N° d'ordre : 3316

THESE

EN CO-TUTELLE

entre

VAST-Vietnam

L'UNIVERSITE BORDEAUX I

ECOLE DOCTORALE

"Sciences du vivant, Géosciences et Sciences de l'Environnement"

et

VIETNAM ACADEMY OF SCIENCE AND TECHNOLOGY

Institut de Biotechnologie

Présentée à

L'UNIVERSITE BORDEAUX I

par Thi Thuy DUONG

POUR OBTENIR LE GRADE DE **DOCTEUR**

SPECIALITE: Ecotoxicologie

Réponses des Diatomées fixées aux pollutions organiques et métalliques dans les hydrosystèmes Nhue-Tolich (Hanoi, Vietnam), et Lot-Riou Mort (France)

Soutenue le : 15 Décembre 2006

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M. Alain BOUDOU	Professeur, Président Université Bordeaux 1, France	Membre invité

- 2006 -

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Defended 15 December 2006

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- 2006 -

Acknowledgements

This thesis is carried out during a period (2003-2006) in which I have been accompanied and supported by many people. Without their help and encouragement, it would not have been finished. It is my pleasure to take this opportunity to thank them.

First of all, I would gratefully acknowledge Dr. ***Josette Garnier***, Prof. ***Georges Vachaud***, Dr. ***Michel Coste***, Dr. ***Agnès Feurtet-Mazel***, Prof. ***Dang Dinh Kim*** and Prof. ***Chau Van Minh***, Prof ***Alain Boudou*** who accepted to be member of my thesis committee to give me many helpful comments and suggestions for the thesis manuscript and also to enrich my scientific knowledge.

I would like to express my sincere gratitude to Prof ***Alain Boudou***, President of the University of Bordeaux 1, for his helps and encouragements through my tenure in France. His wide knowledge and his logical way of thinking have been of great value for me. I also wish to thank him for providing me the opportunity to become a research fellow in the "*Géochimie et Ecotoxicologie des Métaux dans les systèmes Aquatiques* (GEMA)". It was my pleasure to study in his laboratory that has been recently taken over by Dr ***Jean-Charles Massabuau***, another great expert in the field of Physiological Ecology and Aquatic systems, that I would like thank for his support.

With a deep sense of gratitude, I wish to express my sincere thanks to my advisor Dr ***Agnès Feurtet-Mazel***, Associate Professor in the "*Géochimie et Ecotoxicologie des Métaux dans les systèmes Aquatiques* (GEMA)" of the University of Bordeaux 1, who gave me the opportunity to work with her and untiring help during my difficult moments. Her kind support, patience, valuable advices and guidance have been of great value for my thesis and for myself.

I extend my special thanks to my advisor Dr ***Michel Coste***, Director of Research in Cemagref (*Unité de recherche Réseaux Epuration et Qualité des Eaux*, REQE), for his enormous assistance, encouragement, and especially for sharing his huge knowledge in taxonomy and ecology of diatoms. His understanding, comments and personal guidance have provided a good basis throughout this work and were a great force for me to continue with my research.

I would like to gratefully acknowledge to my Vietnamese co-advisor Prof ***Dang Dinh Kim***, Deputy Director of the Institute of Environmental Technology (IET), who gave me stimulating suggestions and encouragements and always helped me in throughout this work of research. With his help, I was always able to over come some of the most difficult issues.

I would express my particular thanks to all my three advisors, they could not even realize how much I have learnt from them.

The research work within this thesis was conducted in Vietnam and in France in the framework of the two projects. I would like to thank Dr. ***Georges Vachaud***, Dr ***Nguyen The Dong*** and Prof. ***Chau Van Minh***, who gave me a chance to work in the framework of ESPOIR project, a French-Vietnamese program for water quality and water treatment in the period from 2002 to 2004. I would like to thank all Vietnamese and French members of the ESPOIR project and all French members of the ECODYN project for my participation. And, of course, I am grateful for the financial support provided by the French Embassy and the National Centre of Scientific Research (CNRS) (BDI fellowship) which allowed me to undertake this research.

Special thanks to my colleagues in GEMA and Cemagref in France from who taught me a lot during my thesis. In GEMA, I thank ***Régine Maury-Brachet*** for sharing with me her extremely valuable technical knowledge in the sampling and analysis of metals. I am deeply thankful to ***Magalie Baudrimont*** for her precious advices as well as to ***Gilles Durrieu*** for his collaboration on the data treatment. I truly thank ***Caroline Gold*** and ***Olivier Herlory*** for their kind guidance and advices. My warm thanks to ***Henry Bouillard***, ***Véronique Duflo*** and ***Bruno Etcheverria*** for their help during the sampling campaign and laboratory techniques. I express my gratitude to numerous following colleagues: ***Alexia Legeay***, ***Patrice Gonzalez, Jean-Paul Bourdineaud, Jean-Pierre Brindamour, Pierre Ciret, Catherine Charlot, Florence Daniel, Nathalie Mesmer-Dudons, Yannick Dominique, Fabien Pierron, Sébastien Cambier, Angélique Bossy, Ika Paul-Pont, Mohamedou Sow…*** for their help and encouragements in various ways during my thesis and my stays in France. I would like to say “grand merci” to them.

I am grateful to the colleagues in Cemagref for their warm welcoming to me. Special thank to ***Soizic Morin*** for her collaboration during all these years. I will never forget the time we spent together in the field. I would like acknowledge to ***Juliette Tison***, ***François Delmas***, ***Young Seuk Park***, ***Catherine Férrière***, ***Marie Helène Montel, Maryse Boudigues, Muriel Bonnet, Mathieu Torre, Henri Beuffe, Timothé Debenest***…for their generous assistance.

I express my warmest thanks to numerous colleagues in the Department of Environmental Biohydrobiology in Vietnam for their support and help, especially ***Ho Tu Cuong*** who accompanied and shared with me the field works in Vietnam. I thank him for his permanent enthusiasm during sampling periods even in the rain and hard condition weather. I extend my thanks to ***Le Thu Thuy***, ***Nguyen Duc Tho*** and ***Dang Thanh Xuyen*** for their help in the sample treatment. Away from

home, this endless list of people in the Department of Environmental Biohydrobiology who provided me the advices and essential moral support: ***Tran Van Tua***, ***Dang Hoang Phuoc Hien***, ***Nguyen Tien Cu***, ***Hoang Thi Bao***, ***Nguyen Sy Nguyen***, ***Bui Thi Kim Anh***… I am deeply grateful to them for all they did for me. And of course, without the assistance from the Department of Environmental Biohydrobiology, this work would not have been completed.

I would like to thank all my friends ***Le Thi Phuong Quynh***, ***Nguyen Thi Thu Hang***...for sharing and cheering me during my stays in France.

I owe my loving thanks to my husband ***Pham Doan Lan*** for all his endless love, support, patience and motivation during my research abroad. Without his encouragements and understanding it would have been impossible for me to realize this work. My special gratitude is due to my parents, my brother, my sister, my relatives and my family in law for their loving support.

Arcachon 30 October 2006

TABLES OF CONTENTS

Résumé

Les diatomées constituent la majeure partie du biofilm qui couvre la surface des substrats de toute nature dans les zones humides et les rivières. De par leur large distribution, leur diversité taxonomique et leur sensibilité aux changements environnementaux, les communautés de diatomées périphytiques sont classiquement utilisées pour la surveillance des conditions de milieu. Dans la présente étude, des biofilms naturels développés sur des substrats artificiels en verre ont été utilisés comme modèle biologique pour apprécier les effets de deux types de pollution (organique et métallique) sur la structure des communautés de diatomées périphytiques. Les caractéristiques de ces communautés au sein du biofilm ont été analysées par des critères généraux (densité totale des diatomées, richesse spécifique et indice de diversité) et par des critères spécifiques (abondance relative et indices diatomiques) en fonction de la nature et du niveau de pollution. Elles ont été complétées par des critères plus globaux intéressant la totalité du biofilm (biomasse de poids sec, poids sec sans cendre et accumulation en métal).

L'hydrosystème Nhue et Tolich, qui reçoit directement les rejets urbains (rejets domestiques et industriels) de l'agglomération de Hanoï (Vietnam), est tout à fait représentatif des rivières fortement anthropisées du delta du Fleuve Rouge. Les effets de la pollution sur le développement du biofilm et sur la structure des communautés de diatomées périphytiques ont été suivis *in situ* le long d'un gradient de pollution organique dans la zone de Hanoï. Les résultats ont montré une inhibition du développement des communautés de diatomées et du biofilm exposés à la pollution. Dès la deuxième semaine de colonisation, les différences entre sites ont été reflétées par une modification claire des communautés de diatomées périphytiques. Les assemblages diatomiques des sites fortement pollués sont apparus caractérisés par des espèces comme *Nitzschia umbonata*, *Nitzchia palea, Eolimna minima* qui sont qualifiées dans d'autres pays d'espèces saprophiles ou tolérantes à une pollution organique forte. Dans les sites modérément pollués, les communautés ont été caractérisées par la présence d'espèces résistantes à la pollution comme *Aulacoseira granulata*, *Sellaphora pupula, Gomphonema parvulum, Navicula venneta* alors que la majorité des espèces sensibles à la pollution organique et les taxa halophiles (*Achnanthidium minutissimum*, *Navicula recens and Bacillaria paxillifera)* ont été trouvés dans le site comparativement non pollué. Enfin, deux indices diatomiques (IPS et DAIPo) ont été appliqués au Vietnam avec succès pour la première fois pour estimer la qualité de l'eau de ces rivières. Leur sensibilité aux changements de qualité d'eau a été confirmée par les brusques changements de composition diatomique des

communautés pendant les expérimentations de transfert illustrant les rejets soudains d'effluents dans le milieu.

A la différence de l'hydrosystème Nhue et Tolich, l'hydrosystème Lot, auquel est associé le Riou Mort, est caractérisé par une pollution polymétallique (principalement cadmium et zinc). Dans les conditions expérimentales de terrain, les communautés de diatomées ont répondu au niveau métallique élevé par la présence en grandes proportions d'espèces adhérentes, de petite taille, et résistantes aux métaux. De fortes corrélations ont également été observées entre l'accumulation du métal dans le biofilm et la sélection préférentielle de certaines espèces de diatomées. Notons que 80-90% des espèces cosmopolites recensées dans les études menées au Vietnam ont aussi été retrouvées dans l'hydrosystème Lot Riou Mort. Des changements dans la composition des communautés de diatomées ont été relevés en fonction de la présence plus ou moins importante de métal dans l'eau. Des modifications saisonnières de la structure des communautés diatomiques sont apparues avec une forte représentation de *Eolimna minima*, *Nitzschia palea* en été et en automne, et de *Achnanthidium minutissimum, Suriella angusta* en hiver et au printemps. Par ailleurs, la fréquence élevée de formes anormales de diatomées collectées dans le site pollué a été associée à la forte concentration en métal dans l'eau et au sein du biofilm. Ces observations ont été confirmées en conditions expérimentales de laboratoire par l'accroissement significatif du développement de valves anormales dans les conditions d'exposition au cadmium, et par une décroissance marquée de la biomasse et de la densité des diatomées, tout en mettant en évidence une augmentation de la tolérance des communautés de diatomées au cadmium par le développement significatif d'espèces tolérantes comme *Nitzschia palea*.

A travers les études développées dans cette thèse, la capacité des communautés de diatomées périphytiques à révéler deux types principaux de pollution a été clairement mise en évidence. Les diatomées apparaissent comme un modèle fiable, qui montre, à travers la structuration de ses communautés, de nombreuses modifications témoignant de l'impact des polluants. Les mécanismes de réponses des diatomées à la contamination métallique évoquent la saturation des sites disponibles à la surface des diatomées, en plus des modifications de communautés. Pour les deux types de pollution, le rôle protecteur du biofilm, qui agit comme une barrière contre la toxicité des métaux, a été confirmé. A l'échelle individuelle, des études cellulaires devraient être désormais considérées pour mieux comprendre les mécanismes extra et intra cellulaires responsables de l'impact des toxiques sur les diatomées.

Abstract

Diatoms constitute a major part of the biofilm that covers surface of substrates of all nature in wetland and rivers. Diatom communities, by their wide distribution, taxonomic diversity and sensitivity to environmental changes are being commonly used for monitoring the environment conditions. In the present study, natural biofilms developed on artificial glass substrates were used as biological model to investigate the effects of two types of pollution (organic and metallic pollution) on structure of periphytic diatom communities. Characteristics of periphytic diatom communities within biofilm according to pollution status were analyzed through general criteria (total diatom density, species richness and diversity index) and specific criteria (relative abundance and diatom indices); and were completed by global criteria involving the whole biofilm matrix (dry weight biomass, ash free dry mass and metal accumulation).

The Nhue and Tolich hydrosystem (Vietnam), receiving directly all the urban wastewaters from Hanoi city (domestic and industrial wastewater), is typically representative of anthropogenic rivers in the Red River delta. Effects of pollution on development of biofilm and structure of periphytic diatom communities were indentified *in situ* along an organic pollution gradient in the area of Hanoi. Results displayed inhibition of the development of diatom communities and biofilm when exposed to pollution. As soon as second week of colonization, water differences were reflected by clear modification of periphytic diatom communities. Diatom assemblages of heavy polluted site were represented by species which have been qualified in other coutries of saprophilous or tolerant to heavy organic pollution such as *Nitzschia umbonata, Nitzchia palea, Eolimna minima*. At moderate polluted sites, communities were characterized by resistant taxa to pollution like *Aulacoseira granulata*, *Sellaphora pupula, Gomphonema parvulum, Navicula venneta*, whereas major diatom sensitive to organic pollution and halophylous taxa were found in comparatively unpolluted site such as *Achnanthidium minutissimum*, *Navicula recens and Bacillaria paxillifera*. Finally, two diatom indices (IPS and DAIPo) were successfully applied in Vietnam for the first time to assess water quality of these rivers. Their sensitivity to water changes were supported by rapid modification of diatom communities during transfer experiment illustrating sudden contaminant and/or organic dischages in the medium.

Different than Nhue and Tolich hydrosystem, the Lot hydrosystem with Riou-Mort River (France) is typical with its polymetallic pollution (mainly cadmium and zinc). In the fields

experiments conditions, diatom communities mainly responded to high level of metal by the presence of high proportions of small, adnate and resistant to metal taxa. High correlations between relative abundance of diatom species and metal accumulation in biofilm were observed. Cosmopolistan species collected in Vietnam and representing 80-90% of the communities were elective too of polluted and non-polluted site in the Riou-Mort River. Besides, modification of diatom communities according to metal presence, seasonal changes in diatoms structure were recorded with a high number of *Eolimna minima*, *Nitzschia palea* both in Summer and Autumn, and *Achnanthidium minutissimum, Suriella angusta* dominant in Winter and Spring. Furthermore, higher frequency of abnomal forms of diatom found at polluted site, was related to high level of metal concentration in water and accumulation in biofilm. It is confirmed in laboratory experimental conditions with a significant increase of abnomal valves in development under Cd exposure accompanied by a clear decrease of biomass and diatom density. However, diatom communities increased their tolerance to Cd by significant development of tolerant species like *Nitzschia palea*.

Studies developed in this thesis confronted capability of periphytic diatom communities to reveal pollution status of hydrosystems subjected two main types of pollutants. Diatoms appear as a reliable model which shows, through the structuration of its communities, numerous modifications signing pollutants impact. Mechanisms, involved in metal contamination as responses of diatoms to perturbations, evoke saturation of binding sites available on diatom surface level, besides communities modifications. For both pollution, the protective role of the biofilm matrix which acts as barrier against metal toxicity was confirmed. At a more individual scale, cellular level study should be now focused to better understand the extra/ intra cellular mechanisms responsible of the toxicants impact toward diatoms.

Introduction

A steadily increasing human population and the associated rise in industrial, agricultural and human activities have led to an increasing pressure on aquatic ecosystems. Anthropogenic activities have changed the global climate and habitats and have increased the input of nutrients and of a large number of chemicals in aquatic system. Increases in nutrients loading combined with human activities have resulted in eutrophication, acidification and metallic pollution in many lotic systems. Water pollution has greatly reduced environmental heterogeneity and subsequently diminished biodiversity by causing reductions in spatial-temporal variability (Wellnitz and Rader, 2004). For sustainable freshwater management, it is essential to know to what extent hydrosystem is affected by pollution.

Two main approaches can be used for monitoring water quality; direct physical and chemical analyses and biological assessments (Vis et al., 1998). The traditional approach to water quality management by using physical and chemical analyses reflects changes in water quality in a rapid and straightforward manner. These measurements provide a simple estimate of the present status, however, especially in streams and rivers, physical and chemical characteristics can be highly variable in time. They also do not display the ecological state of the system. Furthermore, chemical water monitoring is limited by extremely large number of substances which can occur in lotic environment and several of them can be only qualitatively analyzed. Biological assessment, on the other hand, provides better evaluation of environmental changes because biological communities integrate and reflect the environmental effects of physical and chemical that occur over extended periods of time (Stevenson and Pan, 1999). The basis of biological assessment recognises that different species have variable tolerances to environmental stressors. By the examination of the relative abundance or other attributes of communities, environmental conditions characteristics of aquatic systems can be specified. Biological quality can be assessed by different kind of organisms such as bacteria, protozoa, algae, macroinvertebrates, macrophytes and fish. Among them, benthic algae, especially diatoms are routinely used in biomonitoring of lakes (DeNicola et al., 2004), streams (Biggs, 1990), rivers (Eloranta and Soininen, 2002; Fore and Grafe, 2002; Gold et al., 2002; Kelly, 1998) and estuaries (Nayar et al., 2003). In Vietnam, studies on ecological quality of rivers using biological tools are limited and water quality of rivers has been mainly based on the *E. coli* saprobity, phyto - zooplankton and invertebrate communities (Nguyen et al., 2000; Hoang et al., 2001). These studies have provided basic information on regional

taxonomy and their distributions along the river courses (Nguyen et al., 2000). Furthermore, intensive floristic or taxonomic studies on freshwater diatoms in Vietnam are yet incipient.

Elsewhere, diatoms have been used for a long time as a tool to assess variable environmental conditions due to their large number of species, their fundamental role in the food web and their sensitivity to environmental changes. Monitoring environmental conditions in rivers and streams by using diatoms has resulted in the development of several methods used today such as biotic indices (Medley and Clements, 1998; Descy and Coste, 1991; Kelly, 1998). Based on species composition of diatom assemblages, ecological preference tolerances of taxa and indices have been developed to indicate levels of pollution (Lange-Bertalot, 1979). Diatom total density has been suggested as a general indicator of river "health" (Stevenson and Pan, 1999). However, diatom characteristics which respond to water quality changes may vary. For example, certain studies report correlations between diversity index or species autoecology and water quality, whereas others document stronger responses of biomass (Vis et al., 1998). Such differences can be attributed to high natural variability associated with diatom communities or differences between aquatic biotopes in relation with their watershed (rivers, streams, regions, geography). Therefore, knowledge of the ecological status of diatom communities in aquatic system is essential before applying or adapting such biotic indices.

Objective of the thesis

In order to better understand the potential of freshwater periphytic diatoms as indicator of pollution, this study is focused on (i) the effects of pollution (urban and metal) on the structure of periphytic diatom communities and their distribution; (ii) the investigations of the relationships between diatom composition and environmental variables; (iii) furthermore, this study explores the dynamic of periphytic diatom communities related to metal exposure as well as the ability of metal accumulation by the biofilms.

Outline of the thesis

This thesis shows the results of different studies conducted on periphytic diatom communities developed in two different hydrosystems. In the Nhue and Tolich hydrosystems (Vietnam), *in situ* studies have concerned multisources of urban pollution and brought the first data ever presented about taxonomic composition of periphytic diatom flora (Chapter 3, 4 and 5). Investigation conducted *in situ* in the Lot-Riou Mort (France) have been focused on a more

specifically metallic pollution affecting periphytic diatom communities (chapter 6 and 7), whose responses to metal impact have been precised through experiment conducted under laboratory condition (Chaper 8). The eight chapters of the manuscript are described below and are presented as a compilation of paper already published, in press or in preparation.

Chapter 1 is devoted to a general presentation of diatoms and role of environmental factors influencing the distribution and abundances of periphytic algae in rivers and streams. It also gives an overview of the use of diatoms as a tool for water quality assessment.

Chapter 2 presents general information appropriate methodologies on developed in investigated sampling stations. Characteristics of geography, pollution sources of each sampling station are described in this chapter. Methods used to collect and qualitatively and quantitatively analyses periphytic diatom communities are presented as well as physical and chemical analyses of the studied rivers. Data treatments used in this study are also detailed in this section.

Chapter 3 presents the dynamic of the colonization process of diatom communities from its initial stage in three different sites of the Red - Nhue - Tolich hydrosystem submitted to an urban pollution from Hanoi area (Vietnam). This study focuses on the optimal exposure duration of artificial substrates in natural hydrosystems according to the level of pollution and through various general (total diatom density, biofilm dry weight) and specific criteria (relative diatom abundances, indices). Perturbations occurring in the colonization process caused by pollution are disscused and this chapter is accepted as article in the Journal of Ecological Indicators.

Chapter 4 examines the impacts of wastewater (urban pollution) from Red-Nhue-Tolich hydrosystem on periphytic diatom communities and their distribution along the pollution gradient. This study also applies the diatom indices which have being widely used in other countries to assess water quality of these rivers, and the relationships between physico-chemical water quality and diatom indices are described. This chapter is published as article in Hydrobiologia journal, 563: 201-216.

In Chapter 5, the responses of periphytic diatom communities to water quality changes were analyzed by transferring early diatom communities colonized on glass substrates from a non-polluted river to less polluted or polluted rivers and conversely. This study reports the time needed for diatom communities to integrate a change of environmental conditions in their structures and how the periphytic diatom communities can recover their structure from pollution stress. (Publication is in preparation)

Chapter 6 focuses on investigations of kinetics of cadmium accumulation and dynamics of periphytic diatom communities in natural biofilm along pollution gradient in different seasons in the Riou-Mort River (SW France). The relationships between periphytic diatom communities and metal accumulation in biofilm are discussed. (Publication is in preparation)

In Chapter 7 a long term survey of geochemical and diatom survey at a highly metal-polluted site in the Riou-Mort River (SW France) is presented. The distribution patterns of periphytic diatoms under high level of metals are observed and tolerances of diatom species to the heavy metals are confirmed. (Publication is submitted to Enviromental Pollution)

Chapter 8 explores kinetics of cadmium accumulation in freshwater biofilm and effects of cadmium exposure on biofilms as well as on structure of diatom communities under laboratory conditions. The role of a protective layer against cadmium and metal accumulation by diatom communities is discussed. (Publication is in preparation).

Lastly, the general conclusion underlines the main results obtained throughout this thesis and some perspectives are proposed.

CHAPTER 1

LITERATURE OVERVIEW

1.1 General presentation of the diatoms

1.1.1. What are diatoms?

Diatoms are microscopic siliceous unicellular algae which are found in almost all aquatic and semi-aquatic environments. They belong to the algae class Bacillariophyceae of the division or phylum Bacillariophyta. They are photosynthetic micro-organisms and their size ranges from 10 µm to 500 µm. The earlier fossil diatom dates from the early Cretaceous (120 million years ago) and was a marine species. The first known freshwater diatom has been found in the early part of the Tertiary (60 million years ago). Diatoms are usually estimated to contribute about 25-35% of the world net productivity. They are the richnest species group, and it currently includes over 450 genera of living diatoms with over 100,000 species (Werner, 1977; Round et al., 1990; Fourtanieur et al., 1999).

1.1.2 Structure and morphological characteristics of diatom cell

An outstanding fact about diatom cells is their walls mostly consisting of silica in the form of $SiO_2.H_2O$ and organic material (Round et al., 1990). Shaped in two distinctive and interconnected valves called frustule. The ventral valve (hypovalve) and dorsal valve (epivalve) are joined together by a girdle, which is composed of a series of silica bands (copulae) linked together along their margins (Figure 1.1). Each valve with its serie of girdle bands is named hypotheca and epitheca (Round et al., 1990; van den Hoek et al., 1995). The valves exhibit a system of silica ribs which grow out in a circular or linear primary pattern during formation (De Stefano, M and De Stefano, 2005). The shape, morphology and ornamentation of the silica walls are extremely important for exact diatom identification (Dixit et al., 1992). The wall is thinner in planktonic species than and in benthic species (Sládeček, 1986). There are few chloroplasts in a cell; they are typical of heterokonts and contain pigments such as chlorophyll a, c, beta-carotene, fucoxanthin and other pigments giving diatom its yellow, green or brown color. Diatom cells also contain all chemical vegetal cellular structure: a nucleus, mitochondria, golgi apparatus, ribosomes, vacuoles etc. (van den Hoek et al., 1995). Mucilage or Extracellular Polymeric Substance (EPS) is secreted by most diatoms and it covers the exterior of the frustules giving diatoms capacility to adhere to substrate or to form filaments, colonies or gelatinous stalks or pads (Sládeček, 1986; Round 1990; Hoagland et al., 1993).

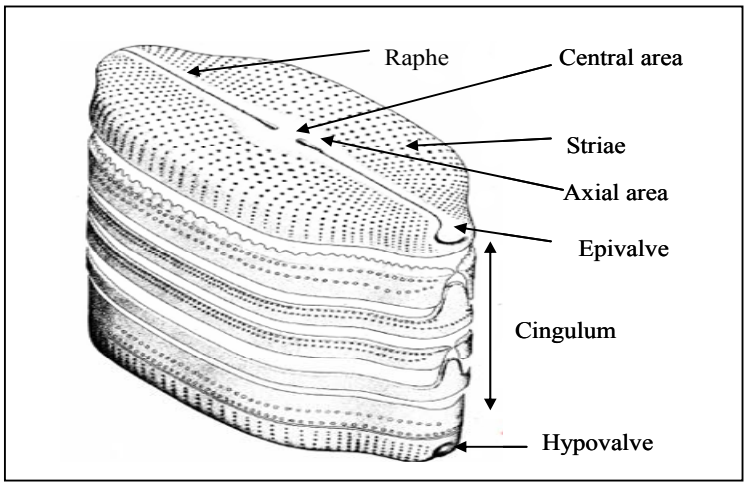

Figure 1.1: Structure of a diatom cell (*Navicula sp*) (modified after Round et al., 1990)

Figure 1.2: Different forms of diatom extracellular polymeric substances. a): stalk of *Cymbella*. b): tube of *Encyonema*. c): Colony of *Melosira varians* and d): Stellate colony *Asterionella formosa*. (Source from http://www.keweenawalgae.mtu.edu)

Thus, diatoms may grow at the end of mucilaginous stalks (Figure 1.2) (e.g. *Achnanthes*, *Gomphonema,* and *Cymbella*) or within mucilaginous tubes (e.g. *Encyonema*). EPS is also employed to hold parts of different cells together in chains (e.g. *Tabellaria*, *Diatoma*) or in stellate colonies (*Asterionella*). Colony formation is also accomplished in diatoms by interdigitation of siliceous spines on the valve margins of adjoining cells (e.g. *Fragilaria*, *Aulacoseira*).

1.1.3 Development cycle of diatoms

Diatoms have short development cycles (a few hours to several days), depending on species and environmental conditions. Their development is largely manifested by cells division (Patrick and Reimer, 1966; John et al., 2000a). Two different modes in diatom reproduction are known: vegetative multiplication and sexual reproduction. The most common type of reproduction of diatoms is accomplished by vegetative reproduction. Vegetative multiplication is acquired by bipartition after an ordinary mitosis. Each daughter cell keeps a valve of the initial cell and produces an hypovalve, smaller than the initial one (Figure 1.3). The average diatom size gets progressively smaller with each round of replication.

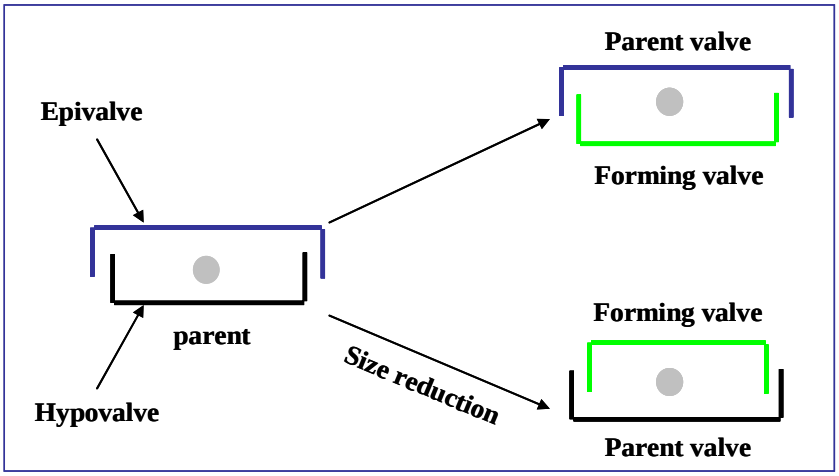

Figure 1.3: A diatom cell division (Modified after John 2000)

Due to the rigidity of cell wall and in order to limit size diminution, the sexual reproduction occurs when the minimal size of valves is reached (about 40% of its maximum size) (John, 2000a). Sexual reproduction can occur in the form of either oogamy (usually found in centric diatoms) or isogamy (usually seen in pennate diatoms). The auxospore is a cell that develops and grows,

eventually proceeding to produce a new frustule. Auxospore formation allows the diatom to restore its size, and vegetative growth can continue once again.

1.1.4 Taxonomy

Identification and taxonomic classification of diatoms are based on the unique, ornamented structure of the valves (Dixit et al., 1992). Different frustules have different features like shape, size, symmetry, structure and density of striae, nature of raphe and processes on the valves (John, 2000a). The determination of morphological features, the presence of special pores and pore structures can be performed using conventional light microscopes techniques using high resolution oil immersion objectives, phase contrast, differential phase contrast, and dark field illumination. Since the invention of the Scanning Electron Microscope (SEM), understandings of the three dimensionality of the diatom frustule and of the shape of processes have been approached in depth.

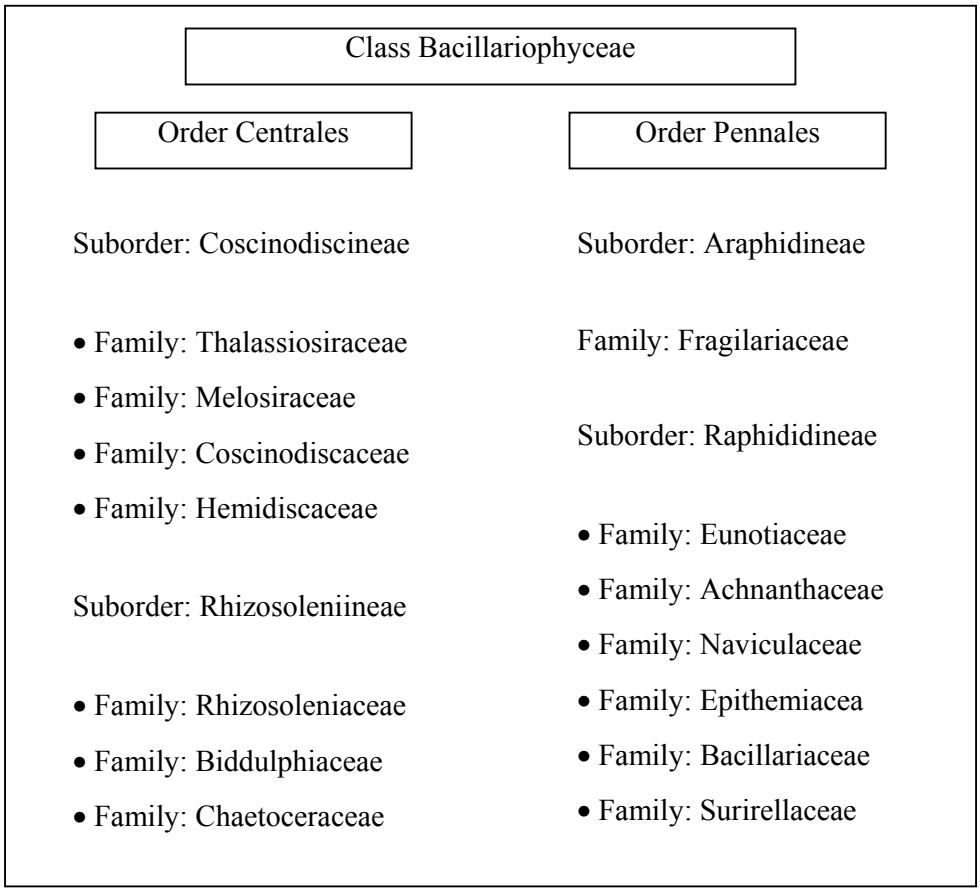

Figure 1.4: Diatom classification system by Kammer and Lange-Bertalot (1986-1991).

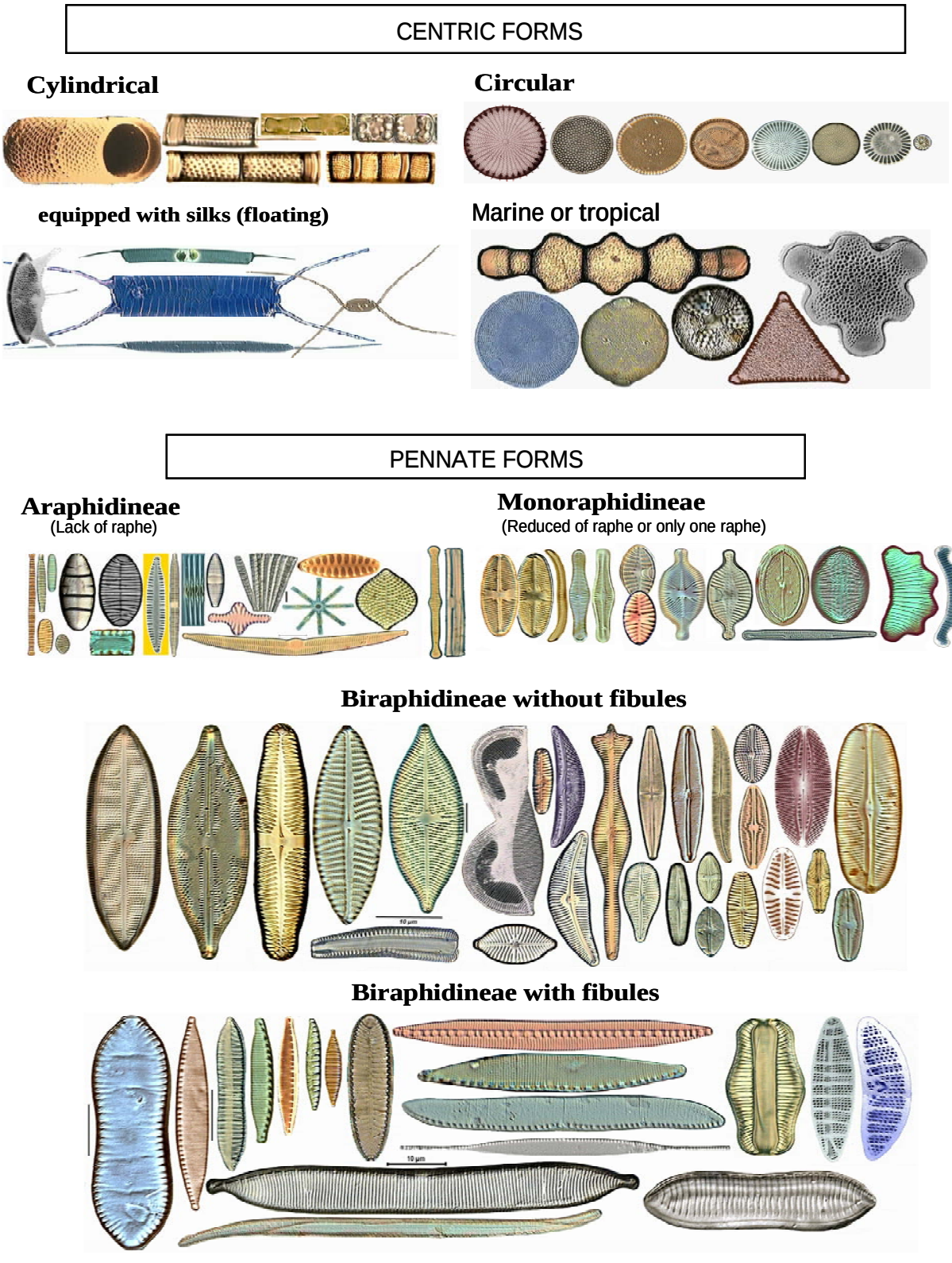

Figure 1.5: Identification key of diatoms (Source: M. Coste, 1999)

Up to now, literature concerning diatom classification is abundant (Hustedt, 1930; Round et al., 1990; Kammer and Lange-Bertalot 1986-1991). In this thesis, we used the taxonomic system proposed by Kammer and Lange-Bertalot (Figure 1.4) with the class Bacillariophyceae subdivided into two groups: centric diatoms which have valve striae arranged basically in a radial symmetry with an annulus or a central areola, and pennate diatoms which have valve striae arranged with bilateral symmetry (Figure 1.5).

1.1.5. Ecology

Diatoms live in different conditions from marine to freshwater environments (they are even found in moist soil, aerial and mosses) and from a wide range of environmental conditions from pristine waters to extreme environments (Elster et al., 2001). The communities which live in water under natural conditions generally consist of a lot more species than those found in aerial habitats or in the soil (Patrick, 1977; van den Hoek et al., 1995). They have several ways of life in aquatic environment. Planktonic diatoms, which live free in the water column are mostly represented by isolated centrics (*Cyclotella, Stephanodiscus*) or associated chains (*Melosira, Aulacoseira*). Besides centrics species, some pennate species exits in phytoplankton such as strip-shape colonies (*Fragilaria*) or the star-shape (*Asterionella, Nitzschia*) and are well represented in aquatic environment. Periphytic diatoms usually are those attached to hard substrates or laid on fine sediments (slit and fine sands). Based on the natural substratum types which periphytic diatom colonize, they can be classified in: epilithon (attached to rock, gravels, and cobbles); epiphyton (living on surface of aquatic plants); epipsammon (developed on the surface of sand); epipelon (living on mud and silt grains); metaphyton (loosely attached to macrophytes and other submerged substrates) (Biggs, 2000). In this study, the term periphyton has been used equally to evoke the different kinds of diatom living communities listed above, which corresponds to the german definition of “aufwuchs”.

1.2 Periphytic diatoms in aquatic systems and factors influencing their development within biofilm

1.2.1 Structure of diatoms communities

In aquatic systems, surface of substratum is rapidly covered by a multilayer structure namely biofilm (Lowe and Pan, 1996; Ledger and Hildrew, 1998; Barranguet et al., 2000; Patil and Anil,

2005). Biofilm is a complex matrix of benthic algae, bacteria, fungi, protozoa, and their secretory products are called extracellular polymeric substances (Sekar et al., 2002; Carr et al., 2005) (Figure 1.6). When light prevails, biofilm is dominanted by photosynthetic organisms (autotrophs) such as algae. Othersewise in low light conditions, it is dominated by bacteria (Burn and Ryder, 2001). Periphytic diatoms are an abundant component of biofilm in streams and rivers, therefore they are an important primary source of energy for higher trophic levels (Biggs, 1996, Stevenson et al., 1996).

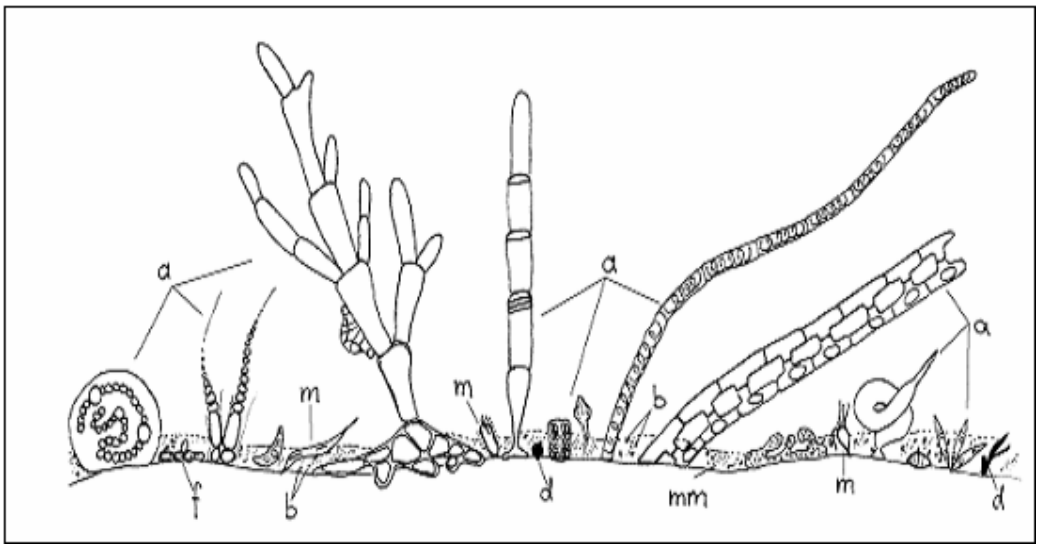

Figure 1.6: The periphytic algal community, an assemblage dominated by benthic algae (a) associated with meiofauna (m), fungi (f), bacteria (b), and organic and inorganic non-living material (detritus) (d) embedded in a mucopolysaccharide matrix (mm). (After Kahlert, 2001)

The structure of a biofilm is characteristic and depends on the development level of the community, hydrological conditions and substrates. Biofilm can have very simple single cell layer or stalked and filamentous species that grow upright, with attached epiphytes (Biggs et al., 1998) or also motile species moving through the matrix (van de Grinten, 2004). The development of a biofilm on substrates starts with the adhesion of an organic matrix and bacteria. The first algae which appear are frequently small pennate diatoms, and then they are followed by others, sometimes planktonic species or filamentous green algae (Stevenson et al., 1996; Ivorra, 2000). Developments of attached diatoms on submerged substrates are supported to follow three phases: phase 1 is characterized by rosette-type species and motile-type species; phase 2 is typical with the stalk producing species and phase 3 is characterized by growth of loosely attached species (Tuji,

2000). After settlement on substrate, the colonizing cells and their activities undergo exponential growth and are regulated by the availability of resources (e.g. light, nutrients) as well as other environmental factors such as substrates nature, disturbances, grazing etc (Stevenson et al., 1996). But, interactions between algae are also important within biofilm and determine species composition of periphyton own to interference competition in relation with limited space available for attachment on substratum.

1.2.2 Factors directly influencing diatoms development within biofilm

Periphytic algae communities are important structural and productive components of freshwater ecosystems. The growth and prospecting of these organisms in streams and rivers is the outcome of complex interactions between hydrological conditions, water quality and biotic factors (Figure 1.7) (Biggs, 1996).

