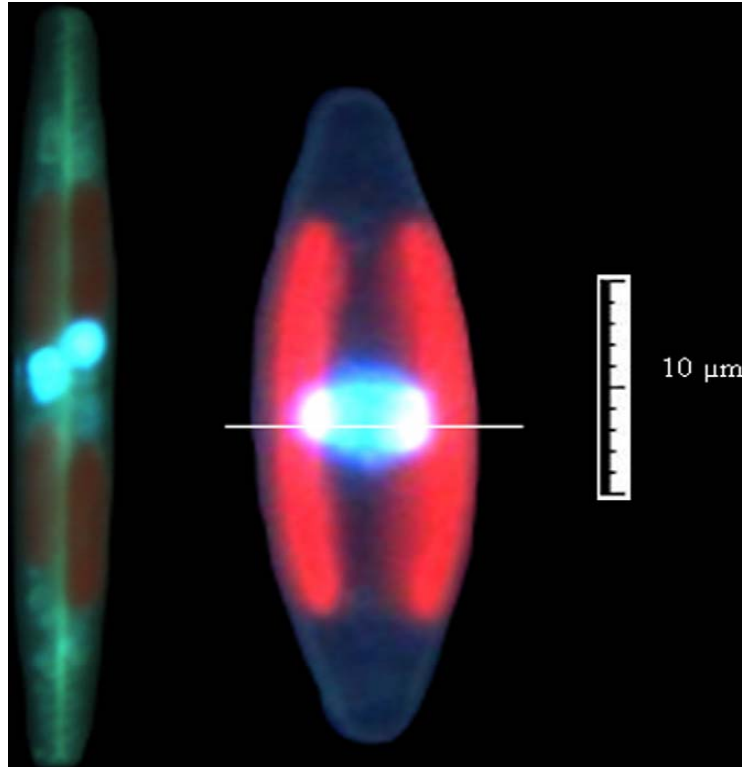


Figure 2.18: Benthic diatoms in division. Anaphase stage (left) and metaphase stage (right, transapical axis in white) observed under fluorescence microscope. Nucleus is stained in blue with Hoechst 33342 and chloroplasts appear red (Debenest *et al.*, 2008).

2.3. Community change

2.3.1. Frustule identification

After transfer in phytotron, cultures were exposed to maleic hydrazide after 14 days. A pesticide exposure of 6 hours was carried out and the change of diatom community was followed after 24 and 48 hours. One day was the minimum period necessary to see alterations in population composition, because it corresponds to one division cycle.

To consent species identification, frustules analysis has been necessary. With this aim, following the French standard for the determination of Diatom Biological Index (IBD) all samples were treated with hydrogen peroxide 30% in weight (CAS No. 7722-84-1) to digest organic components (Figure 2.19). The digester used to prepare the frustule identification has been DigiPREP Jr. (SCP SCIENCE, Canada): samples with treated diatoms and hydrogen peroxide were warmed until the boiling point. After digestion samples were centrifuged and siliceous walls resuspended in water to eliminate the hydrogen peroxide. For all treated samples an aliquot (about 200 μ l) was dried on a glass coverslip. Fixed frustules on the coverslip were than placed on a microscope slide with Naphrax® (a resin with a high refractive index). Slides were scanned with a light microscope (Leica DMRD, Leica Microsystems GmbH, Wetzlar, Germany) at a magnification of 1000X. For each sample exposed to maleic hydrazide and for the control a total of at least 1000 frustules were identified. Diatoms identification was done by Michel Coste (Cemagref, Bordeaux).



Figure 2.19: Samples of diatoms treated with H₂O₂ 30% in the digester (DigiPREP Jr., SCP SCIENCE, Canada) used to prepare the frustule identification.

2.3.2. Flow cytometry technique

Diatoms were also employed with the aim to study the population change after exposure to maleic hydrazide through flow cytometry. For this study the biofilm was collected in the month of July in the Garonne River. Two samples were prepared: one containing diatom cells with nutritive solution (the control) and one other containing cells, nutritive solution and aliquots of maleic hydrazide until obtaining 10^{-7} , 10^{-6} and $5 \cdot 10^{-6}$ M concentrations. Treated samples were exposed to the pesticide for 6 hours and observed through flow cytometry after 24 and 48 hours. Moreover, to have an idea of the community composition, a counting through the Malassez chamber was executed for every sample analysed with the flow cytometer.

Flow cytometry is a technology which allows the individual measurement of cell fluorescence and light scattering. Cells are injected into the centre of a sheath flow. Subsequently, the combined flow is reduced in diameter, forcing cells into the centre of the stream. Finally, the laser hits one cell at a time (Figure 2.20). Cells are conjugated to fluorochromes with the use of a marker. As cells intercept the light source they scatter light and fluorochromes are excited to a higher energy state. This energy is released as photons, characterised by a minor energy and a longer wave length, owning specific spectral properties. Only cells expressing the marker will manifest fluorescence. Scattered and emitted light from cells and particles are converted to electrical pulses by optical detectors. Collimated (parallel light waveforms) light is picked up by confocal lenses focused at the intersection point of cells and light source. Light is sent to different detectors through optical filters. The most common type of detector used in flow cytometry is the photomultiplier tube, PMT (Figure 2.21). The electrical pulses originating from light detected by four PMTs are then processed by a series of linear and logarithmic amplifiers. This type of amplification expands the scale for weak signals and compresses the scale for “strong” or specific fluorescence signals. After the different signals or pulses are amplified, they are processed by an Analog to Digital Converter (ADC) which, in turn, allows for events to be plotted on a graphical scale (one parameter, two parameters histograms) (University of California, 2008).

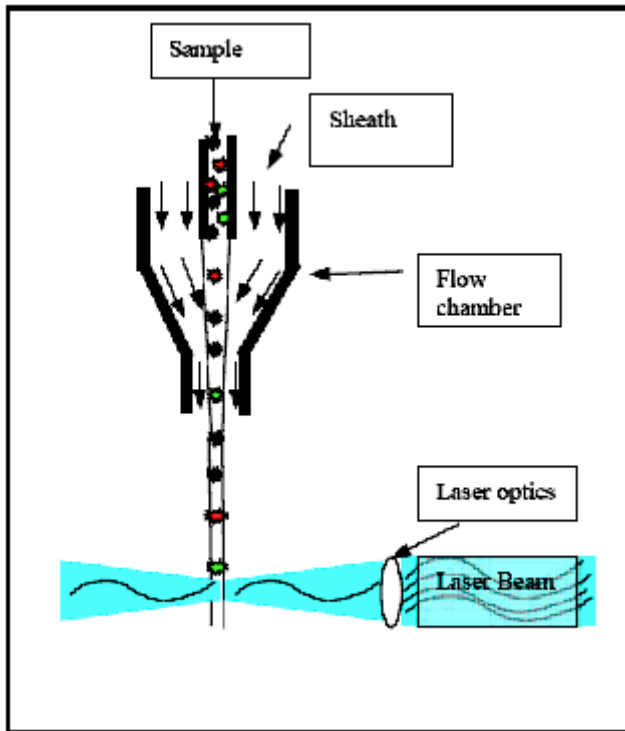


Figure 2.20: Flow cytometry. The sample is injected into the centre of a sheath flow. The flow chamber is conical in shape, the reduced diameter forces the cell into the centre of the stream. In this way one cell at a time passes through the laser (University of California, 2008).

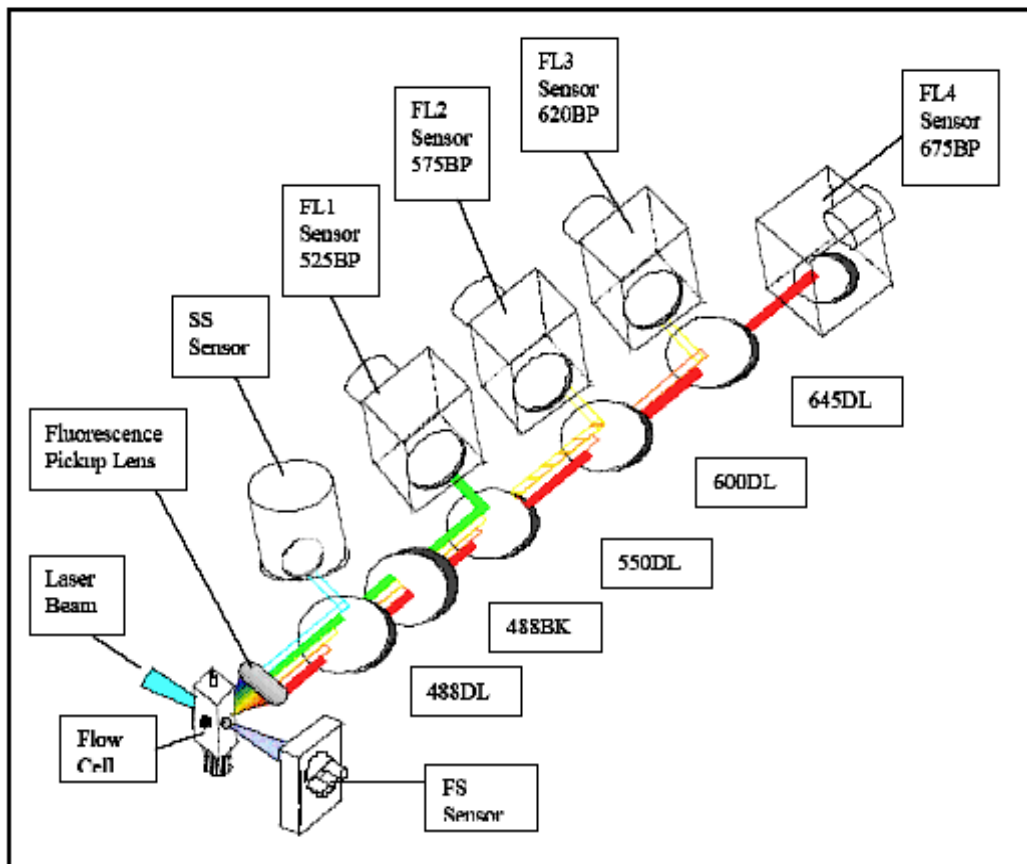


Figure 2.21: The optical schematic system. The SS (Side Scatter) sensor reads a signal which is deviated of 90° allowing a measure of the cell granularity, while the FS (Forward Scatter) sensor reads the not deviated signal, allowing the measure of the cell size (University of California, 2008).

In this study the machine used to follow the population change has been the FACSCalibur™ 15800, a four-color dual-laser system, of Ranguel Hospital, Toulouse.

Propidium Iodide 2% (CAS No. 25535-16-4) has been selected in comparison with chlorophyll autofluorescence and with another cell marker, Hoechst 33342 2% (CAS No. 23491-52-3). In a first proof, a sample of cultivated diatoms has been analysed without marking it to evaluate the autofluorescence of chlorophyll present in diatom chloroplasts. In a second step, a sample of diatoms was labelled with Hoechst 33342 and analysed with flow cytometry. Finally, in a third moment, other diatom cells were stained with Propidium Iodide. A comparative analysis has been carried out to understand the different marking power of the two products and of the cells alone.

Propidium iodide (IP), is a fluorescent molecule that can be used to stain DNA (Lecoeur, 2002). When it bounds itself to nucleic acids, its maximum absorption for IP is 535 nm and the fluorescence emission maximum is 617 nm. It is possible to have red fluorescence at 488 nm (BIOMOL, 2008). Generally, IP fluorescence is detected in the FL2 channel of flow cytometer (Molecular Probes™, 1999). Normally, IP is unable to pass through cell membrane and it is generally excluded from viable cells. Commonly it is used for identifying dead cells in populations and as a counterstain in multicolour fluorescent techniques (Moore, 1998). However, in the case of diatoms, its effectiveness in staining the nuclear content is known.