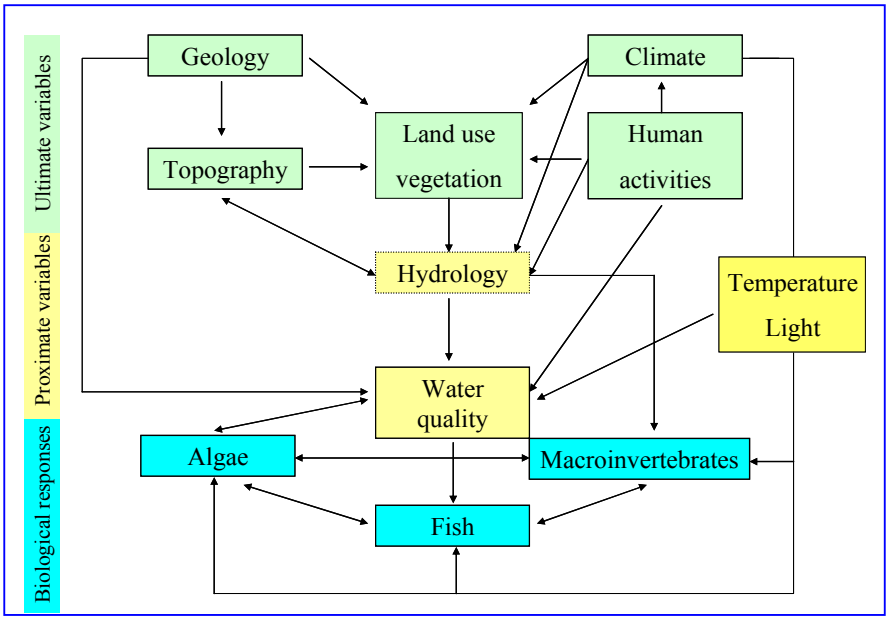

Figure 1.7: Diagram showing how ultimate variable control the proximate variable in streams or rivers, which in turn control biological responses and interactions important to benthic algae (Modified from Biggs 1996).

And it is absolutely nessecery nowadays to take into account environment condition which can be used to establish reference condition and ecological status (EUWFD, 2000). Local "proximate" variables, like discharge regime, are controlled by regional "ultimate" factors like geology, topography or climate operating at spatial scales of catchments or even ecoregions. In addition,

human activities act to change both proximate and ultimate variables in an increasing rate, leading towards variously impacted biological communities, e.g. algal communities with increased amount and biomass of nuisance species, or in general, impoverished biological communities (Biggs, 1996; Soininen, 2004). The main factor that leads to biomass accrual of periphytic algae is the level of resources, particularly nutrients and light, and moderately by other factors such as temperature that influences the rate of metabolism. The main factor which moderates biomass loss is disturbance, often through sloughing or grazing. Factors that potentially influence growth, community structure and distribution of periphytic algae include: temperature (DeNicola, 1996), availability of resources (light, nutrients), current velocity, alcalinity (Stevenson, 1983; Peterson and Stevenson, 1989; Biggs et al., 1998b; Tison et al., 2005), nature of substrate where they develop (Burkholder, 1996; Potapova and Charles, 2005) and grazing (Steinman, 1996).

1.2.2.1 Temperature

Temperature is the ecological parameter which affects all aquatic life. The effects of temperature on periphytic algae may vary from an increase in the metabolic rate of algae to a displacement or even a mortality of sensitive organisms, depending on the temperature in water and the duration the organisms are exposed to (Rajaduraia et al., 2005). Temperature affects algal photosynthesis metabolism through its control of enzyme reaction rate. Moreover, optimum temperature for photosynthesis also varies among algal species, which suggests that taxonomic shifts induced by increased temperatures could lead to increased photosynthetic rates (DeNicola, 1996). Temperature has been also suggested to cause alterations in periphytic algae composition. In general, temperatures below 20^0 C are expected to favor diatom. In addition, temperature has been found to influence sexuality and auxospore formation of sexual reproduction in many algal species and especially in diatoms. Based on preferred temperature of diatom species and the width of their tolerance temperature range, Hustedt (1927-1959) constructed thermal tolerance classification of diatoms which can be applied for to periphyton species.

The seasonal succession of algae communities in temperate zones have been showed by several studies in which distinct patterns of species abundances and taxonomic groups like diatoms, green algae, and cyanobacteria can be described (Mc Cormick and Stevenson, 1991; Sekar, et al., 2002). The effects of temperature on structure of periphytic algae has been found by changes of species richness and diversity with diversity index increase for temperature raising from 25 to 30 ^{0}C, and decrease for higher temperature (>30^0C). Warmer waters (within optimum ranges) are expected too lead to increase algae growth rate and high algae biomass (Finlay et al., 2001). However, interactions of temperature with other factors are complex, making it difficult to separate

specific effects to periphytic algae. We can mention that green house effects can be assessed by mapping the progreesion up north of tropical taxa in temperate European countries (Coste and Ector, 2000).

1.2.2.2 Light

Light is the energy source for all periphytic algae, allowing organisms to transform inorganic compounds into living biomass. Microbenthic algae are exposed in aquatic environments to a light range from <10 µmol photon.m^{-2}.s^{-1} in shaded areas to > 3000 µmol photon.m^{-2}.s^{-1} high sunlight in the subtropical zones (Guasch and Sabater, 1998). Light plays a crucial role in photosynthesis and in the growth of periphytic algae, biomass too and its availability influences a lot primary production in lotic systems as far as nutrients limitation (Hill et al., 1995; Hill, 1996). Thus, as low nutrient concentrations can limit primary production in unshaded streams, primary production in the many streams which are bordered by well-developed terrestrial plants communities appears to be strongly affected by low light levels (Hill et al., 1995). In heavy shaded streams, light availability can be the overriding factor controlling the accrual of periphyton biomass, primary production and composition. At a cellular level, periphytic algae in shaded streams have lower chlorophyll than algal assemblages in unshaded areas (Lowe et al., 1986; Hill and Knight, 1988) and the biovolume may be affected too (Pillsbury and Lowe, 1999). Light conditions have not only an impact on physiological responses of algae (photosynthesis) but also on taxonomic composition and structure of algal communities (Guasch and Sabater, 1998). The effects of light on species composition are often related to different growth forms (Steinman et al., 1992), like an increase of the biomass of small and prostrate alga species in heavily shaded, whereas, vertical growth forms exercise a superior competition for light. In this luminous area, the filamentous and chain-forming algal species therefore tend to dominate (Hillebrand, 2005). In streams, taxanomic diversity and biomass decrease with depth and turbidity (Stevenson et al., 1985). Moreover, sensitivity of algal community to pollutant such as atrazine has been governed by light conditions (Guasch and Sabater, 1998).

1.2.2.3 Nutrients

Nutrients are major factors influencing periphytic assemblages in lotic systems. Nutrient sources for periphytic algal communities include the overlying water, nutrients release by the substrate and internal nutrient recycling (Mulholland, 1996). Nutrients play significant role in controlling primary production in streams where light is not limiting (Hill et al., 1995; Borchardt,

1996). Phosphates, nitrates and silica are generally considered the most important nutrients for autotrophic production, although other chemical constituents also can limit growth under some circumstances. In low nutrients freshwater, inorganic phosphate is often the major factor limiting the growth of algae and other primary producers. Nitrate-nitrogen tends to become limiting when phosphate is plentiful (Allan, 1995). Periphytic algae species have wide-range nutrient requirements for maximum growth rate from 0.5 $\mu g.L^{-1}$ PO_4-P for diatoms and 25–40 $\mu g.L^{-1}$ PO_4-P for filamentous green algae species (Chételat et al., 1999). Nitrogen limitation of benthic algae has been reported when ambient concentration ranged from 55 to 100 $\mu g.\ L^{-1}$ NO_3-N (Borchardt, 1996). Silica concentration has been shown to affect dynamic of diatoms successions in river and streams (Patrick, 1977; Allan, 1995). However, in running waters, silica is rarely in short supply and therefore it may seldom limit diatom growth (Allan, 1995).

Waste discharges with high concentrations of phosphorus and nitrogen commonly increase the occurrence of algal proliferation in rivers and streams (Biggs, 1990b). Enhanced biomass of periphytic algae through increased anthropogenic inputs of N or P has been reported in several studies (Chételat et al., 1999; Greenwood and Rosemond, 2005). Nutrients input may also cause a shift in community structure (change in dominant taxa, diversity and species richness) and function (productivity) of periphytic algae (Havens et al., 1999; Pan et al., 2000). Nutrients addition has been shown to influence algal colonization (Tank and Dodds, 2003). However, effects of nutrients on biomass, growth rate, distribution and structural community of periphytic algae are always related to other factors in lotic system such as light, temperature, disturbance and grazing. Combination of high irradiance and high concentration of nitrogen and phosphorus lead to a significant increase in biovolume of several filamentous species (Rosemond and Brawley, 1996). Nutrients can even play a more important role in streams where the light has been reduced or removed (Mosisch et al., 2001). Nutrients, especially organic enrichments, can affects all forms of periphytic assemblages by favouring filamentous algal species and decreasing the richness of algal taxa when there is sufficient light (Rosemond, 1993). Temperature interacts too with nutrients limitation in a complex manner to influence growth of algae (Borchardt, 1996). Besides, nutrients, light and grazer presence simultaneously limit algal biomass and productivity of stream algal communities in high temperature period (Hill et al., 1992 and 1995; Rosemond, 1993), and higher periphyton biomass and productivity are found when grazers (mainly snails) are removed and nutrients and light are elevated (Rosemond et al., 2000).

1.2.2.4 Current

Current velocity is supposed to controll the distribution and structure of periphytic algae communities in lotic system (Ghosh and Gaur, 1998; Biggs and Close, 1989; Sabater et al., 2002 b; Abe et al., 2000) through antagonistic influences on periphytic accrual; positively, via nutrients uptake, and negatively, via shear stress. Current also impacts algal colonization rate and growth form of periphyton in streams and rivers (Biggs et al., 1998b). Benthic algal immigration rate related to current velocity and higher immigration rates in slow currents (Peterson and Stevenson, 1989). The growth form of periphyton in fast current is often presented by small and adnate diatoms that are tightly attached to the substrata whereas large portion of big forms and stalked cells loosely attached to the substrate may be found in slower currents (Stevenson, 1996b; Abe et al., 2000). Morphology of periphytic algae is also affected by current, for example *Cymbella* and *Gomphoneis* stalks increase in length with increased current velocity (Biggs and Hickey, 1994). In addition, increasing current velocity does stimulate metabolism processes such as nutrients uptake, photosynthesis and respiration of periphyton in streams (Ghosh and Gaur, 1998). Current effects on phosphates uptake by diatom species were examined by Schumacher and Whitford (1965). They indicated that phosphate uptake was 7 times higher in strong current condition.

The degree to which biofilm will respond to current velocity depend on its architecture (Peterson, 1996). Adherent benthic assemblages, for example, were presented above as being resistant to sloughing and benefiting from increased mass transfer with increasing current velocity. The optimum current velocity for periphytic algal assemblages accrual on substrate varies during their development and depends on the dominant species, periphytic diatom assemblages prefer lower current velocity (Stevenson, 1996).

1.2.2.5 Substrate

Periphytic diatoms are present on all kinds of substrates in aquatic systems (Lowe and Laliberte, 1996; Biggs, 2000) as rocks, sands, woody debris, sediments, and aquatic vegetation. Artificial substrates have been successfully used for many types of periphytic investigations such as rate of colonization (Peterson and Stevenson, 1989), community interactions (DeNicola et al., 1990), impact and comparisons of environmental variability (Prygiel et al., 1999a; Whitton and Rott, 1996). Glass and a variety of plastic materials have been used extensively to provide a standard substratum which can be sampled for quantitative measurements of periphytic algae standing crops.

It is evident to suggest that size, surface and type of substrata may influence the distribution and structural of biofilm in streams or rivers (Jüttner et al., 1996; Barbiero, 2000; Potapova and Charles, 2005; Townsend and Gell, 2005). For examples, biomass, diversity and composition

significantly differed between biofilm sampled from dissimilar types of substrates at the same time (Potapova and Charles, 2005). Moreover, larger surface area of substratum shows greater taxa richness than small one (Patrick, 1976). Physical aspects of substrate surface might also affect its suitabiling for biofilm colonization. In general, it would appear that a substrate with coarse surface is favorable for colonization whereas smooth surface is colonized rather more slowly (Whitton, 1975). Additionally, position and slide orientation of substrata in rivers play important factors in determining structure of periphytic communities like, differences in abundance, richness and composition of algae (Kralj et al., 2006).

1.2.2.6 Grazing

Grazing as a biotic factor play an important role in shaping the biofilm in lotic systems. Allan (1995) and Steinman (1996) noted that snails, insects and insect larvae are the most important biofilm consumers and they do not only substantially affect biofilm standing crop, but the species composition as well. This was proved by grazing studies in laboratory streams in which grazing by invertebrate can be the most important factor affecting communities' structure species composition, density and primary production (McCormick et al., 1994 b). Several studies have shown that algae are widely consumed by invertebrate grazers (Chessman, 1986; Rosemond, 1993) and among them diatoms are the most frequently grazed as observed by Rosemond (1993) who highlighted the selection grazing of diatom by snails.

1.3 Use of diatoms as indicator for water quality assessment

The use of biological communities as indicator of water quality is evolving as our understanding of the interactions between water quality and the integrity of biological communities improves (Hill et al., 2000a). A biological indicator is a species or groups of species whose function, populations or status can be used to determine ecosystem level or environmental integrity. Biological indicator can describe water quality and its change over a long time scale and it is considered to have advantages over intermittent physico-chemical analyses (Vis et al., 1998; Soininen, 2004; Salomoni et al., 2006). Biological assemblages provide an integrated response to exposure to the full range of environmental variables occurring at a site. Overall routine monitoring of biological communities is reliable and relatively inexpensive compared to the cost of assessing toxicant pollutants. Biological quality can be assessed by different kinds of organisms such as bacteria, protozoa, algae, macro-invertebrates, macrophytes, molluscs, and fishes. Among these, diatoms have been found to be feasible for water assessment purpose in many studies (McCormick

and Cairns, 1994a; Whitton and Rott, 1996; Prygiel et al., 1999). Many advances in using diatoms for monitoring stream and river quality have been reviewed by Round (1991a), Descy and Coste (1991), Dixit et al (1992), Whitton and Kelly (1995), Stevenson and Pan (1999) who recognise diatoms to have ubiquitous distribution, a well known taxonomy and a high representativeness in periphytic biofilm assemblages. They play an important role in aquatic food web (Lamberti, 1996), geo-chemical cycles of silica and carbon (Patrick, 1977; Stevenson and Pan, 1999). They have the shortest cycle development among bioindicators of rivers water quality (Stevenson and Pan, 1999; Rimet et al., 2005) and they can reach a high rate of diversity in the environment (Prygiel et al., 1999; Poulíčková et al., 2004). They are sensitive and react rapidly to environmental changes. For all above advantages, diatom are often used to monitor the state of rivers and streams as well as ecological, chemical and physical changes and provide early warning indicator of both, pollution increase and restoration success (Lowe and Pan, 1996; Prygiel et al., 1999).

1.3.1 The presence of species and their morphological characteristics as indicators

Indicator species or indicator taxa provide excellent sight of the direction and magnitude of ecosystem degradation (Biggs, 2000). The use of diatom as an indicator species is a fundamental component of many pollution evaluations system such as organic pollution (Sládecek, 1986, Rott et al., 1998), heavy metal pollution (Medley and Clements, 1998; Gold et al., 2002; Gold et al., 2003a and b; Ivorra et al., 2000), eutrophication (Kelly and Whitton, 1998), and this tool has been used extensively in European countries and elsewhere. Numerous analytical studies using multivariable statistics were done on large datasets of diatom abundances from various sites with distinct environmental characteristics to find the main factors which determine the species composition (van de Grinten, 2004). This approach is particularly useful in identifying possible factors influencing communities, where there may be synergistic effects or where the exact causal variables are unknown, and in determining the effects of dissfuse source pollution (Biggs, 2000). In the middle of them, CCA (Canonical Corresponding Analysis), a direct gradient analysis, is widely used. CCA is a powerful tool to determine which environmental factors have the most influence in species distribution (Dixit et al., 1992; John, 1998). Moreover, PCA (Principal Corresponding Analysis), DCA (Detrended Correspondence Analysis) are also applied for ecological analysis purposes. By this way, the species composition of diatom assemblages is strongly correlated to environmental variables (Winter and Duthie, 2000; Soininen, 2004). Nutrients, temperature, substrates types, conductivity, turbidity, pH and various pollutions are all potential key factors which regulate diatom composition and distribution in different studies in lakes (King et al., 2000; Lim et al., 2001), rivers

(Biggs, 1990; Stevenson et al., 1996; Potapova and Charles, 2003). Many of these studies put forward indicator species which are typical for certain environmental characteristics, e.g. for total nitrogen and total phosphate concentration (Winter and Duthie, 2000) conductivity (Potapova and Charles, 2003), pH (Battarbee et al., 1986). Due to their preference and tolerance to various environmental variables e.g. pH, salinity, nutrients, oxygen level, etc., indicator values for hundreds of freshwater diatom taxa have been given (Lange-Bertalot, 1979; Vandam et al., 1994). Specific sensitivities and tolerances of diatoms can be widely used to infer environmental conditions in ecosystems (Whitton and Rott, 1996; Rott et al., 1998; Prygiel et al., 1999). In water quality assessment, the presence and absence of any particular diatom species whose ecological characteristics are well known have been traditionally used (Lange-Bertalot, 1979). Environmental inferences can be based on single indicator species and genera, such as the presence of *Nitzschia palea* or *Gomphonema parvulum* species indicator for water rich in nutriments and high level of metal (Medley and Clements, 1998; Ivorra, 2000; Feurtet-Mazel et al., 2003; Gold et al., 2003; Lai et al., 2003) or high percentage of *Eunotia* genera in habitat in low pH area (Vinebrooke and Graham, 1997; Ledger and Hildrew, 1998), or presence of *Epithemia* and *Rhopalodia* which appear when N is low in streams or rivers.

Beside indicator values of diatom taxa for certain environmental variables, the changes in morphology of diatom species (teratology, size reduction) were suggested for indication of environmental stress (Gold et al., 2002; McFarland et al., 1997). Several studies have been conducted to assess the changes of diatom size, striae density, and shape under alteration of environmental conditions. Acute pollution has often been associaed with appearance of diatom deformation. Diatom deformities have been suggested as indicator of heavy metal pollution and other contaminants (McFarland et al., 1997; Gold et al., 2002; Cattaneo et al., 2004; Morin et al., 2006) (Figure 1.8). For examples, 19% of diatom species in total communities in metal pollution areas had teratology form in Stevenson and Pan, 1999. Diatom deformities have been correlated with presence of genetoxic and toxic chemicals in aquatic systems in several countries (Yang and Duthie, 1993, Dickman, 1998). The changes of morphological form suggest that metals impair normal membrane function and reduce silicic acid uptake and amino acid synthesis leading to abnormalities diatom silica cell wall formation (Fisher and Jones, 1981; Gold, et al., 2002). Changes in size of species equally indicate environmental stress. In algal cultures exposed to high levels of contaminants, uncoupling photosynthesis from cell division may result in increased cell volume. Reduced cell division and marked increase of cell size of *Asterionella japonica* were reported when exposed to elevated level of copper and zinc, and changes in cell size of the

acidophilic diatom *Asterionella ralfsii* var. *americana* species were distributed to aluminum additions (Fisher and Jones, 1981; Gensemer, 1990).

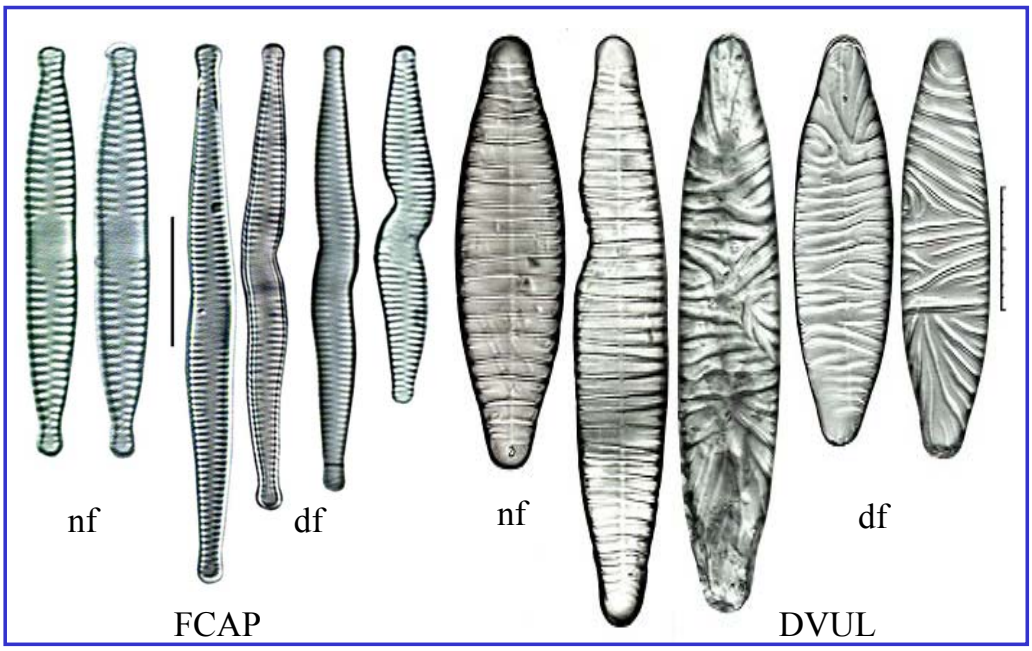

Figure 1.8: Deformed individual of *Fragilaria capucina* (FCAP) and *Diatoma vulgaris* (DVUL) species. (nf: normal form; df: deformed form) and scale bar: 10 µm

1.3.2 Diatom indices

As mentioned above, diatoms are sensitive to pollution and therefore commonly used for assessment of environmental contaminations. To estimate changes of water quality, numerous methods based on the ecology of diatoms have been established, in which large number of diatom indices have been developed in many countries to evaluate the biological quality of running waters (mostly reflecting organic load or/ and nutrient concentration level) (Cemagref, 1982; Watanabe et al., 1986; Descy and Coste, 1991; Kelly and Whitton, 1995; Prygiel et al., 1999). Diatom indices have been derived from diatom profiles and diatom indicator values. Main differences between these indices lie in the number of indicators and the nature taxa used in calculations. The key feature of various methods diatom indices which have been developed in several countries are presented in table 1.1. The first water quality biological index developed in the early 1990, the saprobic system spectrum (Kolkwitz and Marsson, 1908), formed the basic of several indices currently used in European countries and in the United States (Cemagref, 1982; Kelly and Whitton,

1995; John, 2000b, Barbour et al., 1999). This index classified algal species according to their for various level of organic load (polysaprobic, γ mesosaprobic, β mesosaprobic and oligosaprobic).

Index	Author	Date	Comments
	Sládeček	1973	Index based on saprobic system; includes 196 diatom taxa along with representatives of other groups
Id	Descy	1979	Organic pollution index based on 125 taxa
IPS	Coste (Cemagref)	1982 - 2004	Organic pollution index based on 1550 taxa
PTI	Lange - Bertalot	1979	Defined zones based on saprobic system
DAIpo	Watanabe et al.,	1986	Organic pollution index based on 454 taxa of pollution tolerant taxa
GDI	Rumeau & Coste	1988	Generic version of IPS, based on 44 taxa
TDI	Schiefele & Kohmann	1993	Developed from Schiefele Kohmann (1988); index of trophic status based on 50 diatom taxa; variants for P status and N + P status
TDI	Kelly & Whitton	1995	Index of trophic status based on 86 diatom taxa.
	Rott	1997 - 2003	Saprobic and trophic index based on 500 taxa
EPI-D	Dell'Uomo	1999 - 2004	Eutrophication pollution index based on 350 diatom taxa
IBD	Prygiel & Coste	2000	Index take account ecological profile of about 209 diatom taxa established in presence probability for 7 water quality classes.

Table 1.1: Features of various indices based on diatoms (modified after Whitton and Kelly, 1995)

Diatom index (Id) related to organic pollution and based on a formula of Zelinka and Marvan (1961) was constructed by Descy (1979) and is presented below:

$$ID=\frac{\sum_{j=1}^{n} A_j I_j V_j}{\sum_{j=1}^{n} A_j V_j}$$

Where:

A_j = abundance (proportion) of species $_j$ in sample

V_j = indicator value (1-3)

I_j = pollution sensitivity (1-5) of species j

For example *Achnanthes minutissima* has a pollution sensitivity of 4 (i.e. sensitive to organic pollution) and an indicator value of 1 (i.e. widely distributed). On the other hand, *Gomphonema olivaceoides* has a pollution sensitivity of 5 (i.e. very sensitive to pollution) and an indicator value of 3 (i.e. narrowly distributed). *Gomphonema olivaceoides* is therefore a much better indicator of free organic pollution water than A. *minutissima*. The value of this index ranges from 1 (polluted) to 5 (unpolluted) (Whitton and Kelly, 1995). The two diatom indices IPS (Specific pollution sensitivity index) and DAIPo (Diatom assemblage index for organic pollution) have been applied to assess water quality and are discussed frequently in this thesis. DAIPo index was calculated based on relative abundances of pollution tolerance taxa and this index score represents the species' optimum tolerance to BOD (Biological Oxygen Demand: a proxy indicator for organic content) on a scale of 1-100. IPS index was developed based on a large database and an important number of taxa, and the results of this calculation is expressed in a scale ranging from 1 to 5 (Watanabe et al., 1986; Cemagref, 1982). Most of diatom indices mentioned in table 1.1 were developed to be applied in routine water assessment in Europeans and Asian countries and to illustrate water quality of rivers and streams such as organic pollution, eutrophication or trophic status (Cemagref, 1982, Watanabae et al., 1986; Kelly and Whitton, 1995; Kelly and Whitton, 1998). Köster and Hübener (2001) used different indices (the saprobic system of Lange-Bertalot, the saprobic index (SI), trophic diatom index TDI to detect changes in water quality of highly organic and trophic waters. TDI has been used widely in UK for detecting eutrophication in rivers (Kelly, 2003). By using diatom indices, water quality of rivers in central Finland was described and IPS index seemed to give the best results when compared to the general water quality in the different areas, whereas the DAIPo index was responsive in a more specific type of river (Eloranta, 1994). However, these diatom indices were derived and applied principally in temperate areas; there is little information in the tropics and subtropic regions. Consequently, the relevance of such diatom index in the assessment of river water quality for warmer climate needs to be evaluate before the indices can be routinely applied in these areas.

Anyway, diatom indices are found to be good indicators of organic content, nutrient enrichment and acidification and displayed significant correlation with organic pollution, ionic concentration and eutrophication (Prygiel and Coste, 1993; Kwandrans et al., 1998; Köster and Hübener, 2001). Kelly and Whitton (1995) concluded from a study in England and Scotland that correlation was significant between four diatom indices IPS, IDG, TDI-P (which reflects phosphorus concentration) and TDI-NP (which reflects phosphorus concentration plus nitrogen concentration). Several indices such as IPS-IDG, Descy, PTI were successfully applied to indicate the effect of a catastrophic heavy metal spill on Guadiama River, S-W. Spain (Sabater, 2000).

Sensitivities of diatom indices to water quality improvement have been found in study of Rimet et al (2005) in which, some indices have intermediate sensitivities (IBD, IDG), other higher sensitivities (EPI, IPS, TDI).

1.3.3 Structural and functional responses

Structural and functional responses of diatom assemblages have been also used as indicators for environmental conditions of aquatic systems (Stevenson and Pan, 1999). The structure of diatom communities in rivers may be affected by changes of water quality such as high levels of nutrients, inorganic and organic chemical into natural resources. Changes in species composition tend to be the most sensitive responses of diatoms to environmental changes (Gold et al., 2002). Specific-species sensitivity of diatom communities to alterations of environment can be reflected in the changes in the relative abundance of species in communities. Changes in species composition when exposed to pollutants tend to decrease or increase the sensitive or tolerant species (Ivorra, 2000; Gold et al., 2002). Three approaches such as ordination, clustering and community similarity indices are often used to assess variation in species composition among communities (Stevenson and Pan, 1999). Indices of community structure (diversity, richness, similarity) have been used widely in monitoring the impacts of point source pollution on streams (Hill et al., 2000). Diversity index (Shannon-Wiener diversity) has been used as an indicator of changes in community structure under environmental stress (Sabater, 2000). Diversity index appears to be reduced by metal pollution (Foster, 1982). The proposed relationship between diversity and environmental stress has not always been confirmed (Stevenson, 1984) but it seems to better fit for metal pollution (Sabater, 2000; Gold et al., 2002) in which diversity decreases with the increasing concentration of metals.

Pollution-induced community tolerance (PICT) has been proposed for detecting impact of toxic substances in the environment on natural periphyton and to identify agents causing the impact (Blanck and Wängberg, 1988). The PICT concept is based on the fact that communities consist of species with different sensitivity to pollutants. In long term exposure to toxicants, sensitive species will be excluded and resulting restructured community will become more tolerant. The increasing tolerance can therefore be regarded as indicator of environmental disturbance on community. PICT has been validated with controlled microcosm experiments subjected to metal stress (Gustavson and Wängberg 1995; Paulsson et al., 2000; Soldo and Behra, 2000). Physical structure of the periphyton may also be related to sensitivity or tolerance of diatom communities to environmental stressors (Barranguet et al., 2000). Communities of diatom can adapt themself to many environmental stress by changing species composition and thereby achieve biomass and metabolic rate as like those in

un-impacted areas. Changes in structure and metabolic activities are also used to determine effects of discharge from treated wastewater on periphytic communities (Masseret et al., 1998). Photosynthesis and respiration can be used as measures of community productivity and health (Barranguet et al., 2000; Guasch et al., 2003); however these assays are not frequently used in field survey (Stevenson and Pan, 1999). These criteria are used to assess response of periphytic communities to toxicants in several studies. Hill et al (1997) used the response of periphyton respiration rate as an experimental to givethe impact in the habitat. Growth rate (Stevenson, 1996) was used to describe algal biomass production. On the other hand, relative specific growth rate limitation by nutrients was measured using chlorophyll a and has suggested to be a useful tool to determine the effects of point source discharges (Biggs, 1990 b). All the assessments of different responses of species growth rates to various environmental conditions can be enhanced by the simple characterization of the autoecology of species based on changes in their relative abundance (Stevenson and Pan, 1999).

1.3.4 Metals accumulation in biofilm

The capacity of biofilm to accumulate metals from water producing an internal concentration greater than in their surroundings has been reported and discussed in literature (Whitton and Say, 1975; Newman et al, 1985; Clement, 1991; Guanzon et al., 1995; Schorer and Eisele, 1997). The ratio CFs (Concentration Factors) quantifies this ability to concentrate metals in plankton and epilithic diatoms have been proved to show high CFs (Chien, 2004). Uptake of contaminants from surrounding water by algal cells could be the result of several processes. The chemical compound may be metabolically active, act as an essential nutrient or mineral and be transported across the cell membrane and thus enter into biochemical process (Boyle, 1984). In the case of metal, Cambell et al (2002) recogzized the interaction of a metal with an algal cell with thus normally inlvolve the follwing steps: (i) diffusion of the metal from the bulk solution to the biological surface; (ii) sorption/ surface complexation of metal at passive binding sites within protection layer, or at sites on the outer of surface of the plasma membrane; (iii) uptake or "internalization" of the metal (transport across plasma membrane) (Figure 1.9).

Therfore, metals contents in biofilms reflect both their biological avaibility and their ambient concentration over relatively long periods of time (Foster,1982., Berha et al., 2002), even after metal concentrations in aquatic media have decreased background level, through significative levels of metals are still present in organisms (Genter, 1996). Biofilm can be then considered as a potential biological monitor for metallic waste in aquatic systems as suggested by Newman et al

(1985) and Ramelow et al (1992). However, variation of environment factors influencing algal growth may modify toxic concentration of metals in aquatic communities (Hill and Larsen, 2005).

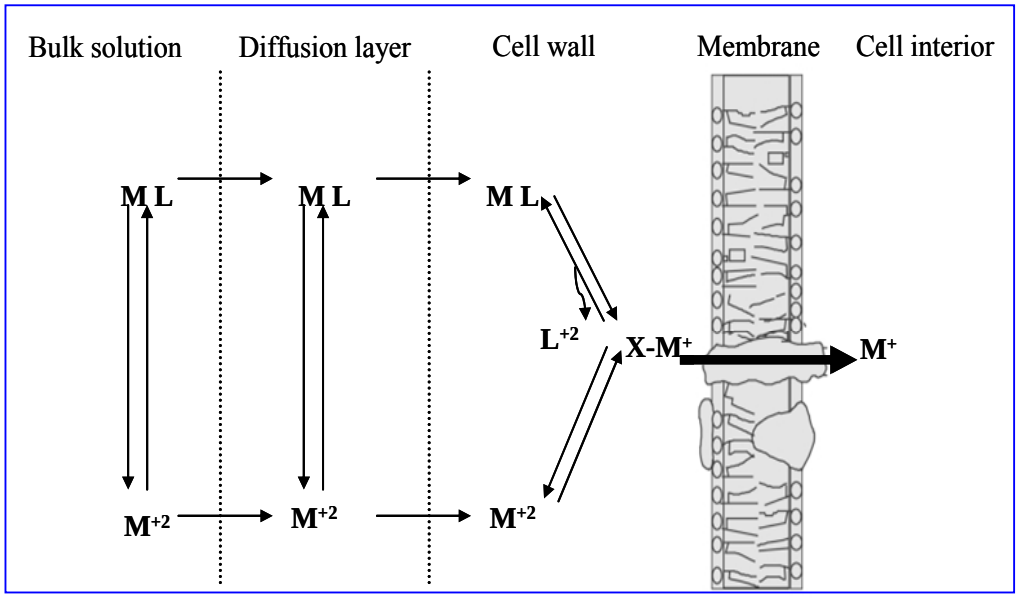

Figure 1.9: Conceptual model of metal-algal interaction. (M^{+2}: free metal ion; ML: metal complex in solution; X-M: surface metal complex) (After Campbell, 2002)

Thus, additions of majour nutrients (P, N and Si) have been shown to influence metal (Cd, Zn, Se) uptake in marine diatoms (Wang and Dei, 2001). Light and current are suggested that to have important simulatory effect on cadmium sorption by biofilm (Hill et al., 2000). Polymeric secretions (polysaccharides), whose nature depends on composition of periphytic assemblage may be rich in iron and manganese and potentially responsible for the metal accumulation when combined with high pH (Newman et al., 1985; Newman and McIntosh, 1989). In return, biofilm itself can modify chemical and physico-chemical conditions in the microlayers via photosynthesis and respiration, an can then enhance sorption of contaminants (Newman and McIntosh, 1989). Consequentely, all these critetia must be taken into account together to understand and explain accumulation levels analyzed in biofilm and diatom organisms in the different aquatic systems. Nevertheless, the role of protection played by the biofilm organized in matrix limites physically inside diffusion of the metals (Hill et al., 2000; Ivorra, 2000; Guasch et al., 2003) and the capacity of biofilm to more strongly accumulate metals. At a more cellular level, tolerance mechanisms inhibition of metal absortiop, development of exclusion detoxification process have been evoked too in literature (Rai et al., 1981; Genter, 1996) as protections against metal contamination.

CHAPTER 2

MATERIAL AND METHODS

2.1 Study sites

2.1.1 Principal characteristics of Red, Nhue and Tolich Rivers system (Vietnam)

2.1.1.1 Geography

Hanoi is the capital of Vietnam and is located 20°53'- 21°23 N 105°44'- 106°02 E. It is surrounded by six provinces (Thai Nguyen, Bac Ninh, Bac Giang, Hung Yen, Ha Tay and Vinh Phuc) (Figure 2.1). Hanoi, situated in the centre of Northern delta of the Red River, occupying an area of 927 km^2, consists of 7 urban districts and 5 sub-urban districts (Trinh and Fredlund, 2000; Horen, 2005). Hanoi topography has an inclination in the direction north-south. Many rivers flow through the city of which the major is the Red River which flows from its source in China to the Gulf of Tonkin (South China Sea). Its section, flowing through Hanoi from Donganh district to Thanhtri district, is 40 km long. Other rivers which run through Hanoi city, include the Duong, Set, Lu, Kimgguu, Tolich and Nhue Rivers.

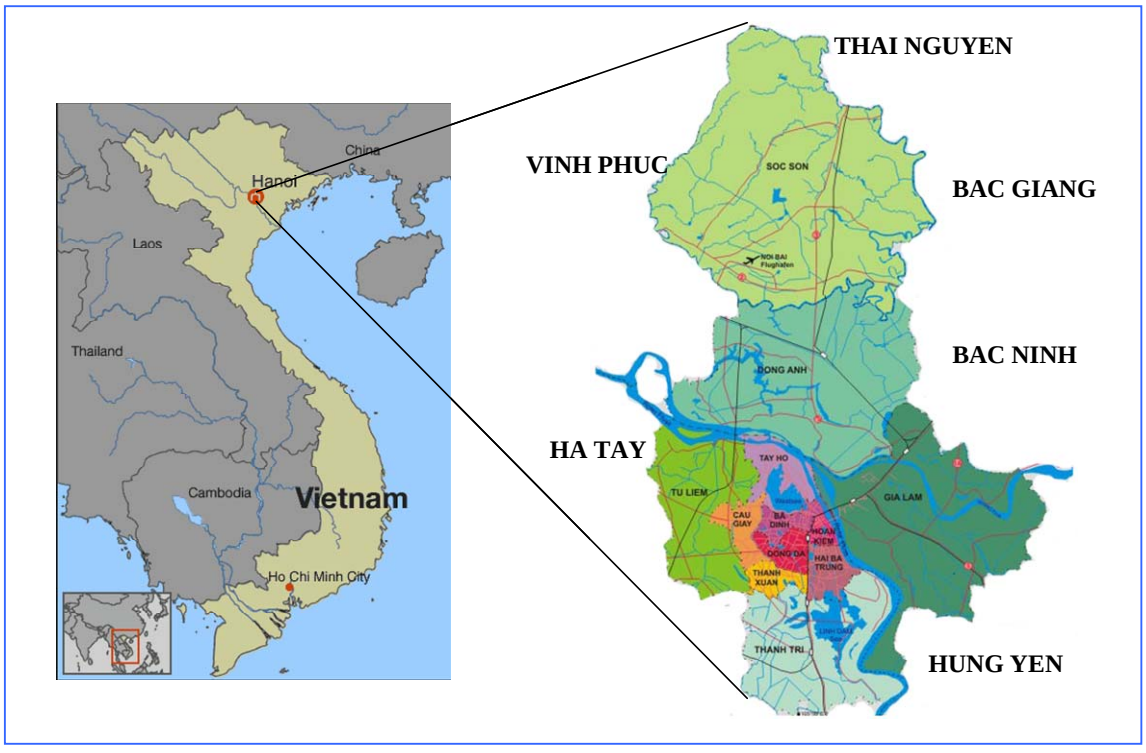

Figure 2.1: Map of Hanoi city

The Red, Nhue and Tolich River system is located in the Red River delta in the Northern part of Hanoi. Red River is the largest river in the Northern part of Vietnam and its role in the agricultural production of the country is as important as that of the Mekong River in the South. The Nhue River is bordered by the Red River in the north, by the Day River in the west and by the Chau Giang River in the south. The Nhue River, a tributary of the Red River, diverges from it at the Thuyphuong dam and receives wastewater from the Tolich River through the Thanhliet dam. The Nhue River, which serves as a major irrigation and drainage canal, flows through the Hanoi area, Hadong city (Hatay province) and Hanam province *via* several dams and finally flows into the Day River. The Nhue River basin area is 74 km long and covers a total of 107,503 ha total of which 75 % is cultivated (Kono and Doan, 1995; Trinh, 2003). The Tolich River originates from West Lake, situated in the Northern part of Hanoi area, flows along 14 km across the Hanoi city to the south before joining the Nhue River. The Tolich River watershed accounts for 7,750 ha including seven urban districts and sub-urban districts of which about 45 % of the total area is used for resident purposes. It receives all the urban wastewaters (domestics and industrial wastewater) from Hanoi area (Trinh et al., 2005).

2.1.1.2 Meteorology

The climate of Hanoi is typical of the Red River delta region, i.e., sunny and tropical along with heavy moonsoon. A local tropical to subtropical monsoon climate leads to an average annual rainfall of 1800-2000 mm, 80% of which occurs during the rainy season from June to October. The incidence of most heavy rains and typhoons happens during July. The dry season prevails from November to April-May (Trinh, 2003; Le, 2005). Relative humidity is very high throughout the year with an annual value of 84.5% (Trinh, 2003; Kurosawa et al., 2004). The most humid months of the year are March, April and August while the least humid months are October, November and December. The annual average temperature ranges from 15°C in the winter (November and January) to 30°C in the summer (June to August) (Trinh, 2003; Le, 2005).

2.1.1.3 Effluent discharges

Urbanization and industrialization are increasing simultaneously everywhere in Vietnam, resulting in a concentration of the pollution in the cities. The process of urbanization has rapidly grown and the population in the Hanoi urban zone has been dramatically risen from 300,000 in 1954 to its current population of more than 4 millions (Trinh and Fredlund, 2000). This rapid urbanization has created significant environmental pressures and unsustainable demand on natural

resources. Hanoi has 20 major industries, the most important of which are chemical, textile, mechanical engineering and food processing. In the year 1996, 5,000 enterprises were operating, of which 4,000 were micro to small-scale private enterprises and 1,000 were medium-scale private enterprises. In parallel with the increasing urbanization and industrialization, infrastructures for wastewater treatment in Hanoi are either absent or of poor quality (UNDP, 1999). A recent study found that 70% of the industries in Hanoi are using technologies which are at least 20 years old and that only a few industrial factories operate waste treatment systems (CEETIA, 1996). Industrial solid wastes are disposed together with municipal wastes at poorly designed dumpsites, allowing pollutants to leak into the groundwater and aquatic adjacent system (Figure 2.2).

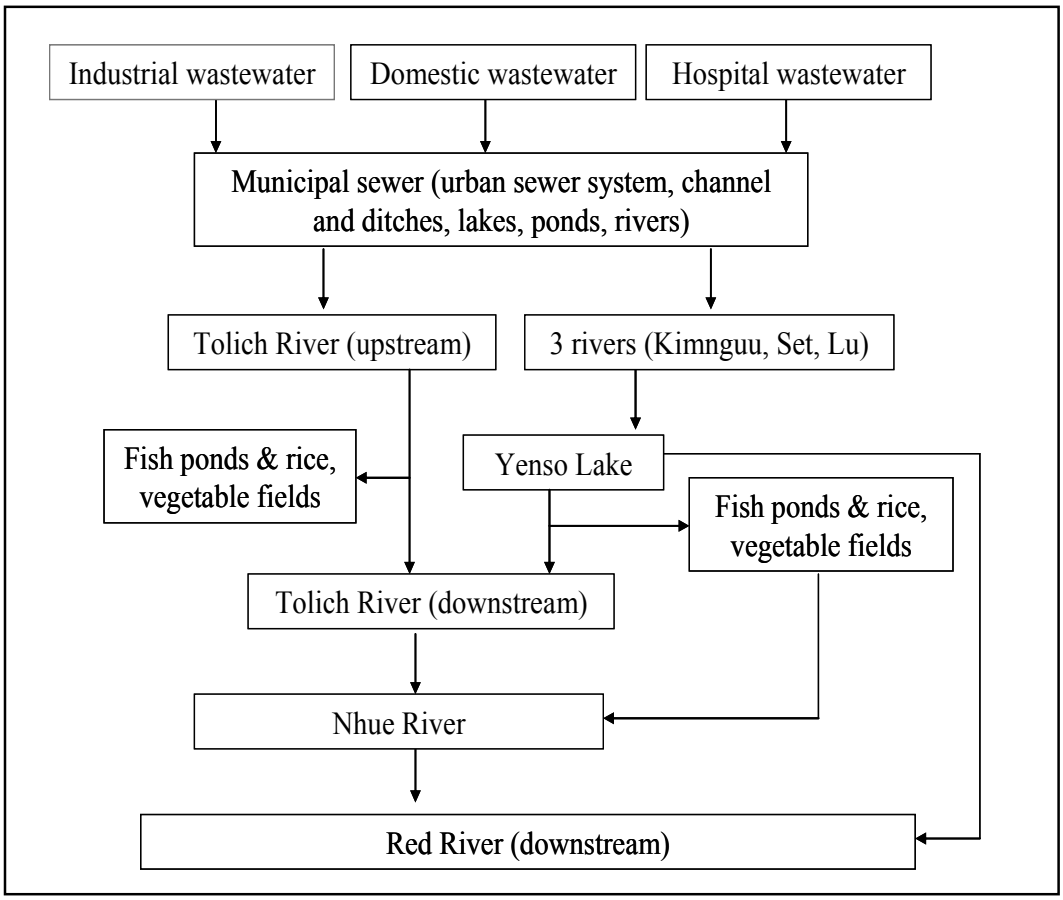

Figure 2.2: Pathway of Hanoi urban wastewater

Furthermore, hazardous industrial wastes are not treated separately, but mixed with inert solid waste. Both domestic and industrial as well as storm water wastewater share the same drainage. The wastewater that flows from the Hanoi city is widely used by the farmers and peri-urban agriculturists living on the edge of the city, and the reuse of wastewater increases in

agriculture and aquaculture systems (Mai et al., 2004). Nowadays, Hanoi discharges about 335,000 m^3 of untreated wastewater into its sewerage and drainage system everyday, of which, 115,000 m^3 per day comes from industries, counting for 27-30% of total wastewater. Domestic wastewater ranges over 220,000 m^3 per day and accounts for the larger volume among the wastewater source. Hospitals discharges are the third group of polluters, and wastewater volume from hospitals is 532 m^3 per day along with more than 26 tonnes per day of solid waste. Although this source of wastewater accounts only 1.4% of the total municipal wastewater, it is a serious threat to human health and environment (Nguyen, 2001; Paladino, 2001; Trinh, 2003). Sewage from Hanoi city is rich in nutrients; BOD_5 concentration ranges from 50 $mg.L^{-1}$ to 100 $mg.L^{-1}$; nitrogen concentration (mainly NH_4-N) ranges from 10 to 12.7 $mg.L^{-1}$; suspended solids (SS) range from 60 to 300 $mg.L^{-1}$. Heavy metals in river sediments reach high level with concentrations (mg/kg) of 2.48 for cadmium, 376 for chromium, 258 for copper, 146 for nickel, 158 for lead and 1040 for zinc (Ho and Egashira, 1999; Ho, 2001).