3. RESULTS

3.1. Genotoxic study

In the study carried out by Debenest *et al.* (2008), diatoms were exposed to maleic hydrazide 10^{-7} , 10^{-6} and $5 \cdot 10^{-6}$ M and a Pearson analysis showed a significant positive correlation between the abundance of nucleus alterations and the abundance of abnormal frustules (0,702, $P < 0,05$). With the aim to confirm this result and the idea supported by these authors, according to which maleic hydrazide disturbs the diatom cytoskeleton, a similar exposure was executed. Moreover, an exposure with another pesticide, chlorpropham (10^{-7} and 10^{-6} M) was carried out. To carry out this study diatoms were collected in the Sor River.

3.1.1. Protocol of staining

After having tested the different solutions, results obtained are shown in Table 3.1. In all cases it was evident that with solution A, the marker entered very well cells, without altering them. However, if chloroplasts were very clear, the nucleus was not evident. In solution B, frustules became yellowish and the marking was not clear. In solution C, the marker was less clear than in solution A; nuclei and chloroplasts were hardly identifiable. In solution D, Hoechst 33342 entered with difficulty through the membranes. Finally, in view of the results, any kind of fixative agent was used and cells were counted alive.

Table 3.1: The four solutions tested, the order of addition of their components and the obtained effects. NS means nutritious solution with diatom cells.

Solutions	Composition	Sequence	Effects
A	500 µl of paraformaldehyde 4% 480 µl of NS	fixation before marking	nucleus not evident
B	500 µl of paraformaldehyde 4% 20 µl glutaraldehyde 25% 460 µl of NS	fixation before marking	yellowish frustules and feeble marking
C	500 µl of paraformaldehyde 4% 480 µl of NS	marking before fixation	feeble marking
D	500 µl of paraformaldehyde 4% 20 µl glutaraldehyde 25% 460 µl of NS	marking before fixation	feeble marking
Control	980 µl of NS	no fixation	good marking

3.1.2. Observations with fluorescent microscope

From fluorescent microscopic observations, alterations found after exposure to tested pesticides are evident in figures reported below. The more abundant anomalies found were: abnormal position and number of nuclei, deformed chloroplasts, micronuclei presence and abnormal morphology of the frustules. In particular, in exposures to maleic hydrazide the presence of each alteration was found, but normal cells (with normal number and position of nuclei and chloroplasts) owning micronuclei were more evident at $5 \cdot 10^{-6}$ M (Figure 3.1, 3.3 and 3.4). Instead, in samples treated with chlorpropham, the presence of abnormal shape of frustules, abnormal location and number of nuclei and chloroplasts were the dominant alterations at every concentration (Figures 3.2, 3.3 and 3.4).

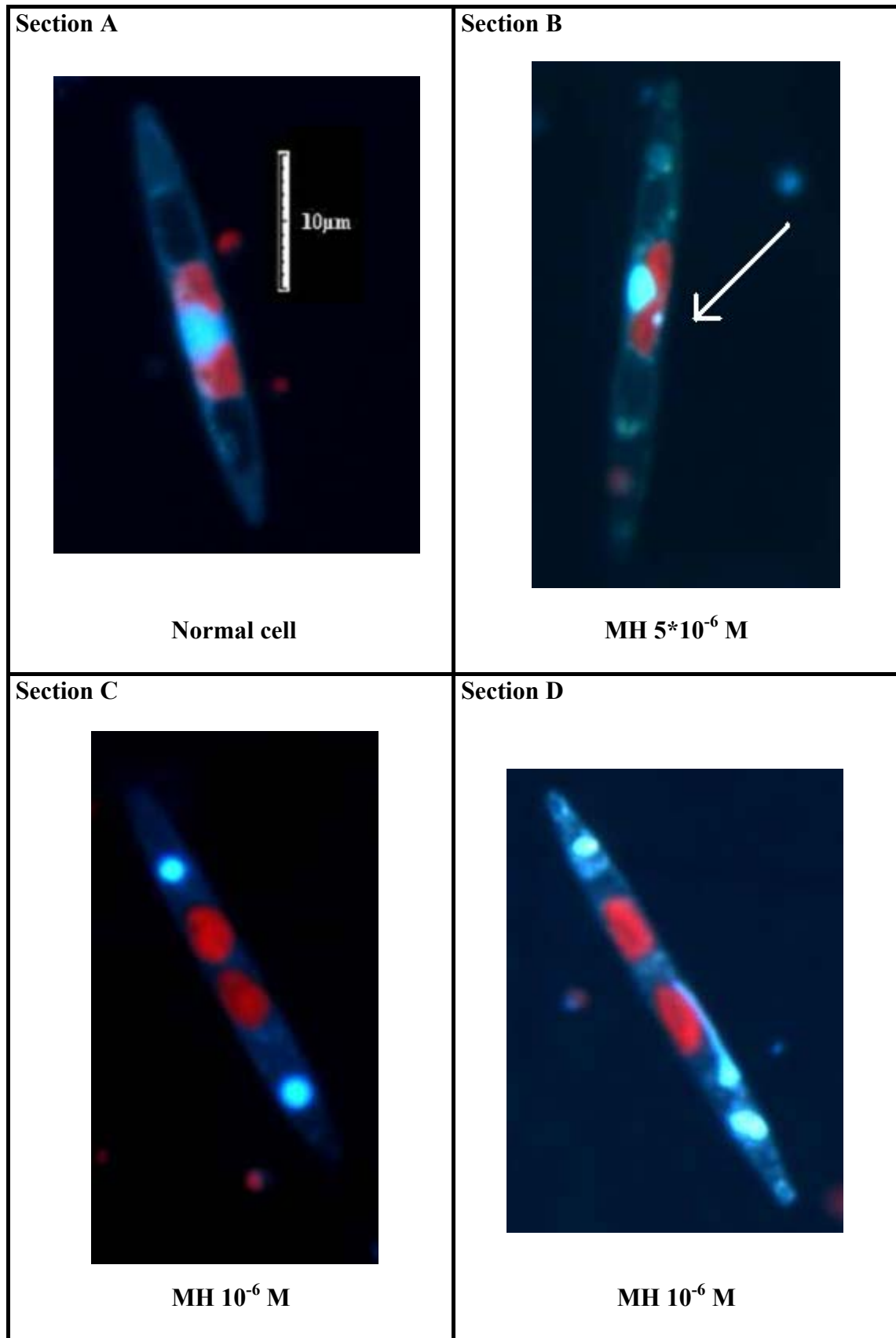


Figure 3.1: A normal diatom cell (Section A), a cell exposed to maleic hydrazide (MH) 5×10^{-6} M presenting a micronucleus (Section B) and cells with abnormal number and position of nuclei exposed to maleic hydrazide 10^{-6} M (Sections C and D). Nuclei are stained in blue with Hoechst 33342, while chloroplasts appear in red (fluorescence microscope).

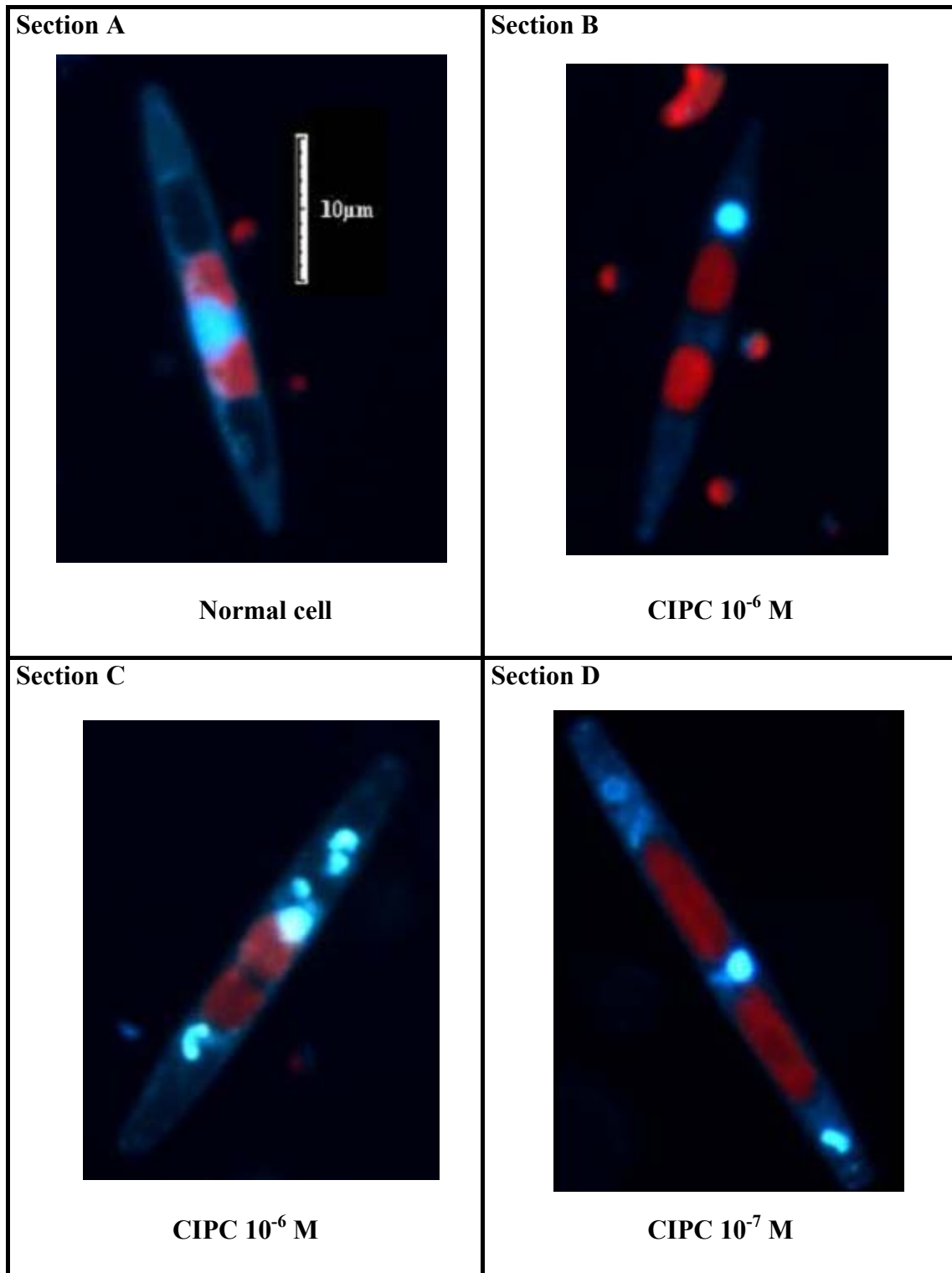


Figure 3.2: A normal diatom cell (Section A) and diatoms with abnormal nuclei position and number after exposure to chlorpropham (CIPC) 10⁻⁶ and 10⁻⁷ M (Sections B, C and D). Nuclei are stained in blue with Hoechst 33342, while chloroplasts appear in red (fluorescence microscope).