2.1.1.4 Sampling sites

In this thesis, five sampling stations have been chosen along the Red-Nhue-Tolich River system (Figure 2.3 and Photo 2.1 to 2.6). Red site (Red, comparatively unpolluted reference site) is located at Trungthon community (Haytay province) (chapter 4) or is situated at Son Tay (Sontay district, Haytay province) (chapter 3 and chapter 5) in the Red River about 4 kilometers upstream or 8 kilometers respectively from the junction with Nhue River. Average annual discharge at Sontay station in period 1997-2004 were 3 400 $m^3.s^{-1}$(Le, 2005). These two stations are located along the Red river bank which has at this point a large width (about > 1 km). Four sampling stations established along the Nhue River include N_1, N_3, NT_1 and NT_2. The N_1 site corresponds to the upper part of the Nhue River, where water from the Red River flows into the Nhue River through the Thuyphuong dam. N_3 is located next to Cauden dam, 5 km upstream from the confluence with the Tolich River. NT_1 and NT_2 positioned in the lower part of the Nhue River at Khetang and Cauchiec (Hatay province), respectively 5 km and 8 km downstream the confluenceof Nhue and Tolich Rivers: N_1, N_3, NT_1 and NT_2 inlustrate moderate and polluted conditions. Depth of Nhue River ranged from 1 to 3 m, and soil in Nhue River basin mainly consists of mud/sand in the areas close to Red and Day River, whereas mud/clay characterized the central areas of the Nhue River (Trinh, 2003). Average discharge of Nhue River is 35 $m^3.s^{-1}$(from 8 to 50 $m^3.s^{-1}$: extreme values). Tolich site (TL, heavy polluted site) is situated in Thanhliet dam, in the Tolich River, 800 m upstream from

the confluence with Nhue River. This site has 4 m width and discharges around 5 $m^3.s^{-1}$ (from 1.5 to 15 $m^3.s^{-1}$: extreme values).

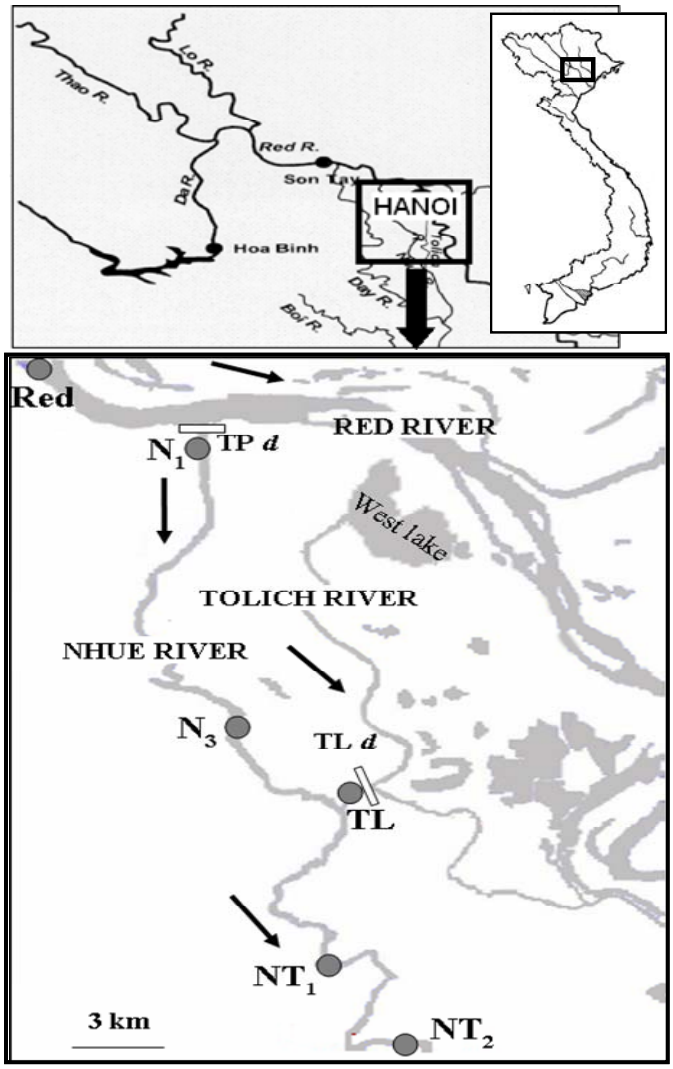

Figure 2.3: Location of sampling stations in the Red, Nhue and Tolich Rivers

Photo 2.1: Red site in Red River (at Sontay district, Hatay province)

Photo 2.2: N_1 site in Nhue River (upstream next to Thuyphuong dam 1 km)

Photo 2.3: N_3 site in Nhue River (before confluence with Tolich River)

Photo 2.4: NT_1 site in Nhue River (downstream)

Photo 2.5: NT_2 site in Nhue River (downstream)

Photo 2.6: TL site in Tolich River

2.1.2 Principal characteristics of metallic pollution in the Lot River (France)

2.1.2.1 Geography and climate

The Lot River is an affluent of the Garonne River and located in the Southwest of France (Figure 2.4). Its source wells up north of Mont Lozère at an altitude of 1295 m. The Lot River is 480 km long and it flows into the Garonne at the Aiguillon (altitude 22 m). It has a watershed of 11,840 km^2 (Thebault and Qotbi, 1999; Audry et al., 2005). The portion of the watershed upstream of Entraygues has steep slopes. Given the fluviometric cycle, the hydrological regime of the river can be characterized by as Mediterrannean. Following heavy precipitations in autumn and winter, the summer is hot and dry (Thebault and Qotbi, 1999). The annual average flow measured over the period 1973-2000 at Villeneuve sur Lot is around 164 $m^3.s^{-1}$ (DIREN, French regional environment department). Geography of the Lot River basin is divided into three zones (i): a higher zone (upstream of Entraygues) with crystal-lophyllian (mica-schist) and eruptive rocks of the Massif Central; (ii): a medium part (from Entraygues to Fumel) which mainly consist of carbonate - rich marine sediments, mostly of Jurassic age; (iii): a lower part (from Fumel to the confluence with the Garonne River) (Audry, 2003). The catchments area of Decazeville is located North-West of Aveyron represents a surface of 165 km². The Riou Mort River, a small tributary of the Lot River, is situated in the industrial basin of Decazeville at 44°N / 2°E (Morin et al., 2006), and it has been known for its polymetallic pollution due to former open-cast coal mining and Zn ore treatment. The mean annual discharge of the Riou Mort River has been 1.9 $m^3.s^{-1}$ from1968 to 2004 and 0.98 $m^3.s^{-1}$ during the year 2004-2005 (DIREN, French regional environment department). The Riou Mort River receives contributions of several tributaries: Riou Viou, Enne, Banel as well as other small streams.

2.1.2.2 Metallic pollution source

The Lot River is known for its chronic metals contamination resulting from mining and industrial development since nineteenth century. Metal pollution was first recognized in 1979 within the framework of the "National Observation Network" which documented high cadmium concentrations in the fresh oysters of the Gironde (up to 100 $\mu g.g^{-1}$ of dry matter and were 10 times higher than those analysed in oysters from non-industrial areas) (Boutier, 1981). The data collected by Lapaquellerie et al., from 1992 to 1995, showed that Zn and Cd loading in the Lot River sediments were respectively of 190 and 19,300 t. $year^{-1}$. During the seven following years, the average annual flow of Cd coming from the Lot River was 7.5 $t.year^{-1}$ with extreme values ranged

from 2 to 20 tons.year^{-1}, as functions of hydrological regime (Andrès et al., 2000).The main metal source has been clearly highlighted in the upper part of the Lot River, where a small tributary (Riou-Mort River) drains waste from the Vieille Montagne factory (Union minière, France), which, for over a century, has produced Zn, first from local and then from imported ores (Andrès et al., 1999; Gold et al., 2002; Audry et al., 2005). Cd input from the anthropogenic point source (Riou Mort River) to the Lot-Garonne fluvial is around 6 tons. year^{-1} (Figure 2.4)

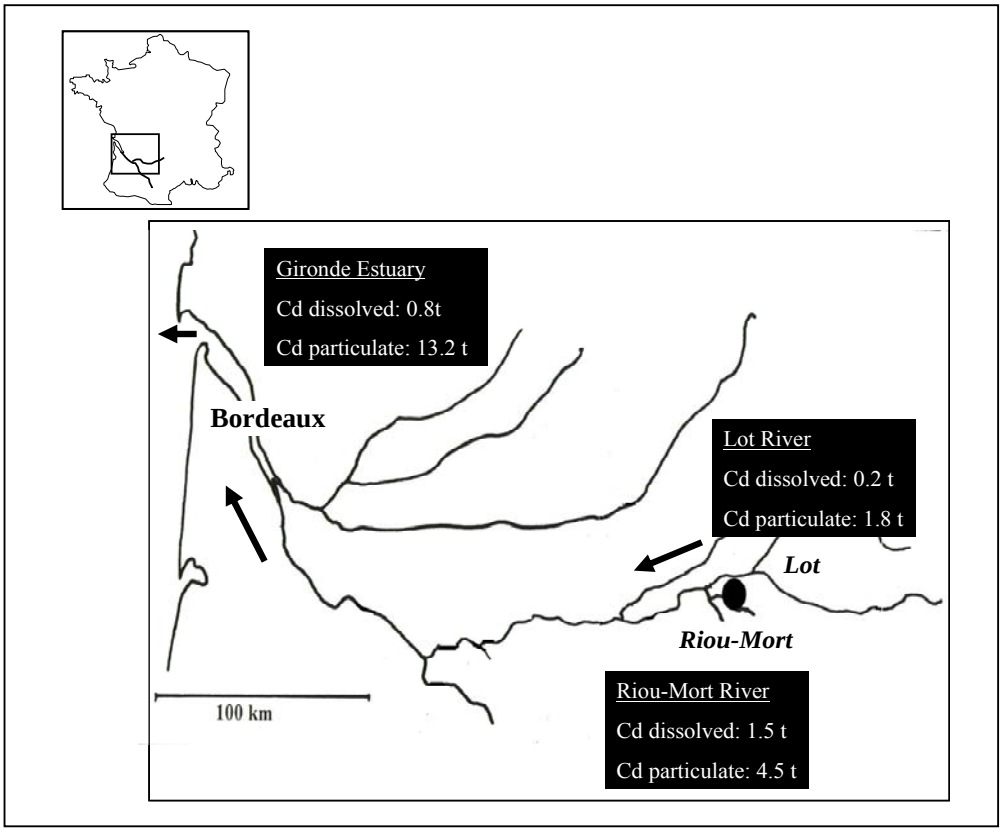

Figure 2.4: Cd in the Lot-Garonne River system in 2000 (Audry et al., 2003)

Approximately 85% of the cadmium in the Lot River is derived from Vieille Montagne factory (Blanc et al., 1999) through Riou-Mort River. This small river is responsible of the metal inputs into the Lot River (Andrès et al., 1999, Gold, 2002). Zinc (Zn) manufacturing industry has been released large quantities of metals mainly cadmium and zinc during the early to mid-1990s (Andrès et al., 2000). Zinc was produced from $ZnCO_3$ until 1922, by thermic reduction and then, from ZnS by roasting and electrolysis. About 35 million tons of high sulphide smelting-waste rich in Zn and Cd (10,000-20,000 mg.kg^{-1}Zn and around 200-400 mg.kg^{-1}Cd) have been produced and deposited on the bed of the river. In 1995-1997, a previous study showed high Cd and Zn level (30 µg Cd.L^{-1}

and 1000 µg $Zn.L^{-1}$) in water samples near Vieille Montagne factory. In parallel, Andrès et al (2000) showed very high Cd and Zn bioaccumulation capacities in freshwater bilvalve and fishes.

2.1.2.3 Sampling sites

The experimentation in the field has been conducted at two sampling stations along the metallic pollution gradient (Figure 2.5).

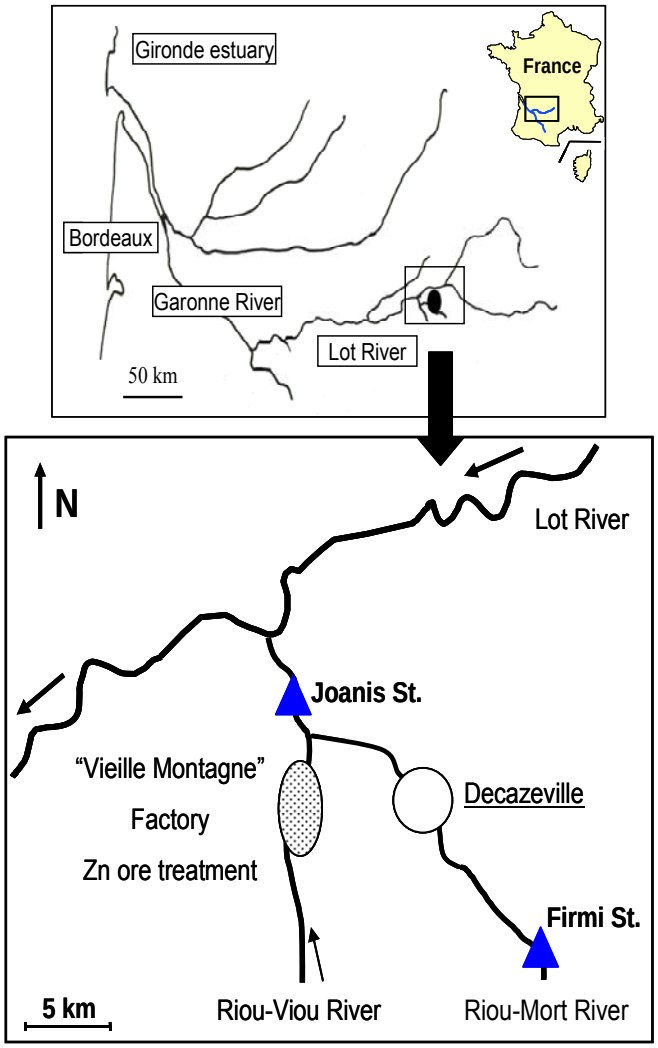

Figure 2.5: Location of sampling stations in the Riou-Mort River

Firstly, the reference station (Firmi) is situated on the Riou Mort River above the confluence with the Riou Viou River, about three kilometers, from the little town of Decazeville (6,787 inhabitants). The site is characterized a relatively low metal concentration in water column (< 1 µg $Cd.L^{-1}$ and < 10 µg $Zn.L^{-1}$), $P\text{-}PO_4$ is 0.25 $mg.L^{-1}$ and $N\text{-}NH_4$ is 1.4 mg. L^{-1} (Morin et al., 2006). At

Firmi station, Riou-Mort is 6 m width and water current is about 0.13 ± 0.01$m^3.s^{-1}$. Secondly, the polluted station (Joanis) is situated downstream of the Riou Mort River, about three kilometers below its confluence with Riou Viou River, and it is characterized by high levels of dissolved cadmium, zinc and nutrients. In 2002, average dissolved cadmium and zinc concentrations in water column were measured at this station and displayed about 16 $mg.L^{-1}$ for Cd and 1300 $mg.L^{-1}$ for Zn (Audry et al., 2004). By analyzing filtered water samples (0.45µm), dissolved forms of two metals Cd and Zn were mostly found (92 % of Cd and 89% of Zn). High concentrations of nutrients were found in this station too with $P\text{-}PO_4$ at 1.28 $mg.L^{-1}$ and $N\text{-}NH_4$ at 2.2 $mg.L^{-1}$. The mean width of Riou Mort River at this level of Joanis station is about approximately 6 m with primarily sandy substrates (see photo 2.7 and 2.8).

Photo 2.7: Joanis site in Riou Mort River

Photo 2.8: Firmi site in Riou-Mort River

2.2 Diatom communities analyses

2.2.1 Samples collection

Artificial substrate is one of several approaches available for collecting samples of periphytic diatoms from a wide variety of environments. They have been successfully used in many types of periphytic investigations (rate of colonization, community interaction, impact and comparisons of environmental variability and collection of algae) (Biggs, 1988). There are many advantages of using artificial substrata in diatom studies including reduced effort and cost of sampling, ease of manipulation, less habitat disruption, redution of heterogeneity (or patchiness) of algae occurring on natural substrates and possibility to obtain many replicates. In addition, the use of artificial substrata is of a great help to measure the quantitative and qualitative criteria of the diatom communities and avoid the biological interaction that may occur between diatom and natural substrata (Gold et al., 2002; Lane et al., 2003). Many kinds of artificial substrate have been used for algae attachment such as coble-stone, plastic, rocks and glasses *etc*. in outdoor and indoor studies (Potapova and Charles, 2005; Rimet et al., 2005). In order to secure standard mean of comparison among periphyton in different stations and between different studies, and to eliminate variability due to differences in colonisation times and surface structure, glass substrates were used in all our studies.

Periphytic samples were obtained by exposing glass slides (18cmx30cm in the field experiment in Vietnam; 6 x 30cm in the field experiment in France and 6 x 15.2cm in laboratory experiment) (Figure 2.6). Time for periphytic diatom development on glass substrate depends on the objectives of each experiment, and our experiment lasted between 20 days and 42 days. In field experiment, glass substrates were maintained vertically separated from each other in a plastic basket equipped with floaters, and then were immersed in water column to be positioned parallel to the current at a depth of 15-20 cm below the water surface, and tied to the bank with a rope. In laboratory experiment, glass substrates were inserted in artificial streams system parallel to the current at a depth of 3-5 cm below the water surface (Gold, 2002). On the day of sampling, substrates were removed, and periphytic diatom samples were scraped by using a toothbrush (in Vietnam) or a blade (in France). All periphytic samples were diluted in a known volume (100 or 200 mL) of mineral water, depending on the biofilm thickness. Each natural periphytic sample was divided into four fractions assigned to various analyses. One fraction (5mL) was preserved with 0.25 mL of formol solution (Formaldehyde 37%, Prolabo, France) in a glass bottle for numeration and diatom identification. The remained fractions were stored in a labelled polyethylene bottle and

placed in a cool (4°C), dark place during transportation to laboratory before different particulate matter analyses: biofilm dry weight (DW), ash free dry mass (AFDM) and metals contents in biofilm.

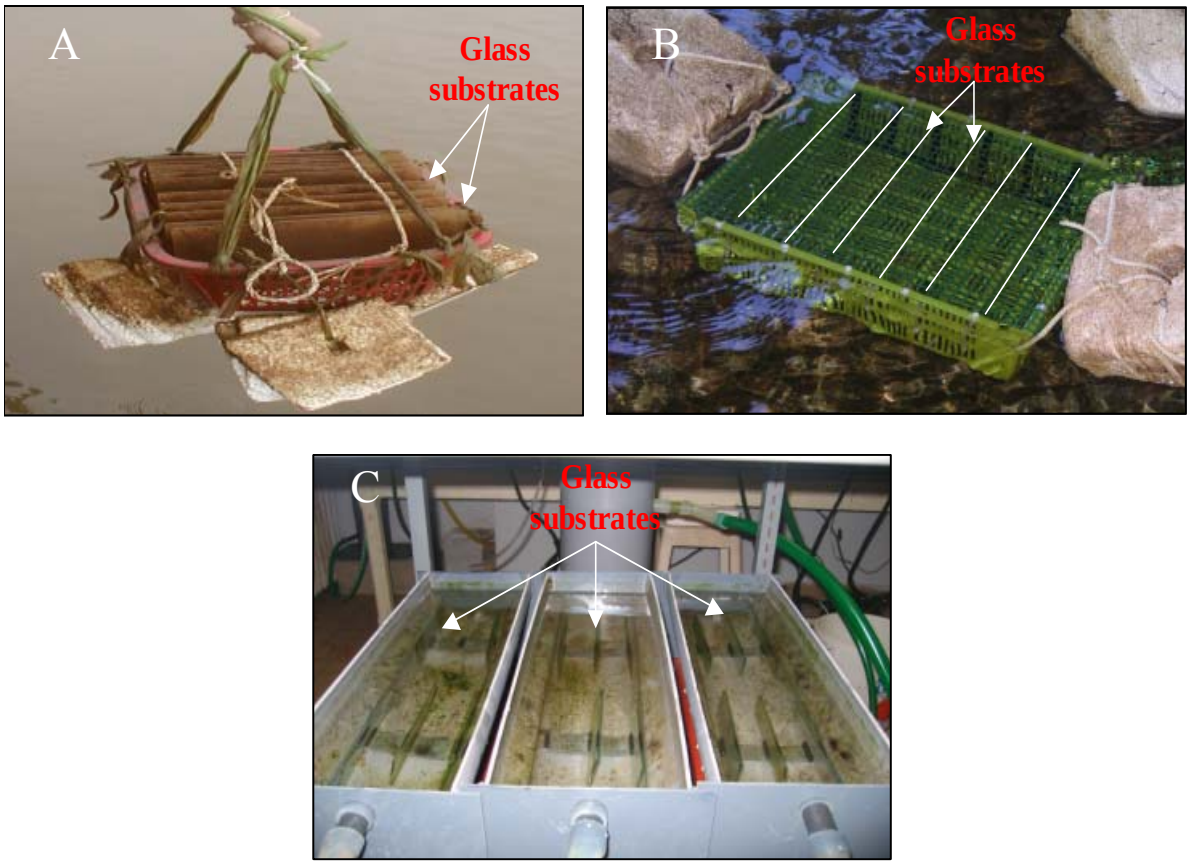

Figure 2.6: Artificial substrates for benthic diatom attachment. A: in Vietnam; B: in France and C: in Laboratory experiment.

2.2.2 Qualitative analyses periphytic diatom communities

2.2.2.1 Diatom preparation

The preparation of diatom samples involves cleaning with strong oxidizing agents, e.g. concentrated acids, to burn away all organic matter. Everything left is mineral matter, including the siliceous parts of diatom frustules (usually by this time dissociated into component valves and girdle elements). Diatom preparation followed the procedure described below (Figure 2.7):

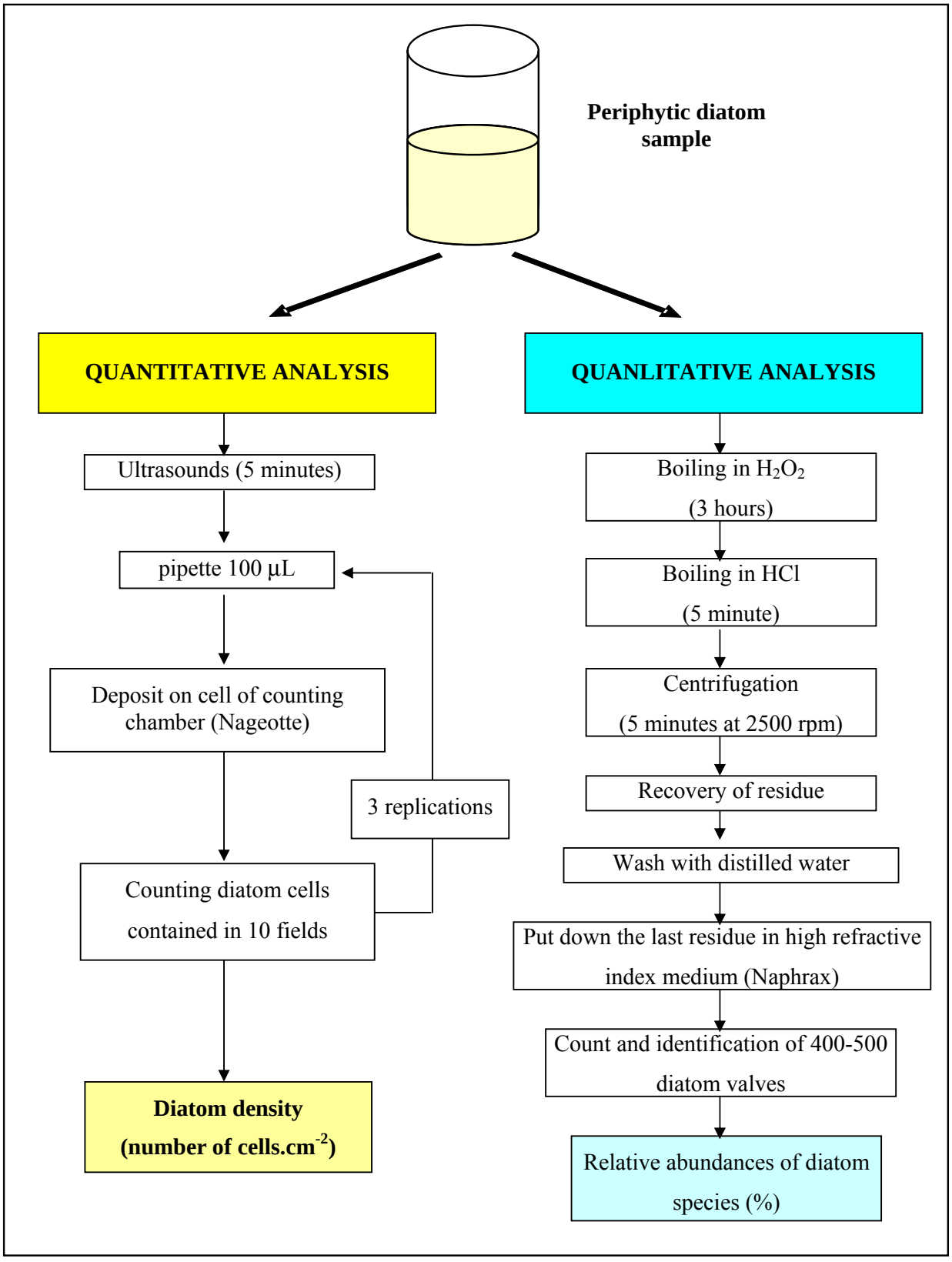

Figure 2.7: Protocol for the quantitative and qualitative analyses of the periphytic diatom communities

- Periphytic diatom samples are well homogenized to break up attached diatoms and to suspend the algae before mixing 2 mL of samples into 10 mL H_2O_2 (30 % Prolabo) in a test tube. The test tubes are placed on electric sand heater untill boiling and evaporation under an extraction hood.
- In each test tube, a volume of concentrate HCl (35% Prolabo) is added to remove organic matter and dissolve carbonate. The samples are boiled in hydrogen hydroxide and chloride acid for 3h.
- After boiling, the diatom samples are stored to cool down.Treated samples are transferred to labelled plastic centrifuge tubes and topped up with deionised water. They are then centrifuged during 15 min at 4000 rpm before decantation of the acid solution and the adding deionised water. The process of centrifuging is repeated three times to ensure all traces of the nitric acid are removed.
- After cleaning, the "cleaned" diatom frustule material is laid on a glass coverslip. The coverslip is placed onto a hot plate at low temperature (60^o C) and the solution is allowed to slowly evaporate. The glass coverslip is placed to glass slide to which a drop of "Naphrax" (Brunel Microscopes Ltd, UK; RI=1.74) had been added. The slide was heated on a hot plate until all the toluene evaporated, leaving the Naphrax. Preparations of diatoms in Naphrax are effectively permanent, enabling them to be kept in a reference collection for future use.

2.2.2.2 Diatom identification

Diatoms in the cleaned and mounted samples were identified to the species level using oil immersion Leitz DMRD microscopy with 1000x magnification. Determination was based on their unique, stable, morphological characteristics. On each slide, up to 400 diatom valves were identified and counted. The flora of Krammer and Lange-Bertalot (1986-1991) were used as a basis for identification and complicated with more recent bibliography. From the diatom inventory, two difference diatom indices IPS (Specific pollution-sensitivity index, Cemagref, 1982) and DAIpo (Diatom Assemblage Index to organic water Pollution, Watanabe et al., 1986) were calculated to assess water quality. IPS index is based on the weighed average equation of Zelinka and Marvan (1961):

$$ID = \frac{\sum_{j=1}^{n} A_j I_j V_j}{\sum_{j=1}^{n} A_j V_j}$$

Where:

A_j: Relative abundance of species j

I_j: Pollution sensitivity of the species j

V_j: Indicator values of the species j

DAIpo index is based on sabrobic requirements were calculated from the following equation:

$$DAIpo = 50 + ½ \left(\sum_{i=1}^{m} Xi - \sum_{j=1}^{n} Yj \right)$$

Where:

$\sum_{i=1}^{m} Xi$ = Sum of relative abundances of Saproxenous taxa from 1 to m

$\sum_{j=1}^{n} Yj$ = Sum relative abundances of Saprophilous taxa from 1 to n

Accorrding to authors, the index shown high correlation between biological oxygen demand and relative abundance of epilithic diatom taxon. To make easier the comparison between the two indices, values were expressed at the same scale (1–20) in which 20 represents the best water quality and 1 the poorest one. Species richness (S, number of species in the community) was counted and Shannon-Weaver diversity index (H') was calculated as well:

$H' = -\Sigma (p_i \log_2 pi)$ with pi = Relative abundance of species i

2.3.3 Quantitative analyses of periphytic diatom communities

Periphytic diatom samples were preserved in a formalin solution (5%) before enumerating the number of cells per surface unit (Figure 2.7). Counting was performed using a Nageotte counting chamber (Marienfeld, Germany) under a light microscope (Olympus BX 50) at 200x magnification. Nageotte counting chamber has 40 rectangular fields and a depth 0.5mm which gives a volume of 1.25 µL in each field. Periphytic diatom samples were ultrasonicated to break up attached diatoms and to obtain a homogenous suspension. 100 µL aliquots were put onto the counting grid of the Nagoette chamber. Diatom valves contained in 10 fields and distributed over the chamber were counted, the procedure was repeated three times to give total diatom cells. The numbers of diatom cells per surface were calculated following a formula below:

Diatom density = [A/ (30 x 1.25) x V)] / S
(in number cells.cm-²)

Where:

A: Total number of diatom cells in 30 fields

30: Total numbers of counted fields

1.25: Volume of 1 field (µL)

V: Initial volume of benthic diatom samples (µL)

S: Total surface of glass substrate (cm²)

2.3 Analyses of environmental parameters and of metals content in biofilm

2.3.1 Analyses of physical and chemical parameters in water samples

Water temperature, pH, dissolved oxygen, conductivity, turbidity were measured in the field by using water quality set, model WQC-22A (TOA, Japan) (experiments in Vietnam) or (WTW, Weilheim, Germany) (experiments in France) during the sampling survey. Before each sampling measurement, the instrument was calibrated using standard solutions. For nutrients quantification, river water samples simultaneously collected at a depth 20-30 cm in the middle of the river bed. Samples were kept in the dark at 4°C during transportation to the laboratory.

In Vietnam, all water samples were analysed by the Institute of Natural Product and Institute of Chemistry (VAST-Vietnamese Academy of Science and Technology, Hanoi, Vietnam). They were treated as soon as they got to laboratory. Whatman GF/F filters paper (0.47 mm) were used to filter river water samples for dissolved nutrients analyses as nitrogen (nitrites, nitrates, and ammonia), phosphorus (phosphates), and dissolved carbon (dissolved organic carbon DOC and dissolved inorganic carbon DIC), and Whatman GF/C fiber filter were used for Chlorophyll a determination (Le, 2005). Phosphates, nitrates, nitrites, ammonium, SPM (Suspended Particulate Matter), BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand) and Chlorophyll a (from phytoplankton) analyses in water followed standard methods for wastewater examination as used by the APHA (American Public Health Association). Total inorganic carbon (TIC) and total organic carbon (TOC) in water samples were measured using ANATOC, series II (Total organic carbon, SGE, Australia). 10 mL samples of dissolved metal concentrations (Zn and Cd) were acidified in 2 % HNO_3 before analysis by atomic absorption spectrophotometry (AAS Perkin-Elmer) (Figure 2.8).

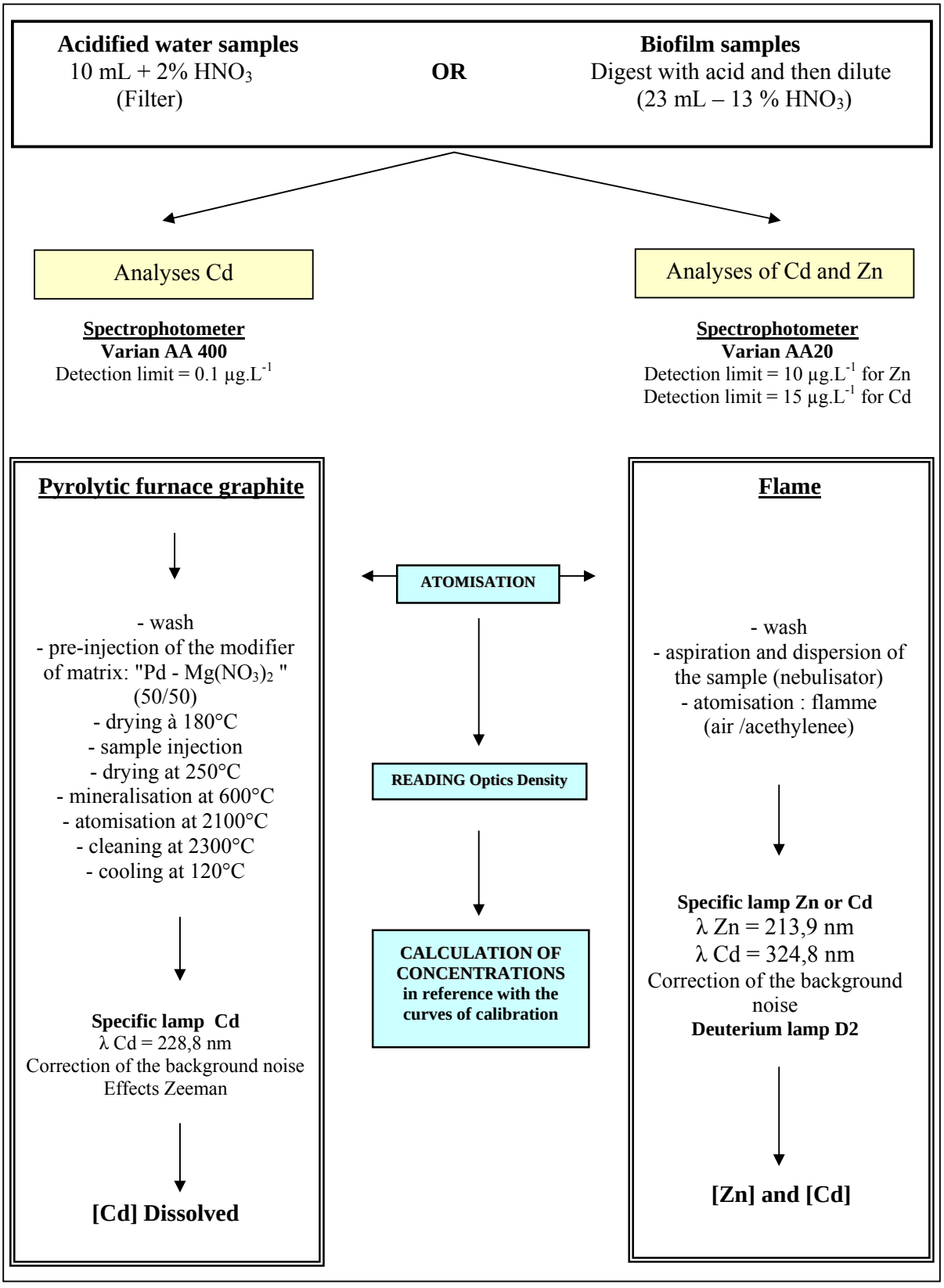

Figure 2.8: The protocol of cadmium and zinc analyses in water and biofilm using atomic absorption spectrometry (Pyrolytic furnace graphite or Flame)

In France, phosphates, silica, ammonium, nitrites and nitrate concentrations were determined according to French and international standards (NF T90-023, NF T90-007, NF EN ISO 11732 and NF EN ISO 13395, respectively) (Morin et al., 2006) and all analyses were carried out by the Cemagref. River water samples for trace metal analysis were sampled using clean techniques. The samples were stored in an acid pre-cleaned polypropylene bottle (1 L), previously rinsed with the river water of the site. Water samples were filtered immediately through 0.2 µm Nucleopore® polycarbonate filters in a glove box (laboratory van). Filtrates were collected in an acid-washed polypropylene bottle after thoroughly rinsing it with an aliquot of the filtrate, acidified (HNO_3 ultrapur 0.1%) and stored in the dark at 4°C until analysis. Metal concentrations of river samples were performed by the TGM[1] laboratory, University Bordeaux1. Details of the analytical procedures to determine dissolved metal concentrations in river samples have been reported previously (Audry, 2003). Metal concentration (Cd) in water samples of laboratory experiment were measured by atomic absorption spectrophotometry (Varian AA 400) equipped with a model GTA 96 graphite tube and atomizer and autosampler. Before measurements, water samples were filtered through 0.20 µm Millex® Millipore filters and then preserved by acidification (2%) and sub-sample 10µL were taken for determination and mix before atomisation with 4 µL of a "50 % palladium + 50% $Mg(NO_3)_2$" solution. The detection limit was 0.1 µg Cd. L^{-1} (DL: mean ± 3 SD) of 10 reagent blanks. Blanks and standards were run with each batch of samples.

2.3.2 Analyses of dry weight, ash free dry mass and metals contents in biofilm samples

In order to determine dry weight of biofilm samples, 20 mL of the homogeneous suspension of biofilm were filtered through a tared metal-free filter paper (0.45 µm membrane Millipore). The filtered samples were then dried at 60°C for 48 hours in incubation tubes. Dried samples were weighed and expressed as $mg.cm^{-2}$. For ash free dry mass (AFDM) determination, 20 mL of biofilm suspensions were filtered using pre-weighed glass fiber filters (47mm and pore size 1 µm, Sartorius, Göttigen, Germany), dried at 105 °C during one hour, and then ashed at 500^0 C for one hour in a muffle furnace (Solax Technology Ltd, China). AFDM was reported as $\mu g.cm^{-2}$.

Methodologically, metal content analyses biofilm samples were performed by simple acidic digestion of dried biofilm sample. Dry weight filters were digested in nitric acid (3 mL of pure HNO_3, Merck, Darmstadt, Germany) in a pressurized medium at 100° C for 3 hours (hot block CAL 3300, Environmental Express, USA). Digestates were then diluted up to 23 mL with ultra-pure water (Milli Q, Bedford, MA, USA). Two different methods were then applied to estimate metal

[1] TGM: Tracer Geochimiques et Mineralogiques

concentration in periphyton due to higher metal levels in natural biofilms than in laboratory biofilms (Figure 2.8). Cadmium and zinc contents in natural biofilm samples were measured by atomic absorption spectrometry (Varian AA20, Australia), with detection limit of 15 $\mu g.L^{-1}$ for Cd and 10 $\mu g.L^{-1}$ for Zn. Cadmium contents in laboratory biofilm were measured by flame atomic absorption spectrophotometry (Varian AA 400, Australia), equipped with a model GTA 96 graphite tube, atomizer and autosampler with detection limit of 0.1 $\mu g.L^{-1}$ Cd . Validity of both methods was checked periodically by measurement of the certified biological reference materials (Tort 2 – lobster hepatopancreas and Dolt 2: dogfish liver from NRCC – CNRC, Ottawa, Canada).

To estimate metals contribute in both intracellular and extracellular spaces, one half of each periphytic biofilm sample was directly analysed and gave the measurement of total metal concentrations distributed inside diatom cell (intracellular) and adsorbed on its surface (extracellular). The second half, being first washed before analysis during 10 minutes at pH 8 (Berha et al., 2000, Meylan et al., 2003) with EDTA 4 mM (Ethylenediaminetetra-acetic acid), a strong metal complexing ligand) which removes all metal adsorbed onto the cell wall gave an estimation of intracellular metal content by diatom/algae and bacteria with the total metal content of biofilm.

2.4 Statistical analyses

Four kinds of data were obtained from all experiments such as characterization of periphytic diatom communities (relative abundance, number of diatom cells, species richness, diversity index, and diatom indices), characterization of biofilm samples (dry weight and ash free dry mass), characterization of environmental parameters in water (nutrients, metal concentration, pH, temperature, dissolved oxygen, turbidity, conductivity etc.) and metals contents in biofilm. Multivariable statistics of computations were peformed and carried out based on average values of all data.

The OMNIDIA software (Lecointe et al., 1993) was used to calculate the two diatom indices IPS and DAIpo. In the OMNIDIA database, two numbers are attached to each diatom species: a sensitivity number (S) and an indicator number (V). The S ranges from 1 (very tolerant species) to 5 (very sensitive species), the V ranges from 1 to 3 according to their ecological significants. Diversity index was also calculated with the OMNIDIA package. Taxonomic differences of periphytic diatom communities between stations during experiment were described using Principal Component Analysis (PCA) with PC-ORD Software (McCune and Mefford, 1999) or SPAD software (v. 5.6, Decisia, Paris, France) on data concerning relative abundances of diatom species.

To reveal relationships between environmental factors and diatom communities collected during experiment, we chose Canonical Correspondence Analysis (CCA). CCA was carried out with Multivariate Statistical package (MVSP, Kovach Computing Services, 2004) on data concerning relative abundances of diatom species and environmental variables (pH, conductivity, turbidity, DO, temperature, SPM, salinity, $N\text{-}NO_3$, $N\text{-}NO_2$, $N\text{-}NH_4$, $P\text{-}PO_4$, TIC, TOC, BOD, COD, Cu, Cd, Pb, Zn, Chlorophyll a). CCA technique allows determination of the environmental variables which are most highly correlated to species distributions.