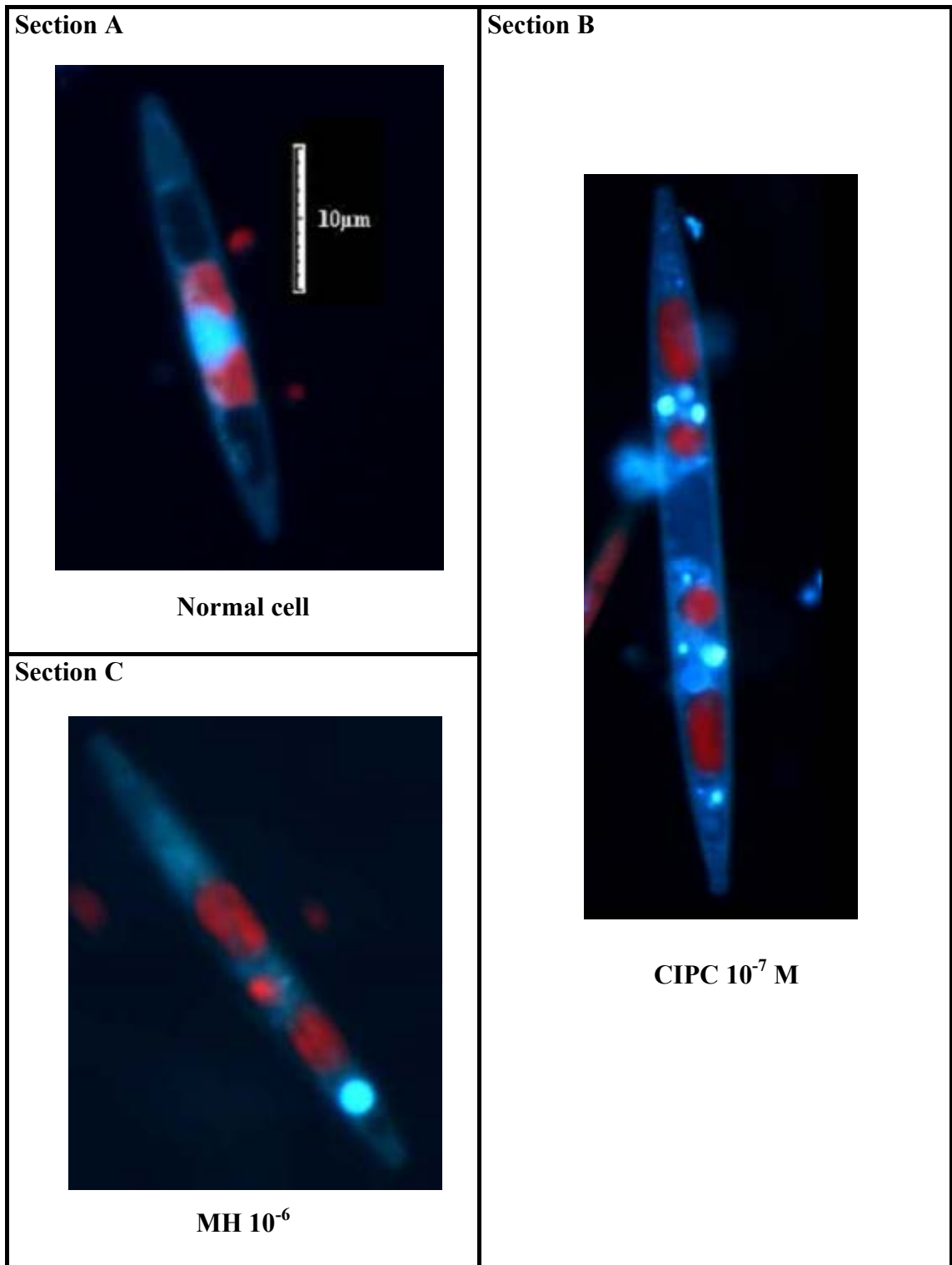


Figure 3.3: A normal diatom cell (Section A) and diatom cells with abnormal nuclei and chloroplast number and position, after exposure to chlorpropham (CIPC) 10^{-7} M (Section B) and maleic hydrazide (MH) 10^{-6} M (Section C). Nuclei are stained in blue with Hoechst 33342, while chloroplasts appear in red (fluorescence microscope).

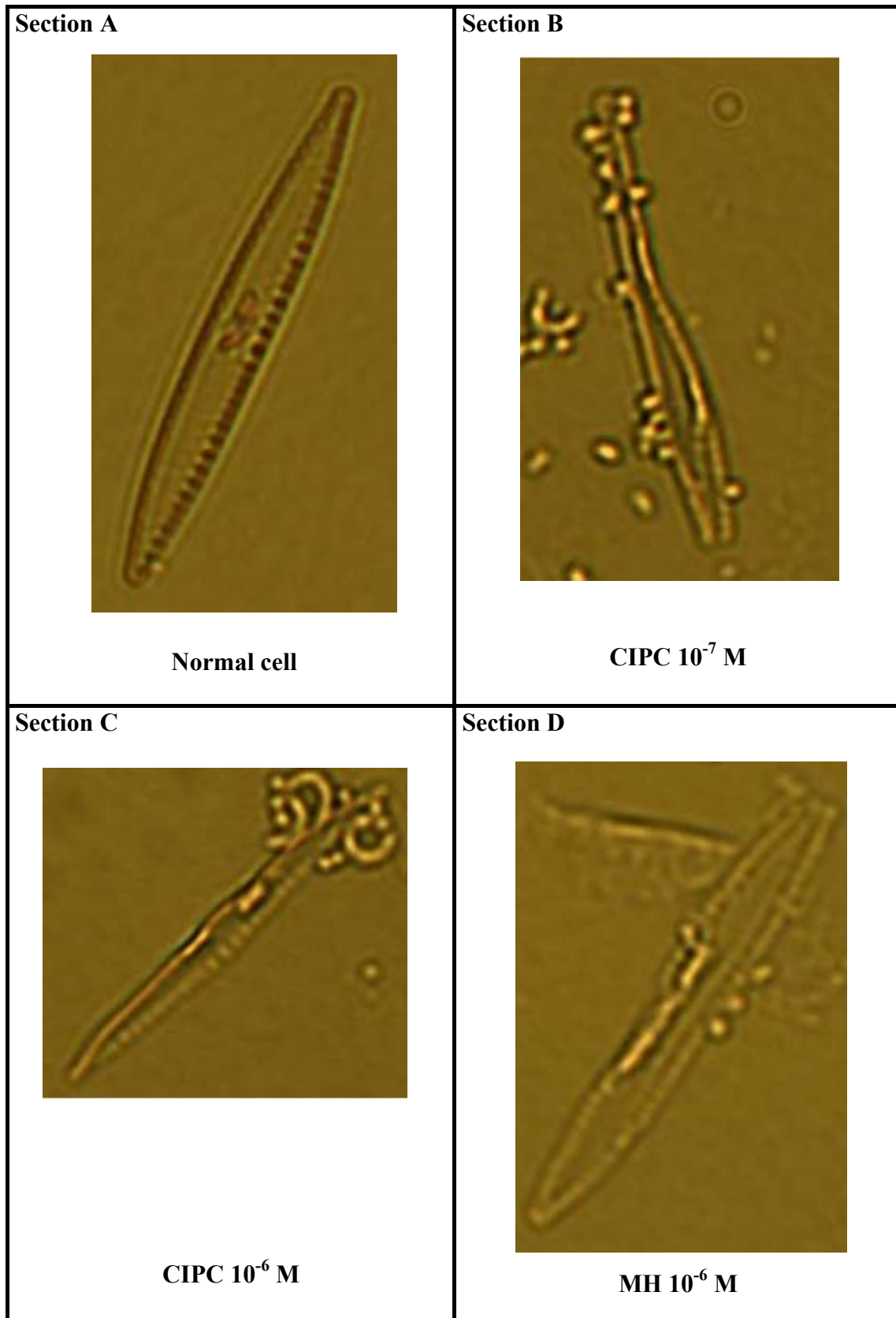


Figure 3.4: A normal diatom cell (Section A) and diatoms seen by light microscope with abnormal frustules after exposure to chlorpropham (CIPC) 10⁻⁷, 10⁻⁶ M (Sections B and C) and maleic hydrazide (MH) 10⁻⁶ M (Section D).

3.1.3. Exposure to maleic hydrazide

Diatoms were exposed to maleic hydrazide concentrations equal to 10^{-7} , 10^{-6} , and $5 \cdot 10^{-6}$ M and results obtained are shown in Table 3.2. Normal and altered diatoms were counted 24 hours after pesticide treatment.

Table 3.2: Effects of three maleic hydrazide (MH) concentrations on the mean abundance of frustule and nuclear alterations (cells with abnormal nuclei number and position), micronuclei presence and on the mean abundance of cells in division (mitotic index). SD means standard deviation.

Treatment (M)	Nuclear anomalies (per 1000 cells)		Cells with micronuclei (per 1000 cells)		Abnormal frustules (per 1000 cells)		Mitotic index (per 100 cells)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	7,95	3,02	0,98	2,23	5,67	1,65	17,48	1,84
MH $1 \cdot 10^{-7}$	16,21	5,64	3,14	5,37	13,83	2,96	20,19	3,02
MH $1 \cdot 10^{-6}$	22,99	5,45	10,08	2,4	16,1	2,58	19,46	1,89
MH $5 \cdot 10^{-6}$	27,18	4,28	12,82	3,6	15,37	3,48	23,42	2,24

A first result was evident: values found after these exposures showed a similar trend to that reported in the study of Debenest *et al.* (2008): with the augmentation of maleic hydrazide concentration, the total number of nuclear alterations (micronuclei, abnormal number and position of nuclei) increased. These alterations appeared in significantly (Tukey test, $P < 0,05$) higher proportions for maleic hydrazide concentrations 10^{-6} and $5 \cdot 10^{-6}$ M.

This trend was shown also in the case of abnormal frustules, but with the raising of a plateau at the higher concentrations (10^{-6} and $5 \cdot 10^{-6}$ M). Moreover, through comparison between abnormal frustules abundance and data obtained regarding the nuclear abnormalities, a positive correlation (0,868) was obtained. If the nuclear anomalies were considered without counting cells presenting micronuclei the positive correlation increased to 0,907.

Cells in mitosis were counted to calculate the mitotic index and, thus, to assess the impact of the pesticide on the cell division frequency. Results showed that no significant variations (ANOVA, $P > 0,05$) in mitosis frequency occurred between the control and the diatom communities treated with the three different concentrations of maleic hydrazide.

3.1.4. Exposure to chlorpropham

In this second culture, diatoms were treated with chlorpropham 10^{-7} and 10^{-6} M. Data obtained are presented in Table 3.3. Normal and altered diatoms were counted 24 hours after the pesticide exposure.

Table 3.3: Effects of chlorpropham (CIPC) concentrations on the mean abundance of frustule and nuclear alterations (cells with abnormal nuclei number and position), micronuclei presence and on the mean abundance of cells in division (mitotic index). SD means standard deviation.

Treatment (M)	Nuclear anomalies (per 1000 cells)		Cells with micronuclei (per 1000 cells)		Abnormal frustules (per 1000 cells)		Mitotic index (per 100 cells)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	7,53	4,97	1,88	1,33	36,40	15,13	2,73	0,24
CIPC $1 \cdot 10^{-7}$	49,83	4,88	9,25	3,58	62,43	21,48	2,70	0,67
CIPC $1 \cdot 10^{-6}$	68,87	14,54	3,06	0,20	89,42	30,93	0,936	0,55

Looking at Table 3.3, it is possible to observe that cell division was not inhibited in the control and in the sample treated with chlorpropham 10^{-7} M. However, in the community treated with the higher dose of chlorpropham, 10^{-6} M, cells decreased visibly their multiplication rate and the mitotic index was less than 2%.

Instead, looking at nuclear alterations (cells with micronuclei, with abnormal number and position of nuclei), it was evident that the negative control presented the lower abundance of abnormal cells, while they increased after exposure to chlorpropham. Among the control

sample and populations exposed to chlorpropham 10^{-7} and 10^{-6} M, the increase in number of nuclear aberrations was linear. Alterations regarding nuclei number and position appeared significantly higher (Tukey test, $P < 0,05$) with chlorpropham concentrations 10^{-6} M.

Finally, a count of the abnormal frustule presence in the negative control and in diatom populations treated with chlorpropham was done. Results showed that at concentration 10^{-6} M the abundance of abnormal frustules was higher than that found in the other cases.