To classify sampling stations, or classify similarity in taxonomic composition of diatom communities, cluster analyses using a single linkage method with Euclidean distance measured with STATISTICA software (StatSoft, 2004) or using PC–ORD software (McCune and Mefford, 1999) were carried out based on relative abundance of diatom species. For all statistics, highly cumulative relative abundances of species were taken into account. In order to find relation between Cd accumulated in biofilm and diatom composition, Pearson correlation matrix between Cd accumulation levels in biofilms and relative abundances of the 20 most abundant diatom species were performed by using PCA, SPAD software (v. 5.6, Decisia, Paris, France). Significant differences in number of diatom cells, species richness, diversity index, diatom indices, dry weigh (DW), ash free dry mass (AFDM), metal accumulation of periphytic samples between stations and colonization time were analysed using one-way or two way analysis of variance model ANOVA, STATISTICA software, after checking assumptions (normality and homoscedasticity of the error term). If a significant effect was observed, we performed *post-hoc* tests (Least Significant Difference test (LSD) and Newman-Keuls test) to isolate significant differences between groups. For all statistical results, significance effects were considered at 0.05 level.

Despite the important roles of diatom and their utility in evaluating and monitoring environmental changes in aquatic ecosystems, no background in floristic or taxonomic studies on freshwater diatoms in Vietnam exists. In order to enhance knowledge of periphytic diatom flora in Vietnam and their distribution and evolution, several experiments were carried out along rivers pollution gradient in the Red-Nhue-Tolich Rivers. They aimed to assess water quality of these hydrosystem through the evolution of periphyton diatom communities as a potential indicator and to examine whether diatom indices developed in several countries could be applied in Red, Nhue and Tolich rivers. Experiments were supported through the ESPOIR project (CNRS-VAST)[2]. The principal objectives of the project included particularly an assessment of the urban hydrosystem around Hanoi i.e. the Nhue-Tolich river hydrosystem, and development of new processes for water treatments (Le, 2005). It has gathered various research groups which have cooperated such as Institute of Geography (VAST) (Director: Ngo, N. C); Institute of Chemistry (VAST) (Directors: Le, L. A; Le, Q. H); Institute of Biotechnology (VAST) (Directors: Dang, D. K; Dang, C. H), Institute of Natural Product Chemistry (VAST) (Director: Chau, V. M), Institute of Ecological and Biological Resource (VAST) (Director: Dang, T. A); LEESA (recently named GEMA[3]) UMR 5805 EPOC, Université Bordeaux 1, CNRS, (Director: A. Boudou and J. C. Massabuau); Sisyphe[4], CNRS-UMR 7619, Université Paris VI (Director: J. Garnier); LTHE-HMG-INPG, CNRS - UMR 5564[5] (Director: G. Vachaud).

The results of the first part of this study are presented in the chapters 3, 4 and 5.

[2] CNRS: Centre National de la Recherche and VAST : Vietnam Academy of Science and Technology
[3] GEMA: Equipe Géochimiques et Ecotoxicologie des Métaux dans les systèmes Aquatiques
[4] Sisyphe : Structure et fonctionnement des systèmes hydriques continentaux
[5] LTHE: Laboratoire d'Etude des Transferts en Hydrologie et Environnement

CHAPTER 3

Dynamics of diatom colonization process in some rivers influenced by urban pollution (Hanoi, Vietnam) *

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Key words: colonization; benthic diatom; artificial substrate; diatom indices; pollution; water quality

* Chapter is accepted as an article in Ecological Indicators Journal

3.1 Abstract:

Periphytic diatom communities' colonization patterns were studied at three sampling stations of the Red-Nhue-Tolich hydrosystem presenting different urban pollution levels by using artificial substrates for six weeks in dry season 2005. Structural characteristics of periphytic diatoms developed on glass substrates at each sampling site were followed and compared. This experiment showed, through various general criteria (total diatom density, dry weight biomass) and specific criteria (relative diatom abundances, indices), that the structure of benthic diatoms developed on these substrates was strongly affected by pollution as early as the second week of colonization. Communities exposed to heavily and moderately polluted sites contained species which are known to be saprophilous or tolerant to organic pollution such as *Nitzschia umbonata, Nitzschia palea, Cyclotella meneghiniana, Eolimna minima*. Growth inhibition of diatom communities at the heavily polluted site was mostly related to a strong increase of organic load rather than to low metallic input, though metallic (Cd and Zn) burdens have been successfully quantified in the biofilms developed at the three studied sites. Nevertheless, no significant difference in species richness and diversity index between colonization duration times was observed. Based on values of diatom indices IPS (Specific pollution-sensitivity index) and DAIPo (Diatom assemblage index to organic water pollution), water quality could be classified as medium quality at Red site, polluted at NT_2 and heavily polluted at TL. Thus, the use of diatoms as a tool for water assessment appears suitable for monitoring rivers in Vietnam as it is in several other countries.

3.2 Introduction

Benthic algae are an important part of the biofilm that coats the upper surfaces of substrates in wetlands and streams. Biofilm consists of a compact association of benthic algae, bacteria, fungi, protozoa, and their secretory products such as extracellular polymeric substances (EPS) and inorganic particles embedded in a slimy matrix (Sekar et al., 2002). Benthic diatoms as an abundant component of biofilms are therefore an important primary source of energy for aquatic food webs in many streams and rivers. They are microscopic unicellular algae characterized by high species diversity (Stevenson et al., 1996; Stevenson and Pan 1999). They grow in a wide range of habitats (soils, lakes, rivers and seas) and in very variable environmental conditions from pristine waters to extreme environments (Elster et al., 2001). Thus, they are an excellent ecological indicator at the species level, sensitive to a number of environmental variables including light, temperature, inorganic nutrients (carbon, phosphorous, nitrogen, silica, iron), organic carbon, organic nitrogen,

oxygen concentration, pH, salinity (Whitton and Rott, 1996). Besides their species behavior towards many environmental factors, the structure of benthic diatom communities in streams is conditioned by other factors such as the nature of the substrate where they develop (Potapova and Charles, 2005), current velocity (Stevenson, 1983; Peterson and Stevenson, 1989), nutrient concentrations (Biggs et al., 1998) and temperature (DeNicola, 1996) within the aquatic environment. Animal presence (small gastropods, oligochaete, and animal micro organisms) responsible for grazing activity may also be episodically noticed in biofilms (Steinman, 1996). Variations of all these factors result in differences in the composition of benthic communities.

River pollution is becoming a critical issue of water management in Vietnam, especially in large urban and industrial cities. Numerous rivers in urban areas of Vietnam have been used for the disposals of both solid wastes and wastewaters, usually untreated, and are consequently seriously polluted. This high pollution status threatens and, in many cases, has already altered the ecological balance of the rivers in Vietnam. The increases in urbanization, industrialization and the use of agrochemicals have caused considerable impact on the river system (Pham et al., 1995). The main sources of river pollution in Vietnam in general and in the Hanoi areas in particular can be classified as urban, domestic, industrial and agricultural. Associated with high concentrations of nutrients (phosphates, ammonium) in rivers which have received untreated wastewater from domestic and industrial activities (Le, 2005), the accumulation of heavy metals in river sediments has been observed (Ho and Egashira, 2001). It is supposed that the considerable accumulations of the six metals Zn, Cd, Cr, Ni, Cu, and Pb in river sediments derive from regular industrial discharges (Ho and Egashira, 2001).

It has long been recognized that the use of diatoms as a tool for monitoring river quality has been applied in many countries. The responses of diatoms to many types of pollution (acidification, eutrophication, organic pollution, metallic pollution) have been observed for a long time (van Dam, 1996; Kelly, 1998; Potapova and Charles, 2005; Rott et al., 1998; Gold et al., 2002; Ivorra et al., 2000; Sabater et al., 2003) and biotic indices have been developed to estimate water quality in European countries and Asian countries (Whitton and Rott, 1996; Watanabe et al., 1986).

Given the sensitivity of diatom communities to environmental parameter modifications due to pollutant or non pollutant conditions, in this study we propose i) to follow the dynamic of the colonization process of diatom communities from its initial stage in three sites of the Red-Nhue-Tolich hydrosystem influenced by urban pollution from the Hanoi area. Long term effects of pollution (six weeks) on the diatom colonization process were studied on artificial glass substrates in order to compare and estimate their growth from a reliable quantitative and qualitative point of view. Such studies in Vietnam rivers are just emerging, although studies involving artificial

substrates have been proven to be comprehensive and of great interest elsewhere (Biggs, 1988; Gold et al., 2003; Potapova and Charles, 2005); ii) to determine the optimal exposure duration of artificial substrates in natural hydrosystems according to the level of pollution and through various general (total diatom density, dry weight biomass) and specific criteria (relative diatom abundances, indices) and thus obtain valuable information on the perturbations occurring in the colonization process, and iii) to present a first quantification of metallic concentrations in biofilms through metal concentration analyses. Metal concentrations in biofilms could thus be considered as a new alternative criterion to assess the incorporation of metallic pollutants within biological microorganisms and aquatic food webs.

3.3 Materials and methods

3.3.1 Study area

The case study was carried out in the Red, Nhue and Tolich rivers, which are located in the Hanoi urban areas (the Red River delta). Red River is the largest river in the Northern part of Vietnam. Its role in the country's agricultural production is as important as that of the Mekong River in the South. The Nhue River, a tributary of the Red River, diverges from it at the Thuy Phuong dam, and receives wastewater from the Tolich River through the Thanh Liet dam. The Nhue River, which serves as a major irrigation and drainage canal for the surrounding Hanoi and Hatay areas, runs through the Hanoi area, Hadong city (Hatay province) and Hanam province *via* several dams and finally flows into the Day River. The Nhue River basin area is 74 km long and covers a total catchment area of 107,503 ha of which 75 percent is cultivated (Trinh, 2003; Kono & Doan, 1995). Tolich River originates in West Lake, flows across Hanoi city 14 km to the south before joining the Nhue River. It receives directly all the urban wastewaters from the Hanoi city (domestic and industrial wastewaters) (Trinh, 2003).

Three study sites presenting different pollution levels were selected in this study area according to previous studies conducted along the same hydro-system (Duong et al., 2006a) (Figure 3.1): (i) the comparatively unpolluted reference site (Red) situated at Son Tay (Son Tay district, Haytay province) on the Red River, about 8 km upstream from the junction with Nhue River, and characterized by low nutrients and metal concentrations (Le et al, 2005); (ii) the polluted site (TL) located upper from the Thanh Liet dam (TL *dam*) on Tolich River, receiving major discharges and characterized by black, foul-smelling waters; (iii) the moderate polluted site (NT_2) located on the Nhue River about 7 km downstream from the junction with the Tolich River.

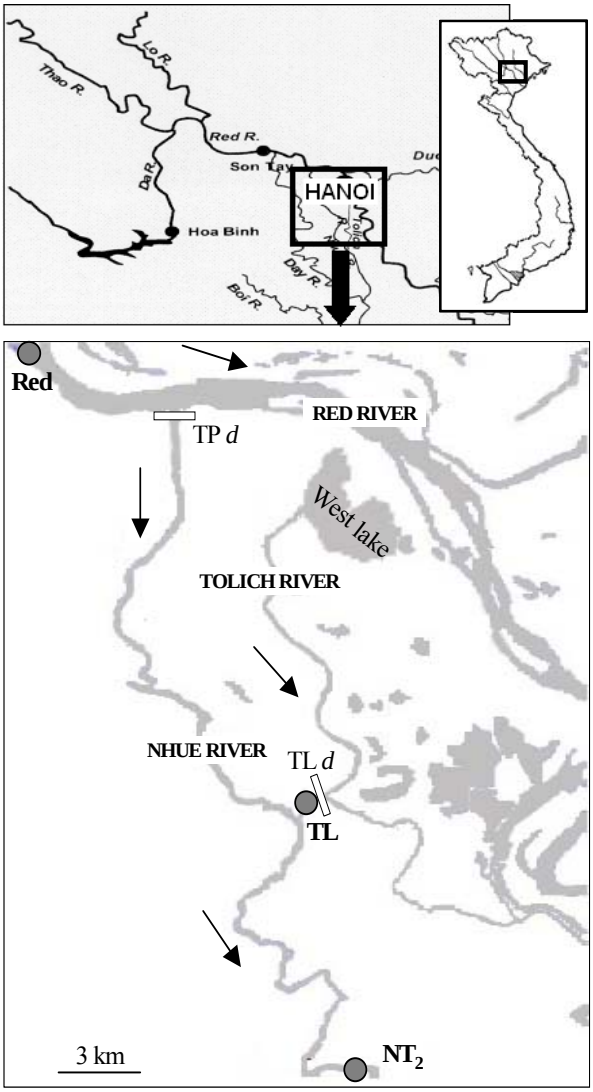

Figure 3.1: Location of sampling stations (*TPd*: Thuy Phuong dam; *TLd*: Thanh Liet dam).

3.3.2 Experimental set-up, sampling procedure and water analyses

The experiment was carried out during the dry season from 9th January to 20th February 2005. During this season, all three rivers are connected, dams being kept open to provide normal irrigation in the basin for agricultural purposes. However, during the 2005 dry season (from November 2004 to April-May 2005), the water level of the Red River was low and water support to the Nhue River was quite limited. At each site, a plastic basket equipped with floaters, containing 18 separate vertical glass substrates (30 x 18 cm, 540 cm² surface for both sides), as an artificial substrate for algal attachment, was immersed in the water column, parallel to the current, at a depth of 15-20 cm below the surface, and tied to the bank with a rope (Gold et al., 2002). To study the

colonization process of periphytic benthic diatoms, the glass substrates, all subject to similar light and current conditions within the basket, were sampled every week for six weeks. At every sampling date and at each site, three glass slides, considered as independent samples, were randomly removed from the basket. They were then thoroughly rinsed with filtered river water. Biofilms were collected from the glass slides using a nylon brush, washed with distilled water and diluted in a known volume (100 or 200 mL) of distilled water depending on the biofilm thickness. In order to determine biofilm dry weight, a known volume of samples was stored in a labelled polyethylene bottle and placed in a cool (4°C), dark place during transportation to the laboratory. The remaining natural biofilm samples were preserved in a labelled polyethylene bottle with 5% formalin solution (Formaldehyde 37%, Prolabo, France) for delayed diatom identification.

Water temperature, dissolved oxygen, conductivity, and pH were measured in the field by multi-parameter sensors (Model WQC-22A, TOA) during the experiment. Water samples were analyzed in the laboratory for NH_4^+, NO_3^-, NO_2^- and PO_4^{3-} (APHA, 1995). Nutrient analyses were performed by the Institute of Natural Products Chemistry (VAST - Vietnamese Academy of Science and Technology, Hanoi, Vietnam).

3.3.3 Specific laboratory analyses

Diatom preparation

In the laboratory after homogenization, 2 mL aliquot of each diatom sample from each site was heated with hydrogen peroxide (30 %) and hydrochloric acid (35 %) to remove organic matter and dissolve calcium carbonates. The cleaned frustules were then mounted on a microscope glass slide in a high refractive index medium (Naphrax, Northern Biological Supplies Ltd, UK; RI = 1.74) (Gold et al., 2002; Charles et al., 2002). Up to 400 diatom valves were identified and counted in each sample using a Leitz DMRD light microscope at 1000 X magnification. Krammer & Lange – Bertalot' floras (1986-1991) were used as reference for identification. Relative abundances of the diatom species (in percentage) were estimated. Species richness (S) was calculated and biological diversity was estimated using the Shannon-Weaver index (H') (Shannon and Weaver, 1963).

A Nageotte counting chamber (Marienfeld, Germany) was used to estimate total diatom density in each sample by counting the total number of diatoms in 30 fields (1.25 µL each, 0.5mm depth) using a light microscope (Olympus BX 50) at 200x magnification. Data are expressed in cells per unit area of artificial substrate (cells.cm $^{-2}$).

Biofilm manipulations to determine weight and metal concentration

Samples of biofilm suspensions were filtered with an aspiration pump, through a tared metal-free filter paper (0.45 µm membrane, Millipore) to obtain the dry weight (dw) of each sample after drying at 60°C for 48 hours in incubation tubes (values expressed as mg $dw.cm^{-2}$). Acquired dry weight filters were digested for metal analysis, by nitric acid (3 mL of pure HNO_3, Merck, Darmstadt, Germany) in a pressurized medium at 100° C for 3 hours (hot block CAL 3300). Digestates were then diluted up to 23 mL with ultra-pure water (Milli Q, Bedford, MA, USA), and metal concentrations in the biofilms were measured by flame atomic absorption spectrometry (Varian AA20), with detection limits of $10\mu g.L^{-1}$ for Zn and $15\mu g.L^{-1}$ for Cd. The validity of the method was checked periodically with certified biological reference materials (Tort 2-lobster hepatopancreas and Dolt 2: dogfish liver from NRCC-CNRC, Ottawa, Canada); values were consistently within the certified ranges (data not shown).

3.3.4 Data treatment

Diatom indices IPS (Cemagref, 1982) and DAIPo (Watanabe et al., 1986), often used in western countries to determine biological water quality, were calculated using the OMNIDIA software (Lecointe et al., 1993). Diatom indices were transformed to range from 1 to 20 for comparability. Statistical computations were performed with STATISTICA software (StatSoft, 2004) using one or two way analysis of variance model (ANOVA) to reveal the effect of time (weeks) and stations (Red, NT_2 and TL) on total diatom density, diatom indices and biofilm dry weight. If a significant effect was observed, *post-hoc* tests (Least Significant Difference test (LSD) and Newman-Keuls test) were performed to isolate the significantly different station. Significance effects were considered at $p \leq 0.05$ level. A principal component analysis (PCA) was performed using PC-ORD Software (McCune and Mefford, 1999) on data concerning relative abundances of diatom species to display taxonomic differences between the three sites. In order to reduce the effects of high variable population densities on ordination scores and minimize the influence of rare taxa, only taxa presenting relative abundance ≥ 1% were included in the analyses.

3.4 Results

3.4.1 Physical and chemical characteristics of water in the study area

Physical and chemical characteristics of water at the three sampling sites Red, NT_2 and TL are shown in table 3.1. Average values are expressed, with physical and chemical parameters remaining fairly stable at each site during the six weeks experimental period. pH values do not show significant differences, although they present values slightly inferior to neutral pH.

Parameters	Red	NT_2	TL
pH	6.7 (6.5 – 7.5)	6.9 (6.7 – 7.2)	6.3 (6.2 – 6.5)
O_2 (mg L^{-1})	7.3 (6.9 – 8.1)	3.12 (2.7 – 4.6)	2.06 (1.7 –2.8)
Conductivity (µS/m)	19.5 (16.4 – 29.5)	52.2 (37.8 –73.3)	75.3 (63.6 – 98.6)
Water temperature °C	18.5 (18.1 – 20)	20.9 (19.5 – 21.4)	20.8 (19 – 21)
NH_4-N (mg L^{-1})	0.18 (0.1 – 0.2)	5.9 (2.2 – 7.8)	26.8 (24.5 – 28.7)
NO_3-N (mg L^{-1})	0.7 (0.6 – 0.8)	1.2 (0.9 – 1.7)	2 (1.2 – 2.5)
NO_2-N (mg L^{-1})	0.014 (0.009 – 0.02)	0.05 (0.01 – 0.08)	0.024 (0.013 – 0.04)
PO_4-P (mg L^{-1})	0.16 (0.15 – 0.18)	1.7 (0.96 – 2.66)	3.17 (1.89 – 6.7)
Dissolved Cd (µg L^{-1})*	0.32 (0.21 – 0.5)	0.35 (0.3 – 0.4)	0.99 (0.91 – 1.18)
Dissolved Zn (µg L^{-1})*	13.3 (10 – 19)	20 (9 – 36)	34.3 (20 – 48)

Table 3.1: Physical and chemical characteristics of the water from the three sampling sites: Red, NT_2 and TL during (January - February 2005) period (average values and min - max values, n = 6). (* Duong et al., 2006a).

Lower values of dissolved oxygen are found at NT_2 and TL sites (3.12 and 2.06 mg. L^{-1} respectively) whereas the Red site value is higher by a factor of two to three.Water shows low thermic amplitude between sites (18.5 - 20.9 °C), but conductivity is 2 to 3 times higher in the TL and the NT_2 sites respectively, than in the Red site; ammonia, nitrate, nitrite and phosphate average concentrations showed similar trends. Dissolved metal concentrations (Cd and Zn) measured in the water are not high (Duong et al., 2006a); however, the TL site presents the highest values followed by the NT_2 and then Red site.

3.4.2 General criteria to study periphytic diatom communities

Diatom total density, dry weight and metal concentration in biofilms were taken into account to give a general pattern of the development and of the characteristics of diatom communities on biofilms during the six weeks of the experiment in the study area. After week 1, at the Red and NT_2 sites, artificial substrates were uniformly but sparsely coated by a thin layer of

biofilm composed of detritus, algae, bacteria, and suspended particular matter, and the glass substrate surface was still visible through the film. Total diatom densities (Figuge 3.2) reached at that time were similar at both sites (Red site: 622 ± 34 cells.cm^{-2} and NT_2 site: 673 ± 128 cells.cm^{-2}). From week 2 to the end of the experiment (week 6), the development of diatom density increased quickly with the glass substrates being densely covered, and their surfaces no longer visible. However, from the beginning of colonization until the end of the experiment the increase in diatom density was more marked at the Red site than at the NT_2 site, with the highest value found in week 6 at the Red site (27,851 ± 2,235 cells.cm^{-2}) in comparison with the NT_2 site (20,740 ± 2,492 cells.cm^{-2}). Moreover, at the NT_2 site, diatom density growth appeared slower during the first three weeks of colonization, then suddenly increased to reach a maximum value at week 6, close to the Red site levels. The TL site is distinctive, with poor growth during the whole experiment from 258 ± 48 cells.cm^{-2} (first week) to 872 ± 132 cells.cm^{-2} (last week) after six weeks of colonization, developed in a tenuous black transparent layer. Significant differences ($p < 0.05$) in cell densities are found between colonization time and sites, according to the two-way ANOVA results.

Biofilms collected on glass substrates provided sufficient biomass, and their dry weights showed distinct developments at the three sites (Figure 3.3). Biofilms collected on glass substrates provided sufficient biomass, and their dry weights showed distinct developments at the three sites (Figure3.3). Significantly high values at the Red site and overall at NT_2 site versus very low values at the TL site were observed. At the TL site, biofilm dry weights stayed low throughout the experiment (< 1mg.cm^{-2}), but still increased slightly. At the Red and NT_2 sites, during the first week of exposure, biofilm developments were low (respectively 0.32 ± 0.03 mg.cm^{-2} and 0.2 ± 0.05 mg.cm^{-2}), then increased sharply at week 2. While biofilm development was maintained approximately at this level at the Red site (between 1.49 ± 0.08 mg.cm^{-2} at W_4 and 2.52 ± 0.09 mg.cm^{-2} at W_6), it continued to increase at a high rate at the NT_2 site during W_3 (2.3 ± 0.6 mg.cm^{-2}) to reach its peak values and stabilize (7.92 ± 0.5 mg.cm^{-2}) at W_5.

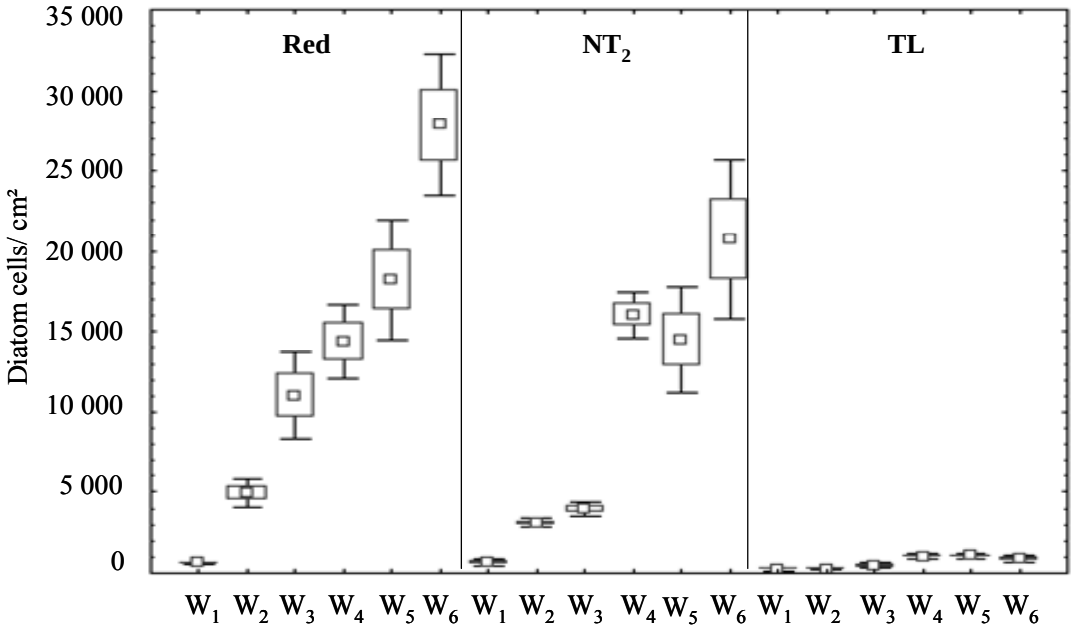

Figure 3.2: Evolution of diatom density on artificial substrates (cells.cm $^{-2}$) during 6 weeks (W_1 to W_6) colonization at the three stations: Red, NT_2 and TL. Boxplot: mean value (n = 3) with its confidence interval at 95 % denoted by the two horizontal lines outside the box.

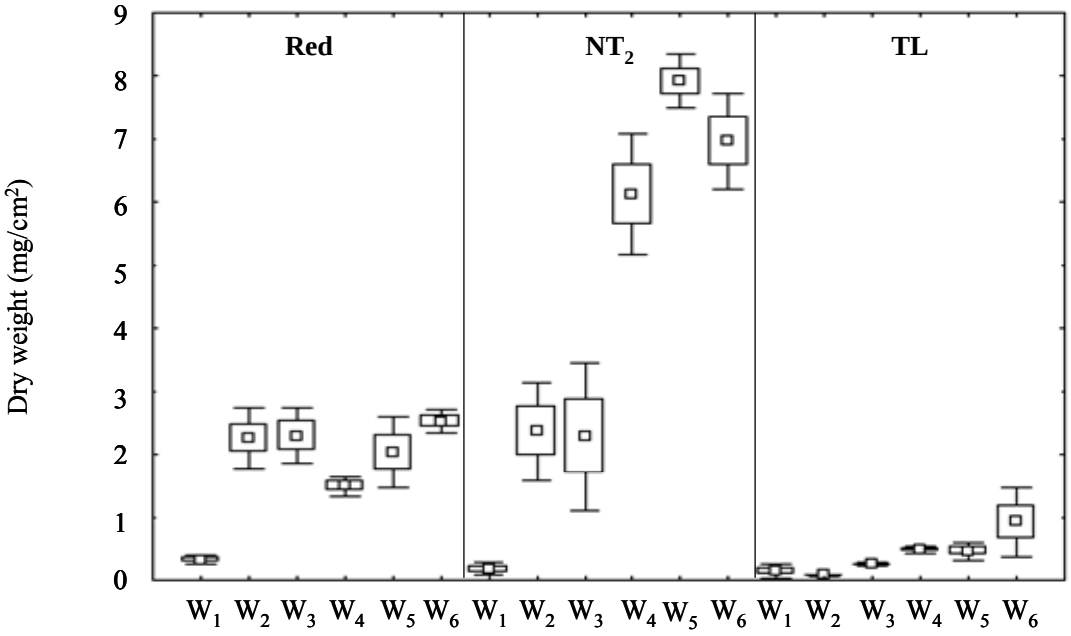

Figure 3.3: Dry weight of biofilms collected from Red, NT_2 and TL after 1, 2, 3, 4, 5, 6 weeks (W) of colonization. Boxplot: mean value (n = 3) with its confidence interval at 95 % denoted by the two horizontal lines outside the box.

Although sufficient biomass was developed on substrates, it proved necessary to devise a method for metal concentration analyses within such light biomass biofilm developments. Accumulation of Cd and Zn by the biofilms (per unit dry weight, dw) at the Red, NT_2 and TL sites was successfully analyzed and results are presented in figure 3.4.

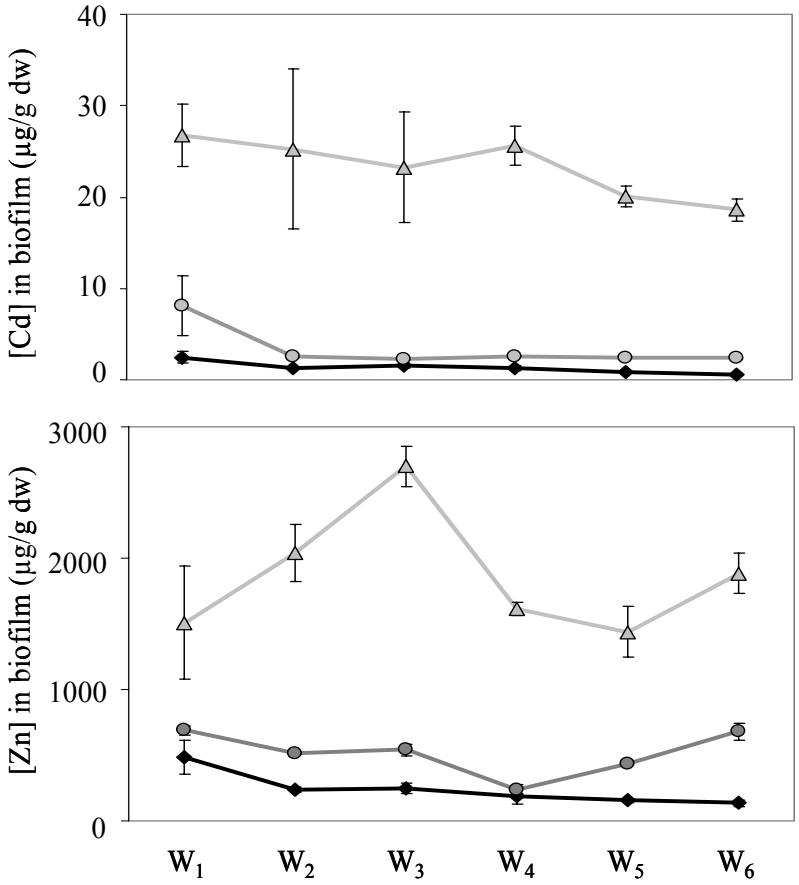

Figure 3.4: Cd and Zn concentrations in biofilms developed on glass substrates after 1, 2, 3, 4, 5, 6 weeks (W) of colonization at Red, NT_2 and TL sites. (mean ± standard deviation; n = 3).

Cd concentrations at the Red and NT_2 sites present an average value of 1.34 ± 0.37 $\mu g.g^{-1}$ and 3.4 ± 1.33 $\mu g.g^{-1}$ respectively, significantly different from the TL site value of 23.2 ± 1.89 µg/g; but concentrations do stay constant in the three sites during the six weeks of the experiment. Zn concentrations in biofilms at the three sites show a similar trend, with high concentrations being measured at the TL site (1,863.4 ± 270 $\mu g.g^{-1}$ dw) and lower values estimated at the NT_2 and Red sites (517 ± 96 $\mu g.g^{-1}$ and 241± 73 $\mu g.g^{-1}$ dw respectively), that i.e. 3.5 times less at the NT_2 site and 7.7 times less at the Red site in comparison with the TL site.

3.4.3 Specific criteria to study the composition of the periphytic diatom communities

The features of diatom communities at the three sites and during the whole experiment are presented in figure 3.5a and b by their species richness (S) and Shannon-Weaver diversity index (H'), with IPS and DAIPo indices results.

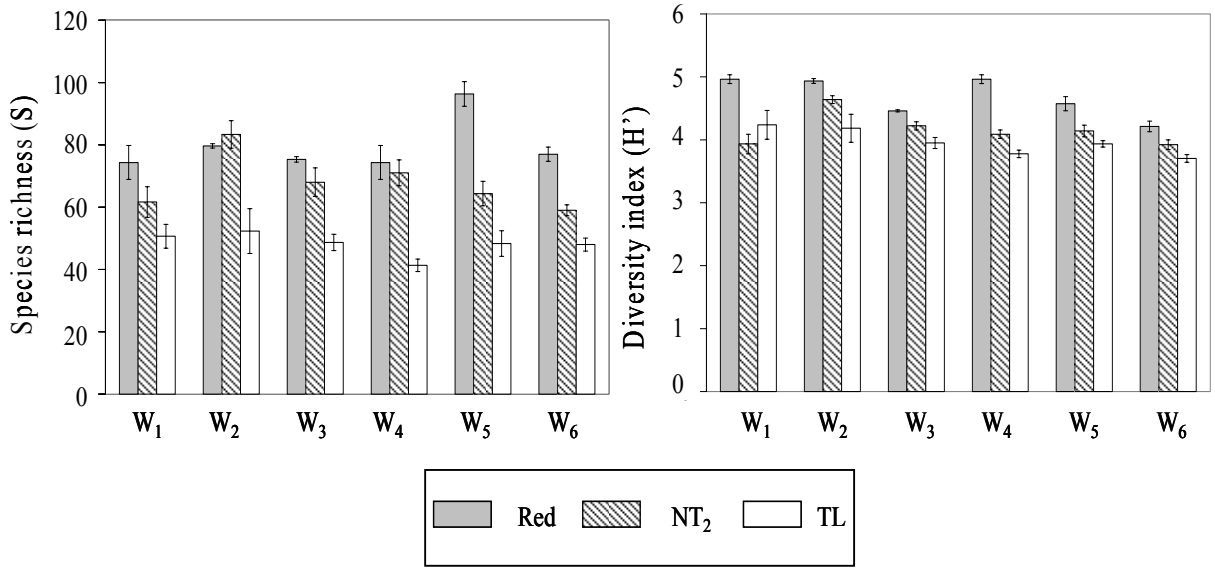

Figure 3.5 a: Species richness and diversity index of diatom communities at Red, NT_2 and TL sites (mean ± standard deviation; n = 3) after 1, 2, 3, 4, 5, 6 weeks (W) of colonization

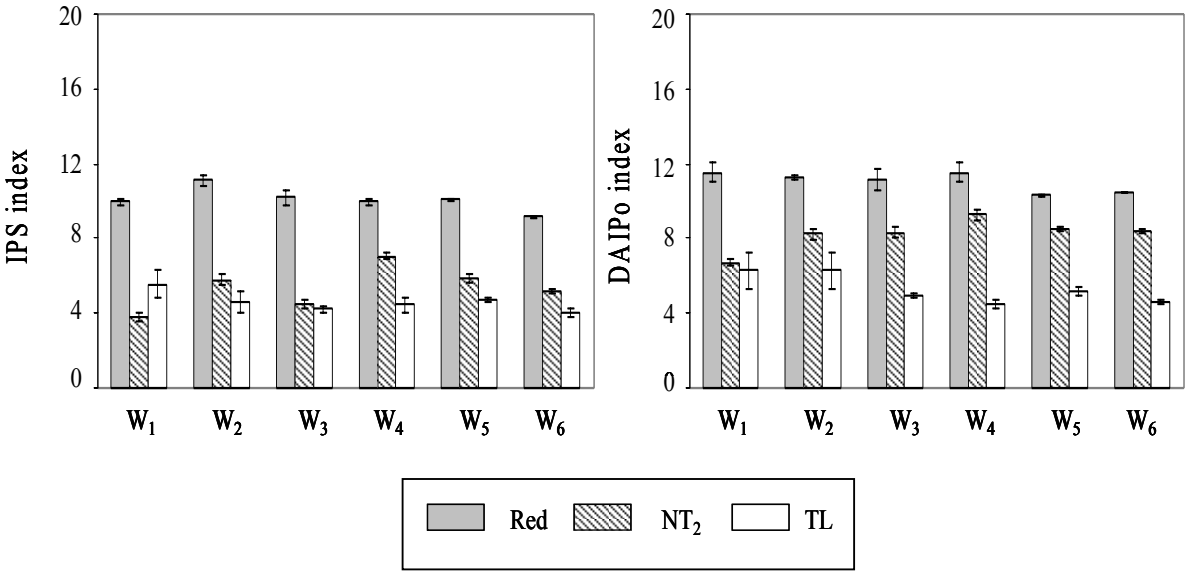

Figure 3.5 b: IPS and DAIPo index of diatom communities at Red, NT_2 and TL sites (mean ± standard deviation; n = 3) after 1, 2, 3, 4, 5, 6 weeks (W) of colonization.

There is no significant fiffirence in S between stations was observed ($p<0.05$). Nevertheless, a significant difference in S between stations was observed ($p < 0.05$) with higher values of S at both Red and NT_2 sites (from 74 to 96 at Red site, and from 59 to 83 at NT_2 site), where more species were found, whereas these values ranged from 41 to 52 at TL site. During the succession development, Shannon-Weaver diversity index with H' around 4.3 is not particularly high.

Based on the values of diatom indices, water quality at the three sampling sites is estimated (Figure 3.5b). Both diatom indices are almost constant at the three sites throughout experiment. Water quality at the comparatively unpolluted reference site (Red) could be characterized as medium, with IPS index varying from 9.1 to 11 and DAIPo index ranging from 10.3 to 11.5. Heavily polluted water quality is recorded at TL with mean values of 4.6 (IPS) and 5.3 (DAIPo) and polluted water quality is determined at NT_2 with mean values of 5.4 (IPS) and 8.2 (DAIPo).

In this investigation, 264 diatom taxa from 58 genera were identified through all the attached diatom assemblages collected. Temporal variations in relative abundances of seven major periphytic diatom species (≥ 6%) are shown in figure 3.6. At the Red site, *Navicula recens* (NRCS) is dominant during the first week (11.2%) and increases significantly throughout the colonization process to reach a contribution of 32.1 %. *Bacillaria paxillifera* (BPAX) appears as early as W_1 and reaches 15.5% at W_6 while *Gyrosigma scalproides* (GSCA) shows a reverse development and decreases to 2% during the second half of the experiment. At NT_2 and TL sites, *Nitzschia palea* (NPAL), *Nitzschia umbonata* (NUMB), *Cyclotella meneghiniana* (CMEN) and *Aulacoseira granulata* (AUGR) are relatively stable during the six week experiment, except *Nitzschia palea* (NPAL) at W_1 in NT_2 site with a larger proportion. These species are also present at the Red site but in low proportions. Differences between the NT_2 and TL sites are perceived in the proportion of these 4 species with *Nitzschia umbonata* (NUMB) (from 17.8% to 20.7%) and *Aulacoseira granulata* (AUGR) (from 3.9% to 9.2%) overriding at the NT_2 site and *Nitzschia palea* (NPAL) (from 19.2% to 28.3%) and *Cyclotella meneghiniana* (CMEN) (from 11.4% to 18.3%) at the TL site.

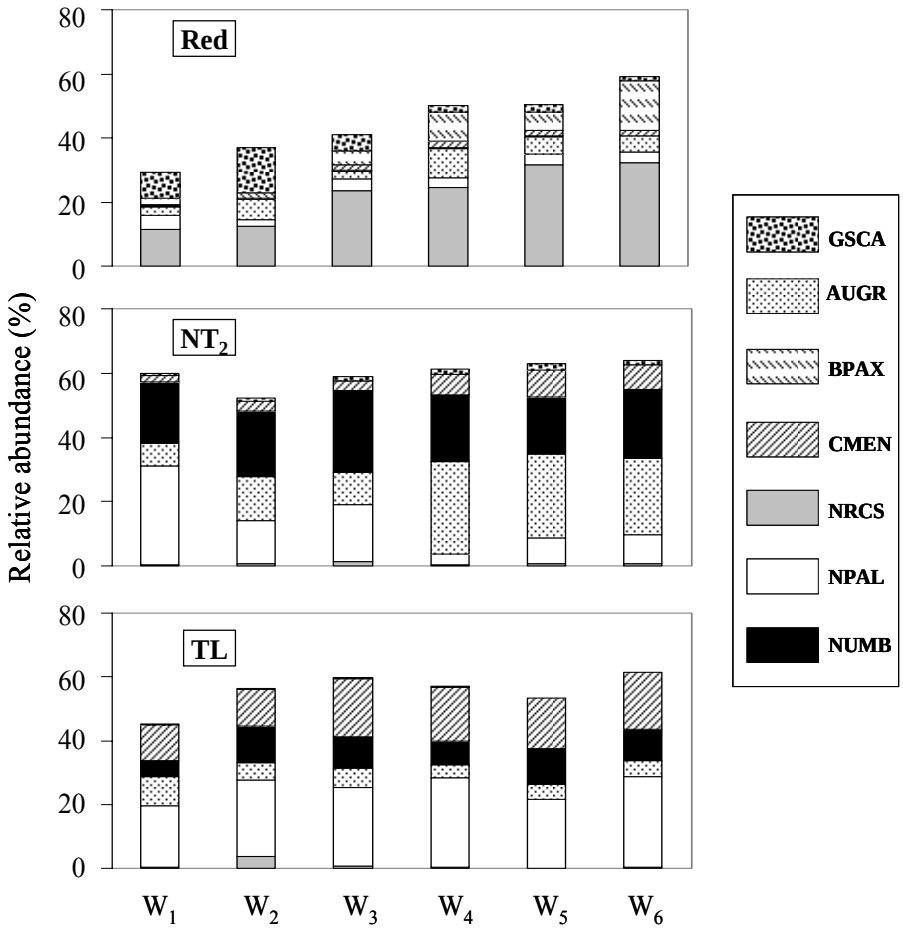

Figure 3.6: Relative abundances of diatom species (mean values, n = 3) with relative abundances ≥ 6 % after 1, 2, 3, 4, 5, 6 weeks (W) of colonization at Red, NT_2 and TL stations. (NUMB: *Nitzschia umbonata*, NPAL: *Nitzschia palea*, AUGR: *Aulacoseira granulata*, GSCA: *Gyrosigma scalproides*, NRCS: *Navicula recens*, CMEN: *Cyclotella meneghiniana*, BPAX: *Bacillaria paxillifera*.)

Of the 264 diatom taxa identified in this investigation, 55 taxa, with relative abundance ≥ 1%, were included in data analysis using Principal Component Analysis (PCA) (Figure 3.7). Results of the PCA enable us to relate diatom distribution to diatom succession during the colonization experiment at the three sites. The first two axes account for 54 % of the variance and ordination clearly separates the three sites, characterizing three different diatoms community structures. The first axis (accounting for 34 % of variance) separates diatom communities in their six week colonization process with the Red site in the half negative axis side, and the TL site in the positive side. Axis 2 (accounting for 19 % variance) isolates diatom communities from the NT_2 site. Including the 7 major diatom taxa mentioned above, figure 3.7 points out groups of taxa such as *Navicula recens* (NRCS), *Seminavis strigosa* (SMST), *Bacillaria paxillifera* (BPAX),

Achnanthidium minutissimum (ADMI), *Gyrosigma scalproides* (GSCA) which characterize the Red site too. At the NT_2 site, *Nitzschia umbonata* (NUMB), *Navicula veneta* (NVEN), *Aulacoseira granulata* (AUGR), *Navicula cryptocephala* (NCRY) and *Fragilaria ulna varacus* (FUAC) are grouped. The TL site, which mainly contains dominant taxa such as *Nitzschia palea* (NPAL), *Lemnicola hungarica* (LHUN) and, *Nitzschia clausii* (NCLA), also shows some plankton diatom species such as *Cyclostephanos invisitatus* (CINV) and *Cyclotella atomus* (CATO) which are well presented at this site.

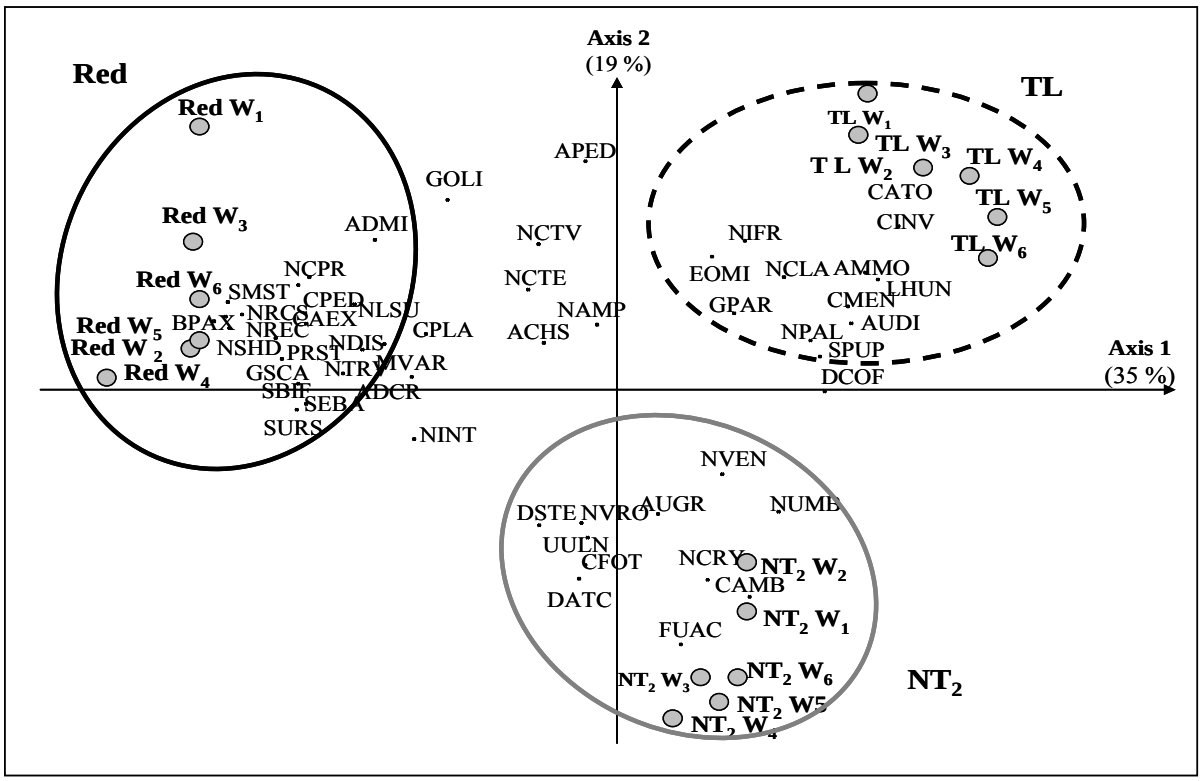

Figure 3.7: Principal Component Analysis (PCA) based on relative abundances of 55 diatom species (relative abundance > 1% and 3 replicates per site) from the three sites: comparative unpolluted (Red), moderate polluted (NT_2) and heavy polluted (TL) during 6 weeks of colonization. Colonization duration is indicated on the graph (weeks; W_1, W_2, W_3, W_4, W_5, and W_6).

3.5 Discussion

This investigation showed a diversity of structural diatom communities between rivers and displayed the diatom indicator species groups for each river. In addition, two diatom indices (IPS and DAIpo) which have been widely used in many countries were successfully applied for the first

time to assess water quality of the three rivers in Vietnam. In this study, the dynamics of diatom colonization were followed over a 6-week period and a minimum of two weeks colonization has been shown to be sufficient and relevant, as colonization duration did not appear as a discriminating criterion in the results. This rather short period reveals the main characteristics of the diatoms communities at each selected site, since the structure of diatom assemblages developed on glass slides remained quite stable, after six weeks at the three levels of pollution, despite a large increase in their densities. This is confirmed by Hoagland et al. (1982) who consider that mature communities can be established in equilibrium with the environment from two weeks; Biggs (1988), however, suggests that four weeks are suitable for periphyton biomass in enriched rivers. Biofilm colonization was rapidly visible and quantifiable as early as week 1, with a thin layer on artificial substrates forming a mucilage biofilm, enhancing diatom immigration rate (Stevenson 1983). After week 2, a thick brown-yellow layer covered the glass surfaces at the Red and NT_2 sites. Nevertheless, colonization rate proceeded slowly at NT2 for the first three weeks, before showing a marked increase related to a high growth rate of *Aulacoseira granulata* (AUGR) from week 4 until the experiment ended. This growth of colonial planktonic taxa *Aulacoseira granulata* (AUGR) at NT_2, concomitant with a severe decrease in *Nitzschia palea* (NPAL) development at this site could be explained by the passive trapping of the dominant planktonic taxa (AUGR) on the slides among mucilaginous, filamentous algae and bacteria. AUGR was also found to be dominant in the phytoplankton composition of the Nhue River (Duong et al, unpublished). Stevenson and Peterson (1989) noted that benthic algal succession has been hypothesized to be a change from species which rapidly colonize but are not competitive, to species of higher competitive abilities but lower immigrating abilities. Succession in diatom species composition during the colonization of artificial substrates could also be caused by shifts in dominance from species with rapid migration rates to dominance by species with high reproductive rates. Hoagland et al. (1982) describe three stages in diatom assemblage succession where development of an adnate species layer attached to substrate surface was the first stage of diatom community development. *Nitzschia palea* (NPAL) can be assumed to correspond to some of these criteria with its strong capability to colonize new substrate forming a new living layer which can host new species like the plankton *Aulacoseira granulata* known for its high productive rate. This competition shift was not observed at the heavily polluted site TL, where the evolution of diatom density was very different from the Red and NT_2 sites, with low diatom density observed after six weeks of colonization (Figure 3.2). There, the glass slides were coated by a black layer containing three main diatom species: *Nitzschia palea* (NPAL), *Cyclotella meneghiniana* (CMEN) and *Nitzschia umbonata* (NUMB) (Figure 3.6). Throughout the experiment, the development of these diatom species was stable at the TL site, due

mostly to high concentrations of nutrients and other contaminants as well as low dissolved oxygen (Trinh, 2003; Le, 2005), all these factors contributing to inhibit diatom growth at this site.

Diatom species considered as characteristic of each of the three sites from figure 6 are also indicated in PCA results (Figure 3.7), and are in agreement with the assemblages described in Duong et al. (2006). The diatom assemblages at the Red site were characterized by halophilous taxa such as *Navicula recens* (NRCS), *Bacillaria paxillifera* (BPAX) and *Seminavis strigosa* (SMST) and other species known to be pollution sensitive like *Nitzschia dissipata* (NDIS) and *Achnanthidium minutissimum* (ADMI) (Lange-Bertalot, 1979). Diatom communities at the TL site are found to be typical of heavy organic pollution (Lange-Bertalot, 1979; van Dam et al., 1994), knowing that *Nitzschia palea* (NPAL) and *Gomphonema parvulum* (GPAR) have likewise been reported as metal-resistant species in polluted rivers (Gold et al., 2001, Ivorra et al., 2002, Feurtet-Mazel et al., 2003). At NT_2, other species such as *Navicula cryptocephala* (NCRY) and *Fragilaria ulna var. acu*s (FUAC) are frequently considered as moderately tolerant to pollution or β-mesosaprobic species (van Dam et al., 1994). However, valuable information on metal or even pesticide tolerant diatom species is rather scarce and not as abundant as data on organic pollution. Physical and chemical data give basic and very important information on the present status of the water quality but do not display the ecological state of the system which can be pre-received by the study of diatom community development over an extended period of time. The use of diatom composition to assess multi-source pollution (metal or pesticide etc.) in situ could be improved by using some biological traits such as deformities and size reduction to evaluate toxic effect (metal and pesticide pollution). Complementary laboratory studies could support these investigations by isolating diatom response to a specific contaminant.

The diversity of benthic algae assemblages has important implications for ecosystem processes in streams (Biggs and Smith, 2002) and estimating this could be a valuable tool to reveal the dynamics of diatom community colonization. However, our experiment did not display effect of colonization time on the diversity and species richness of benthic diatoms (Figure 3.5a). In fact, diversity index and species richness varied moderately over the six weeks of colonization. This result is in good agreement with those obtained from the response of benthic microalgae to colonization time, eutrophication (Hillebrand and Sommer, 2000) and temporal succession of benthic algae in Sonoran Desert stream studies (Fisher et al., 1982), where diversity did not vary significantly from early to mature stage of benthic development. Although species richness was similar at different stages of colonization at each site, this criterion showed the lowest value at the TL site when compared to the Red and NT_2 sites. As a major source of urban pollution, the TL site is affected by discharges of metal and organic effluents, which can induce a decrease in species

diversity as described by Ivorra et al. (2002), and also changes in community for the benefit of the most tolerant taxa (Gustavson and Wängberg, 1995) like *Nitzschia palea* (NPAL) and *Nitzschia umbonata* (NUMB) which were dominant at the TL site. These detrimental effects of pollution on the development of diatom density and species composition are not only visible at the TL site, but also on the biofilms accrual rates during colonization time (Juji, 2000), underscoring the tight positive correlations between the amount of benthic diatom communities and the quantity of detritus, silts, organic matter, bacteria and other microbenthic algae in biofilms. Thus, in contrast to the lowest values of biofilm dry weight at the TL site, after six weeks' colonization, corresponding values in both Red and NT_2 sites increased (Figure 3.3), especially at the NT_2 site, and showed a considerable increase in periphytic diatom communities embedded in a proportional growing and dense matrix of various matters and microorganisms within the biofilms.

The fact that diatom communities appear to build their assemblages precociously at each site with very little modifications occurring thereafter, leads us to question whether or not this observation is accompanied in the study area by an evolution in pollutant incorporation within diatom cells. Consequently, analyses of metal concentrations in biofilms have been attempted after adapting a technique traditionally used with tissue materials to fit very small biomasses. The capacity to accumulate metal from water column is found to be one of the singular properties of periphytic biofilms (Ivorra et al., 2000), even when ambient concentrations are admitted too low to be accurately detectable by routine analyses. In the studied area, our data nevertheless show that concentrations of metals in biofilms at the Tolich site are much higher than those analyzed at the NT_2 and Red sites (Figure 3.4), underscoring the fact that biofilms can provide an integrated picture of metal pollution over time, and emphasizing their accumulation capacities. These observations are in agreement with Ho and Egashira (2001) who analyzed heavy metal pollution in river-sediment samples collected from Tolich and Nhue Rivers, and found differences between sites that were similar but not so great. Significant correlations were found between EDTA extractable biofilm and exchangeable sediment fraction for Cd, Cu and Zn (Holding et al., 2003). Similar concentrations of metals were reported in biofilms of polluted areas with heavy metals (Cu, Zn, Pb and Cd) in Kakehashi River (Nakanishi et al., 2004); however, much higher values of metals accumulated in natural biofilms were found in acute metal pollution conditions by Admiraal et al (1999).

The thin biofilms developed in the early stages of colonization present large areas exposed to metals and, according to Ivorra et al. (2000) and Barranguet et al. (2000), these accumulate more Cd per unit of dry weight than thick biofilms in later phases. Vymazal (1984) upholds the same process by demonstrating that metal accumulation by periphyton is mostly active during the first few hours of exposure. Within the thickness of the biofilms, the bottom cells may be protected by

the upper diatom cells against pollutants and metallic water input. Our results may confirm, such an interpretation but more support is needed through complementary field and indoor studies, taking into account very short-term bioaccumulation analyses.

To monitor water quality levels in rivers and streams and to standardize evaluations, considerable research has been carried out using periphytic diatom communities, resulting in their being selected as a model to develop biological methods and indices. The two diatom indices applied in this study (Figure 3.5b) to estimate water quality, (IPS and DAIPo) showed similar trends in their results, despite the fact that they differ in the number of indicators and the list of taxa used in calculation. The DAIPo index was based on relative abundances of pollution tolerant taxa. The IPS was developed on large databases and important number of taxa (Watanabe et al., 1986; Cemagref, 1982; Rimet et al., 2005). These indices have been widely used in many countries, and their good fit in our study, confirmed by clear PCA results (Figure 3.7), proves that the application of quantitative diatom indices is a worthwhile means of assessing water quality. Within the studied area, three distinct water quality levels have been identified ranging from highly polluted at the TL site (4.6 for IPS index and 5.3 for DAIPo index) to polluted at the NT_2 site (5.4 for IPS index and 8.2 for DAIPo index) and moderate water quality at the Red site (10 for IPS index and 11 for DAIPo index), with no site referenced as being of really good water quality. As shown by PCA data, diatom communities are clearly distinct within the plan according to their sampling locations, whereas their exposure duration in the three sites does not appear to be dominant in differentiating them. Therefore, the whole set of data supports the idea that water quality may be diagnosed in the environment after a short exposure period.

3.6 Conclusion

In Vietnam, the dynamic colonization process of benthic diatom communities on three rivers subjected to an urban pollution gradient was monitored using artificial substrates during a six week period. This experiment showed, through various general criteria (total diatom density, dry weight biomass) and specific criteria (relative diatom abundances, indices), that the structure of benthic diatoms developed on these substrates was strongly affected by pollution as early as the second week of colonization. Communities exposed in the heavily polluted and moderately polluted sites presented species which are known to be saprophilous taxa or tolerant to organic pollution such as *Nitzschia umbonata, Nitzschia palea, Cyclotella meneghiniana*. Growth inhibition of Tolich diatom communities was mostly related to an increase in organic load rather than to the level of metal concentrations in the water, and water quality indices, currently applied in other countries but used

for the first time in Vietnam, have enhanced this conclusion. The use of diatoms as a tool for water assessment has therefore been demonstrated in this study to be suitable for monitoring rivers in Vietnam.

However, more studies on the development of benthic diatom communities on artificial substrates are still necessary and should be focused on detailed analyses of relationships between communities and environmental conditions in these rivers influenced by multiple organic or metallic pollutant sources. Even if low metallic concentrations are noted in these rivers, bioaccumulation has been analyzed in biofilms, and further experiments in microcosm conditions would improve our understanding of how diatom biofilms are affected by such contaminants according to microalgae localization within biofilms and according to their species.

Acknowledgements

The authors would like to thank the Institute of Natural Product (VAST-Vietnamese Academy of Science and Technology) for carrying out the chemical analyses. This study was supported by grants from BDI fellowship (CNRS France) and the ESPOIR programme within a framework of a French -Vietnamese cooperation. The authors also thank many individuals for assisting in collecting samples in field. Thanks to Georges Vachaud, Research Director at the CNRS for the coordination of the ESPOIR programme (CNRS - VAST). We thank H. Koziol, formerly the Department of Foreign Language (University of Bordeaux 2) for improvements to the native style of the manuscript.

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CHAPTER 4

Impact of urban pollution from the Hanoi area on benthic diatom communities collected from the Red, Nhue and Tolich rivers (Vietnam) *

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Keywords: benthic diatoms, biological assessment, water pollution, Vietnam

* Article published in Hydrobiologia 563: 201-216, 2006

4.1 Abstract

The effects of urban pollution from Hanoi city on the benthic diatom communities of the Nhue-Tolich river system were studied during the 2003 dry season. Benthic diatoms were allowed to grow on glass slides suspended in the water flow for four weeks. To reveal the relationship between water quality and diatom communities, Canonical Correspondence Analysis (CCA) was used on data concerning relative abundances of diatom species and environmental variables. Two diatom indices, IPS and DAIpo, were applied to evaluate water quality in the three rivers. A total of 291 diatom taxa were found in the Red, Nhue and Tolich Rivers. These were mainly cosmopolitan taxa, with some tropical, subtropical and endemic taxa. The most abundant taxa at the Red site were *Aulacoseira granulata*, *Achnanthidium minutissimum*, *Encyonema minutum, Navicula recens* and other halophilous taxa such as *Nitzschia kurzii, Seminavis strigosa, Entomoneis paludosa, Bacillaria paradoxa*. Diatom assemblages at the Tolich site consisted mainly of *Nitzschia umbonata, Nitzschia palea* and *Eolimna minima.* Diatom density ranged from 660 to 30,000 cells/cm^2. Environmental variables and diatom assemblage composition at all sites were significantly correlated. Two diatom indices gave similar results and indicate the Tolich River with the lowest values as a highly polluted site.

4.2 Introduction

Water pollution is a major environmental problem. Rapid urbanization and economic development have resulted in unfavourable changes in the hydrology and ecology of river systems. Today, Vietnam faces the challenge of effectively managing the ever increasing number of wastewater sources due to increasing urbanization (population of Hanoi area, approximately 4 million inhabitants) (Trinh & Fredlund, 2000) and industrialization, with only poor quality infrastructure for wastewater treatment. Most wastewater is still discharged directly into canals or rivers with no efficient collection or treatment, thus affecting aquatic life and human health. The French – Vietnamese project on Water Quality and Treatment (FVWQT) set up in 2001, therefore plans to examine the impact of pollution from the Hanoi area on water quality in the Nhue and Tolich River systems (Figure 4.1) using periphytic diatom communities as an indicator of water quality.

Alongside traditional methods of physico-chemical analysis, biological methods have been applied to water bodies to measure the effect of anthropogenic and natural impacts, and living organisms have been widely used to assess environmental conditions. Many groups such

as benthic macro-invertebrates, molluscs, fish, and diatoms have been considered for biological assessment of rivers (Vis et al., 1998; Growns, 1999). Of these, diatoms, which are photosynthetic unicellular organisms belonging to the *Bacillariophyta*, present several advantages. They are found in almost every aquatic and semi–aquatic habitat, where they play an important role in the food web and in the geo-chemical cycles of silica and carbon (Patrick, 1977). Diatom cells present a short cycle development (a few hours to several days) (Wan Mazahn & Mansor, 2002); they support high development increase within short periods of time and can reach a high rate of diversity in the environment (Prygiel et al., 1999; Poulíčková et al., 2004). They are sensitive and react rapidly to environmental changes such as eutrophication and organic pollution (Rott et al., 2003; Prygiel et al., 1999). There are abundant references as to their identification and information about their ecological requirements is readily available (Soininen, 2002). For all these reasons, diatoms are often used to monitor the state of rivers and streams as well as ecological and chemical changes in aquatic systems in many countries (Wan Mazahn & Mansor, 2002; Potapova et al., 2003, Soininen, 2002). An in-depth study of diatom communities in Vietnam hydrosystems is of great interest for the determination of water quality and for follow up projects on running freshwaters.

Several diatom indices have been developed to estimate water quality in European countries and Asia countries (Whitton & Rott, 1996; Watanabe et al., 1986) where they have been widely used to monitor the impact of disturbance and organic pollution on streams (Rott et al., 2003; Prygiel & Coste, 1993, Wu & Kow, 2002). Indicator values have been published for one hundred freshwater diatom taxa for pH, salinity, organic nitrogen enrichment, saprobity, oxygen level, etc. (Van Dam et al., 1994).

In Vietnam, the water quality of rivers is currently determined using physical and chemical analyses, complemented by studies of micro-algal and zooplankton communities as bioindicators for changes in water quality. The use of periphytic diatom assemblages as indicators of water quality is a new field of investigation. Consequently, the main objective of this study is to evaluate the impacts of wastewaters from Hanoi city on periphytic diatom communities. The Nhue River constitutes a major irrigation and drainage canal for the Hanoi area, receiving all domestic and industrial wastewaters from the cities of Hanoi, Cau Dien and Hadong *via* its tributaries and the Tolich River (through the Thanh liet dam) (Figure 4.1). These results will allow us to establish relationships with physico-chemical water quality in the Nhue, Tolich River system and ultimately test the efficiency of diatom indices commonly used in other countries for an initial water quality assessment.

4.3 Material and methods

4.3.1. Sampling sites

The sampling area is located in the Red River delta in the North of Vietnam (Figure 4.1). The Nhue River flow is formed on the right bank of the Red River and is regulated upstream through the Thuy Phuong dam. It flows through the Hanoi area, Hadong city (Hatay province) and Hanam province *via* several dams and finally flows into the Day River. The topography of the Nhue basin slopes down from north to south.

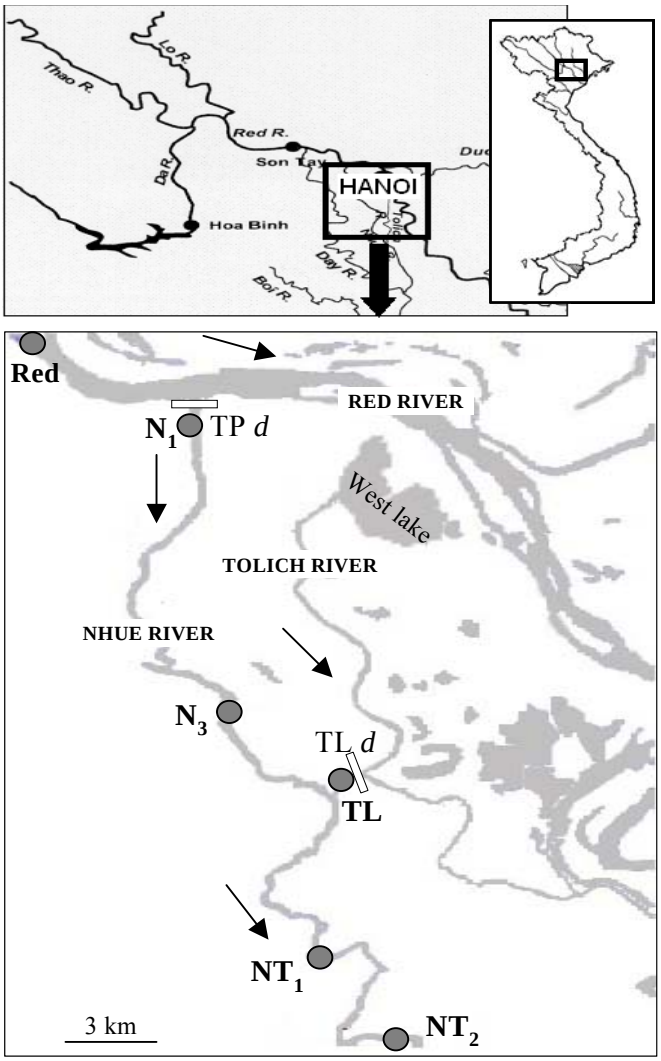

Figure 4.1: Location of sampling stations (TPd: Thuy Phuong dam; TLd: Thanh Liet dam)

The soil textures of the Nhue River watershed change with topography from sandy loam to clayey loam in the lower part (Kono & Doan, 1995). The Nhue River basin area is 74 km long and covers a total of 107,503 ha (Trinh, 2003). Average annual rainfall is about 1,800-2,000 mm, 80% of which occurs during the rainy season from June to October. The dry season lasts from November to April-May. Temperatures increase from 15°C in the winter (December and January) to 30°C in the summer (June and July) (Trinh, 2003). There are two water flow conditions in the Nhue River, related to precipitation: the free flow regime and the closed dam regime, regulating water current parameters throughout the year. In winter and spring, during the dry season, dams are opened to release water from the Red River to irrigate winter/spring crops, and during this period, water inflow values of 30 m^3/s are currently measured. During the rainy season, water inflows are notably higher as dams are closed to prevent flooding in Hanoi and other areas of the Nhue basin.

The study area is located in the Red/Nhue/Tolich hydrosystem. The 6 selected study sites (Figure 4.1) are distributed along an increasing urban pollution gradient. In the Red River, Red site is the local reference site; it is situated on the right bank just upstream from the Nhue River and Hanoi city. N_1 is located in the upper part of the Nhue River (below Thuy Phuong dam, TPd). N_3 (Cau Den) is in the lower part above the Tolich River junction. Tolich (TL), situated just downstream from the Thanh Liet dam (TLd), 21 km from the Red site, represents the polluted site, as it receives discharges directly from various sources (factories, domestic, hospitals…), and is characterized by black, foul-smelling waters, and slow flow conditions estimated at about 5 m^3/s in the dry season (Trinh, 2003). Downstream from the confluence of the Tolich and Nhue rivers, NT_1 and NT_2 stations receive combined waters from both rivers.

4.3.2 Environmental data

Environmental variables (water temperature, pH, conductivity, turbidity, dissolved oxygen, salinity) were measured monthly *in situ* by multi-parameter sensors (Model WQC-22A, TOA) during the dry season from December 2002 to March 2003. Water samples for nutrients and dissolved metal determinations were collected near the surface, filtered through Whatman GFC filters (0.45 µm) and kept in plastic boxes in the dark at 4°C, before immediate transfer to the laboratory for further analyses. Samples for metal concentrations (Cu, Zn, Pb, and Cd) were acidified in HNO_3 before analysis by atomic absorption spectrophotometry (AAS Perkin-Elmer). Phosphate, nitrate, nitrite, ammonium, SPM (Suspended Particulate Matter), BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand), Chlorophyll (from phytoplankton)

analyses in water followed standard methods for wastewater examination as used by the APHA (American Public Health Association). Total inorganic carbon (TIC) and total organic carbon (TOC) in water samples were measured using ANATOC, series II (Total organic carbon, SGE, Australia). All analyses were performed by the Institute of Natural Products and Institute of Chemistry (VAST – Vietnamese Academy of Science and Technology, Hanoi, Vietnam) which is also involved in the ESPOIR programme supporting our study (French – Vietnamese research programme on water pollution of the urban hydrosystem around Hanoi: health consequences of pollution and elimination of pollutants).

4.3.3 Diatom sampling and analysis of communities

Periphytic diatom samples were obtained during dry season by exposing glass slides (30 cm x 18 cm – 1,080 cm^2 surface for both sides) in water flow for 4 weeks in February 2003. Dry season was selected to avoid variables effects of rainy season like great variations of water level, floods and inundations, which affect diatom development, specially its growth rate and its relative abundance of different species. Furthermore, in dry season, water level being lower, and rivers regime being reduced, influences of organic matter and nutrients concentration on diatom communities can be detected more easily (Eloranta & Andersson, 1998).

The glass slides were maintained vertically and separated from each other in plastic baskets equipped with floaters. They were positioned in the water column parallel to the current at a depth of 15-20 cm below the surface and tied to the bank with a rope (Gold et al., 2002). At each station, three glass slides were used to give three replicates. Diatom samples were removed from the glass slides at the end of the four weeks colonization period using a toothbrush and were then washed with distilled water. All diatom samples were then transferred into plastic vials and preserved in 5% formalin solution (Formaldehyde 37% Prolabo France).

A Nageotte counting chamber (Marienfeld, Germany) was used to estimate diatom density in each sample by counting the total number of diatoms in 30 fields (1.25 µl each, 0.5 mm depth) using a light microscope (Olympus BX 50) at 200x magnification. Data are expressed in cells per unit area of artificial substrate (cells /cm^2). Three replicates (each corresponding to a glass slide sample) per site were treated separately. After homogenization, 2 ml aliquot of each diatom sample from each site were exposed to hydrogen peroxide (30%) and hydrochloric acid (35%) to remove organic matter and dissolve calcium carbonates, then mounted on a microscope glass slide in a high refractive index medium (Naphrax, Northern Biological Supplies Ltd, UK; RI=1.74) (Gold et al., 2002; EN 13946, 2003; EN 14407, 2004). Diatom valves were identified

under Leitz DMRD microscope at 1,000x magnification. On each slide, 400 diatom valves were identified following European standards and the Süßwasserflora nomenclature (Krammer & Lange – Bertalot, 1991-97). The relative abundances of the diatom species (in percentage) and diatom indices (IPS, and DAIpo index) were estimated in order to classify the six sampling sites according to their water quality. The Van Dam classification (1994) was used in parallel to obtain diatom nitrogen demand, oxygen demand and saprobity. Species richness (S) was calculated and biological diversity was estimated using the Shannon-Wiener diversity index (H').

4.3.4. Data treatment:

To reveal relationships between environmental factors and diatom communities collected during the experiment, Canonical Correspondence Analysis (CCA) was carried out with Multivariate Statistical package (MVSP, Kovach Computing Services, 2004) on data concerning relative abundances of diatom species and environmental variables (pH, conductivity, turbidity, DO, temperature, SPM, salinity, $N\text{-}NO_3$, $N\text{-}NO_2$, $N\text{-}NH_4$, $P\text{-}PO_4$, TIC, TOC, BOD, COD, Cu, Cd, Pb, Zn, Chlorophyll). Cluster analyses using a single linkage method with Euclidean distance measured were applied to classify sampling sites based on diatom species abundance using statistical software STATISTICA (StatSoft, 2004). The relative abundances of only 120 out of a total of 291 identified diatom species in the studied area were considered by selecting those with the highest cumulative relative abundances, all the samples being taken into account. Statistical computations were performed with STATISTICA software (StatSoft, 2004) using one way analysis of variance model (ANOVA) to reveal the effect of station (Red, N_1, N_3, TL, NT_1 and NT_2) on the diatom density, diatom indices and environmental parameters. If a significant effect was observed, we performed *post-hot* tests (Least Significant Difference test (LSD) and Newman-Keuls test) to isolate the significantly different station. Significance effects were considered at 0.05 level. Omnidia 1 software (Lecointe et al., 1993) was used to calculate diatom indices.

4.4. Results

4.4.1. Environmental characteristics of water in the Red, Nhue and Tolich rivers

Parameters	Red	N_1	N_3	TL	NT_1	NT_2
SPM (mg l^{-1})	103 (46 - 197)	109 (34 - 208)	89 (31 - 141)	46.4 (39 - 58)	56.3 (36 - 89)	51.3 (23 - 94)
O_2 (mg l^{-1}) *	7.6 (7.1 - 8.1)	7.85 (7.7 - 8)	6.2 (4.2 - 7.4)	1.9 (0.1 - 5.8)	3.9 (0.7 - 6.4)	4.9 (2.4 - 6.6)
pH	7.6 (7.3 - 7.9)	7.6 (7.5 - 7.7)	7.2 (6.9 - 7.4)	7.4 (7 - 8)	7.2 (7 - 7.4)	7 (6.4 - 7.4)
Temperature °C	20.4 (19.2 - 21.3)	21 (19 - 21)	20.5 (19 -21)	22.1 (20 - 23)	21(19 - 22)	21.1 (19.4 - 21.1)
Conductivity (µS/m) *	20.2 (17.1 - 22.3)	20.8 (17.2 - 22.3)	28.7 (23 - 43.2)	65.4 (23.5 - 89.4)	35 (24.6 - 38.9)	37 (25 - 66.7)
Salinity (%)	0.01 (0.01- 0.011)	0.011 (0.01 - 0.011)	0.015 (0.01-0.02)	0.03 (0.01 - 0.04)	0.02 (0.02 - 0.019)	0.02 (0.01 - 0.03)
Turbidity (NTU)	123 (58 - 196)	138 (25 - 270)	101 (36 - 158)	57.3 (15 - 76)	77.8 (50 - 109)	67.5 (27 - 121)
NO_3-N (mg l^{-1})	0.3 (0.12 - 0.6)	0.24 (0.16 - 0.34)	0.22 (0.05 - 0.48)	0.15 (0.03 - 0.48)	0.15 (0.04 - 0.31)	0.15 (0.06 - 0.21)
NO_2-N (mg l^{-1})	0.03 (0.004 - 0.04)	0.03 (0.004 - 0.05)	0.01 (0.003 - 0.016)	0.01 (0.006 - 0.02)	0.02 (0.002 - 0.04)	0.03 (0.003 - 0.06)
NH_4-N (mg l^{-1}) *	0.04 (0.02 - 0.08)	0.04 (0.01 - 0.09)	0.6 (0.3 - 1.2)	7.9 (5 – 12)	1.4 (0.5 - 2)	1.3 (0.6 - 2.3)
PO_4-P (mg l^{-1}) *	0.02 (0.003 - 0.06)	0.01 (0.003 - 0.02)	0.12 (0.04 - 0.4)	1.8 (0.04 - 2.6)	0.34 (0.05 - 0.8)	0.36 (0.04 - 1.1)
TOC (mg l^{-1})	2.46 (1.9 - 3)	2.5 (1.5 - 3)	4 (1.9 - 8.9)	9.2 (1.9 - 16.9)	4.7 (2.6 - 9.2)	5.1 (3.2 - 10)
TIC (mg l^{-1})	10 (9.2 - 15.5)	10.3 (2.8 - 13.6)	11.7 (3.5 - 15)	26.6 (6.8 - 45)	12.5 (3.4 - 20)	12.1 (5.8 - 16.6)
BOD (mg O_2 $l^{-1)}$ *	4.3 (2 - 5.9)	4.9 (2 - 6.8)	6.9 (4 - 8.8)	47 (26 - 68)	10.5 (8 - 14.7)	18.2 (5.5 - 44)
COD (mg O_2 l^{-1}) *	19.2 (16.2 - 21)	16.8 (11.3 - 19)	21.2 (20.2 - 21.4)	100 (98.4 - 114)	32.4 (30.3 - 34.4)	30.4 (28.4 - 32.7)
Chlorophyll (mg/m^3)	5.7 (3.3 - 7.6)	5.5 (3.3 - 7.5)	8.9 (3.3 - 18.2)	30 (4 - 61)	9.7 (5.3 - 12.7)	9.8 (4 - 14.3)
Dissolved Cu (µg l^{-1})	2.4 (1.7 - 2.9)	2.5 (2 - 3.3)	1.8 (0.9 - 2.9)	1.2 (0.9 - 1.6)	1.5 (0.9 - 1.9)	1.4 (1 - 2)
Dissolved Pb (µg l^{-1})	1.1 (0.9 - 1.3)	1.26 (0.7 - 1.6)	1.2 (0.8 - 1.5)	2.9 (2.6 - 3.1)	1.28 (0.6 - 2.5)	1.2 (0.9 - 1.8)
Dissolved Cd (µg l^{-1}) *	0.3 (0.21 - 0.5)	0.4 (0.27 - 0.5)	0.4 (0.28 - 0.6)	1 (0.9 - 1.9)	0.5 (0.3 - 0.6)	0.4 (0.3 - 0.4)
Dissolved Zn (µg l^{-1})	13.3 (10 - 19)	17 (10 - 22)	22.8 (9 - 47)	34.3 (20 - 48)	20.3 (12 - 27)	20 (9 - 36)

Table 4.1: Environmental parameters of the three rivers at six studied stations: combined observations from Decembre 2002 to March 2003 (average values and max-min values, n = 4) *: $p < 0.05$

The main environmental parameters measured in this study area during the dry season (December 2002 to February 2003) are shown in Table 4.1. All sites present an average temperature of 21°C, and neutral pH level. SPM concentrations were not significantly difference between sites but were high in the upstream sites, with an average value of 100 mg/L at Red and N_1, and these regularly decrease downstream to reach half their initial values. Among the whole set of sampling sites, Tolich (TL) stands out with the lowest dissolved oxygen (DO) values, as low as 1.92 mg/L, whereas upstream values are at least 3 times higher at Red, N_1 and N_3. Downstream, DO values at NT_1 and NT_2 are intermediate, coinciding with the low oxygenated wastewater input from the Tolich area. NH_4, PO_4, TOC, TIC concentrations, BOD and COD reach their lowest values in the upstream part (Red, N_1, N_3), then significantly increase ($p<0.05$) below the confluence with the Tolich River, where the highest values are found. NO_2 and NO_3, on the other hand, do not differ between sites, but conductivity still points out major nutrient (NH_4 and PO_4) input from Tolich, which affects the downstream sites NT_1 and NT_2 by nearly doubling their conductivity values (35 and 37 mS /m, respectively). Chlorophyll concentration in water follows the same profile as BOD and COD along the area of the Red-Nhue-Tolich hydrosystem, highest value at TL, and slightly less at N_3, N_1 and Red sites in decreasing order. Finally, no major metallic contaminations are revealed through measurements taken from the whole area (<5 µg/L), and among the dissolved metallic compounds analysed, Cu is the only one showing significantly higher values in the Red River, while again Pb, Cd and Zn mark Tolich as the most polluted site in the area.

4.4.2. Characteristics of diatom assemblages

4.4.2.1 Global and taxonomic composition:

After four weeks of colonization, the glass substrates were covered by a yellow-brown coloured layer at every site except TL. This layer was a lot thicker at the Red, NT_1 and NT_2 sites than at the N_1 and N_3 sites. At the TL site, a very thin black layer colonized the immersed substrates. These qualitative observations are confirmed (Figure 4.2), which shows the number of diatom cells at each site, with the lowest densities (< 5,000-cells/ cm^2) observed upstream in the Nhue River (N_1, N_3 sites) and in the Tolich River (TL site). The highest densities range from 20,000 to 30,000 cells/cm^2 for the Red, NT_1 and NT_2 sites. Neither H' (Diversity index) nor S (Species richness) provides any interesting information and both show high variability between

sites without any relation with the studied gradient. H' varied from 3.07 to 4.48 and S from 57 to 80. The highest H' and S values were recorded at the N_3 site and the lowest at N_1.

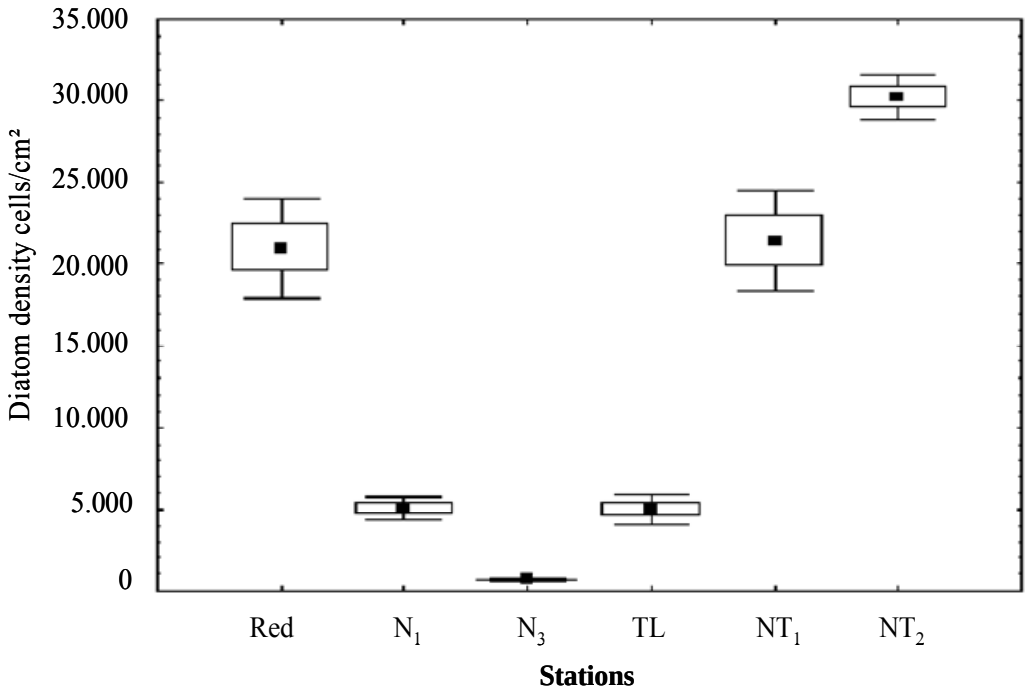

Figure 4.2: Diatom density on artificial substrate (cells.cm^{-2}) after 4 weeks colonization at the 6 stations along the Red-Nhue and Tolich Rivers. Boxplot: mean value (n = 3) with its confidence interval at 95 % denoted by the two horizontal lines outside the box. ($p < 0.05$)

Among the 6 sites studied, after four-week colonization, a total of 291 species and subspecies belonging to 8 different families were enumerated. Some genera contain numerous species, such as *Navicula* (42 species), *Nitzschia* (36 species), *Gomphonema* (16 species) and *Pinnularia* (11 species); however, most of the species collected are cosmopolitan taxa, accompanied by some tropical, subtropical and endemic taxa such as *Cyclotella asterocostata* Xie Lin & Cai (Xie et al.,1985), *Cymbella sinensis* Metzeltin & Krammer, *Luticola sp.aff. seposita* (probably new for science), and *Encyonopsis leei* var *leei* Krammer (Krammer, 2002-2003), *Gomphonema chubichuensis* Jüttner & Cox (Jüttner & Cox, 2000), *Pinnularia valdetolerans* Mayama & Kobayasi (in Idei et al., 2001). These data, which represent the taxonomic composition at each site by assessing relative abundances of the 8 main species, with mean relative abundances $\geq$ 9 %, are summarized (Figure 4.3).

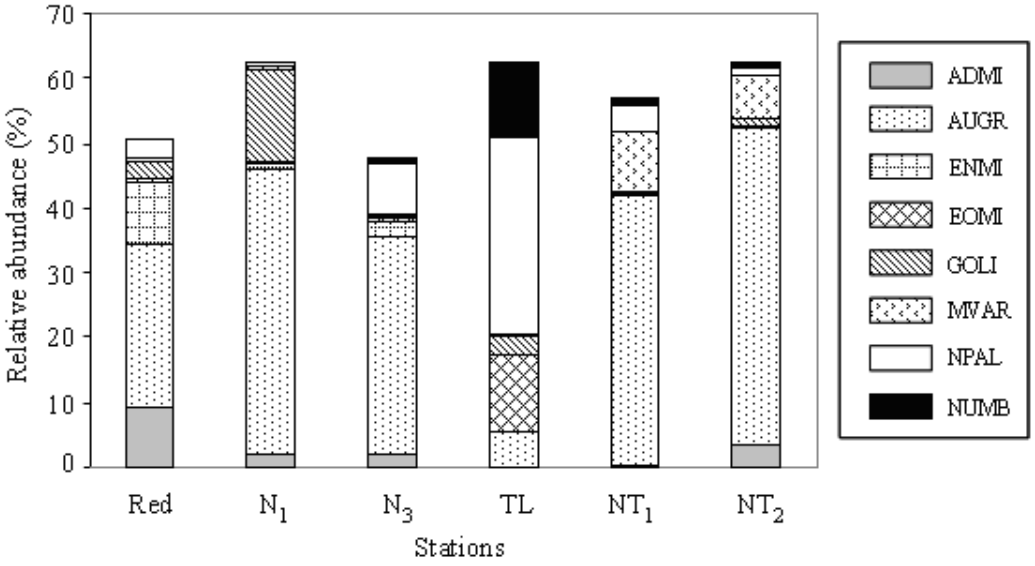

Figure 4.3: Relative abundances of diatom species (mean value, n=3) with mean relative abundances ≥ 9 % after 4 weeks colonization in Red, Nhue and Tolich Rivers at the six stations. (NUMB: *Nitzschia umbonata*, NPAL: *Nitzschia palea*, MVAR: *Melosira varians*, GOLI: *Gomphonema olivaceum*, EOMI: *Eolimna minima*, ENMI: *Encyonema minutum*, AUGR: *Aulacoseira granulata*, ADMI: *Achnanthidium minutissimum*)

In the Red River, diatom assemblages are characterized by the presence of many halophilous taxa: *Nitzschia kurzii* (NKUZ), *Seminavis strigosa* (SMST), *Entomoneis paludosa* (EPSU), *Bacillaria paradoxa* (BPAR); however the most abundant taxa are *Aulacoseira granulata* (AUGR) with 25.2%, *Achnanthidium minutissimum* (ADMI) with 9.1% and *Encyonema minutum* (ENMI) with 9.8%. Other species such as *Nitzschia palea* (NPAL) are also present at the Red site in low proportions (2.9 %), but these increased at N_3 and NT_1. Upstream in the Nhue River, sites N_1 and N_3 present quite similar diatom assemblages to the Red site, yet with the specific presence of *Gomphonema olivaceum* (GOLI) (14.2%) at N_1. Down the Nhue River at NT_1 and NT_2, the dominant diatom taxa are *Aulacoseira granulata* (AUGR), *Nitzschia palea* (NPAL) and *Achnanthidium minutissimum* (ADMI), as already cited above, but to these are added *Melosira varians* (MVAR) (41.6 % and 48.7 % respectively), which are characteristic of these 2 sites, and *Nitzschia umbonata (*NUMB) in a lesser proportion. Nevertheless, *Nitzschia umbonata* (NUMB), *Nitzschia palea (*NPAL) and *Eolimna minima* (EOMI) are very dominant at the polluted site TL, which displays a very different diatom assemblage from the other 5 sites.