From a comparison with previous exposition to maleic hydrazide 10^{-7} , 10^{-6} and $5 \cdot 10^{-6}$ M, it was possible to see that nuclear and frustule alterations in samples exposed to chlorpropham 10^{-7} and 10^{-6} M were more abundant. Moreover, a comparison between micronuclei abundance found after maleic hydrazide and chlorpropham exposure, showed that the bigger number of these anomalies was found after treatment with maleic hydrazide $5 \cdot 10^{-6}$ M.

3.2. Community change

3.2.1. Frustule identification

The relative abundance of species distribution was followed in a culture studied before and after treatment with three maleic hydrazide concentrations (10^{-7} , 10^{-6} and $5 \cdot 10^{-6}$). The same doses were selected in the study at the cellular level.

Before diatom exposure to maleic hydrazide, species identification was made in three different periods: the day of sampling in the Garonne River (to have an idea of the original community composition), the first day of culture in the plastic box (three days after the sampling) and two days before diatom exposure (i.e. after twelve days of culture), to understand the population change due to culture conditions (Figure 3.5).

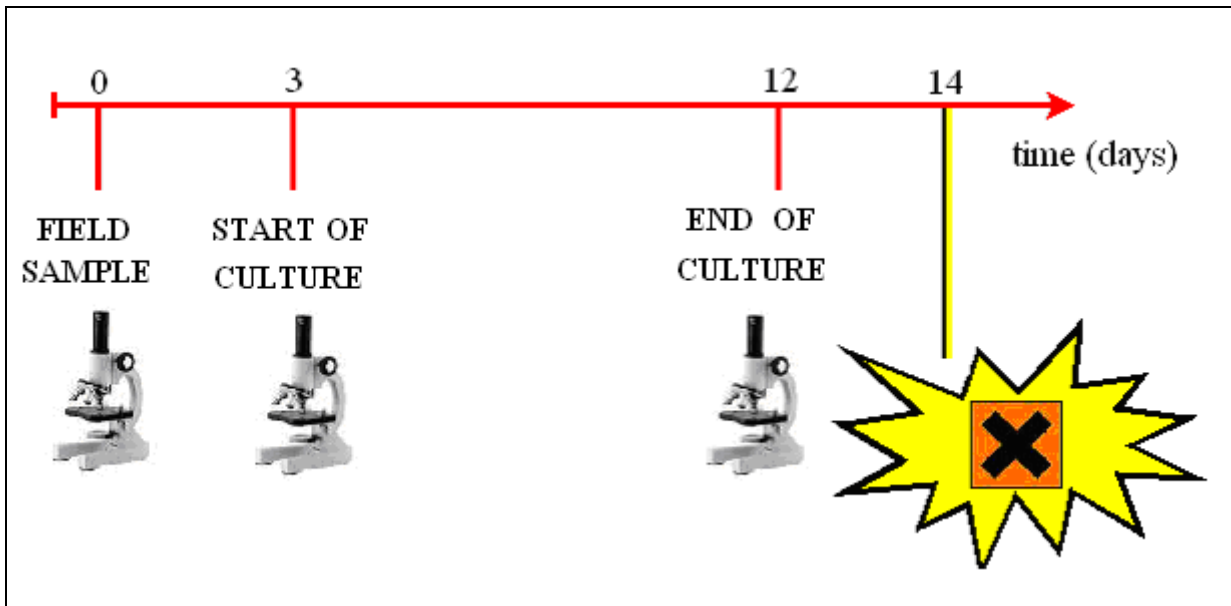


Figure 3.5: Sequence of the operations. Diatom identification events are signalled by a microscope and the day of diatom exposure by a danger symbol.

The initial composition of diatom community collected in the Garonne River and its change during a two weeks period of culture is presented in Figure 3.6. In this histogram the relative abundance of species is reported in percentage: for every sample 400 total cells were initially counted, however, for a better comprehension of data, they were expressed on percentage. Only diatoms over 3% found in the communities were considerate, the others were not abundant enough to visualise a population shift. Appendix A reports the list of abbreviations used for diatom species.

The dominant species present in natural conditions was ENMI, followed by NFON, NDIS, GOLI, NTPT and by other less numerous species. Diatoms did not seem to increase their cell density after transfer under controlled condition culture. Rather, their abundance decreased visibly after the third day, thus, since the transfer of diatoms in the apposite flasks of culture. However, this trend is not confirmed for NPAL species: it was present in the day of sample and in the third day of culture at undetectable abundance and after about two weeks became the more abundant species.

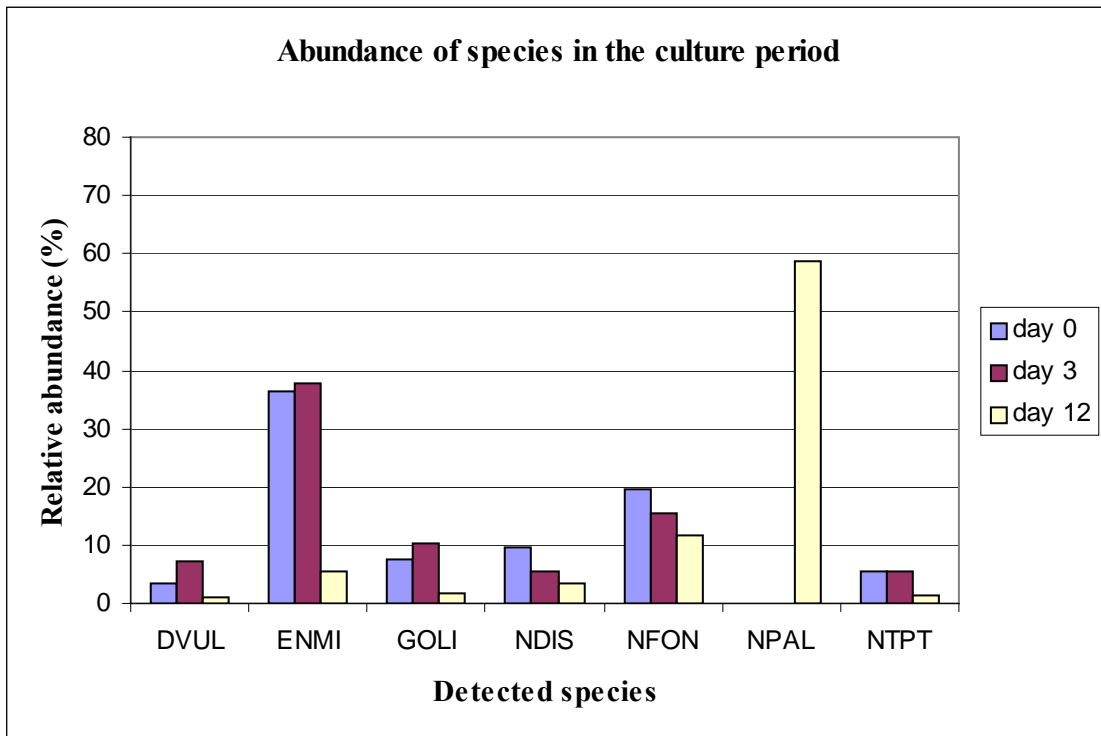


Figure 3.6: Community change during the culture period. Diatom identification was made the day of collection in the Garonne River (day 0), the third and twelfth day of culture.

The population change was also analysed 24 and 48 hours after maleic hydrazide exposure (Figure 3.7).

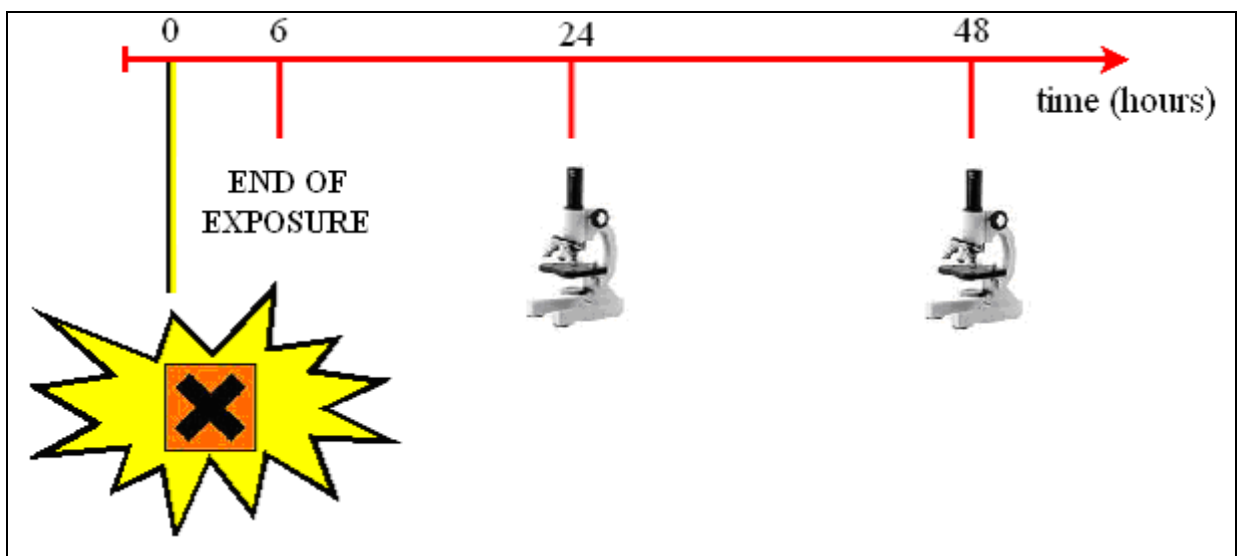


Figure 3.7: Diatom identification events are signalled by a microscope and the day of diatom exposure by a danger symbol.

After 24 hours from the exposure (Figure 3.8) results showed that in the controls there was not a particular abundance variation from the twelfth day of culture. However, some species present before maleic hydrazide exposure (DVUL, GOLI, NDIS and NTPT) disappeared and one other, initially under the detection threshold, became more abundant (GPAR). Looking at the exposed populations, some species seemed to increase from the lower pesticide concentration (10^{-7} M) to the higher one ($5 \cdot 10^{-6}$ M), like ENMI and GPAR, while one other species decreased, NFON. Looking at the dominant species, i.e. NPAL, it is possible to see that it seemed not to be affected by the pesticide.

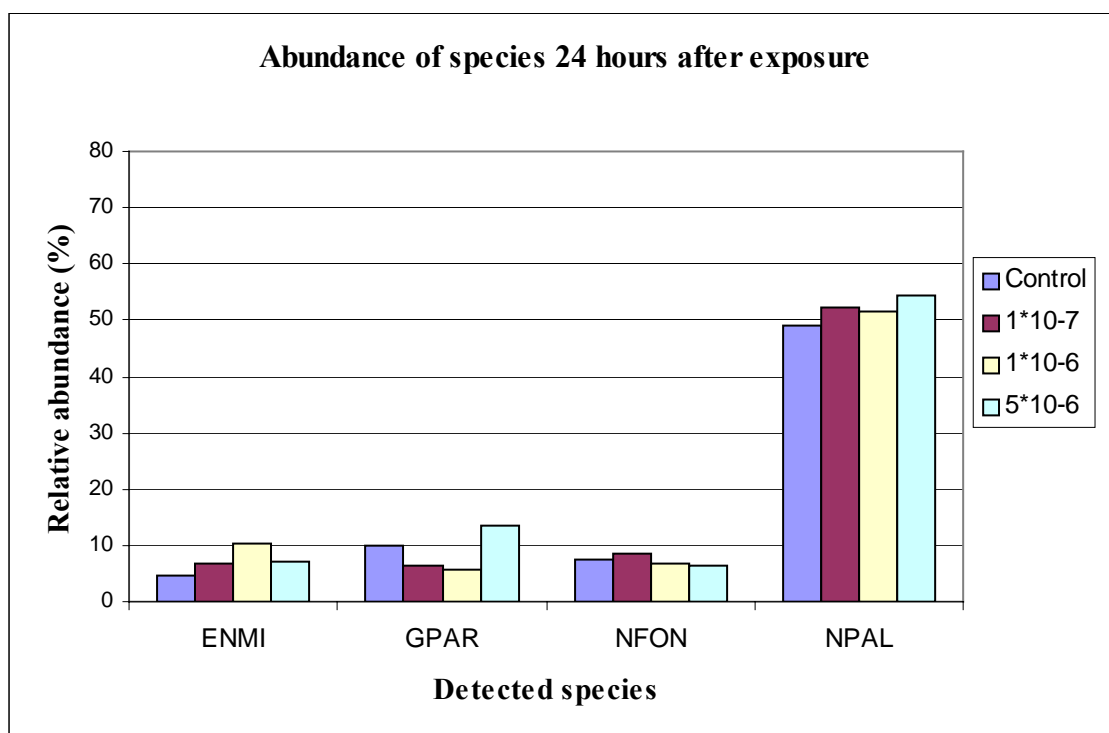


Figure 3.8: Community change after 24 hours from maleic hydrazide exposure.

At 48 hours from treatment (Figure 3.9), by comparison between the controls and the samples exposed to maleic hydrazide $5 \cdot 10^{-6}$ M, every detected species (ENMI, GPAR and NFON) decreased their relative percentage. The more evident change regards NPAL species, whose abundance in sample treated with maleic hydrazide $5 \cdot 10^{-6}$ M increased from 24 to 48 hours after exposure.

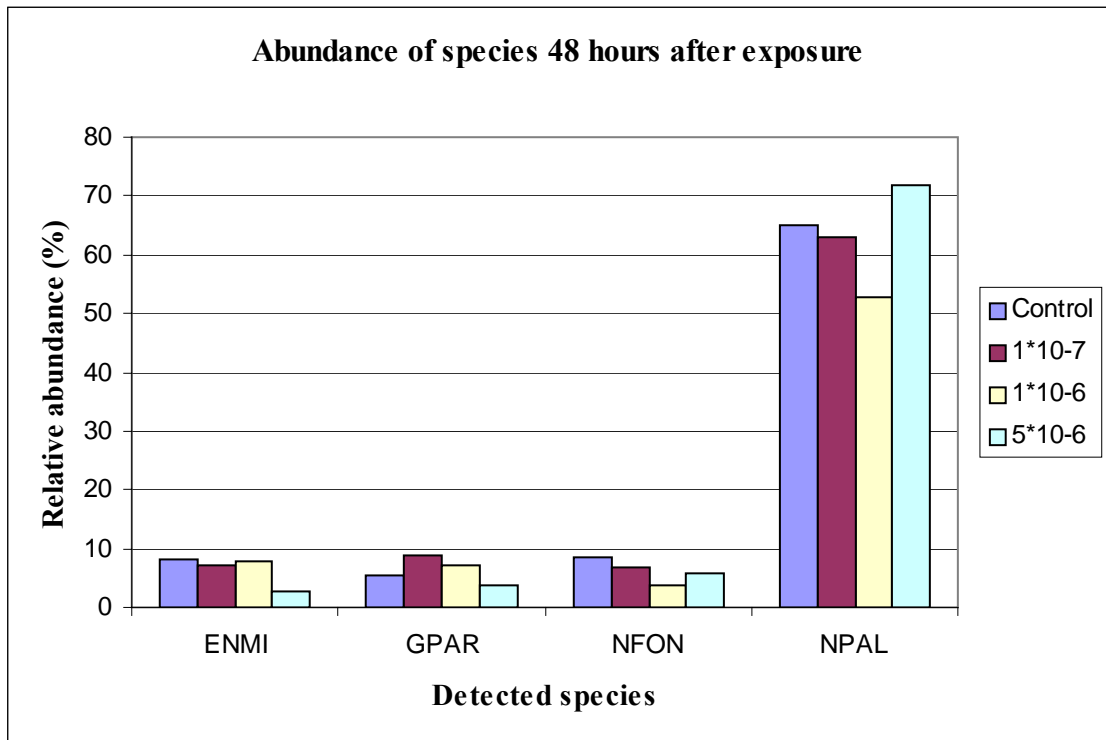


Figure 3.9: Community change after 48 hours from maleic hydrazide exposure.

3.2.2. Flow cytometry technique

The diatom community collected in the Garonne River in the month of July 2008 was exposed for 6 hours to maleic hydrazide 10^{-7} , 10^{-6} and $5 \cdot 10^{-6}$ M concentrations after two weeks of cultivation and studied through flow cytometry technique one and two days from the pesticide treatment. Before flow cytometry analysis a count of cells present in the culture was carried out to assure the presence of diatoms in the samples and to have an idea of their density. Through the use of the Malassez chamber results showed that the main organisms present in samples were green algae, followed by diatoms. Also other organisms as bacteria have been found.

Through flow cytometry, populations were analysed in a first moment without the use of a label and, in a second one, with Hoechst 33342 and Propidium Iodide (IP) separately. Results showed that with IP the identified populations were more evident and easy to distinguish, while cells alone and cells labelled with Hoechst 33342 appeared less clear to individuate. For this reason, in the following studies, the stain used to visualize diatom cells has been IP.

Two different graphs presenting two measurement parameters on the x- and y-axis (“Dot Plot” histogram) were made after every flow cytometry analysis. The first kind of graphic represents the value FSC-H on the x-axis (the cell size) and the SSC-H value (the cell granularity) on the y-axis. The second kind of graphic obtained after the sample analysis with flow cytometry presents on the x-axis the FL1-H value, corresponding to an emission wavelength equal to 530 nm. This axis shows the natural autofluorescence of chlorophyll contained in diatoms and green algae; moving to right from the origin point the intensity of this value grows up. Instead, the y-axis represents the emission wavelength of Propidium Iodide, equal to 585 nm and corresponding to the filter FL2-H. UL, UR, LL and LR represent respectively to the upper left, upper right, low left and low right side of this kind of graphic. It is important to say right now that in all the obtained graphics the described parameters are exclusively qualitative ones: they are not referred to any unit of measure. They have only the aim to give an idea of the studied parameters to compare the investigated populations.

In a first step a flow cytometry was carried out to understand the general composition of the untreated sample labelled with Propidium Iodide (Figures 3.10). Figures 3.11, 3.12 and 3.13 show respectively the composition of samples exposed to MH 10^{-7} , 10^{-6} and $5 \cdot 10^{-6}$ M, after 24 hours from exposure. In every graphic showing cells distributions depending on their size and granularity (“FSC-H vs. SSC-H”) it was possible to individuate two different groups. One group presenting a smaller cell size and granularity was situated in the left side and one other with a bigger cell size and a more developed granularity, was situated in the upper right. These two populations were found also in graphics, “FL1-H vs. IP”: cells belonging to the population situated in the left side of graphics “FSC-H vs. SSC-H”, were distinguishable because of their lower fluorescence (LL and UL) and cells situated in the right of graphics “FSC-H vs. SSC-H” were characterised by a more evident fluorescence (LR and UR). Population placed in LL and UL was found in higher proportions if confronted with the population situated in LR and UR sides of graphics .

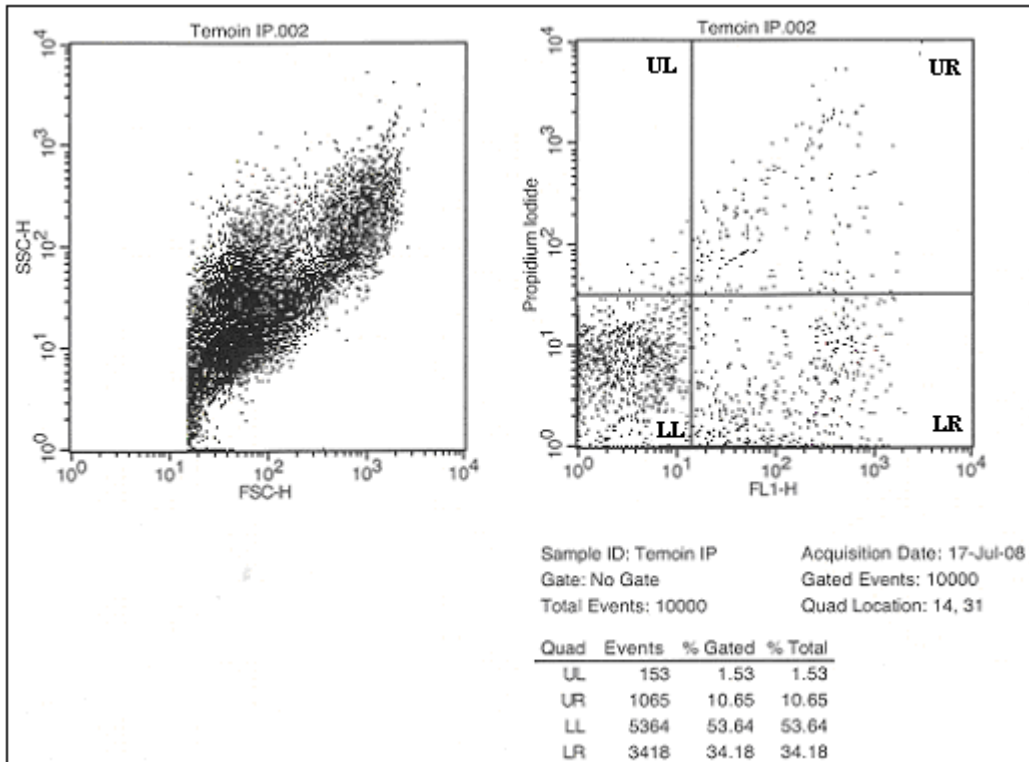


Figure 3.10: Control sample after IP labelling, 24 hours from the exposure.

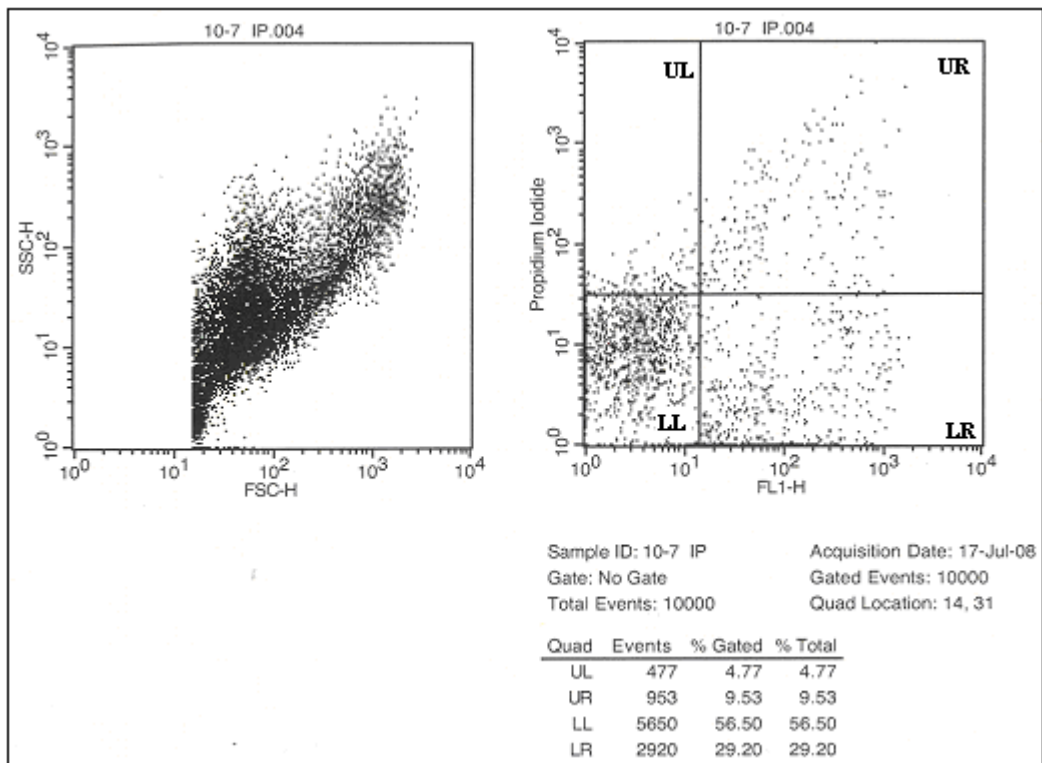


Figure 3.11: Sample exposed to MH 10^{-7} M after IP labelling, 24 hours from the exposure.