Cluster analysis of diatom communities (Figure 4.4) highlights these clear differences between sampling sites. TL, which is a tributary, is completely different from other study sites

appearing in the different groups in the cluster analysis. NT_1 and NT_2 are very closely grouped, while N_1 and N_3 are grouped in the relatively large linkage distance level. Red site, a branch, is close to the N_1, N_3, NT_1 and NT_2 sites and there is a clearly defined separation of the Red site from the TL site.

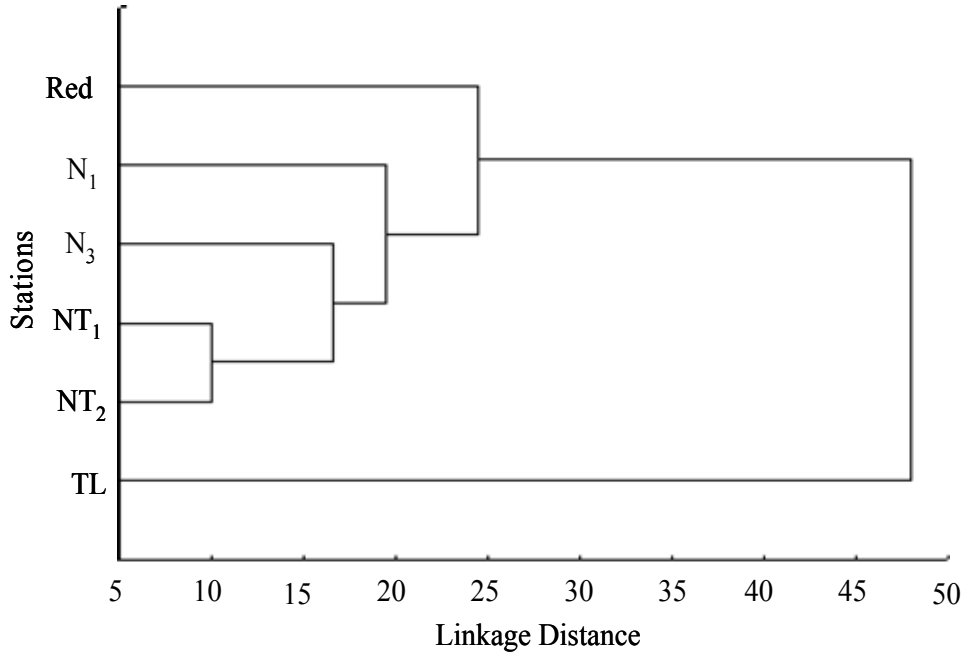

Figure 4.4: Cluster dendrogram analysis based on relative abundances (%) of diatom species in the six studied stations.

4.4.2.2 Diatom communities and water quality

Relations between diatom communities and environmental parameters are enhanced through the Canonical Correspondence Analysis (Figure 4.5). Axis 1 of CCA accounts for 47% of the explained variability, in which diatom composition is significantly correlated with environmental variables such as BOD_5, COD, Zn, Pb, Cd, N-NH_4, PO_4^{3-}, total organic carbon, total inorganic carbon, chlorophyll, conductivity, whereas axis 2 accounts for 22% of the explained variability in which various diatom assemblages are related to turbidity, dissolved oxygen, Cu, SPM, NO_3 and NO_2. Study sites appear clearly separated in the CCA plan with the TL site isolated on axis 1 in the right area, the Red site on axis 2 in the upper left area, and N_1, N_3, NT_1, and NT_2 in the central area of the plot. These distribution results are in agreement with the cluster analysis results (Figure 4.4).

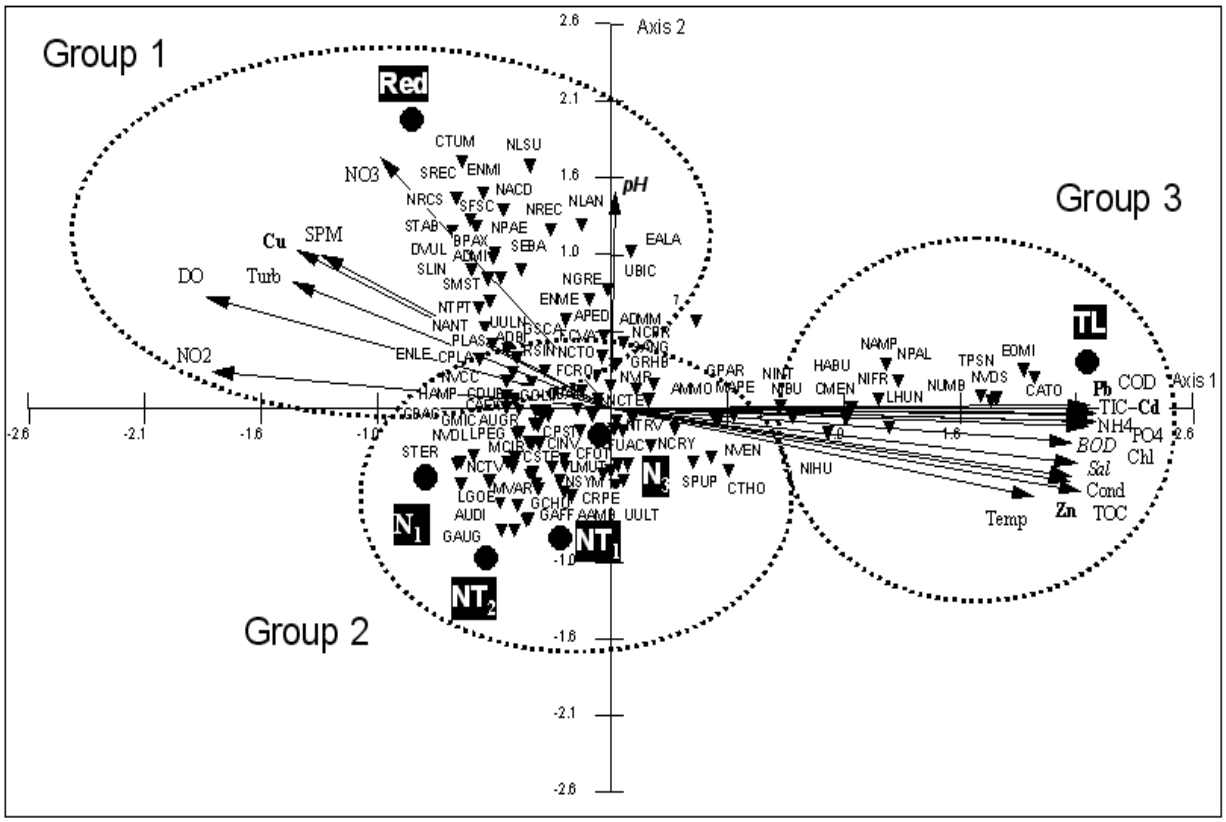

Figure 4.5: Canonical correspondence analysis based on environmental parameters and relative abundances of 120 diatom species. (Cf Appendix 1: List of 120 diatom taxa using in figure 4.5)

Regarding environmental variables, these are distributed in two sets along axis 1. Ranking clearly separates environmental factors and diatom composition into three groups: Group 1-TL site, which is represented by dominant taxa *Eolimna minima (*EOMI*), Nitzschia umbonata* (NUMB), *Nitzschia palea* (NPAL), *Cyclotella atomus* (CATO), *Lemnicola hungarica* (LHUN); Group 2 - Red site, which is located on the upper left side of the plan, is found to be related to *Cymbella tumida* (CTUM), *Navicula recens* (NRCS), *Encyonema minutum* (EMIN), *Achnanthidium minutissimum* (ADMI), *Nitzschia recta* (NREC), *Nitzschia linearis var.subtilis* (NLSU), *Diatoma vulgaris* (DVUL); Group 3 - N_1, N_3, NT_1, NT_2 sites, in the lower left part of the CCA, where *Melosira varians* (MVAR), *Luticola goeppertiana* (LGOE*), Navicula trivialis* (NTRV*), Sellaphora pupula* (SPUP), *Gomphonema affine* (GAFF), *Caloneis bacillum* (CBAC), *Cyclotella pseudostelligera* (CPST) represent the diatom communities of the Nhue River from upstream to downstream.

4.4.2.3 Diatom distribution according to their ecological preferences

To represent the ecological preferences of the diatoms for each sampling site, the Van Dam classification (1994) was considered for three criteria: Saprobity, Nitrogen heterotrophy, dissolved Oxygen. The distribution of diatom species presented in Figure 4.6a indicates that the polysaprobous taxa dominate in Tolich but are markedly reduced in the Red and Nhue rivers. At the Red site, oligosaprobous taxa make up only around 3% and only 0.4% at TL. Figure 4.6b shows the distribution according to nitrogen requirements. The nitrogen autotrophic sensitive taxa, which tolerate very low concentrations of organically bound nitrogen, are more numerous in the Red River and in the upstream part of the Nhue River. According to dissolved oxygen content in water, polyoxybiontic taxa, which need a high concentration (100% saturation), are abundant at the Red site, where they reach around 18% of total relative abundances, as shown in Figure 4.6c. In the Tolich River, they do not exceed 3%, while species characterized by low and very low oxygen requirements represent more than 74%. Diatoms associated with moderate oxygen requirements dominate in the downstream sites on the Nhue River at NT_1 and NT_2, reaching respectively 73 % and 76 %.

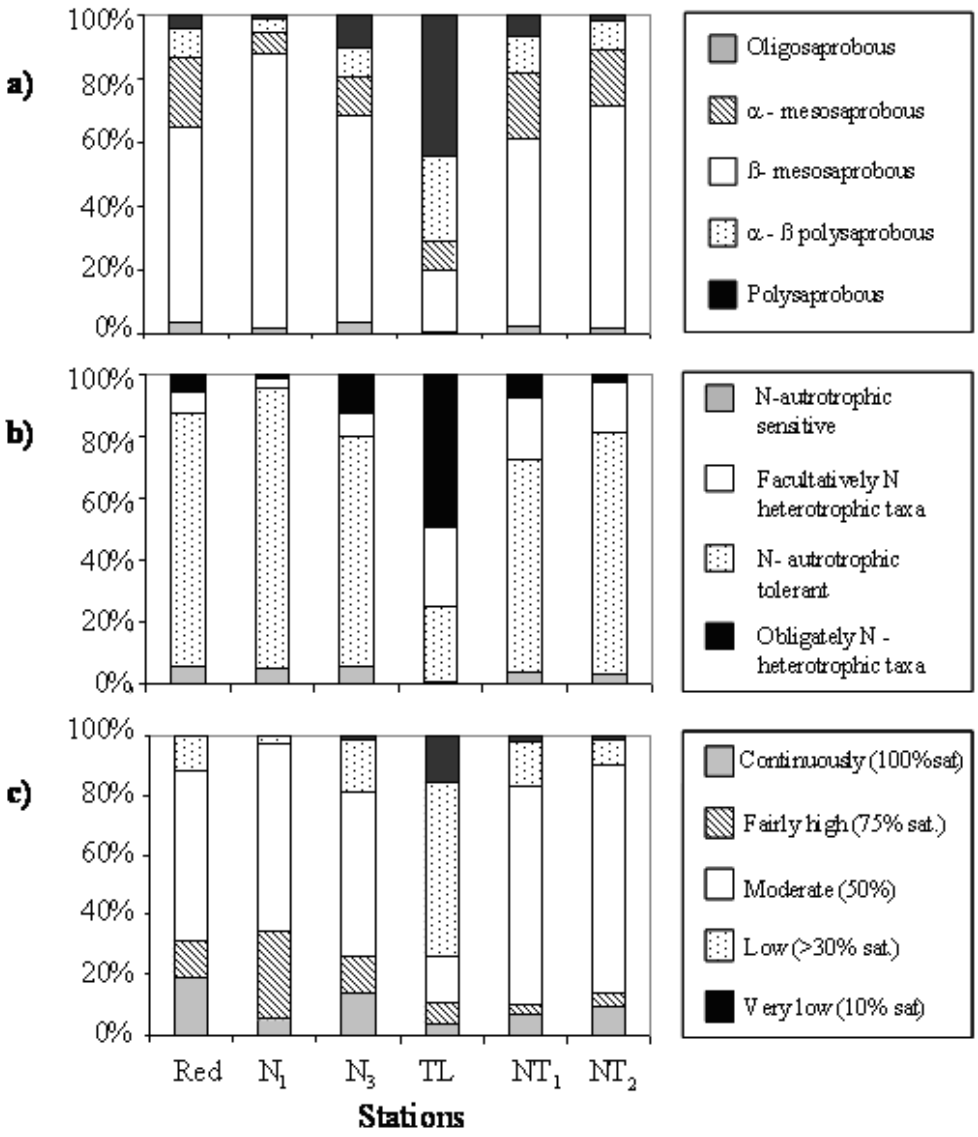

Figure 4.6 a, b and c: Distribution of Diatoms according to Saprobity, Nitrogen requirements, Oxygen requirements in Red, Nhue and Tolich Rivers.

Finally, to evaluate water quality in the Red, Nhue and Tolich rivers, IPS and DAIpo indices were applied (Figure 4. 7) and they give fairly similar results. They highlight the TL site, which shows the lowest values, as a highly polluted site. According to IPS and DAIpo index, the rivers were grouped into three quality classes: (i) highly polluted water in the Tolich River (IPS 3.9 and DAIpo 5.7); (ii) a less polluted class with IPS and DAIpo ranging from 9 to 10.3, including N_3 and the two downstream sites on the Nhue river (NT_1 and NT_2); (iii) least polluted water, expressed by IPS and DAIpo values between 11.4 to 12.4, corresponding to the N_1 and Red sites.

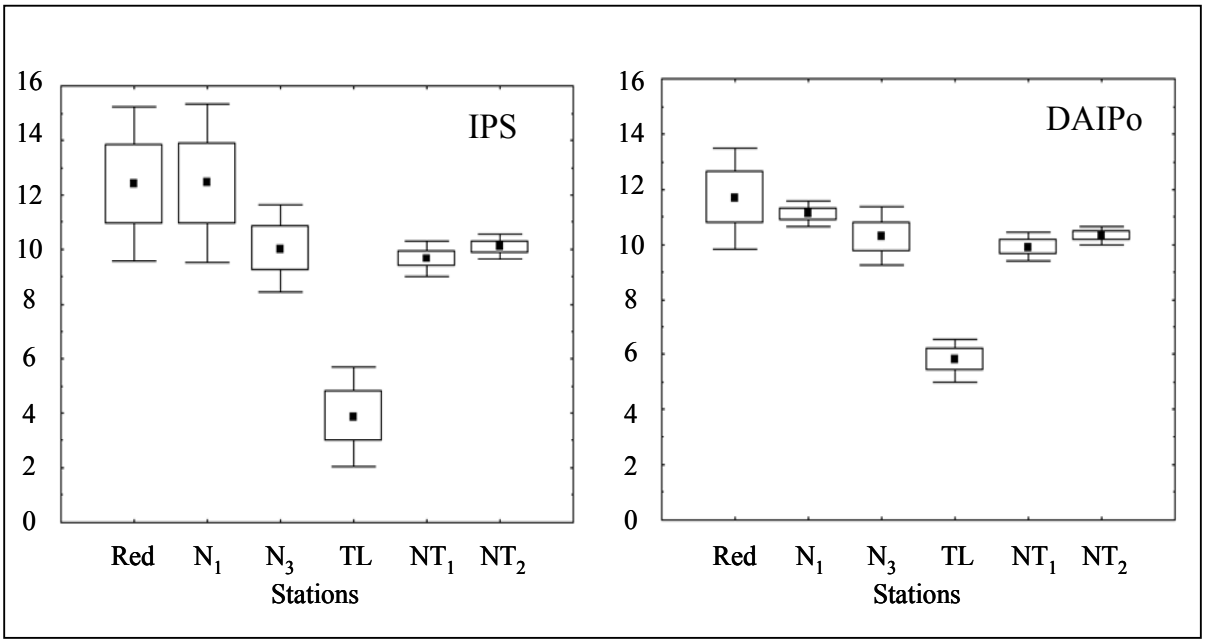

Figure 4.7: IPS and DAPo index in Red, Nhue and Tolich Rivers after 4 weeks colonization. Boxplot: mean value (n =3) with its confidence interval at 95 % denoted by the two horizontal lines outside the box.

4.4 Discussion

This study of the characterization of freshwater diatom assemblages in relation to physico-chemical measurements has produced a first report on the periphyton ecology and environmental status along the Red-Nhue-Tolich hydrosystem during the dry season (February 2003). Due to the dry conditions, the open dams along the Nhue River regularly linked up the 6 different stations studied. Upstream, the Red site is representative of the Red River, which is a very broad and turbid river, well oxygenated, with flow rates around 1600 m^3/s in the dry season. The highest turbidity level measured at this site, in relation to the greatest concentrations of SPM weighed, was observed to gradually decrease in the Nhue River under the effects of sedimentation, and was amplified downstream by water flow dilution from the Tolich River (TL). Dissolved oxygen follows the same trend as SPM because of the slower water current in the Nhue River compared with Red River. Nevertheless, the TL site emerged as a very unusual one, revealing anoxic levels, whereas downstream, the NT_1 and NT_2 sites reached hypoxic levels as a result of the dilution effect below the Nhue-Tolich confluence. Many factors can influence dissolved oxygen concentration: seasonal variations, chemical reactions, anthropogenic inputs, decay processes, respiration of living organisms. Thus, during the dry season, associated with

these frequent extremely low oxygen levels, the Tolich River presents a constant flow of black-coloured waters (Trinh, 2003) allowing only a poor development of diatom cells. At the same time, however, high chlorophyll concentrations occur at this site due to the strong abundance of phytoplankton mainly *Cyanobacteria* and *Euglenophyta* (data not shown) capable to tolerate very low oxygenated conditions.

Anthropogenic input may also be responsible for low dissolved oxygen levels by increasing organic degradation from wastewater. Thus, conductivity and concentrations of N-NH_4, P-PO_4, BOD, COD reached their highest values in the Tolich River, then downstream in the Nhue River, at NT_1 and NT_2 by decreasing order, probably because most of the wastewater from Hanoi City, reaching 335,000 m^3/day, is discharged directly without treatment into the Tolich River, and diluted downstream. Of this total amount of wastewater, domestic waste water accounts for 50%, and industrial release represents the remainder (115,000 m^3/day), with 5,321 m^3 /day from hospitals and also more than 26 tons /day of solid waste (Trinh, 2003). These results clearly show that the Hanoi area is affected by multi sources of pollution along the studied gradient, reaching its most acutely polluted sector in the Tolich River.

This study of diatom assemblages using artificial substrates immersed in rivers for 4 weeks is a first step in the process of evaluating ecological impact on diatom communities. Total diatom densities reflect their developmental potential over time within a physico-chemical environment. Regarding the very low total density values (< 5,000 cells/cm^2) found in the upstream Nhue section (sites N_1, and overall N_3) and in the Tolich River, a probable sporadic presence of various effluents in the Nhue River from surrounding agricultural zones in the Nhue basin (rice cultivation) can be supposed, despite no specific differences between the environmental data analyzed here and at the other sites located upstream. In fact, physico-chemical analysis gives instantaneous data, which can show wide variations especially when several pollution sources are being discharged into rivers (urban, industries, crop treatment, sewage). This assumption is enhanced by the IPS and DAIpo index, which define the N_3 sector as a slightly polluted zone, whereas its instantaneous nutrient records are rather low, and the Tolich (TL) site is identified as a highly polluted site. Other traditional factors like light condition, water temperature, turbidity, affect the colonization process (Patrick, 1977; Chessman, 1985; Peterson and Stevenson, 1989). Conductivity, total P, pH and humus content are also considered to influence diatom community structure (Soininen, 2002). They have all been discussed at length by many researchers; several factors differ significantly between the Nhue-Tolich sites, notably nutrients from probable anthropogenic sources, as already mentioned above, with levels which are a lot higher in the Tolich River and downstream from the confluence with

the Nhue River. There, they support a considerable periphytic development, even in the very drastic environmental conditions of the TL site, but they are not especially highlighted at the N_3 site.

Several water velocity measurements taken during the sampling period indicate that the average values are higher in the Nhue River (30 cm/s) than in the Tolich River (15 cm/s); this difference may explain higher total density values at TL compared to N_3, as increasing water current is recognized to increase its erosive role on periphytic biofilm colonization (Peterson & Stevenson, 1989; Ghosh & Gaur, 1998). Nevertheless, the effects of toxic substances can be considered to explain the low diatom density at site N_3 (near by Ha Dong town where most of factories and traditional craft villages of Ha Tay province are concentrated). The range of total diatom density values obtained in this study (from 660-cells/cm^2 to 30,000-cells/cm^2) is anyway comparable to earlier outdoor experiments carried out in the Lot river (South-West France; Gold et al., 2002) also after 4 weeks of colonization, in similar water current conditions, density ranges are not so different (8,000 - 30,000 cells/cm^2). Though, this global quantitative analysis of diatom communities through total density criterion is not clear enough in this study to be considered as efficient. It has been strengthened by qualitative criteria to evaluate the compared evolution process of diatom assemblages. Relations between environmental factors and diatom assemblages, identified along the hydrosystem, show specific diatom communities preferring different water quality zones. This corresponds to what Potapova & Charles (2003) observed in their study of numerous American rivers where the ionic composition gradient accounted for most variation in diatom assemblage structure. Even if, at first glance, diatom composition is found to be diversified at every investigated site, the diatom taxa found in this study are mainly cosmopolitan. Only a few endemic and tropical taxa have been recorded, particularly in the Red River. This river contains many forms of halophilous taxa already reported in the Mekong River (Coste & Pateron, 2004), such as *Bacillaria paradoxa*, *Nitzschia kurtzii, Navicula recens*, whose presence and abundances might explain a decrease in water quality estimated by diatom indices along the Nhue river (Figure 4.7). In the Nhue River, especially in the lower part, planktonic and alkaliphilic taxa are well represented, indicating moderate pollution. Polysaprobic taxa such as *Nitzschia umbonata* and *Nitzschia palea,* which usually reveal polluted water, are dominant in the Tolich River. It should be mentioned that several *Placoneis* species have been found in the Red and Nhue rivers,some of which have been described as new species (in press). If the composition of diatom communities is examined in more detail (Figure 4.3 and Figure 4.5), major species assemblages can be highlighted at each site, and completely assumed by pollution levels by showing 3 groups of sites. The polluted site (TL) appears to be dominated by taxa that

are resistant to organic pollution like *Nitzschia umbonata* (NUMB), *Nitzschia palea* (NPAL) and *Eolimna minima* (EOMI), which are frequently reported in polluted waters that are rich in nutrients and poorly oxygenated (Takamura et al., 1990; Round, 1991). In the Nhue River, which is supposed to illustrate an increasing pollution gradient compared with reference site in the Red River, the upstream site (N_1) is characterized by dominant species such as *Aulacoseira granulata* (AUGR) and *Gomphonema olivaceum (GOLI*). These are replaced at the N_3 station by resistant taxa such as *Nitzschia palea* (NPAL), which reflect a certain level of organic polluted water quality, as already mentioned above. Downstream in the Nhue River (NT_1 and NT_2) the dominant diatom taxa recorded are *Aulacoseira granulata* (AUGR), *Melosira varians (*MVAR), *Nitzschia palea (*NPAL), *Nitzschia umbonata* (NUMB). They are considered as tolerant to organic pollution and an increasingly heavy metal load (Round, 1991) and highlight the influence of the polluted Tolich waters, after dilution in the Nhue River flow, by contributing specific Tolich diatom species to the downstream Nhue River where they mix with Nhue River diatom species. Finally, at the Red site, a majority of taxa that are more sensitive to organic pollution, like *Encyonema leei, Sellaphora rectangularis Navicula recens*, *Diatoma vulgaris* or tolerant to organic pollution such as *Cymbella tumida, Nitzschia recta, Nitzschia linearis var.subtilis,* were collected.

Among identified communities in each site, diatom response to pollutants is variable not only at community level through the number of dominant taxa, but also at individual level, with morphological alterations. Cattaneo et al., (2004) show a significant reduction in diatom cell length accompanied by an increase in the percentage of valve deformations in Lac Dufault (Canada, Québec), contaminated by heavy metals. In the Red-Nhue-Tolich system few deformities affecting diatom shapes or striae patterns were noticed at the TL site and downstream in the Nhue River. These concern mainly *Fragilaria capucina* and *Diatoma vulgaris* species. The abundance of such abnormal forms has been attributed to heavy metal concentrations by several authors (Gold et al., 2003; Dickman, 1998; Gómez et al., 2003), and is taken into account by the IPS index and will be used by further indices for a better assessment of water quality, even if metallic concentrations are not actually so important in the area studied here.

However, as the IPS and DAIpo indices (Figure 4.7) applied in this study present similar trends and give results in agreement with physico – chemical analysis, they reveal their possible application in this type of hydrosystems. The Tolich River appears to be highly polluted, and the Nhue River shows a water quality that is slightly polluted. The performance of diatom indices have been demonstrate in European and tropical countries (Kwandrans et al., 1998; Wu & Kow,

2002; Kelly & Whitton, 1995) to evaluate water quality in rivers which already shown a high correlation between them and organic load (COD), oxygen concentration, conductivity and most ions. Consequently, such indices should be given further consideration in association with Japanese indices, because some endemic taxa such as *Luticola aff. seposita*, *Gomphonema chubichuensis*, *Encyonopsis leei* and various *Placoneis* are frequently encountered in the Red River and upstream in the Nhue River, and not taken into account by IPS and DAIpo.

4.5 Conclusion

Based on diatom composition and growth on artificial substrata in three rivers, this study was able to classify water quality. The Tolich River, with the highest concentrations of NH_4, PO_4, TOC, TIC, BOD, COD and minimal dissolved oxygen levels, was characterized by the dominant polysaprobic taxa *Eolimna minima, Nitzschia umbonata*, *Nitzschia palea*. The Nhue River (downstream), which is supposed to illustrate an increasing pollution gradient, presented resistant taxa such as *Aulacoseira granulata, Melosira varians, Nitzschia palea, Nitzschia umbonata* and *Gomphonema parvulum*. In the Red River, the dominant taxa were *Encyonema minutum, Achnanthidium minutissimum* and many forms of halophilous taxa such as *Bacillaria paradoxa*, *Nitzschia kurtzii*. IPS and DAIpo were applied to evaluate water quality for the three rivers. Both gave similar results and indicate that the Tolich site, which shows the lowest values, is a highly polluted site.

Further investigations are needed to increase our knowledge of the ecological requirements of these taxa and reference sampling sites. They have to be collected from the whole river catchment area, by increasing the number of sites upstream and downstream, in order to monitor urban pollution in the Hanoi area so that water treatment plants can be set up in the future. To this end, complementary experiments are being carried out. They are based on the transfer of mature and non mature diatom communities (from polluted sites to unpolluted sites and vice versa) to evaluate their recovery potential after exposure to polluted conditions or their response to pollution through the application of modified and adapted diatom indices, taking into account new data from this study.

4.6 Acknowledgements

The authors would like to thank the Institute of Natural products and Institute of Chemistry (VAST – Vietnamese Academy of Science and Technology) for carrying out the

physico-chemical analyses. This study was financed by the ESPOIR programme within a framework of a French-Vietnamese co-operation. Thanks to Georges Vachaud, Research Director at the CNRS for the coordination of the ESPOIR programme (CNRS-CNSTV). We thank H. Koziol, from the Department of Foreign Language (University of Bordeaux 2) for improvements to the native style of the manuscript.

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Taxa name	Abbreviation	Taxa name	Abbreviation
Aulacoseira ambigua	**AAMB**	*Geissleria decussis*	**GDEC**
Achnanthidium biasolettianum	**ADBI**	*Gomphonema gracile*	**GGRA**
Achnanthidium convergens	**ADCG**	*Gomphonema lagenula*	**GLGN**
Achnanthidium minutissimum	**ADMI**	*Gomphonema micropus*	**GMIC**
Adlafia minuscula var. muralis	**ADMM**	*Gomphonema minutum*	**GMIN**
Achnanthidium saprophila	**ADSA**	*Gomphonema olivaceum*	**GOLI**
Achnanthidium subatomus	**ADSU**	*Gomphonema parvulum*	**GPAR**
Amphora montana	**AMMO**	*Gomphonema rhombicum*	**GRHB**
Amphora pediculus	**APED**	*Gyrosigma scalproides*	**GSCA**
Aulacoseira alpigena	**AUAL**	*Hantzschia abundans*	**HABU**
Aulacoseira distans	**AUDI**	*Hantzschia amphioxys*	**HAMP**
Aulacoseira granulata	**AUGR**	*Luticola goeppertiana*	**LGOE**
Bacillaria paxillifera	**BPAX**	*Lemnicola hungarica*	**LHUN**
Cymbella excisa	**CAEX**	*Luticola mutica*	**LMUT**
Cymbella affinis	**CAFF**	*Luticola peguana*	**LPEG**
Cyclotella asterocostata	**CATC**	*Mayamaea atomus var. permitis*	**MAPE**
Cyclotella atomus	**CATO**	*Meridion circulare*	**MCIR**
Caloneis bacillum	**CBAC**	*Melosira varians*	**MVAR**
Cymbopleura amphicephala	**CBAM**	*Nitzschia acidoclinata*	**NACD**
Cymbopleura naviculiformis	**CBNA**	*Nitzschia amphibia f.amphibia*	**NAMP**
Cyclostephanos dubius	**CDUB**	*Navicula antonii*	**NANT**
Cyclotella fottii	**CFOT**	*Navicula capitatoradiata*	**NCPR**
Cyclostephanos invisitatus	**CINV**	*Navicula cryptocephala*	**NCRY**
Cyclotella meneghiniana	**CMEN**	*Navicula cryptotenella*	**NCTE**
Cocconeis pediculus	**CPED**	*Navicula cryptotenelloides*	**NCTO**
Cocconeis placentula	**CPLA**	*Navicula caterva*	**NCTV**
Cyclotella pseudostelligera	**CPST**	*Nitzschia dissipata*	**NDIS**
Craticula perrotettii	**CRPE**	*Nitzschia filiformis var. conferta*	**NFIC**
Cyclotella stelligera	**CSTE**	*Navicula gregaria*	**NGRE**
Cymbella turgidula var.bengalensis	**CTBE**	*Nitzschia bulnheimiana*	**NIBU**
Cymbella turgidula var. turgidula	**CTGL**	*Nitzschia frustulum*	**NIFR**
Cyclostephanos tholiformis	**CTHO**	*Nitzschia gracilis*	**NIGR**
Cymbella tumida	**CTUM**	*Nitzschia hungarica*	**NIHU**
Diadesmis contenta var. biceps	**DCBI**	*Nitzschia intermedia*	**NINT**
Diadesmis confervacea	**DCOF**	*Navicula lanceolata*	**NLAN**

Taxa name	Abbreviation	Taxa name	Abbreviation
Diatoma vulgaris	**DVUL**	*Nitzschia linearis var. subtilis*	**NLSU**
Entomoneis alata	**EALA**	*Nitzschia paleacea*	**NPAE**
Encyonopsis leei	**ENLE**	*Nitzschia palea*	**NPAL**
Encyonema mesianum	**ENME**	*Navicula radiosa*	**NRAD**
Encyonema minutum	**ENMI**	*Navicula reichardtiana*	**NRCH**
Encyonema perpusillum	**ENPE**	*Navicula recens*	**NRCS**
Eolimna minima	**EOMI**	*Nitzschia recta*	**NREC**
Fragilaria capucina	**FCAP**	*Navicula symmetrica*	**NSYM**
Fragilaria crotonensis	**FCRO**	*Navicula tripunctata*	**NTPT**
Fragilaria capucina var. vaucheriae	**FCVA**	*Navicula trivialis*	**NTRV**
Fragilaria ulna var. acus	**FUAC**	*Nitzschia umbonata*	**NUMB**
Gomphonema augur	**GAUG**	*Naviculadicta laterostrata*	**NVDL**
Gomphonema chubichuensis	**GCHU**	*Navicula seminulum*	**NVDS**
Navicula veneta	**NVEN**	*Synedra fasciculata*	**SFSC**
Navicula viridula	**NVIR**	*Surirella linearis*	**SLIN**
Navicula viridula var. rostellata	**NVRO**	*Seminavis strigosa*	**SMST**
Placoneis sp.	**PLAS**	*Sellaphora pupula*	**SPUP**
Planothidium frequentissimum	**PLFR**	*Sellaphora rectangularis*	**SREC**
Pinnularia obscura	**POBS**	*Synedra tabulata*	**STAB**
Planothidium lanceolatum	**PTLA**	*Asterionella formosa*	**STER**
Reimeria sinuata	**RSIN**	*Thalassiosira pseudonana*	**TPSN**
Surirella angusta	**SANG**	*Ulnaria biceps*	**UBIC**
Surirella brebissonii	**SBRE**	*Ulnaria ulna*	**UULN**
Sellaphora bacillum	**SEBA**	*Ulnaria ulna* abnormal form	**UULT**

CHAPTER 5

Responses and structural recovery of periphytic diatom communities after short-term disturbance in some rivers (Hanoi, Vietnam)*

5.1 Abstract:

Field transfer experiments of periphytic diatom assemblages developed on artificial substrates were set up to assess responses of periphytic diatom communities to environmental disturbances. Glass substrates were positioned for colonization in comparatively unpolluted site (Red, in Red River) and heavily polluted site (TL, in Tolich River) at the beginning of the experiment. After a period of two weeks colonized glass substrates were transferred from Red site to TL site and to a moderate polluted site (NT_2 in Nhue River) and conversely, from TL site to Red site, and to NT_2 site. Responses and capacity of periphytic diatom communities to adapt environmental changes were assessed by using cells density, diversity index, species richness, taxonomic composition and diatom indices after 2 and 4 weeks transfer periods and varied for each site. For transfers from Red to NT_2, TL to Red and TL to NT_2, diatoms density significantly increased till the end of experiment whereas growth inhibition of diatom cells was found in transferred biofilms from Red site to TL site. Thus, diatom communities have expressed their pollution tolerance or sensitivities by changing their composition to adapt themselves to changes of environment. In transferred biofilms, from Red to NT_2, characteristic species of Red site were replaced by *Nitzschia palea, Nitzschia umbonata,* and *Aulacoseira granulata* species typical of NT_2 site. Relative abundances of typical diatoms species of Red site proliferated in biofilm transferred from TL site to Red site. Replacement of periphytic diatoms communities after transfer appeared from two weeks in the different sites. Slowly shift of Red species by typocal TL species could be in relation with the organized structure of the biofilm before transfer. Species richness and diversity index were not clearly reflecting responses of periphytic diatom to disturbance. Shifts in values of IPS and DAIPo indices, throughout the experiment indicated sensitivity of these indices to water quality changes.

Key words: responses, Disturbance, Periphytic Diatom, Recovery, Artificial substrate, pollution.

* This chapter is prepared as an article to be submitted under the reference:
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5.2 Introduction

The increasing population and the associated rise in industrial and agriculture activities results in an ongoing pressure on aquatic ecosystems. Anthropogenic activities play a major role in increasing nutrients input, and a large number of chemicals discharged into rivers cause unfavorable changes in ecological systems. The biological integrity of an aquatic ecosystem depends upon its physical and chemical component which is also controlled by the local geology besides land use activities within the watershed. In many streams and rivers, periphytic algae, which are primary producers, are considered to be a major source of energy to aquatic food webs (McCormick and Stevenson, 1998). Because they are attached to substrates, their characteristics are affected by physical, chemical and biological disturbances which occur in streams or rivers during the time in which they developed. Moreover, periphytic algae integrate ecological variations over time and can provide information of changes in water quality (Lowe and Pan, 1996; Gold et al., 2002). Periphytic algae in general and diatoms in particular have been widely used as organisms for monitoring and assessing water quality owing to their wide distribution and well-studied ecology (Potapova and Charles 2005). Diatoms are considered to be excellent biological indicators for many types of pollution in aquatic systems such as organic pollution (Descy and Coste, 1991), acidification, metallic pollution (Cattaneo et al., 2004) and eutrophication (Dixit et al., 1992). Analysis of diatom communities can be used to discriminate the impacts of organic pollution and inorganic nutrients enrichment such as treated urban wastewater and diffuse nutrient sources from farmland (Rott et al., 1998). Diatom indices based on selected sensitivities or tolerance of diatom species to organic pollution or acidity are being routinely and widely used in many European and Asian countries (Whitton and Rott, 1996; Watanabe et al., 1986) for rivers quality assessment. Communities in streams and rivers are subjected too to natural stress such as floods besides their exposition to multiple anthropogenic inputs as well.

It has been well documented that multi-sources of pollution greatly affect structure and function of periphytic algae communities. These effects include reduced photosynthetic ability (Soldo and Behra, 2000; Barranguet et al., 2000), reduced growth rate (Gibson and Fitzsimon, 1991; Genter, 1996), interruption of cell division and deformation of diatom frustules (Gold et al., 2003a, Cattaneo et al., 2004). At a cellular level, algae may tolerate pollutant stress by showing a decreased number of binding sites at the cell surface, physiological development of exclusion mechanisms, genetic adaptation, morphological changes, and internal detoxifying mechanisms (Genter, 1996). At a community level, periphytic algae increase their tolerance by

shifting its composition to more tolerant species when they are exposed to pollution (Kasai, 1999), which can result in structural changes such as decreased species diversity and richness (Genter, 1996). Many manipulative stream experiments have been extensively performed in both natural and laboratory conditions to assess the impacts of short and long term environmental changes by using periphytic algae communities. The effect of long term copper exposure on structure of periphytic communities was monitored in outdoor flow and indoor by Soldo & Behra (2000). Barranguet et al (2000) used indoor experiment to assess the short term metal effect on communities' tolerance in photosynthesis process. Pan et al (2000) used mesocosms to study structural and functional changes of epiphytic algal assemblages due to increased P loading.

Up to now, a few transfer experiments have been performed to assess alters of periphytic diatom assemblages caused by changes in water quality. Earlier studies have suggested that translocation of periphytic diatom communities on artificial substrates between different pollution sites was a suitable method to assess *in situ* effects of metallic pollution. (Ivorra et al., 1999; Gold et al., 2002). Differences or changes in the biomass, productivity, and structure of periphyton communities can provide a sensitive measure of the trophic and wholeness status of streams and rivers.

In the present study, through transfer outdoor experiments, we aimed to test the appropriativity of periphytic diatom communities as a reliable and efficient tool for a fast diagnosis of biological conditions in streams and rivers, first by assessing the response of benthic diatom communities to environmental stress via transfers of early stages of benthic diatom communities developed on artificial substrates from reference site to heavy polluted site and moderate polluted site and reversely; then by estimating the time needed for diatom communities to integrate a change of environmental conditions in their structures; and finally by studying how benthic diatom communities recover their structure from pollution stress.

5.2 Materials and methods

5.2.1 Study area and design

The sampling area is located in the Red River delta in the north of Vietnam, and includes three rivers: Red, Nhue and Tolich rivers (Figure 5.1). Red River is the largest river and has an important role in agricultural production of the country. Nhue River, as a tributary of Red River, diverges from it at Thuy Phuong dam, runs through the Hanoi area, Hadong city (Hatay

province) and Hanam province *via* several dams and finally flows into the Day River. Nhue River basin area is 74 km long and covers a total of 107,503 ha of which 75% is cultivated (Trinh, 2003; Kono & Doan, 1995). At 20 km from its confluence with Red river, Nhue River receives water discharges from Tolich River through Thanh Liet dam. Tolich River originates from West Lake and flows 14 km across Hanoi city. Untreated waste water of Hanoi city and Hatay province is discharged directly into Tolich River. Situated in a densely population (population of Hanoi area, approximately 4 millions inhabitants), Nhue and Tolich rivers serve as a major drainage canal and irrigation for surroundings of the Hanoi, and Hatay areas.

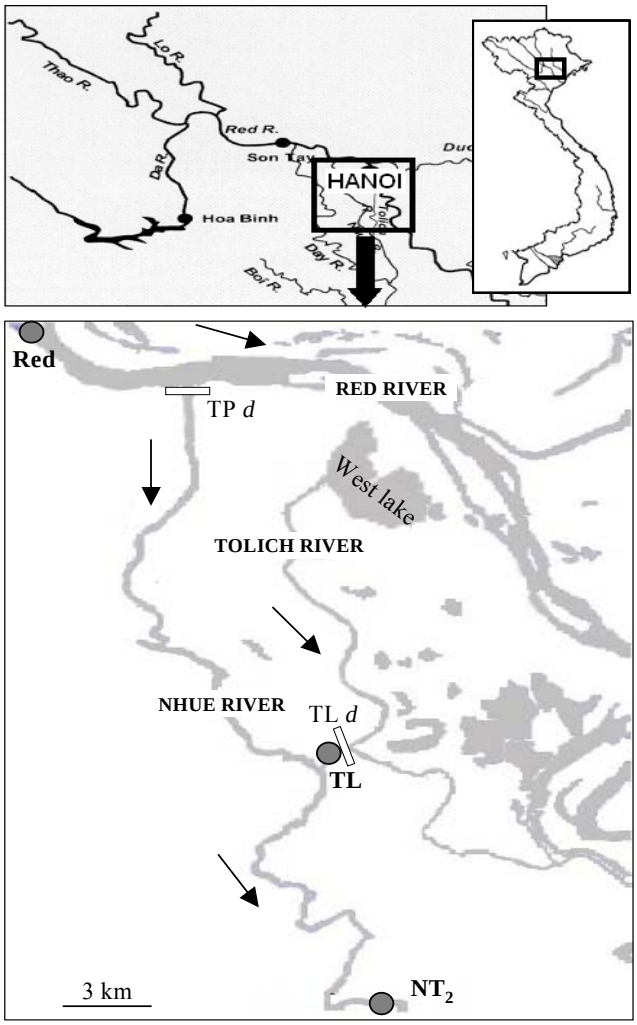

Figure 5.1: Location of sampling stations (*TPd*: Thuy Phuong dam; *TLd*: Thanh Liet dam).

The wastewater source of Nhue and Tolich system comes from four main supplies: (i) 335,000 m^3/day of industrial and municipal wastewater from Hanoi area; (ii) disposal of municipal waste

in the riverbank; (iii) waste water from numerous traditional villages along the river banks in Hatay province (craft villages produce papers, food, leather, texture, timber...etc); (iiii) agricultural run-off (fertilizers and pesticides) and soil erosion from agricultural activities along the riverbank (Trinh, 2003; Nguyen, 2001). A local tropical to subtropical monsoon climate leads to an average annual rainfall of 1800-2000 mm, 80% of which occurs during the rainy season from June to October. Dry season lasts from November to April-May. Relative humidity is very high throughout the year with an annual value of 84.5% (Trinh, 2003; Kurosawa et al., 2004).

Within this area, three study sites, presenting different water pollution levels, were selected (Figure 5.1) and subjected to transfer experiment during dry season: (i) the comparatively unpolluted reference site (Red) situated at Sontay (Sontay district, Hatay province) in the Red River, about 8 km upstream from the Nhue River and Hanoi city, shows low nutrients and metal concentrations (Trinh, 2003; Le, 2005); (ii) the heavy polluted site (TL) located upper the Thanhliet dam (*TL dam*) and downstream Tolich River, is characterized by low dissolved oxygen, high nutrients and metal concentrations with black, foul-smelling waters; (iii) the moderate polluted site (NT_2) is positioned downstream Nhue River about 7 km below its confluence with the Tolich River.

5.2.2 Field sampling

The experiment was performed during the dry season from 9th January to 20th February, 2005. During this season, the three rivers are connected, dams being opened to normally irrigate downstream basin for agricultural purposes. However, in dry season 2005, water level of Red River was low and water support to Nhue River was quite limited. At both sites, Red and TL, one set of two plastic baskets equipped with floaters were immersed in water column parallel to the current at a depth of 15-20 cm below the surface and tied to the bank with a rope (Gold et al., 2002). In each plastic basket, 18 glass slides were placed separately and vertically to be used as artificial substrates for algae attachment (30 cm x 18 cm-1080 cm^2 surface for both sides). One set was left at Red and one at TL sites, at the beginning of the experiment, for a period of two weeks to allow the development of biofilms on glass slides. After two weeks of colonization at Red site, one of the two plastic baskets containing glass slides was transferred to heavy polluted site (TL); the other basket was moved to the moderate site (NT_2). Same procedure was followed at TL site, with one plastic basket containing glass slides transferred to reference site (Red) and one basket to moderate site (NT_2). During transportation, glass slides were kept immersed in

their initial river water within a container box (1-2 h travel time). After transfer, glass slides were left in their new locations for an addition period of four weeks. Periphytic samples developed on glass slides were sampled before transfer at week 2 (W_2), and after transfer at week 4 (W_4) and week 6 (W_6). On the day of sampling, at each site, three glass slides, considered as independent samples, were randomly removed from each plastic basket. Glass slides were then thoroughly rinsed with filtered river water. Biofilms were collected from the glass slides by using a nylon brush, and then washed and diluted in a known volume (100 or 200 mL) of distilled water depending on the biofilms thickness. In order to determine dry weight of biofilms, a known volume of samples was stored in a labelled polyethylene bottles and placed in a cool (4 - 10°C), dark place during transportation to laboratory. Remainders of natural biofilm samples were preserved in labelled glass bottles with 5% formalin solution (Formaldehyde 37% Prolabo France) for delayed identification of diatoms composition.

Water temperature, dissolved oxygen, conductivity and pH were measured *in situ* during the whole experiment by using multi-parameter sensors (Model WQC-22 A, TOA). Water samples were taken near the surface (30 cm below the surface) for nutrients determination and stored on ice box (4-10^{o} C) during sampling. Samples were filtered through Whatman GF/C filter (glass micro-fiber filters 0.45 µm) using hand pump filtration with high pressure and high flow rate. The analyses of phosphate, ammonia, nitrate, nitrite concentrations in water samples followed American Public Health Association (APHA, 1995). These analyses were performed by the Institute of Natural Products (VAST - Vietnamese Academy of Science and Technology, Hanoi, Vietnam).

5.2.3 Laboratory analyses

Diatom preparation

After homogenization, 2 ml aliquot of each diatom sample from each site were digested with concentrated hydrogen peroxide (30%) and hydrochloric acid (35%) to remove organic matter and dissolved calcium carbonates, then rinsed several times and diluted with deionized water. The cleaned diatom frustules were then mounted on a microscope glass slide using Naphrax, high resolution mounting medium (Brunel Microscopes Ltd, UK; RI = 1.74) (Gold et al., 2002; EN 13946, 2003; EN 14407, 2004). Diatoms were identified with a Leitz DMRD light microscope at 1000x magnification. Approximately 400 valves were identified to species level from each slide of the three replicates. The Süßwasserflora nomenclatures (Krammer and Lange-

Bertalot, 1986-1991) were used as references for diatom taxonomy. Relative abundance of diatom species (in percentage) was estimated. Species richness (S) was calculated and biological diversity was estimated through Shannon-Weaver diversity index (H'). Diatom indices (IPS and DAIPo index) were applied in order to classify water quality in each sampling site.

A Nageotte counting chamber (Marienfeld, Germany) was used to estimated diatom density in each sample by counting the total number of diatoms in 30 fields (1.25 µl each, 0.5 mm depth) using a light microscope (Olympus BX x 50) at 200x magnification. Data were expressed in cells per unit area of glass slide (number diatom cells.cm^{-2}).

Biofilms dry weight determination

A known volume of homogenous biofilm suspension sample was filtered with a manual aspiration pump, through a previously weighed filter membrane (0.45µm Millipore corp) for biofilms dry weight (dw) (values expressed as mg dw.cm^{-2}) determination. The filters covered with drained biofilms were then thoroughly dried at 60° C for 48 h in incubation tubes, and dry weight of natural biofilms was measured afterwards.

5.2.4 Data treatment

Diatom indices IPS (Index of Polluosensitivity Specific) (Cemagref, 1982) and DAIPo (Diatom Assemblage Index to organic Pollution) (Watanabe et al., 1986) were calculated using the OMNIDIA software (Lecointe et al., 1993). These diatom indices were transformed to range from 1 to 20 to be comparable. In order to study significant differences in diatom density, structure of diatom communities (species richness, diversity index) and diatom indices between the local and transferred diatom communities, we first performed ANOVA method (one way or two way) using STATISTICA software (StatSoft, 2004) after checking assumptions (normality and homoscedasticity of the error term). If the null hypothesis was rejected, *post-hoc* tests (Least Significant Difference test (LSD) were applied in order to find significant differences between groups. For all statistical results, a probability of $p < 0.05$ was considered significant. Taxonomic differences between the sites were displayed using Principal Component Analysis (PCA) with PC-ORD Software (McCune and Mefford 1999). PCA was performed on relative abundances of only 53 species out of a total of 277 identified diatom species, which had the highest cumulative relative abundances within local and transferred diatom communities collected through out the experiment.

5.3 Results

5.3.1 Physical and chemical characteristics of sites

Physical and chemical characteristics of water measured at the three sampling sites Red, NT_2 and TL are shown in table 5.1. Most of them were stable at each site throughout the experiment. At all sites, pH values vary from 6.4 to 7.5 showing quite neutral pH. Water temperature values are around 18.2°C to 20.6°C with lowest values measured at Red site, which reversely shows the highest dissolved oxygen values, lower mean values of dissolved oxygen being recorded at NT_2 and TL sites (3.1 and 1.73 respectively).

Parameters	Red			NT_2			TL		
	W_2	W_4	W_6	W_2	W_4	W_6	W_2	W_4	W_6
pH	7	6.8	7.5	nm	6.9	7.1	6.4	6.5	6.5
Temperature °C	18.2	18.9	19	nm	20.6	20.3	19	20.5	21
O_2 ($mg.l^{-1}$)	8.01	7.4	7.9	nm	2.3	4.3	1.9	1.7	2.7
Conductivity (µS/m)	20	18.6	19.2	nm	71.9	69.3	98.6	69	72.6
NO_3-N ($mg.l^{-1}$)	0.8	0.6	0.7	nm	0.9	1.7	2.5	2.1	2.2
NO_2-N ($mg.l^{-1}$)	0.029	0.012	0.011	nm	0.019	0.047	0.025	0.027	0.018
NH_4-N ($mg.l^{-1}$)	0.1	0.2	0.1	nm	19.2	5.9	30.2	30.1	26.8
PO_4-P ($mg.l^{-1}$)	0.15	0.18	0.15	nm	1.40	2.66	2.75	2.80	6.78

Table 5.1: Physical and chemical characteristics of the three sampling sites Red, NT_2 and TL (January - February 2005) during study period. (nm: not measured).

Highest nutrients concentrations are recorded by decreasing order at TL site, NT_2 site, then Red site. Mean conductivity value is approximately four times higher at TL and NT_2 sites than at Red site, and points out the high organic pollution level of these two sites. Nevertheless, NH4-N and PO_4-P are differentiated among other parameters by a sharp decrease of NH4-N values at the end of experiment at NT_2, and a clear increase of PO_4-P at TL, both associated with a raise in dissolved oxygen values.

5.3.2 Diatom density

Quantitative characterization of diatom communities assemblages are reflected by numeration of diatom cells. Diatom densities developed on glass substrates before (W_2) and after transfer (W_4 and W_6) are illustrated in figure 5.2 for the three sites.

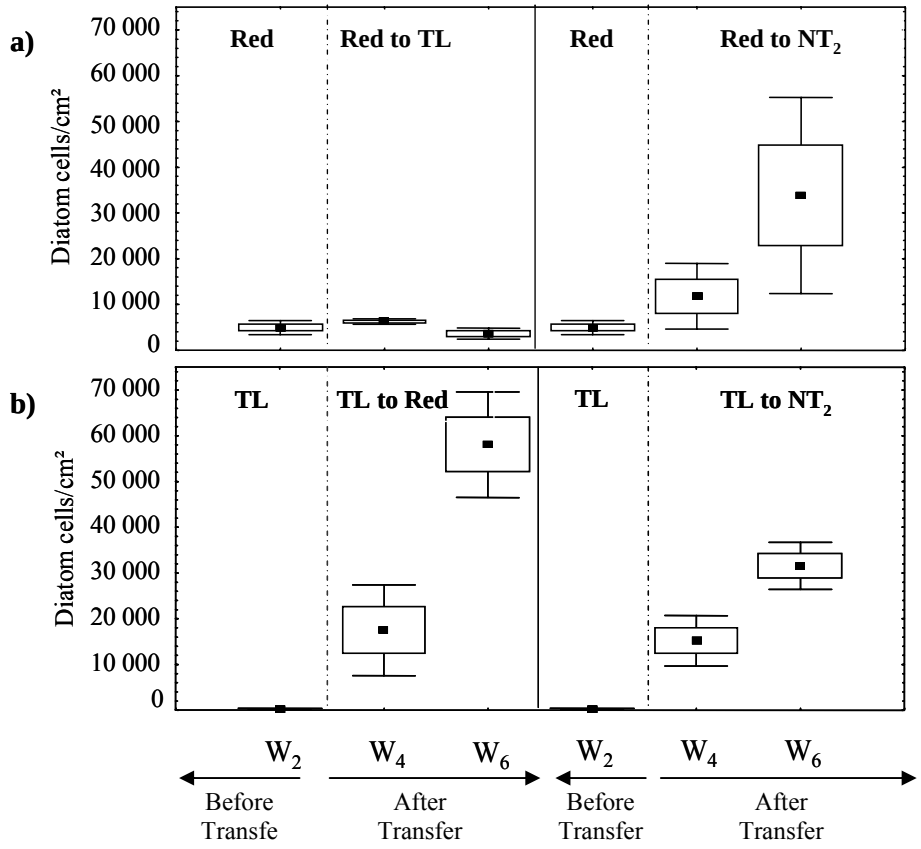

Figure 5.2: Diatom densities on artificial substrates (cells.cm^{-2}) collected from the three stations Red, NT_2 and TL at the week 2 (W_2) (during initial colonization on site before transfer), at week 4 (W_4) and week 6 (W_6) (after transfer at their new locations) of the experiment. Boxplot: mean value (n = 3) with its confidence interval at 95 % denoted by the two horizontal lines outside the box.

Figure 5.2a figures diatoms density coming from biofilms previously colonized in Red site during two weeks, then transferred to either TL site or NT_2 site during four extra weeks. Figure 5.2b shows same evolution, but for biofilms previously developed at TL site then transferred to Red or NT_2 site. Total diatom density at Red site shows a higher number of cells during the two weeks initial period of colonisation than those at TL site (4,928 ± 446 cells.cm^{-2}; 240 ± 19 cells.cm^{-2}, respectively). A yellow-brown colour layer including detritus, algae, bacteria, and suspended particular matters covered glass substrates at Red site after two weeks, whereas a thin black coloured layer was visible on its substrates at TL site. After transfer of the

glass substrates from Red site to NT_2 and TL sites, development of diatoms at these two sites are different (figure 5.2a). Densities at TL site range from 3,595 ± 351 cells.cm^{-2} to 6,248 ± 182 cells.cm^{-2}, showing low development of diatom communities. Meanwhile at NT_2 site, diatom densities developments after transfer from Red site increase significantly ($p < 0.05$) till the end of the experiment. For glass substrates transferred from TL site to Red and NT_2 sites, densities of diatoms on substrates grow a lot at their new location and reach maximum values during the last week with 31,539 ± 1511 cells.cm^{-2} at NT_2 site and 58,034 ± 3401 cells.cm^{-2} at Red site. Despite the difference in diatom density from the initial period of colonization in Red or TL site, diatom growth at NT_2 site after both translocations shows similar trend of evolution, ranging from 11,786 ± 2,110 cells.cm^{-2} at W_4 to 33,810 ± 6,313 cells.cm^{-2} at the W_6 for transferred diatom communities from Red site to NT_2 site, and from 15,176 ± 1,621 cells.cm^{-2} at W_4 and 31,539 ± 1,511 cells.cm^{-2} at the W_6 for transferred diatom communities from TL site to NT_2 site. Qualitative observations of biofilms at NT_2, after 4 weeks of transfer, indicated the development of a thick, yellow-brown and mucilaginous coat on their outside, black and colored aspect on their inside. According to two-way ANOVA results, significant differences in diatom density between sites and colonization durations are observed ($p < 0.05$).

5.3.3 Species richness, diversity of diatom communities and diatom indices

Evolution of species richness (S), diversity index (H') and diatom indices occurring along the experiment are shown in table 5.2. A significant difference is noted between communities previously developed at Red and TL site during the first two weeks with 22% more species initially developed at Red site than at TL site. On the other hand, no significant difference is detected in S between local diatom communities (Red, TL) at W_2 and transferred diatom communities during the four last weeks of experiment, although S seems to decrease for the condition Red to NT_2, and to increase for both transfers from TL when compared to week 2 values. Concerning Shannon diversity index (H'), only transferred diatom communities from Red to NT_2 shows a significant difference during the course of the experiment, when compared to local diatom communities according to one-way ANOVA result ($p < 0.05$). Both water quality diatom indices, IPS and DAIPo, reveal fairly similar profiles (table 5.2). After transfer from Red site, IPS and DAIPo indices gradually and significantly ($p < 0.05$) decrease with exposition duration to reach a minimum value at W_6 (4,5 and 7,3 at NT_2 site and 7,9 and 8,6 at TL site,

respectively). Table 5.2 results also show a considerable increase of the indices (mainly IPS) after transfer from TL site to Red site, although they do not reach the high values obtained at the local site (Red) during the first period of colonization. After transfer from TL to NT_2, diatom indices increase too at W_4 but show different patterns after, with a decrease during the last week of experiment for IPS and a stabilization for DAIPo.

Stations	Weeks	S	H'	IPS	DAIPo
Red to NT_2	W_2	80 (1)	4.9 (0.04)	11.1 (0.3)	11.3 (0.1)
	W_4	73 (6)	4.3 (0.1)	6.4 (05)	8.6 (0.2)
	W_6	70 (2)	4.2 (0.03)	4.5 (0.2)	7.3 (0.2)
Red to TL	W_2	80 (1)	4.9 (0.04)	11.1 (0.3)	11.3 (0.1)
	W_4	81 (3)	5.1 (0.2)	8.3 (0.4)	9.1 (0.3)
	W_6	83 (2)	5.1 (0.1)	7.9 (0.4)	8.6 (0.4)
TL to NT_2	W_2	52 (7)	4.2 (0.23)	4.6 (0.6)	6.3 (1)
	W_4	67 (2)	4 (0.1)	7.0 (0.5)	9.3 (0.3)
	W_6	62 (2)	3.9 (0.1)	4.2 (0.5)	10 (0.1)
TL to Red	W_2	52 (7)	4.2 (0.23)	4.6 (0.6)	6.3 (1)
	W_4	69 (1)	4.2 (0.03)	8.1 (0.4)	7.9 (0.9)
	W_6	64 (3)	3.8 (0.1)	8.6 (0,2)	7.1 (0.5)

Table 5.2: Values of Species richness (S), Diversity index (H') of diatom communities and values for the two diatom indices IPS and DAIPo collected from the three stations Red, NT_2 and TL at the week 2 (W_2) (during initial colonization on site before transfer), at week 4 (W_4) and week 6 (W_6) (after transfer at their new locations) of the experiment (mean value and standard error, n = 3)

5.3.4 Dry weight of biofilms

During the first two weeks colonization period, mean value of dry weight of biofilms is higher at Red site than at TL site by a factor 29 (figure 5.3). However, transferred biofilms from Red to TL and from Red to NT_2 do not follow the same pattern of development (Figure 5.3a). A continuous increased development is observed in biofilms transferred from Red site to NT_2 site, and the highest dry weights values of the study are then recorded with 6.2 $mg.cm^{-2}$ at W_4 and 7.2

mg.cm^{-2} at W_6. In contrast, transferred biofilms from Red site to TL site maintain an average biomass of 2 mg.cm^{-2} during the last 4 weeks period after transfer. Very thin biofilms, initially constituted at TL, site grow a lot after transfer to their new location ($p < 0.05$) with a stronger development at NT_2 site (dry weight average value: 5.3 ± 0.6 mg.cm^{-2} at W_4 and 4.6 ± 0.4 mg.cm^{-2} at W_6) than at Red site (dry weight average value: 2.5 ± 0.26 mg.cm^{-2} at W_4 and 2.8 ± 0.5 mg.cm^{-2} at W_6), but without reaching the outstanding development observed at NT_2 for the transfer Red to NT_2.

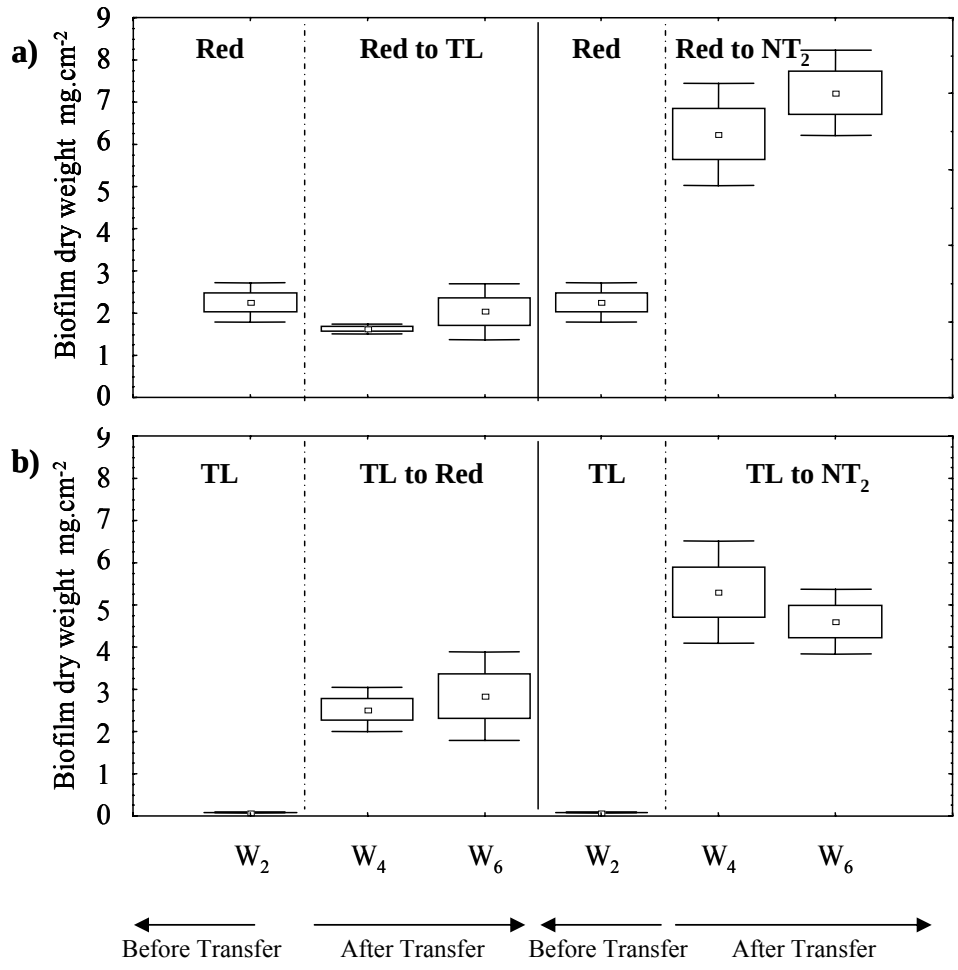

Figure 5.3: Dry weight of biofilms collected from Red, NT_2 and TL at week 2 (W_2) (during initial colonization site before transfer), week 4 (W_4) and week 6 (W_6) (after transfer at their new locations). Boxplot: mean value (n = 3) with its confidence interval at 95 % denoted by the two horizontal lines outside the box.

5.3.5 Diatom composition

Composition of diatom communities of local and transferred diatom assemblages which colonized the substrates through out the experiment is presented through relative abundances of 7 diatom species (mean relative abundances ≥ 8%) in figure 5.4a and b. Diatoms community of Red site at W_2 is dominated by *Gyrosigma scalproides* (GSCA) and *Navicula recens* (NRCS) in the range from 14.1% to 12.5% respectively (figure 5.4a).

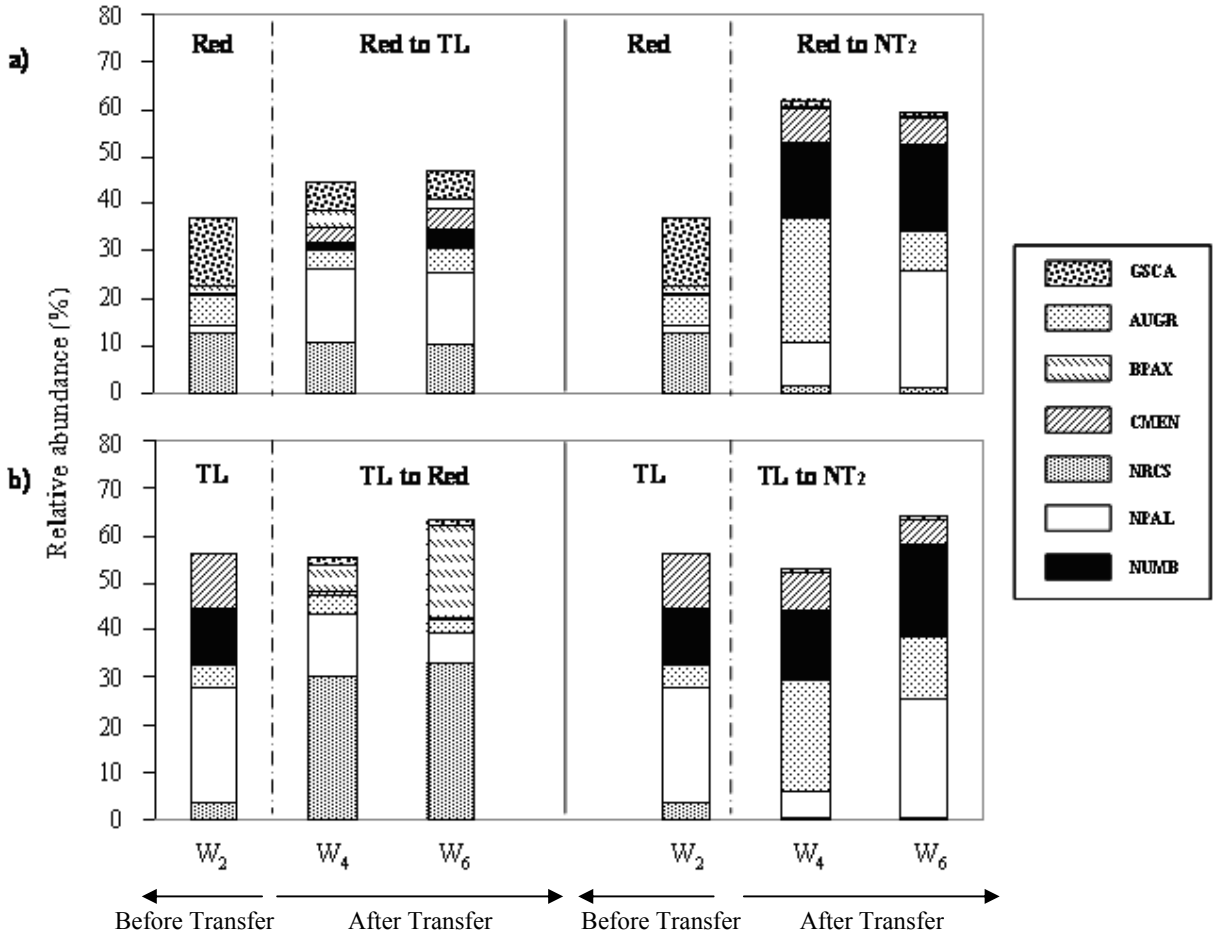

Figure 5.4: Relative abundances of diatom species (mean value, n = 3) with mean relative abundances ≥ 8 % at week 2 (W_2) (before transfer), at week 4 (W_4) and 6 week (W_6) (after transfer) of the experiment for glass substrates collected at Red, NT_2 and TL stations. (NUMB: *Nitzschia umbonata*, NPAL: *Nitzschia palea*, AUGR: *Aulacoseira granulata*, GSCA: *Gyrosigma scalproides*, NCRS: *Navicula recens*, CMEN: *Cyclotella meneghiniana*, BPAX: *Bacillaria paxillifera*.)

After transfer, the composition of transferred diatom communities is modified. When periphytic diatom communities from Red site are transferred to NT_2 site, high proportion of *Gyrosigma*

scalproides (GSCA) and *Navicula recens* (NRCS) are rapidly replaced by polysaprobic taxa like *Nitzschia palea* (NPAL) (W_4: 8.9 %; W_6: 24.6%) *Nitzschia umbonata* (NUMB) (W_4: 16.7%; W_6: 18.3%), the planktonic taxa *Cyclotella meneghiniana* (CMEN) (W_4: 7%; W6: 5.4%) and *Aulacoseira granulata* (AUGR) (W_4: 26.2%; W_6: 8.4%). In contrast, initial diatom communities of Red site transferred to TL site are still present till the experiment ends; pollution tolerant taxa *Nitzschia palea* (NPAL) and *Nitzschia umbonata* (NUMB) being clearly settled with mean relative abundances around 15.6% and 2% respectively. Besides, these two species *Nitzschia umbonata* (NUMB) and *Cyclotella meneghiniana* (CMEN) appear as predominant species at TL site on the week 2 (before transfer), with mean relative abundance $\geq$ 10% (figure 5.4b). After transfer from TL to Red and from TL to NT_2, diatom composition of the communities is manifestly modified. For the diatom assemblages transferred to Red site, the initial dominant taxa *Nitzschia palea* (NPAL) is still persistent on substrates, but with lower relative abundances (ranged from 13.5% to 6.2%), until the end of the experiment, whereas *Nitzschia umbonata* (NUMB) and *Cyclotella meneghiniana* (CMEN) are replaced by the dominance of *Navicula recens* (NRCS) (30% at W_4 and 33 % at W_6) and *Bacillaria paxillifera* (BPAX) (5.4% at W_4 and 19.5 % W_6). The assemblages of the biofilms transferred from TL site to NT_2 are modified too and proportions of the 4 main diatom species such as *Nitzschia palea* (NPAL), *Nitzschia umbonata* (NUMB), *Cyclotella meneghiniana* (CMEN) and *Aulacoseira granulata* (AUGR) differ from initial colonization period.

Through Principal Component Analysis (PCA) of 53 diatom species upon a total of 277, modifications in the composition of transferred diatom communities in comparison with local diatom communities are figured in terms of duration time and sampling site (figure 5.5). The graph shows a projection of plan 1/2 explaining 53.9% of total variability, and includes all species data and transfers conditions. Communities from Red site are more rapidly discriminated when transferred to NT2 site than to TL site. In all the other cases, discrimination from initial colonization site (Figures 5.5b and 5.5c) occurs as soon as week 4 (after 2 weeks of transfer), indicating a clear shift of the communities to adopt same type of assemblages than those of their transfer site. Diatom communities transferred to NT_2 (from Red and TL) are characterized by species such as *Nitzschia umbonata* (NUMB), *Navicula veneta* (NVEN), *Sellaphora pupula* (SPUP), *Navicula cryptocephala* (NCRY), *Aulacoseira granulata* (AUGR), *Ulnaria ulna* (UULN). Group of diatom assemblages positioned on the left half plane are representative of Red site, communities transfered from TL to Red and from Red to TL such as *Gyrosigma scalproides* (GSCA), *Navicula recens* (NRCS), *Seminavis strigosa* (SMST), *Cymbella excisa* (CAEX), *Achnanthidium minutissimum* (ADMI), *Sellaphora bacillum* (SEBA), *Bacillaria*

paxillifera (BPAX). Meanwhile, planktonic and polysabrobic taxa e.g. *Cyclotella atomus* (CATO), *Amphora montana* (AMMO), *Cyclotella meneghiniana* (CMEN), *Lemnicola hungarica* (LHUN), *Nitzschia palea* (NPAL), *Gomphonema parvulum* (GPAR) and *Eolimna minima* are (EOMI) of the TL site (W_2) mainly separate communities from Red and NT sites.

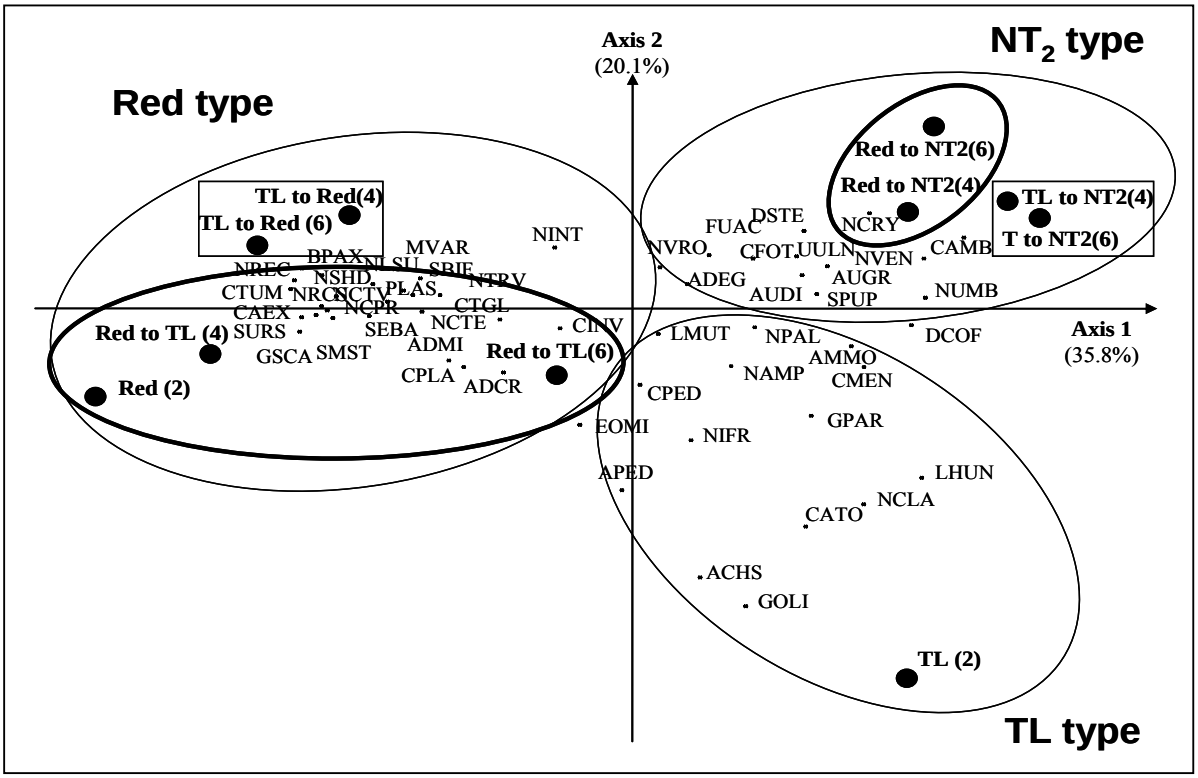

Figure 5.5: Principal Component Analysis (PCA) based on relative abundances of 53 diatom species (>1% and 3 replicates per site) at week 2 (W_2) (before transfer) and week 4 (W_4), week 6 (W_6) (after transfer) of the experiment on glass substrates collected at Red, NT_2 and TL stations. (Week is indicated in bracket)

5.4 Discussion

Qualitative and quantitative characteristics of aquatic organisms organized in communities depend on various physical, chemical and biological factors. These factors determine the whole responses of aquatic organisms communities in general and algae communities in particular. In the present study, alterations of structural characteristics of periphytic diatom communities to adapt themselves to a new environment were illustrated by

measurements of density of diatom cells, diversity index, species richness and estimated changes of diatom indices values and diatoms composition.

The structural adaptability of periphytic diatom communities to environmental disturbances was observed after transfer of early stages of periphytic diatom communities developed on artificial substrates from comparatively unpolluted site (Red) or heavily polluted site (TL); to moderate polluted site (NT_2); and transfer between Red and TL site conversely. In our study, multiple sources of pollution in the rivers and directions of transfer determine responses of structural periphytic diatom communities are clearly illustrated by densities of diatom. Significant increase of diatom densities after transfer are already observed when periphytic diatom communities are moved from TL site to Red site and to NT_2 site and from Red site to NT_2 site. Some communities are differently altered and increase their biomass; others show reduction in cells density after short time period of disturbance (figure 5.2a and 5.2b). Equally, an active immigration and growth rate of cells could also play an important role and contribute to increase the number of diatoms cells on substrates, in both early and late stage of colonization in our study (Stevenson and Peterson, 1991; Stevenson et al., 1991). Conversely, high concentrations of nutrients and other contaminants accompanied by low dissolved oxygen showed a marked influence on the accumulation of diatom on substrates in TL site where a slow development was reported in diatom density and biofilms dry weight communities transferred to TL, though they were higher than those at local site before transfer. Same observations were made in previous experiment when dynamic of diatom colonization was followed in similar conditions (Duong et al., in press). In Red site before transfer (W_2) a complex layer containing algae, bacteria, detritus and polysaccharide exudates which constitutes the biofilm matrix covering densely the substrates (figure 5.2 and 5.3), and permitted the development of higher number of diatoms cells in comparison with local site (TL).

Diversity and species richness are classical indicators of changes in communities structure to disturbances (Jüttner et al., 1996; Sabater, 2000). Sabater (2000) showed a marked decrease in diversity between references sites in comparison with affected sites of the Guadiamar River, S-W Spain. Stress effects of from mine drainage on diversity of primary producers in mountain stream were observed by Niyogi et al (2002), who suggested that physical stress did not strongly affect diversity of primary producers in streams like chemical stress. Hill et al (2000) asserted that periphyton taxa richness was not related to environmental stressors, and Hoagland (1983) reported that, periphytic diatom diversity did not reflect adverse environment conditions as clearly as biomass. In our study, we found that response of structural communities (diversity index and species richness) to water quality changes was not clear (table 5.2). No

significant difference in species richness and diversity index was found between local and transferred diatom communities in all cases except transferred communities from Red to NT_2. This result is not surprising as diatom communities are deeply transferred after a disturbance. The assemblages are rearranged by changing their composition from sensitive species to more tolerant species to cope environmental altered condition. In this case, numerous replacements occur as long as polluted condition stays bearable for newly assemblages to settle without conducting to a notable and significant decrease of diversity. This adaptation can reversely happen with the replacement of resistant taxa by more sensitive taxa in aquatic stress (Kassai, 1999) and in return communities facing stress may modify their structure and function on their own (Niederlehner and Cairns, 1992). Thus, diatom communities transferred from Red to NT_2, TL to Red and TL to NT_2 changed quickly their composition to adapt themselves to new conditions (figure 5.4a and 5.4b). Communities characterized by rich nutrients, high conductivity and low dissolved oxygen with dominant taxa such as *Nitzschia umbonata* (NUMB), *Cyclotella meneghiniana* (CMEN), *Nitzschia palea* (NPAL) are replaced by less tolerant taxa to pollution such as *Navicula recens* (NRCS), *Bacillaria paxillifera* (BPAX) and sensitive taxa *Achnanthidium minutissimum* (ADMI) when diatom assemblages are moved from TL to Red site. The increase of diatom density and taxa less tolerant to pollution in communities transferred to Red site reflect considerable improvement of water quality. On the other hand, complete replacement of pollution-tolerant taxa in transferred communities to NT_2 site (Red to NT_2 and TL to NT_2) showed capacity rapid recovery of periphytic diatoms which succeed to a structural stability after environmental changes. Our results are in agreement with those of Gold et al (2002), who observed structural community adaptation to new environmental conditions within two weeks after exposure to metal pollution. However, after four weeks of transfer, diatom communities did modify their structure but not completely for assemblages transferred from Red site to TL site (Figure 5.5). At Red site before transfer, biofilm matrix densely covering substrates seems to limit the settlement of indigenous diatom species of polluted site (TL) onto to substrates. This results in the retention of initial species of Red site, *Navicula recens* (NRCS), *Bacillaria paxillifera* (BPAX), *Gyrosigma scalproides* (GSCA) still represented in a minor proportion of transferred communities polluted site although some native diatom species of the TL site were already settled and gradually increased at W_6 (after 4 weeks transfer) such as *Nitzschia palea* (NPAL) and *Cyclotella meneghiniana* (CMEN). In this case, four weeks prove to be necessary for transferred diatom communities to TL site to acclimate to TL water quality conditions. Nutrient enrichments are not only parameters affecting diatom assemblages communities, increased metals concentration and other contaminants present in TL site play a

possible and important role in the responses of communities structure (Soldo and Behra, 2000), nature and source of wastewater effluents being diversified but with no precise data.

Diatom indices (IPS and DAIPo) have already been applied successfully to assess water quality rivers of Vietnam (Duong et al., 2006a and b). In this study, both of them give a similar trend of improvement in water quality when diatom communities were transferred from polluted site to unpolluted and to moderate sites; and worsening of water quality when communities were transferred from unpolluted site to heavily polluted and moderate polluted sites (table 5.2). Recovery of water quality was clearly observed very soon only after 2 weeks transfer. A slow decrease of diatom indices occurred when diatom communities were moved from Red to TL even if a proportion of diatoms taxa characterized of Red site still remained in its biofilm. Changes of diatom indices according to assemblages transfer direction showed a sensitivity of indices to notice alteration of water quality. This result is in agreement with observation of Rimet et al (2005) who reported diatom indices sensitivity to water quality changes by using transferred biofilms from several polluted rivers to an unpolluted stream. According to them, several indices such as IPS (Index of pollution sensitivity), ROT (Saprobic index of Rott), SHE (Schiefele and Schreiner index), EPI (Eutrophication Pollution Index) and CEE (European index) are well reflecting the improvement in water quality. Thus, diatom indices appear as relevant global criteria to assess water quality in rivers.

In conclusion, this study indicates that responses of periphytic diatom communities to environmental changes varied and greatly depended on specific environment of each site. Species richness and diversity index did not clearly reflected responses of periphytic diatom to disturbance. Shifts in values of IPS and DAIPo indices throughout the experiment indicate sensitivity of these indices to water quality changes. Recovery of periphytic diatoms to new conditions appeared from two weeks in unpolluted site and moderate polluted sites. During this two week periods necessary for the shift in communities, diatom assemblages transferred from TL still keep initial characteristics of tolerant species; they are accompanied in their new location by new colonization of more sensitive species which can incorporate contaminant adsorbed within biofilm. This possible availability of contaminant could lead to a favorised incorporation of contaminants in sensitive taxa newly settled which do not have the same defense or tolerance mechanisms against pollutants than tolerant taxa. Trophic transfer could take in charge contamination and transfer it to higher trophic levels for contaminants like metals. Set up of new transfer experiment including contaminant quantification at the different steps of the process that is: in polluted site before transfer and after transfer to non polluted site and reversely could be great interest to evaluate the importance of the potential of trophic transfers during the period of

time necessary to the assemblages to adapt to new conditions. *In situ* experiment with declared metal pollution and indoor laboratory experiment are question to study throughly.

Acknowledgments

This study forms part of the PhD thesis of T. T. Duong. This work was financially supported by CNRS (Centre National de la Recherch Scientifique) and the ESPOIR programme as part of a French-Vietnamese co-operation project. Thanks to Georges Vachaud, Research Director at the CNRS for the coordination of the ESPOIR programme (CNRS - VAST). The The authors thank Institute of Natural Products Chemistry (VAST - Vietnamese Academy of Science and Technology, Hanoi, Vietnam) for chemical analyses. The authors also thank many individuals for assisting in collecting samples in field.

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As this thesis was conducted in co-supervision between France and Vietnam, and after the studies performed in Vienam, it appeared interesting to conduct other studies in France in an hydrosystem submitted to different pollution than urban pollution to test the suitability of periphytic diatom communities as a diagnose tool of water quality. Thus, the second part of this thesis focused on the metallic pollution of the Riou-Mort River with analyses of metal level contents in natural biofilms and the impacts of metal on the structure of periphytic diatom communities. The experiments in this part were set up within framework of the multidisciplinary ECODYN program managed in cooperation with GIS ECOBAG project based on geochemical and ecotoxicological studies related to metal contamination. The principal objectives of the program were to study mechanisms of metal contamination in the Decazeville mine drainage basin, characterizations of the chemical speciation of metals, their bioavailability, and their several toxic impacts on biological components of the hydrosystem. This program has gathered research groups such as two laboratories of the University of Bordeaux 1 UMR CNRS 5805 EPOC: GEMA[6] group (Director: J. C. Massabuau) and TGM[7] group (Director: G. Blanc); Cemagref group (Director: M. Coste); LPTC, UMR 5472[8] (Director: E. Parlanti); GEO TRANSFERT, UMR 5805 (Director; E. Maneux); TCEM, UMR 1220[9] (Director: F. Denaix); LEH, UMR 5177[10] (L. Gauthier); LMTG,UMR 5563[11] (Directors: O. Pokrovsky /B.Dupré); LCABIE, UMR 5034[12] (Director: G. Lespes); LSTM[13] (Director:B. Dreyfus).

The results presented in the chapters 6 and 7 concern *in situ* investigations and periphytic diatom communities examined in relation with their responses to metal exposure in the Riou-Mort River. In the last chapter (chapter 8), an experiment channels study was designed to focus impacts of metal on biofilm and on structure of periphytic diatom communities.

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[7] TGM: Traceur Géochimiques et Mineralogiques.
[8] LPTC: Laboratoire de Physico-Toxicochimie des Systemes Naturels
[9] TCEM : Transfert sol-plante et Cycle des Eléments Minéraux dans les écosystèmes cultivés
[10] LEH : Laboratoire d'Ecologie des Hydrosystèmes
[11] LMTG : Laboratoire des Mécanismes de Transfert en Géologie
[12] LCABIE : Laboratoire de Chimie Analytique
[13] LSTM: Laboratory of Tropical and Mediterranean Symbioses

CHAPTER 6

Seasonal effects of cadmium accumulation on freshwater biofilms and periphytic diatom communities*

6.1 Abstract:

The relationships between diatom species and cadmium (Cd) accumulated in biofilms of the Riou-Mort River (SW, France) were studied in July 2004 and March 2005. Biofilms were sampled from artificial substrates immersed along metallic pollution gradient during twenty days. Dynamic of diatom communities and cadmium accumulation were followed by collecting samples after 4, 7, 14 and 20 days of biofilm colonization. Cd accumulation in biofilms increase during experiment was significantly higher in Cd polluted station (Joanis) than in reference station (Firmi) for both seasons. Periphytic diatom composition varied beteen sites and seasons. At Firmi station, dynamic of diatom communities was stable with dominance of *Cyclotella meneghiniana* and *Melosira varians* in July and *Surirella brebissonni* and *Navicula gregaria* in March. At Joanis station, diatom communities mainly responded to high level of metal by presence of high proportion of small, adnate species. Positive correlation between *Eolimna minima*, *Nitzschia palea*, *Encyonema minutum, Surirella angusta*, *and Gomphonema parvulum* and cadmium accumulation was observed and indicated that these species are tolerant to high level of cadmium. On the other hand, negative correlation of *Cyclotella meneghiniana*, *Navicula gregaria, Navicula lanceolata, Melosira varians* and *Nitzschia dissipata* with cadmium qualifies them as sensitive diatom species. Periphytic diatom composition through presence of specific species highlight metal tolerant indicator diatom group which will be meaningful for biomomitoring pollution in natural aquatic systems.