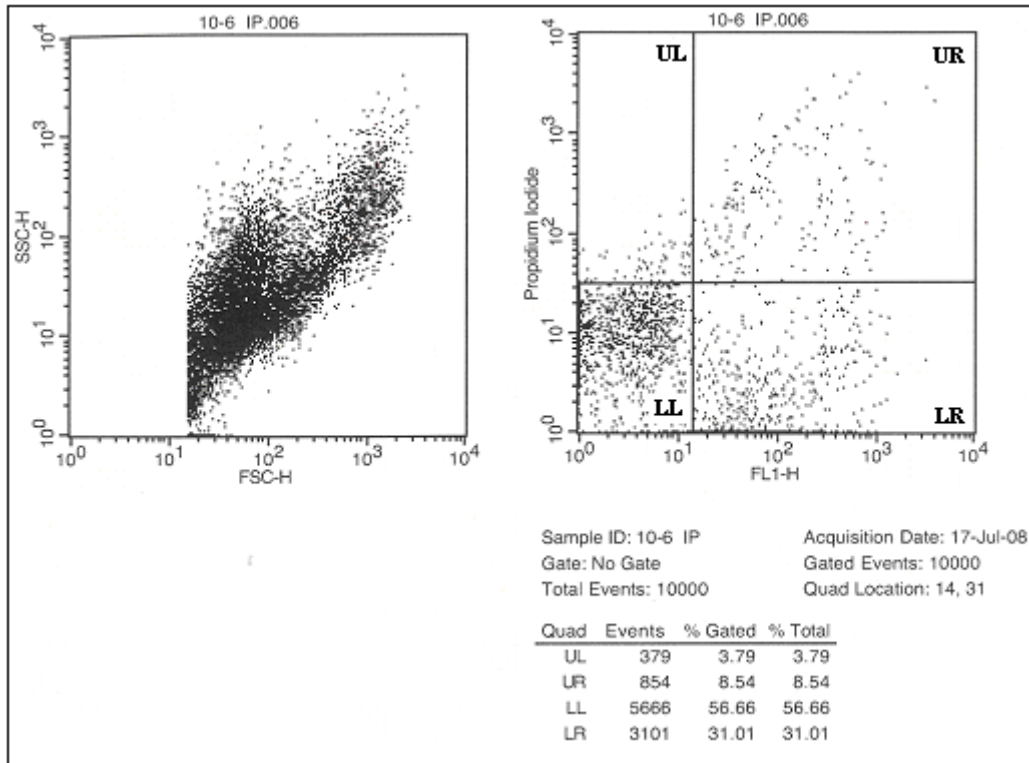


Figure 3.12: Sample exposed to MH 10^{-6} M after IP labelling, 24 hours from the exposure.

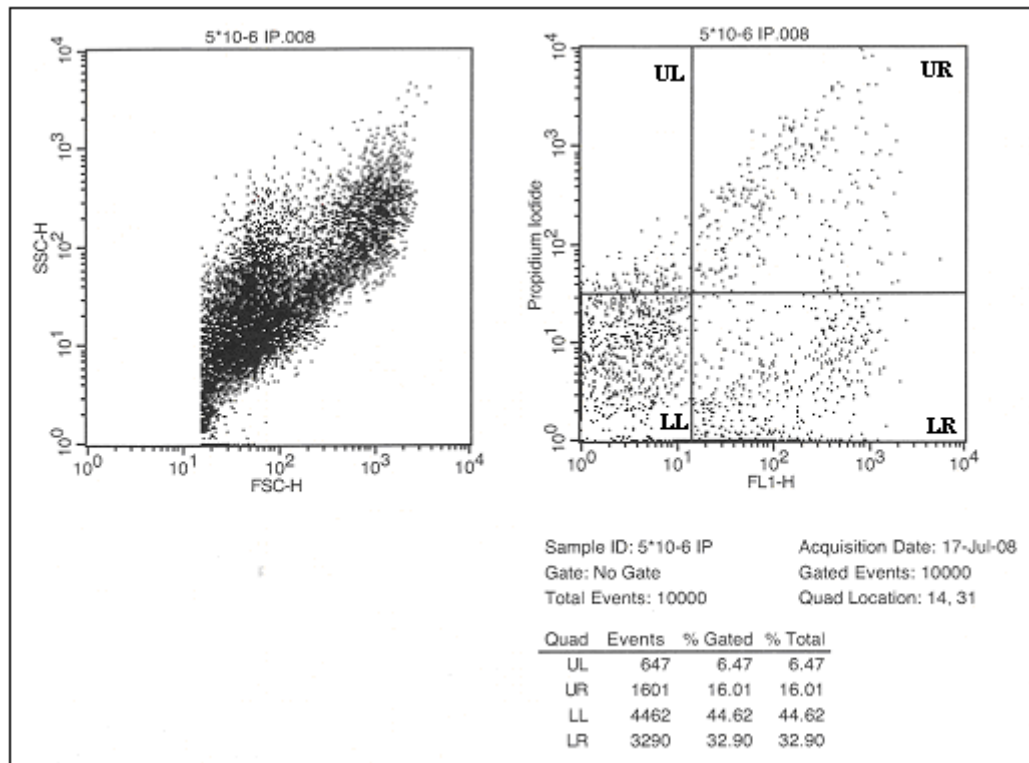


Figure 3.13: Sample exposed to MH $5 \cdot 10^{-6}$ M after IP labelling, 24 hours from the exposure.

Results obtained 48 hours after maleic hydrazide exposure are presented below. Figure 3.14 shows the composition of the untreated sample, while Figures 3.15, 3.16 and 3.17 show samples exposed to 10^{-7} , 10^{-6} and $5 \cdot 10^{-6}$ M. The black population present in graphics “FSC-H vs. SSC-H” is also visualized in the left side of graphics “FL1-H vs. IP”. The red population corresponds to R1 group (LR side in “FL1-H vs. IP” graphics) and the green one to R2 group (UR side in “FL1-H vs. IP” graphics). Red and green cells are considered as the same population. From all graphics “FSC-H vs. SSC-H” it is possible to note the same two populations individuated after 24 hours. Also after 48 hours the population situated on the left of graphics “FL1-H vs. IP” was more abundant than that situated on the right.

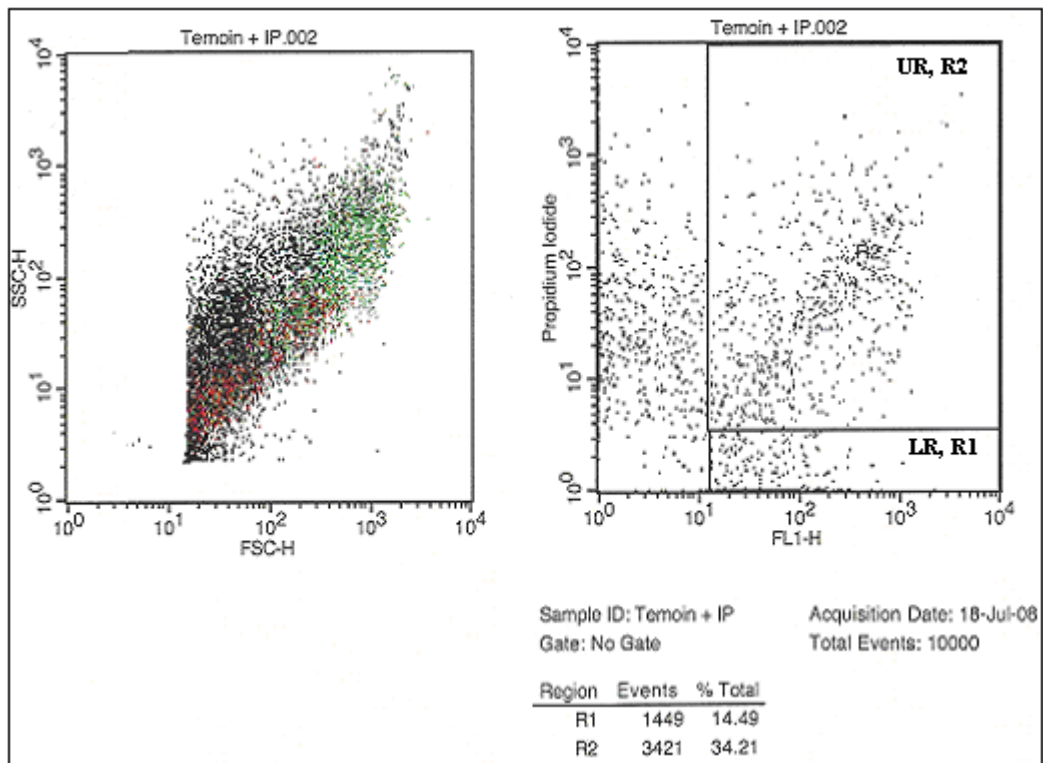


Figure 3.14: Control sample after IP labelling, 48 hours from the exposure.

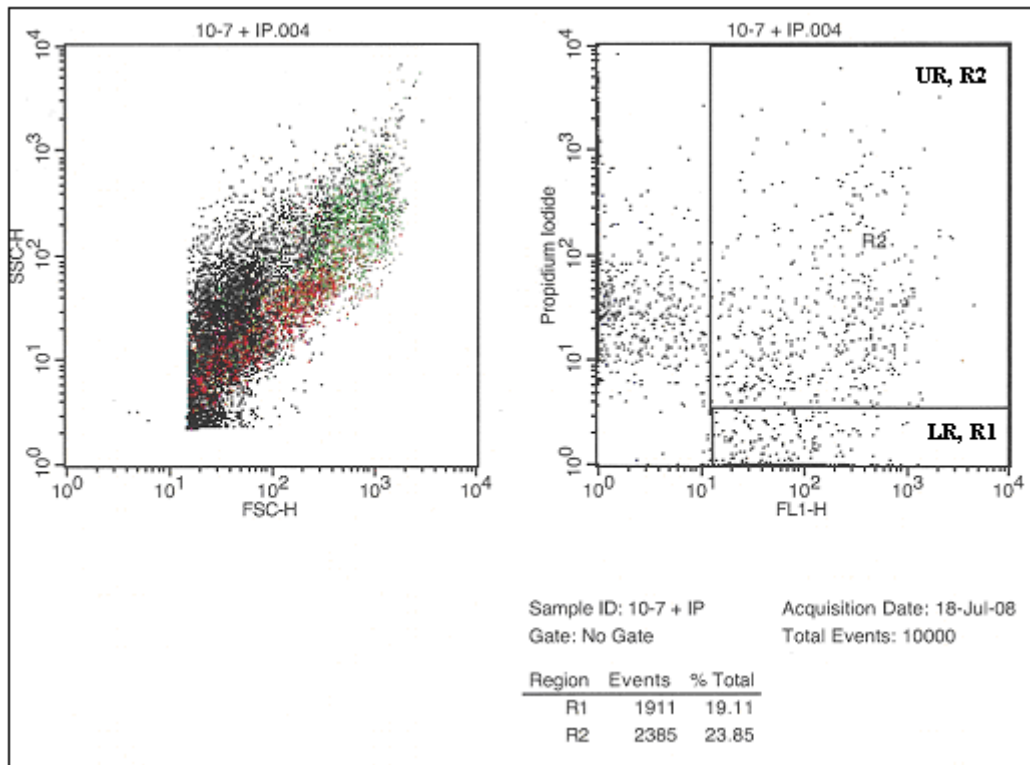


Figure 3.15: Sample exposed to MH 10^{-7} M after IP labelling, 48 hours from the exposure.

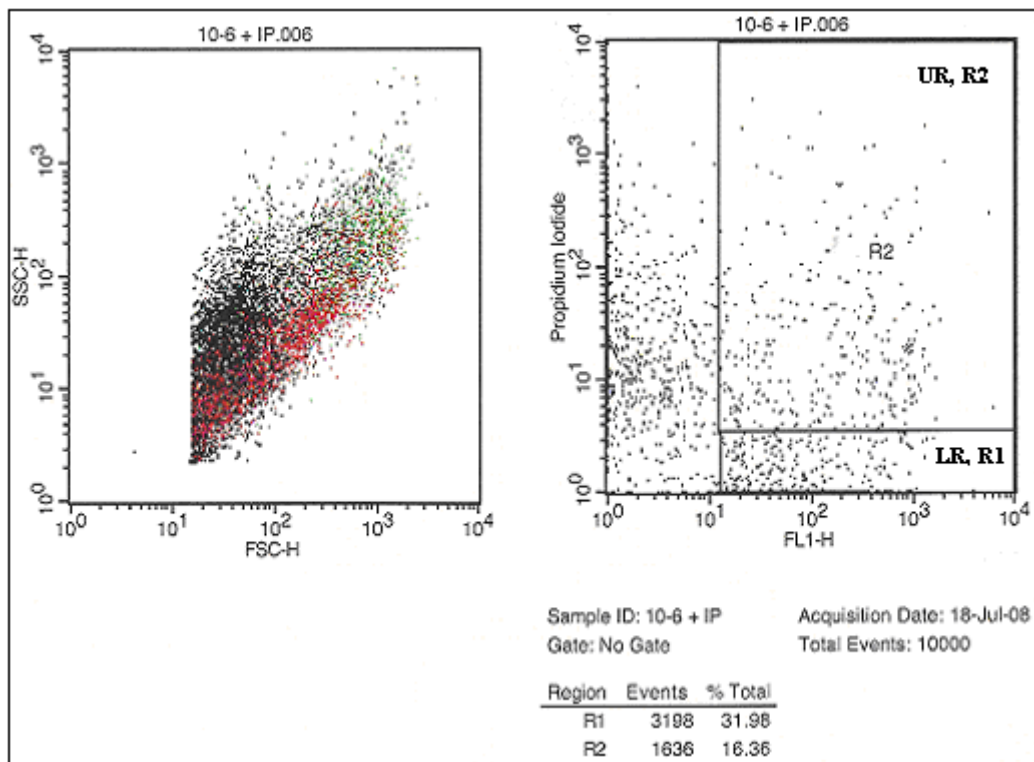


Figure 3.16: Sample exposed to MH 10^{-6} M after IP labelling, 48 hours from the exposure.

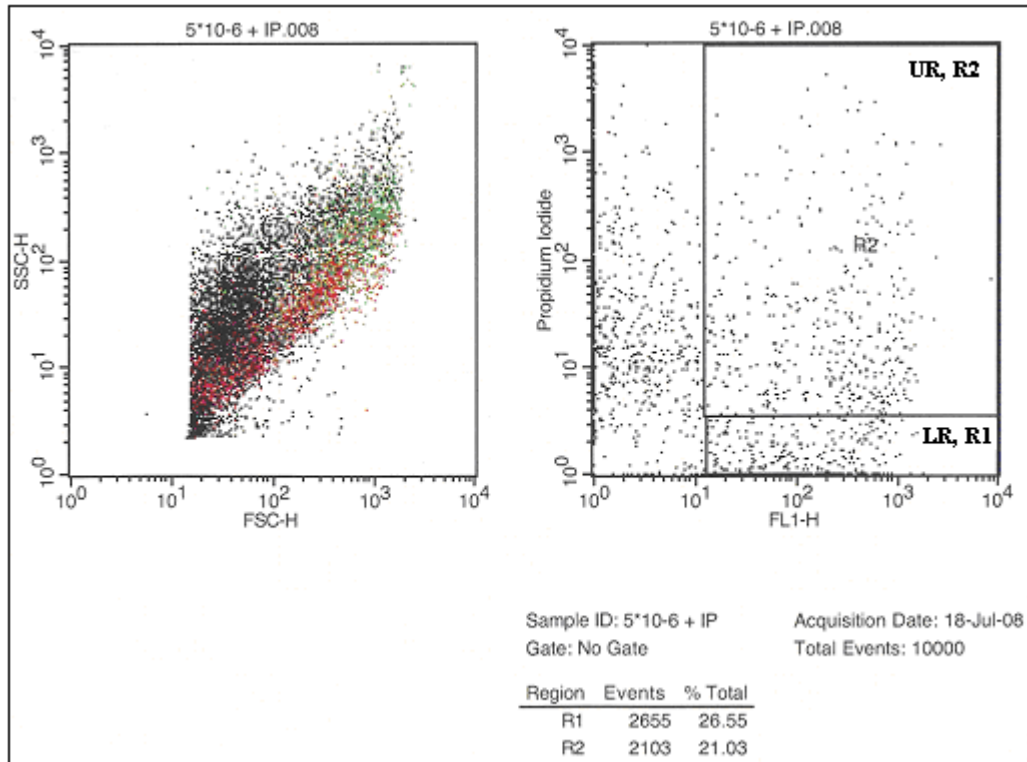


Figure 3.17: Sample exposed to MH $5 \cdot 10^{-6}$ M after IP labelling, 48 hours from the exposure.

If values collected 24 and 48 hours after exposure through Malassez chamber and flow cytometry were compared, some results appeared more evident. First of all it is to explain that data referred to flow cytometry events counted in the right side of graphics “FL1-H vs. IP” were considered diatoms, while cells situated in the left were considered as green algae. This assumption was done because diatoms own some pigments, xanthophylls, which make them appear more fluorescent than green algae.

Both the analysis techniques showed that from the control to the sample treated with the higher concentration of maleic hydrazide diatom abundance decreased and that of green algae increased. From Malassez chamber, regarding diatom population change after 24 hours (Table 3.4), it was evident a decrease in cells abundance, from the control to the $5 \cdot 10^{-6}$ M sample. Also after 48 hours the same trend was evident (Table 3.5): diatoms appeared more abundant in the control than in the $5 \cdot 10^{-6}$ M sample. For green algae the opposite trend was found: they increased in abundance from the control to the $5 \cdot 10^{-6}$ M sample. Finally, it was possible to see that in the control, but also in the treated samples, diatoms appeared more abundant after 24 hours than after 48 hours. However, these results were less clear and congruent in the case of count through flow cytometry.

Table 3.4: Average composition after 24 hours from maleic hydrazide exposure. Count executed through Malassez chamber and flow cytometry technique (F. C.).

Treatment (M)	Diatoms		Green algae		Total	
	Malassez (%)	F.C. (%)	Malassez (%)	F.C. (%)	Malassez (n/1 µl)	F.C. (n)
Control	36	45	64	55	625	10 000
MH 1*10 ⁻⁷	23	39	77	61	121	10 000
MH 1*10 ⁻⁶	22	40	78	60	317	10 000
MH 5*10 ⁻⁶	26	49	74	51	130	10 000

Table 3.5: Average composition after 48 hours from maleic hydrazide exposure. Count executed through Malassez chamber and flow cytometry technique (F. C.).

Treatment (M)	Diatoms		Green algae		Total	
	Malassez (%)	F.C. (%)	Malassez (%)	F.C. (%)	Malassez (n/1 µl)	F.C. (n)
Control	22	49	78	51	185	10 000
MH 1*10 ⁻⁷	31	43	69	57	339	10 000
MH 1*10 ⁻⁶	20	48	80	52	152	10 000
MH 5*10 ⁻⁶	18	48	82	52	186	10 000

4. DISCUSSION

4.1. Genotoxic study

4.1.1. Observations with fluorescent microscope

Photos collected during the counting of anomalies showed different cases of cells presenting micronuclei together with normal numbers of nuclei and chloroplasts after maleic hydrazide exposure at 10^{-6} and $5 \cdot 10^{-6}$ M concentration, suggesting a disrupting effect on the diatoms DNA.

On the contrary, with every chlorpropham exposure, the presence of abnormal number and position of nuclei, chloroplasts and abnormal frustules suggested that the internal microtubule architecture was disrupted. In abnormal cells even 7-8 nuclei were counted. This phenomenon suggested also the appearance of polyploid cells.

The only observation of the collected photos induced to hypothesize a double way of action of maleic hydrazide (which seemed to affect the cytoskeleton integrity at every concentration and the DNA structure only at the higher doses) and a single way of action of chlorpropham (affecting the cytoskeleton). To discover the involved mechanisms in the formation of these anomalies, the count and analysis of aberrations was carried out.

4.1.2. Exposure to maleic hydrazide

Regarding the first results obtained, data collected after maleic hydrazide exposure confirm results discovered by Debenest *et al.* (2008), but also by Coombs *et al.* (1968), Duke and Reimann (1977) and Lee and Li (1992), who assumed that cells treated with microtubule inhibitors developed multinuclear cells and abnormal frustules. The experiment described in

this thesis work reported a similar behaviour. In fact, at increasing maleic hydrazide concentrations the number of abnormal frustules increased visibly in relation with the nuclear alterations (without considering cells presenting micronuclei). These observations, together with that of fragmented chloroplasts, support the idea that maleic hydrazide disturbs the diatom cytoskeleton. Frustules with deformed morphology and abnormal ornamentations seem to be caused by cytoskeleton disturbance, because of an indirect genotoxic effect of maleic hydrazide (the aneugenic effect). Disruption of the microtubules seems to be the first target of maleic hydrazide: this would lead to abnormal fragmentation of the nucleus and chloroplasts during cell division and to a poor silica supply for the formation of new valves at the end of mitosis. This report suggests two hypotheses to explicate the comparison of these anomalies. In the first case, if the internal network of filaments and microtubules is irreversibly altered, the division capacity is probably lost. Cells cannot follow a normal mitosis because during anaphase the chromosomal heritage cannot separate in a good way: one or more chromosomes are excluded by the nucleus formation and the cell cannot end the division and becomes multinucleated. In the second case, the cell does not lose the division possibility and from the diatom mother two daughters with abnormal number and position of the internal organelles are originated. This last hypothesis would explicate why the mitotic index did not decrease after treatment of always higher maleic hydrazide concentrations.

Maleic hydrazide seems to have a double way of action in diatoms cells, causing disruption in the microtubular architecture at concentrations included between 10^{-7} and 10^{-6} M (at $5 \cdot 10^{-6}$ M abnormal frustules stop their augmentation raising a plateau) and micronuclei appearance at the higher dose ($5 \cdot 10^{-6}$ M). At $5 \cdot 10^{-6}$ M concentration about 12,82 cells per 1000 show micronuclei, against 0,98 found in the control. This suggest that maleic hydrazide direct genotoxicity is more acute at the higher concentration ($5 \cdot 10^{-6}$ M). To confirm this behaviour, it is possible to see that the correlation between abnormal frustules and nuclear alteration is greater if micronuclei are not considered. The origin of micronuclei seems to be different to that of the other nuclear alterations. Some micronuclei would be originated from problems in the cytoskeleton architecture, but the bigger part would be probably due to a clastogenic effect (DNA strand breaks or chromosome bridges). Bibliographic data confirm this behaviour of maleic hydrazide (Cotelle *et al.*, 1999; Grant, 1999; Kong and Ma, 1999; Marcano *et al.*, 2004; Hajjouji *et al.*, 2007). According to this hypothesis, with maleic hydrazide $5 \cdot 10^{-6}$ M the DNA present in the mother cell would be broken, causing the origin after the division of a

normal cell and of a second one presenting a micronucleus (with a fragment of original DNA).

4.1.3. Exposure to chlorpropham

From results obtained after exposure of the diatom culture to chlorpropham 10^{-7} and 10^{-6} M it is possible to draw different conclusions. First of all it is clear that chlorpropham at concentration equal to 10^{-7} and 10^{-6} M has disrupting effects on the diatom cytoskeleton and, as a consequence, an inhibition power on diatom mitosis stopping the division cycle. This evidence is given from data regarding the mitotic index and from data regarding the nuclear alteration (abnormal number and location of nuclei), which indicate that the cell possibility of multiplication is lost. Therefore, it is probable that this pesticide acts with an aneugenic way on diatoms, like maleic hydrazide, but with two differences. The first one regards the number of nuclear anomalies, which is higher in the exposure to chlorpropham if equal concentrations of the two pesticides are compared (10^{-7} and 10^{-6} M). Chlorpropham has an effect more important on microtubules than maleic hydrazide. Moreover, by comparison between abnormal frustules abundance and nuclear abundance (micronuclei and abnormal position and location of nuclei), it is evident that the two aberrations increase simultaneously. These data suggest that their origin is the same. The second difference concerns the micronuclei presence: in the exposure to chlorpropham micronuclei were less abundant than in treatments with maleic hydrazide. From these data it is possible to conclude that chlorpropham acts principally in an aneugenic way, without affect directly the nuclear patrimony. Micronuclei are present with high probability for disruption in the cytoskeleton integrity.

4.2. Community change

4.2.1. Frustule identification

Some discussions can be advanced after the analysis of data regarding the exposure of the Garonne cultures to three concentrations of maleic hydrazide equal to 10^{-7} , 10^{-6} and $5 \cdot 10^{-6}$ M. It is clear to understand that NPAL species is the more tolerant one to culture conditions and to maleic hydrazide exposure. In fact, after a recovery time of 24 and 48 hours this species increased in its quantity until becoming the more numerous. In sample treated with the higher pesticide concentration, after 48 hours, species NPAL alone represented the 70% of the sample, against other 30% composed of all the other species.

Moreover, it was evident that the number of detected populations fell from the culture period to the community study 48 hours after exposure. After a maleic hydrazide exposure of 6 hours the diversity within the freshwater diatom community decreased. Because of the stress caused by the culture conditions and by the pesticide, NAPL colonized the flask of culture with detriment of the other species. An evidence of this results, which confirms the ability of NPAL species to adapt itself to polluted environments, comes from bibliographic data. Debenest (2007) showed that freshwater benthic diatom communities were populated in particular from species known for their preference to eutrophic environments, rich in nutrient elements. One of these species was exactly NPAL. Moreover, other authors saw that NPAL species is tolerant to environments polluted by pesticides (Kosinski *et al.*, 1984; Kasai, 1999; Dorigo *et al.*, 2004).

4.2.2. Flow cytometry technique

From observations of results concerning the community change after one and two days from maleic hydrazide exposure, some hypothesis can be advanced. Data collected with Malassez chamber and flow cytometry showed similar values and, therefore, same population changes within the different samples. With regard to data coming from the Malassez chamber, it was

clear that green algae were more abundant than diatoms after 24 and 48 hours from treatments. However, data coming from flow cytometry were less distinct between diatoms and green algae abundance. Flow cytometry has probably not been able to well differentiate the two communities. It is only possible to affirm with high probability that events counted in the left side of graphics “FL1-H vs. IP” belonged to green algae populations and that counted in the right one belonged to diatoms and green algae together. This proportion is due to the wrong month of sampling, July, when diatom communities are supplanted by green algae. Therefore, the more abundant community, situated in the left side of graphics “FL1-H vs. IP”, was probably composed of green algae, while the community situated on the right, was composed of diatoms because less numerous. Moreover, one other consideration that confirms the hypothesis according to which diatoms belonged to the more fluorescent population situated in the right side, is that these organisms own some pigments of the family of xanthophylls (fucoxanthin, diatoxanthin, diadinoxanthin), not present in green algae. In particular the adsorption wavelength of fucoxanthin is 453 nm, of diadinoxanthin 448-478 nm and of diatoxanthin 453-481 nm. On the x-axis of graphics the filter FL1-H is characterised by an absorption wavelength equal to 488 nm. Because of the presence of these pigments, diatoms could appear more fluorescent than green algae. It is also possible to think that death cells or bacteria individuals composed these detected populations. However, cells like these ones owning a low content of genetic material are normally found near the origin of the two axes of graphic “FL1-H vs. IP” because of their feeble fluorescence. However, this kind of bottom noise was not represented in the exposed graphics.

Regarding effects of maleic hydrazide on the community change one and two days after the exposure, from count with Malassez chamber it is possible to conclude that this pesticide had an inhibiting power on the diatom community. It decreased in abundance from the control to the sample with maleic hydrazide $5 \cdot 10^{-6}$ M, consenting to green algae to become more numerous. However, data collected with flow cytometry after 24 hours did not confirm this trend, on the contrary it seemed that the events counted on the right side of the graphic (considered as diatom population) increased. This is explicable with a hypothesis. In the right side of graphics “FL1-H vs. IP”, where diatoms and green algae were probably mixed together, it is possible that a green alga species with a fluorescence perceptible in the right side was selected and successively become more abundant than the other algae and diatoms.

A last observation has to be done: the difference noticeable in the community composition between treated and untreated samples in the two days of study were not really pronounced because diatoms and green algae probably had a kind of immunity, explicable taking in consideration the period of sampling. In fact to carry out this flow cytometry study, diatoms were collected in the Garonne River at the beginning of July. In this period the biofilm was already treated with high quantity of phytotoxic products, coming from the agricultural activity and washed in rivers after rain events (very numerous in spring and summer 2008 at Toulouse). This reflection suggests that at the beginning of summer organisms found in the biofilm were already selected and only the more resistant were present. For this reason it is possible that green algae and diatoms in the culture did not change visibly their composition, because already adapted to relatively polluted environments.

5. GENERAL CONCLUSIONS AND PERSPECTIVES

In a context of important degradation of the water quality in rivers, not only in France but also in whole the planet, and for the absence of adequate tools to use in the detection of pollution by pesticides, this thesis work had the aim to ameliorate the knowledge about effects on diatoms of pesticides.

Through the studies exposed in this report it is possible to confirm that freshwater benthic diatom cytoskeleton is the first target of chlorpropham and maleic hydrazide. However, this last pesticide, if used at high concentrations has also a clastogenic effect, inducing micronuclei formation. Anyway, other concentrations and pesticides with different ways of action (e.g. inhibition of cell division) should be tested in order to evaluate their impacts on cultivated diatoms and to have other confirmations of the supposed mechanisms involved in the anomaly formation. Understanding the precise mechanisms responsible of micronuclei presence, of abnormal frustules, nuclei and chloroplasts, for example labelling the actin present in the cytoskeleton, will consent the creation of biomarkers within freshwater benthic diatom cells. Nuclear and frustule alterations will facilitate the study of the running water quality, the detection of the more degraded sites by phytosanitary products and the identification of adequate environment restoration actions.

Looking at the two studies carried out at a community level, it is possible to confirm the diatom biomass sensibility to maleic hydrazide exposure. After two days from the exposure, the number of diatoms and species decreased, probably because of the disturbances induced in the cytoskeleton and, therefore, in the cell division capacity. At a cellular level the mitotic index did not decrease under the 2% threshold probably because cells of NAPL species were mostly counted. Only NAPL species seemed to adapt itself to the polluted environment. From the two studies of the community change after 24 and 48 hours from pesticide exposure, frustules identification was the more useful to understand populations dynamics. Flow cytometry technique is still not adequate for this kind of study: after analyses there is not a distinction between diatom populations and other organisms present in samples. To better exploit flow cytometry it would be useful to try to individuate populations after analyses. In this way the content would be studied to have the confirmation of the hypotheses made on the community composition and change. Moreover, a flow cytometry study should be done using

only some monoclonal diatom populations, to real understand if this technique would be able to differentiate species.

Present devices used in monitoring rivers quality through benthic diatoms, like IBD and IPS, have many limits. They are not able to differentiate the trophic pollution form that caused by pesticides. As a consequence, these indices are still not adequate to understand the total effect of agricultural pollution. The amelioration of knowledge about pesticide effects on diatom cells and of population change within the community, will help in a future the existing devices based on diatoms to evaluate the water pollution in rivers also after flood episodes.

APPENDICES

Appendix A: list of the abbreviations used for diatom species

Abbreviations	Taxa names
ADMI	<i>Achnantheidium minutissimum</i> (Kützing)
AFOR	<i>Asterionella formosa</i> (Hassall)
ALFR	<i>Achnanthes lanceolata</i> (Brébisson in Kützing) Grunow
AMIN	<i>Achnanthes minutissima</i> (Hustedt)
CPLA	<i>Cocconeis placentula</i> (Ehrenberg)
DVUL	<i>Diatoma vulgare</i> (Bory)
ENMI	<i>Encyonema minutum</i> (Hilse in Rabh.) D.G. Mann
FCRO	<i>Fragilaria crotonensis</i> (Kitton)
FCVA	<i>Fragilaria capucina</i> Desmazieres var. <i>vaucheriae</i>
GOLI	<i>Gomphonema olivaceum</i> (Hornemann) Brébisson
GPAR	<i>Gomphonema parvulum</i> (Kützing)
NDIS	<i>Nitzschia dissipata</i> (Kützing) Grunow
NCRY	<i>Navicula cryptocephala</i> (Kützing)
NFON	<i>Nitzschia fonticola</i> Grunow in Cleve et Möller
NHAL	<i>Navicula halophila</i> (Grunow) Cleve
NLAN	<i>Navicula lanceolata</i> (Agardh) Ehrenberg
NMIN	<i>Navicula minima</i> (Grunow)
NPAL	<i>Nitzschia palea</i> (Kützing) W. Smith
NTPT	<i>Navicula tripunctata</i> (O.F.Müller) Bory
SACU	<i>Synedra acus</i> (Kützing)
SSEM	<i>Sellaphora seminulum</i> (Grunow) D.G. Mann

Appendix B: the nutritive solution Chu No. 10 used for the diatom culture

Products	Initial concentration (g/L)	Final concentration (g/L)	ml to introduce in 1 L of distilled water
MgSO ₄ , 7H ₂ O	25	0,025	1
K ₂ HPO ₄	40	0,01	0,25
Ca(NO ₃) ₂ , 4H ₂ O	10	0,04	4
Na ₂ CO ₃	22,10	0,0221	1
Na ₂ SiO ₃ , 9H ₂ O	58	0,058	1
Fe-EDTA			0,1
Vitamins:			
Biotin	1	0,00005	0,05
Thiamine (B1)	10	0,01	1
Vitamin B12	0,01	0,00001	1
Oligoelements			0,1

Appendix C: the oligoelements used in the nutritive solution Chu No. 10

Oligoelements	mg in 100 ml of distilled water
H ₃ B0 ₃	310
MnSO ₄ , 4H ₂ O	223
Na ₂ WO ₄ , 2H ₂ O	3,3
(NH ₄) ₆ Mo ₇ O ₂₄ , 4H ₂ O	8,8
KBr	11,9
KI	8,3
ZnSO ₄ , 7H ₂ O	28,7
Cd(NO ₃) ₂ , 4H ₂ O	15,4
Co(NO ₃) ₂ , 6 H ₂ O	14,6
CuSO ₄ , 5H ₂ O	12,5
NiSO ₄ (NH ₄) ₂ SO ₄ , 6H ₂ O	19,8
Cr(NO ₃) ₃ , 7H ₂ O	3,7
V ₂ O ₄ (SO ₄) ₃ , 16 H ₂ O	3,5
Al ₂ (SO ₄) ₃ K ₂ SO ₄ , 24 H ₂ O	47,4

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