Key words: Biofilms; Diatom communities; Cadmium; Seasonal effect

* This chapter is prepared as an article to be submitted under the reference T. T. Duong, S. Morin, O. Herlory, A. Feurtet-Mazel, M. Coste, A. Boudou.

6.2 Introduction

Pollution of aquatic systems by heavy metals is an important environmental problem because of their potential accumulation and transfer along the food chains, leading to more or less severe toxic effects on the different biological levels, from the cellular and molecular basis to the communities and biocenosis. Metal sources for freshwater systems result from natural processes (weathering of soils and rocks, volcanic eruptions, etc.) and from a variety of human activities (mining, smelting and agricultural fertilizer) (Audry et al., 2004; Ruangsomboona and Wongrat, 2006). Trace metals such as cadmium (Cd) are considered to be non-essential elements for living organisms; Cd is one of the most toxic metals, with a high solubility in water and a great bioaccumulation capacity in many aquatic species, notably algae and bivalves (Lee et al., 1996; Torres et al., 1998; Baudrimont et al., 1997a).

In freshwater ecosystems, biofilms are complex matrix attached to submerged substrata, made of periphytic algae, bacteria, fungi and their secretory products such as extracellular polymeric substances (EPS) and organic and inorganic non living materials (Newman and McIntosh, 1989; Sekar et al., 2002; Burkholder, 1996). The capacity of freshwater periphytic algae to accumulate metals has been reported and discussed in several papers (Whitton and Say, 1975; Newman et al., 1989; Clements, 1991; Ramelow et al., 1992). Metals content in algae are used to reflect their bioavailability from the aquatic biotopes, especially when their concentrations in water are so low to be undetectable by routine analyses (Foster, 1982; Clements, 1991; Berha et al., 2002). Three main processes are involved in metal accumulation by periphytic algae: (i) binding to EPS; (ii) cell surface adsorption; (iii) intracellular uptake (Holding and Carter, 2003).

The use of freshwater algae in general and of periphytic diatoms in particular as indicators for water quality led to the definition of several diatom indices which are currently applied in many countries (Cemagref, 1982; Watanabe et al., 1986; Kelly, 1998; Prygiel and Coste, 1999). The use of the structure of diatom communities to assess impacts of metal pollution on freshwater system has been discussed by several authors (Medley and Clements, 1998; Ivorra et al., 1999; Gold et al., 2002). Numerous studies, based on biofilm samples collected along pollution gradients or within indoor or outdoor artificial streams, have investigated metal impacts on periphyton communities, the majority of the studies being devoted to diatom communities (e.g. Rushforth et al., 1981; Gustavson and Wängberg, 1995; Soldo and Behra, 2000; Gold et al., 2003a; ; Gold et al., 2003b; Guasch et al., 2003). The polymetallic pollution of the Lot River in the South-West of France (Figure 6.1), essentially based on Cd and

zinc (Zn) discharges from a zinc ore treatment factory via a small tributary (Riou-Mort), represents a remarkable field site for ecotoxicological studies. Gold et al. (2002; 2003a and 2003b) have investigated metal impacts on periphytic diatom communities after colonization of artificial substrates introduced along the pollution gradient (Stations 1, 2 and 3: Figure 6.1). The confrontation between field data and experimental data obtained from indoor artificial streams enriched with diatom communities collected at the reference station on the Lot River (St. 1) and contaminated with metals added isolely or combination (Cd, Zn, Cd+Zn), has revealed the key role played by cadmium towards toxic effects on the diatom communities: significant decrease of cell density and diversity; presence of abnormal diatom frustules (Gold, 2002; Gold et al., 2003a).

In this paper, we present an additional field study focused on the upstream zone where metal effluents are originated in order to investigate the relationships between Cd accumulation levels in biofilms and the structural characteristics of periphytic diatom communities after 4, 7, 14 and 20 days of colonization on artificial substrates in two different seasons. Besides metal pollution impact, this zone is subjected to anthropic pressure by a constant, even if not massive, touristic and urban development along Riou-Mort River which generates an organic overload. All the previous studies have solely taken into account the contamination pressure via the determination of metal concentrations in the dissolved fraction of the water column during the different colonization phases. Cd concentrations in biofilms result both from the metal bioavailability and the structural and functional properties of the biofilms; both set of factors being highly linked to environmental conditions and therefore to seasonal variations. In order to investigate these seasonal effects, this comparative study was set up between July 2004 and March 2005, using identical artificial substrates introduced in the reference upstream zone of the Riou-Mort River (Firmi station) and in the Cd polluted zone (Joanis station) located downstream the metallic factory discharges from the Riou-Viou tributary.

6.3 Material and methods

6.3.1 Study area and sampling stations

The study area is located in the industrial basin of Decazeville (SW France), in the middle section of the Lot River (Figure 6.1). Since the end of the 19th century, the Riou-Mort River, a small tributary of the Lot River, is contaminated by direct discharges of percolation

water coming from the industrial site of Vieille Montagne, specialized in zinc ore treatment. Two sampling stations were selected (Figure 6.1):

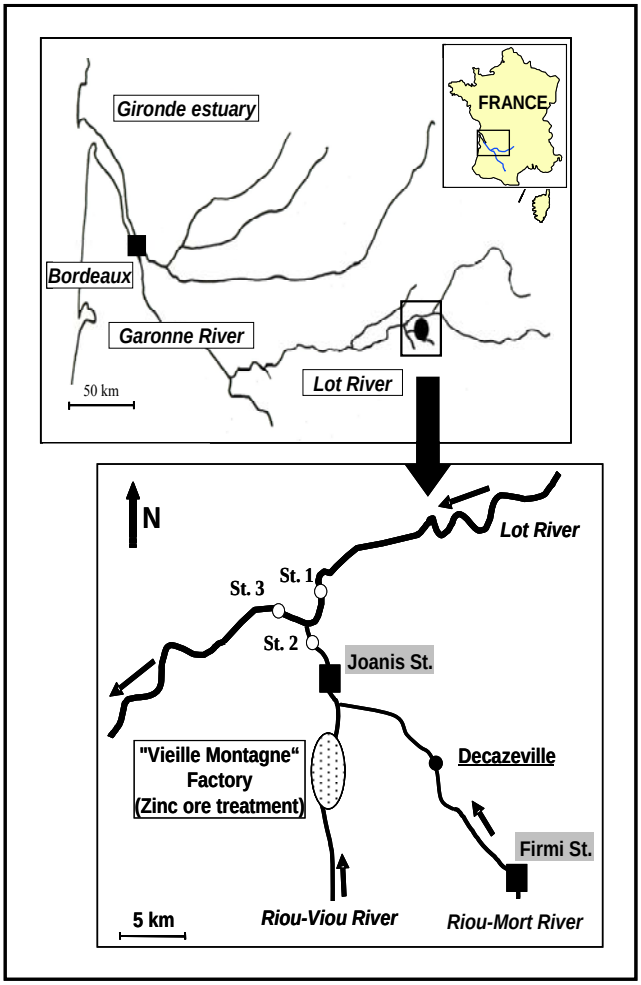

Figure 6.1: Sampling station in Riou-Mort River

The reference station (Firmi), located on the upstream zone of the Riou-Mort River, with very low metal background levels in the water; the polluted station (Joanis), located in the downstream zone of the Riou-Mort River, at about 3 km after its confluence with the Riou-Viou River, characterized by high concentrations of Cd and Zn in the water column (Audry et al., 2004; Blanc et al., 1999). All along the Riou-Mort River, organic discharges coming from urban and touristics activities are observed.

6.3.2 Biofilm collection

Glass slides (6 x 30 cm - 300 cm^2 for both sides) were used as artificial substrates for biofilm attachment. At each sampling station, three plastic racks containing four vertical glass slides were immersed in the water column, parallel to the current at about 10-15 cm below the water surface (see Gold et al., 2002 for details). Biofilm samples were collected after 4, 7, 14 and 20 days of colonization, in July 2004 (07/01 to 07/21) and March 2005 (03/03 to 03/23). At each sampling day, one glass slide was removed randomly from each rack (3 replicates), then scratched using a cutter blade and washed with mineral water. All biofilm samples were diluted in a standard volume of 100 mL and divided after homogenisation into three fractions assigned to various analyses: 5 mL were preserved with 1 mL of formol solution (Formaldehyde 37%, Prolabo, France) for diatom identification; 20 mL were used for ash free dry mass (AFDM) determination; 50 mL for biofilm dry weight measurement and Cd concentration determination. The 25 ml remained sample were maintained for additional analyses (if necessary)

6.3.3 Physico-chemical characteristics of the water and Cd concentrations in the dissolved fraction of the water column

Temperature, conductivity, pH and dissolved oxygen concentration were measured *in situ* at each sampling date using a multi-probe analyser (WTW, Weilheim, Germany). Water samples were also collected for nutrients analyses and phosphates, nitrates, nitrites, ammonium and silica concentrations were determined according to French and International standard methods (NT T 90-023, NF T 90-007, NF EN ISO 11723 and NF EN ISO 13395, respectively). Dissolved Cd concentration was measured using ICP-MS (X7, THERMO, Elemental, UK) with external calibration. Indium was used as internal standard and after each batch of five samples, a calibration blank and one calibration standard were measured to control potential sensitivity variations or memory effects. The analytical method employed was continuously quality checked by analysis of certified reference river waters (SLRS-3, SLRS-4). Accuracy was within 5% of the certified values and the analytical error (relative standard deviation) was generally better than 5% for concentrations ten times higher than detection limits (see Audry, 2003 for details). Detection limit were 0.1 µg $Cd.L^{-1}$.

6.3.4 Biofilm ash free dry mass (AFDM)

AFDM was determined following the European standard procedure (NF EN 872): after filtration using pre-weighted glass fibre filters (47mm and pore size 1 µm, Sartorius, Göttinggen,

Germany) and drying at 105^0C during one hour (dry weight measurement, dw), biofilm samples were then ashed at 500^0C for one hour in a muffle furnace (ash weight measurement) (Solax Technology Ltd, China). AFDM were expressed in $\mu g.cm^2$ (Morin et al., 2006). In this paper, results are expressed in AFDM percentages (AFDM/total dw) x 100).

6.3.4 Cd accumulation levels in biofilm

Biofilm samples (50 mL) were filtered through metal-free filters (47 mm, 0.45 µm pore size, Millipore). Filters were dried at 60^0C for 48 h and weighed, to determine the total dry weights (dw), expressed in $\mu g.cm^{-2}$. The filters were digested for Cd analysis by nitric acid attack (3 mL of HNO_3, Merck, Darmstadt, Germany) in a pressurized medium at 100^0C for 3 h (hot block CAL 3300, Environmental Express, USA). Digestates were then diluted up to 23 mL with ultra-pure water (Milli Q, Bedford, MA, USA). Cd concentrations were determined by flame atomic absorption spectrometry (Varian AA20, Australia), with detection limit of $15\mu g.L^{-1}$. The validity of the method was checked periodically with certified biological reference materials (Tort 2, lobster hepatopancreas; Dolt 2, dogfish liver from NRCC-CNRC, Ottawa, Canada). Values were consistently within the certified ranges (data not shown). Cd concentrations in biofilms were expressed in $ng.cm^{-2}$.

6.3.5 Samples preparation for diatom studies

In the laboratory, after homogenization, each sample collected for diatom studies were heated at 100 °C with hydrogen peroxide (30 %) and hydrochloric acid (35 %) to remove organic matter and dissolve calcium carbonates. The cleaned frustules were then mounted on a microscope glass slide in a high refractive index medium (Brunel Microscopes Ltd, UK; RI = 1.74). Up to 400 diatom frustules were counted and identified on each slide at 1000x magnification, following the Süßwasserflora classification (Krammer and Lange-Bertalot, 1986-1991). Relative abundances (%) of each diatom species and species richness were estimated and diversity index was calculated using the Shannon-Weaver index (Shannon and Weaver, 1963). Deformation forms of diatom species with abnormal general shape and/or species with deformed valve wall ornamentation were estimated.

6.3.6 Data treatment

Statistical analysis was carried out using one-way variance model (ANOVA) to reveal the effects of colonization duration (days) and sampling stations (Joanis and Firmi) on biofilm dry weight, and Cd accumulation in biofilm. Test for normality and homogeneity of variance were verified by using Cochran's test. If a significant effect was observed, *post-hoc* tests (Least Significant Difference test, LSD; Newman-Keuls test) were performed to isolate difference at a significant level of $p < 0.05$. All statistical investigations were performed using *STATISCA* version 6.1 software. Results expressed corresponding to mean values ± standard error (SE). A principal component analysis (PCA) using SPAD Software (version 5.6, Decisia, Paris, France) was performed on the relative abundances of diatom species, in order to reveal taxonomic differences between communities collected from the two stations during the two seasons (July 2004 and March 2005). Pearson correlation matrix between Cd accumulation levels in biofilms and relative abundances of the 20 most abundant diatom species were done by PCA, SPAD software (v. 5.6, Decisia, Paris, France). Indices (IPS and Diversity) were calculated using OMNIDIA software (Lecointe et al., 1993).

6.4. Results

6.4.1 Environmental characteristics of the two sampling stations

Values of physico-chemical parameters measured at Firmi and Joanis stations during the two 20 days' sampling periods in July 2004 and March 2005 are summarized in table 6.1. The average values for pH were 7.6 for Firmi and 7.8 for Joanis, without significant difference between the two seasons. Extreme water temperatures ranged from 2.8 °C (Joanis, March, + 4 days) to 24.8 °C (Joanis, July, + 20 days). The average values for Firmi in July and March were 19.4 and 7.8 °C, respectively; for Joanis, they were 19.7 and 8.2 °C, respectively, without significant differences between the two stations. No significant difference was observed neither between the two stations for the oxygen concentrations during each sampling periods: 10.0 $mg.L^{-1}$ in March and 7.5 $mg.L^{-1}$ in July. For the conductivity, no significant difference was observed between the two stations in July (Firmi: 1580 ± 259 µs.cm and Joanis: 1359 ± 46 µs.cm); but in March, values from Joanis were significantly higher (1451 ± 240 and 608 ± 31 $\mu s.cm^2$, respectively). Marked differences were observed between ammonium, nitrate, nitrite and phosphate concentrations, in favour of Joanis: overall for $N\text{-}NO_3$. Silica determination in water

samples showed weak differences between the two stations and seasons: a significant difference was observed between the two seasons (7.4 ± 0.7 in March and 12.4 ± 0.7 in July).

Parameters	Firmi (R)				Joanis (P)			
	4 days	7days	14days	20days	4 days	7days	14days	20days
July 2004								
pH	7.4	7.4	7.4	7.3	8.0	7.7	7.7	7.7
T (^{0}C)	17.6	16.4	19.4	24	19.7	17.6	16.7	24.8
Cond (µS.cm)	1377	1223	1410	1427	2350	1426	1290	1257
O_2 (mg. L^{-1})	7.0	8.0	9.3	nm	7.2	7.3	6.4	nm
NH_4 (mg. L^{-1})	0.54	0.71	1.33	2.34	0.77	1.95	2.5	3.43
NO_3 (mg. L^{-1})	3	3.6	2.5	2.9	23.7	54.4	38.4	36
NO_2 (mg. L^{-1})	0.18	0.18	0.23	0.24	0.9	1	0.9	1.3
PO_4 (mg. L^{-1})	0.11	0.1	0.1	0.04	0.58	0.87	0.82	2.86
Si (mg. L^{-1})	10.5	13.5	12	15	12	11.5	12	12.5
Cd (µg. L^{-1})	< 0.1	< 0.1	< 0.1	< 0.1	26	26	27	24
March 2005								
pH	7.6	7.6	7.9	8	7.5	7.8	7.9	8.2
T (^{0}C)	3,4	4,5	10,3	13	2,8	6,1	10	13,9
Cond (µS.cm)	678	538	639	578	735	1602	1723	1744
O_2 (mg. L^{-1})	11,1	9,4	9,1	10.5	10,3	9,3	9,9	10.1
NH_4 (mg. L^{-1})	0.88	0.39	0.61	0.62	0.92	3.44	3.54	2.98
NO_3 (mg. L^{-1})	5	3.9	2.8	2.7	33.8	11.6	26.6	37.3
NO_2 (mg. L^{-1})	0.07	0.04	0.08	0.1	0.7	0.5	1.1	1.1
PO_4 (mg. L^{-1})	0.24	0.08	0.2	0.1	0.2	1.5	1.4	1.3
Si (mg. L^{-1})	7	7.5	5.5	6.5	6.5	9	9.5	8
Cd (µg. L^{-1})	< 0.1	< 0.1	< 0.1	< 0.1	17.1	19.8	21.6	11.2

Table 6.1: Physical and chemical characteristics of water quality at Firmi (R) and Joanis (P) stations during experimental period in July 2004 and March 2005. (nm: not measure)

Cd concentrations measured in the dissolved fraction (< 0.45 µm) of the water column were systematically below the detection limit (0.1 µg.L^{-1}) at the reference station (Firmi). Higher Cd concentrations were measured at the polluted site (Joanis), with mean values of 25.6 and 17.4 µg.L^{-1} in July 2004 and March 2005, respectively.

6.4.2 Biofilm total dry weight and AFDM

Evolutions of biofilm dry weight during the 20 days' colonization periods from the two stations in July 2004 and March 2005 are shown in figure 6.2. After 14 and 20 days of colonization, significant differences were observed between the two stations in favour of Joanis: at the end of the experiment, the mean values were 824 ± 80 against 405 ± 128 µg.cm^{-2} in July

and 446 ± 29 against 287 ± 28 µg.cm^{-2} in March. No significant difference was observed after 4 and 7 days. The dry weight increase was progressive, with tendencies close to linearity, excepted Joanis in March where a decrease was observed between 14 and 20 days' colonization. AFDM percentages showed a weak variability during the different colonization periods. Significant differences were observed between Joanis and Firmi stations: 60.4 ± 8.7 and 36.2 ± 3.9 % in March, respectively; 47.8 ± 4.2 and 32.6 ± 2.3 % in July, respectively.

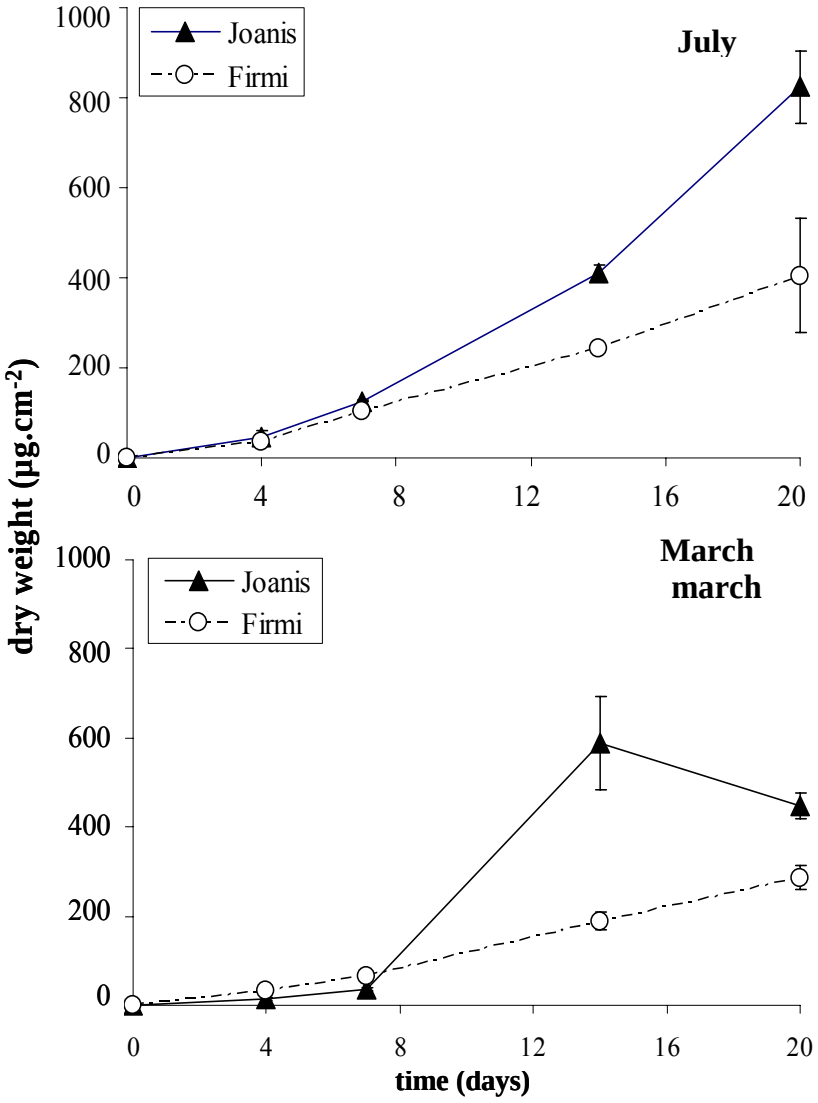

Figure 6.2: Dry weight of biofilms collected from Firmi and Joanis stations in July 2004 and March 2005 after 4, 7, 14 and 20 days of colonization. Mean ± standard error (n = 3).

6.4.3 Cd accumulation in biofilms

Kinetics of Cd accumulation in biofilm samples are shown in figure 6.3. At the reference station (Firmi), Cd levels were very low, close to background levels: after 20 days' colonization, mean values were 0.75 ± 0.52 ng.cm^{-2} in July and 1.8 ± 0.4 ng.cm^{-2} in March.

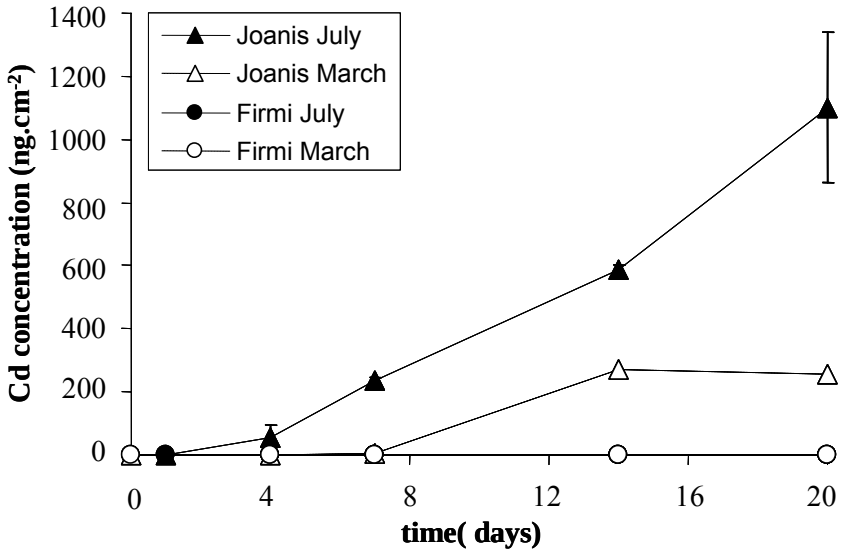

Figure 6.3: Cd accumulated in biofilms at Firmi (R) and Joanis (P) stations in summer 2004 and winter 2005 after 4, 7, 14 and 20 days of colonization. (mean ± standard deviation; n = 3).

These accumulation levels, expressed on the biofilm dry weight basis, were 1.8 and 6.3 ng.mg^{-1}, respectively. At the opposite, very high values were observed at Joanis at the end of the experiment: 1100 ± 237 ng.cm^{-2} (corresponding to 1327 ng.mg^{-1}) in July and 254 ± 88 ng.cm^{-2} (corresponding to 784 ng.mg^{-1},) in March. The evolution tendencies are different between the two seasons at the polluted station: in July, Cd accumulation levels increase progressively between + 4 and + 20 days; in March, the mean values measured after 14 and 20 days are not significantly different.

6.4.4 Diatom community characteristics

Over 200 diatom taxa were identified from the different biofilm samples collected. Species richness (S: total number of species per sample) and diversity index (H') were quite similar between the two stations and significantly higher in March (Table 6.2). Mean values of

IPS index ranged from 7.2 (Joanis, July) to 10.7 (Firmi, March), and indicated water poor to moderate water quality. The calculated IPS values were higher in Firmi station than in Joanis station and IPS values obtained in July were lower than in March.

	July 2004		March 2005	
	Firmi	Joanis	Firmi	Joanis
Species richness (S)	41 ± 0.5	46 ± 4	54 ± 4	55 ± 3
Diversity index ($H^{'}$)	3 ± 0.02	3.2 ± 0.1	4.2 ± 0.1	4.1 ± 0.2
IPS	8.5 ± 0.34	7.2 ± 0.34	10.7 ± 0.21	10 ± 1.03

Table 6.2: Species richness, diversity index and IPS (mean value and standard deviation n = 3) of the reference Firmi (R) and polluted Joanis (P) in July 2004 and March 2005.

Taxonomic composition and relative abundance of diatom species were markedly different between the two stations and the two seasons (Fig 6.4). In July, at Firmi station, the relative abundances of the seven most abundant species were quite similar during the 20 days' colonization: *Cyclotella meneghiniana* (CMEN) was the dominant species, with more than 50 % of the total diatom communities. In addition, *Navicula gregaria* (NGRE) and *Melosira varians* (MVAR) represented 8.4 and 9.2 % respectively. In Joanis, still in July, *Eolimna minima* (EOMI) were dominant, with a mean relative abundance close to 50 %. For *Cyclotella meneghiniana* (CMEN), *Nitzschia palea* (NPAL), *Ulnaria ulna* (UULN) and *Gomphonema parvulum* (GPAR), the mean values were 12.1%, 5.4%, 4.6% and 2.7%, respectively.

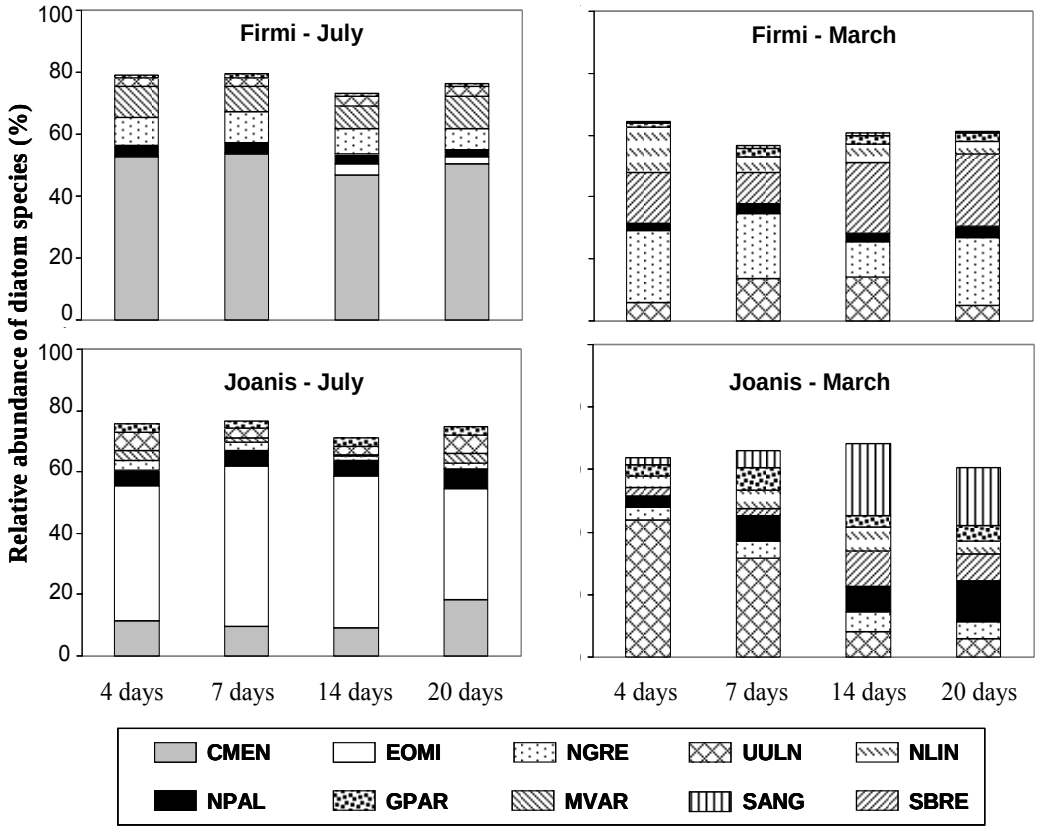

Figure 6.4: Relative abundances of diatom species (mean values, n = 3) with relative abundances ≥ 10 % in summer and > 5% in winter after 4, 7, 14 and 20 days of colonization at Firmi (R) and Joanis (P) stations. (CMEN: *Cyclotella meneghiniana; EOMI: Eolimna minima; NGRE: Navicula gregaria; UULN: Ulnaria ulna; NLIN; Nitzschia linearis; NPAL: Nitzschia palea; GPAR: Gomphonema parvulum; MVAR: Melosira varians; SANG: Surirella angusta; SBRE: Surirella brebissonii*)

In March, relative abundances of the main diatom species at Firmi station were comparable after 4, 7, 14 and 20 days' colonization. The dominant planctonic species CMEN in July was replaced by two species: *Surirella brebissonii* (SBRE, 18 % on average) and *Ulnaria ulna* (UULN, 10 %), with a marked increase of *Navicula gregaria* (NGRE, 19 %). At Joanis station, a marked decrease of UULN relative abundance was recorded (44 % after 4 days and 6 % after 21 days) with in parallel an increase of the relative abundance of *Surirella angusta* (SANG) from 2 to 19 %, respectively. The two species *Nitzschia palea* (NPAL) and *Gomphonema parvulum* (GPAR) were still represented in March: 8 and 5 % respectively. A global approach via a principal component analysis (PCA), was based on the relative abundances

of the 50 diatom species with the highest cumulative abundances from the two stations and the four sampling dates during the two seasons (Figure 6.5a and b).

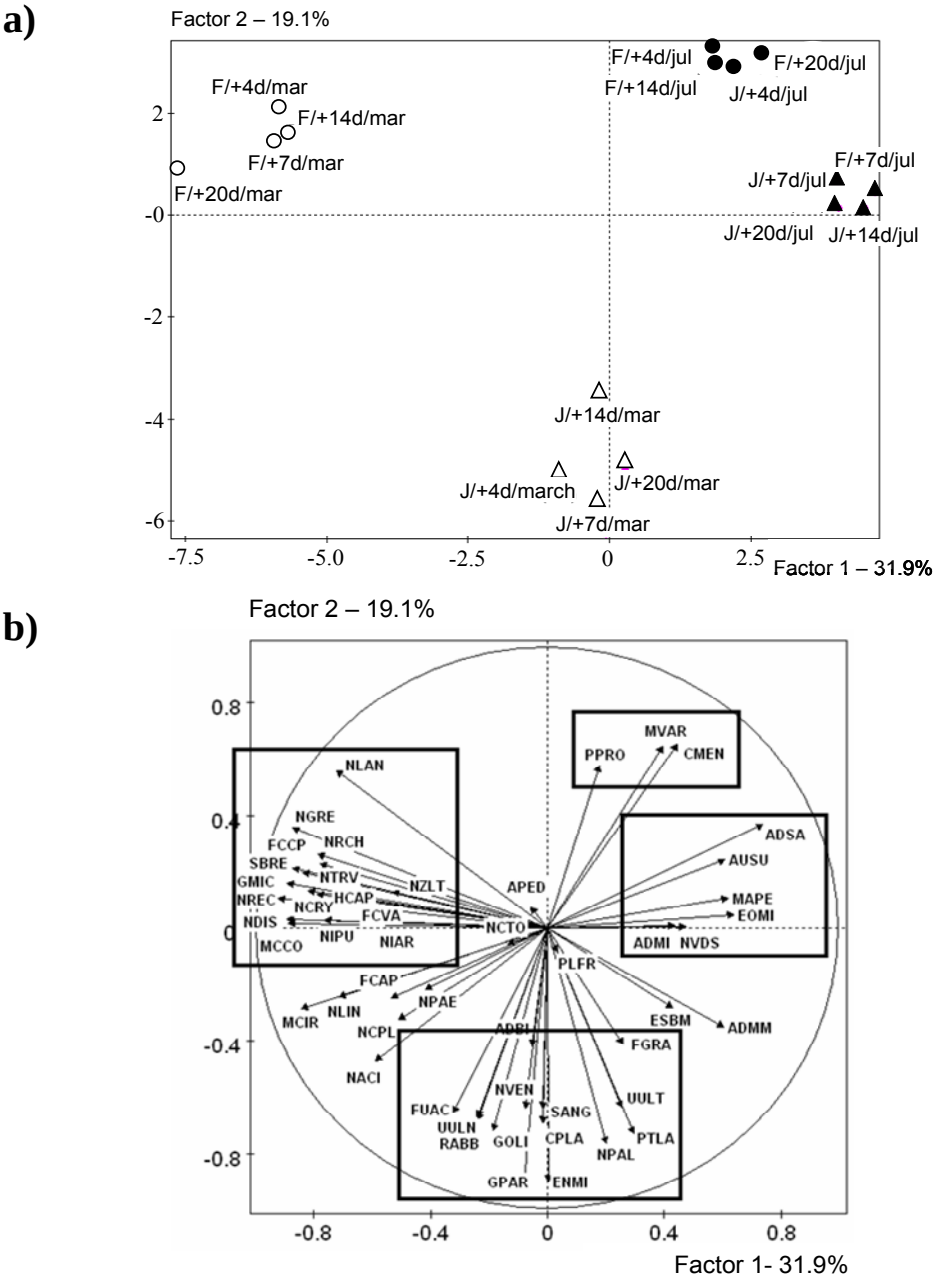

Figure 6.5a and b: Principal component analysis (PCA) based on the taxonomic composition of the diatom communities collected at Firmi (R) and Joanis (P) along metallic pollution during two periods (July 2004 and March 2005) after 4, 7, 14 and 20 days colonization. (a): Projection of the communities on the two first principal components axes (3 replicates per site). Colonization duration (4, 7, 14 and 20 days) of each site and sampling period is pointed out in bracket. b: Projection of the diatom species on the correlation circle and 50 diatom species which highest accumulate are plotted on the graph.

On the first plan defined by the two axis 1 and 2, which represents more than 50 % of the total variance, the four conditions are clearly individualized: the two stations Firmi (R) and Joanis (P) in July are localized in the right superior quarter; Joanis station in March in the middle of the inferior part; Firmi in March in the left superior quarter (Figure 6.5a). The correlation circle (Figure 6.5b) indicates the links between diatom species and the four studied conditions. Besides the seven species which were dominant according to their relative abundances, Joanis' diatom communities in July were characterized by small species such as *Navicula seminulum* (NVDS), *Mayamaea atomus var. permitis* (MAPE), *Achnanthidium saprophila* (ADSA) and *Achnanthidium minutissimum* (ADMI). In March, numerous species were associated to this polluted site: the dominant ones *Surirella angusta* (SANG), *Nitzschia palea* (NPAL), *Ulnaria ulna* (UULN) and *Encyonema minutum* (ENMI), *Cocconeis placentula* (CPLA), *Gomphonema olivaceum* (GOLI) or *Navicula veneta* (NVEN). For the reference site (Firmi), only three species were associated with the July period: *Cyclotella meneghiniana* (CMEN) (dominant species), *Melosira varians* (MVAR) and *Parlibellus protracta* (PPRO). At the opposite, more than fifteen species were representative of the March period, with the dominant taxa such as *Surirella brebissonii* (SBRE), *Navicula gregaria* (NGRE), *Navicula lanceolata* (NLAN), *Nitzschia dissipata* (NDIS), *Gomphonema micropus* (GMIC), *Nitzschia recta* (NREC).

The relationships between relative abundances of diatom species and Cd accumulation levels in biofilms were investigated via Pearson correlations, one for each season: among them twenty diatom species have relative abundances higher than 10 % in July and 5 % in March (Table 6.3). Species (*Eolimna minima* (EOMI), *Nitzschia palea* (NPAL), *Encyonema minutum* (ENMI), *Gomphonema parvulum* (GPAR) and *Surirella angusta* (SANG)) are strongly positively correlated to Cd content in biofilms and species (*Cyclotella meneghiniana* (CMEN), *Navicula lanceolata* (NLAN), *Navicula gregaria* (NGRE) *Surirella brebissonii* (SBRE) and *Melosira varians* (MVAR)) are negatively correlated to Cd accumulation in biofilms.

Teratological frustules of diatom species which consisted in twisted valves in their medium or irregularity in striaes arrangement were rather frequently observed at Joanis station in both seasons (Table 6.4) (Figure 6.6). The percentage of deformations in the total assemblages at Joanis station was higher in July than March and ranged around 27.3‰ and 23‰ (average the abundance) respectively. Among them, araphids diatom species such as *Ulnaria ulna* (UULN) occupied larger proportion with 14.5‰ (mean value) in July and 16.7 ‰ (mean value) in March.

	July 2004																			
	CMEN	**EOMI**	**NPAL**	**NGRE**	**MVAR**	**UULN**	**GPAR**	**SBRE**	**NLAN**	**MAPE**	**ADMI**	**PLFR**	**ADSA**	**PTLA**	**NVDS**	**NDIS**	**ESBM**	**AUSU**	**ADMM**	**ENMI**	**[Cd]**
[Cd]	-0.57	0.55	0.83	-0.72	-0.60	0.79	0.73	-0.64	-0.73	0.31	-0.52	0.15	0.53	0.68	0.17	-0.21	-0.01	0.33	0.35	0.67	1.0
	March 2005																			
	UULN	**NGRE**	**NPAL**	**SBRE**	**NLIN**	**GPAR**	**SANG**	**NACI**	**NLAN**	**NZLT**	**ENMI**	**CMEN**	**GMIC**	**FUAC**	**EOMI**	**NDIS**	**PLFR**	**PTLA**	**NCPL**	**NIPU**	**[Cd]**
[Cd]	-0.38	-0.49	0.81	-0.18	-0.05	0.20	0.98	-0.42	-0.59	-0.32	0.76	-0.44	-0.52	-0.50	0.98	-0.72	0.68	0.22	0.49	-0.10	1.0

Table 6.3: Pearson correlations between relative abundances of 20 diatom species from the two stations: Firmi (R) and Joanis (P) during two sampling periods (July 2004 and March 2005) and the cadmium accumulation levels in biofilms ([Cd], ng.cm^{-2}).

Colonization duration (days)	July 2004		March 2005	
	Firmi	Joanis	Firmi	Joanis
4	0	8 ± 0.7	0.7 ± 0.6	13.7 ± 0.3
7	1 ± 0.7	19.5 ± 3.2	4.3 ± 1.3	43 ± 9.3
14	0	33.5 ± 0.4	2.3 ± 2	17.7 ± 3.1
20	0	48.5 ± 1.1	6 ± 0.9	17.3 ± 1.3

Table 6.4: Abnormal diatom frustules (‰) at two sampling stations: Firmi (R) and Joanis (P) along metallic pollution gradient during two sampling periods (July 2004 and March 2005) after 4, 7, 14, and 20 days of colonization.

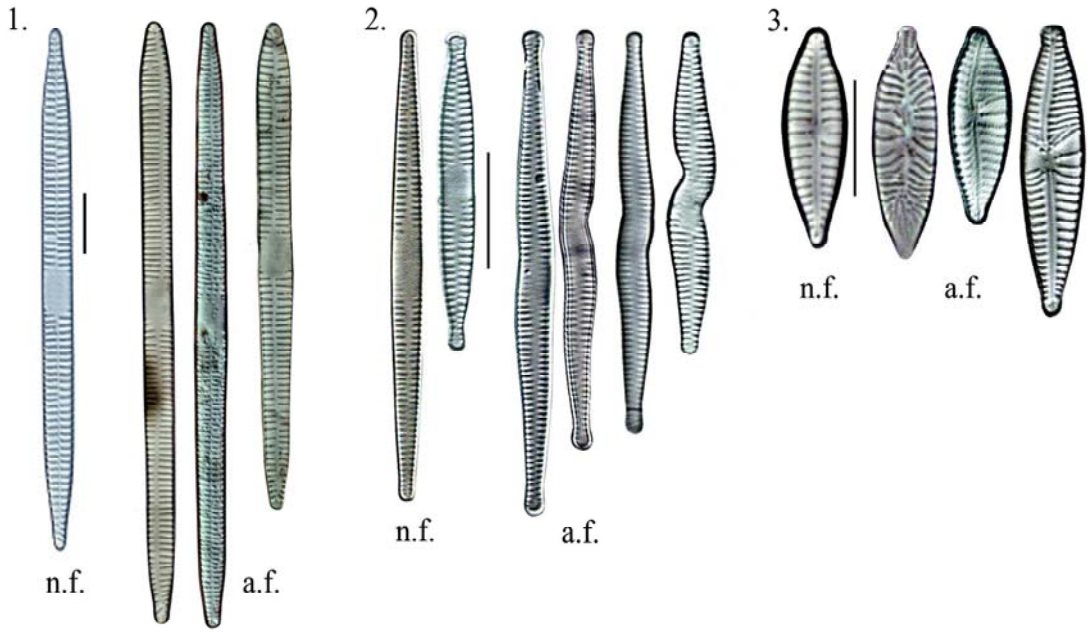

Figure 6.6: Abnormal forms of *Ulnaria ulna* (UULN) (1), *Fragilaria capucina* (FCAP) (2) and *Gomphonema parvulum* (GPAR) (3) found in Joanis station (nf: normal form and ab: abnormal form)

In addition, other abnormal frustules belonging to raphid diatom species were also quite abundant in biofilm samples at Joanis station at both seasons, with *Gomphonema parvulum* (GPAR) which presented a percentage of 2.3 ‰ in July and 1.6 ‰ in March; *Eolimna minima* (EOMI) which showed (2.6 ‰ in July and 0.5 ‰ in March and *Nitzschia palea* (NPAL) in July and March assemblages of appearing with 1 and 0.3 ‰ respectively. Besides the abnormal forms of species cited above, araphids, monoraphids and centric diatom deformed appeared in both seasons with low proportion of *Diatoma vulgaris* (DVUL) *Achnanthidium minutissimum* (ADMI), *Achnanthidium saprophila* (ADSA), and *Cyclotella meneghiniana* (CMEN).

6.5 Discussion

In the present study, the development of periphytic biofilm along metallic pollution gradient was investigated at two stations during two seasons. The dynamics of periphytic diatom communities and cadmium accumulation in natural biofilm varied considerably between sites and seasons. Biofilm dry weight gradually increased and reached a maximum value after 20 days of colonization in both stations and seasons. Despite a high level of metal in Joanis station (table 6.1), biofilm biomass in this station was higher than in of Firmi station, and corresponded to a

higher availability in nitrates in Joanis which seems to contribute to an increasing growth of biofilm at this station. Although dissolved Cd concentrations in water were not detectable in the reference site (Firmi), metal levels were detected in natural biofilms at Firmi station, however not as much as in Joanis station which allows biofilm to accumulate metal overall in July , an underlines its utilization as a useful tool to indicate metallic pollution (Ramelow et al., 1992). Cd accumulation in natural biofilm reflected their exposure history (Figure 6.3). In our study, Cd content in biofilm from polluted station (Joanis) increased gradually and reached its maximum value at the end of the experiment in July whereas in March Cd content in biofilm increased till the day 14 and then was reached an equilibrium. This could be related to the saturation of binding sites in biofilm leading to a limitation of Cd accumulation in biofilm on the day 20. As large number of metal binding sites in the biofilm (organic matrix: surface of cells, organic particles) have found to play an important role in metal sorption from water column (Pistocchi et al., 1997; Decho, 2000; Barranguet et al., 2000). Increase of biofilm dry weight (containing: bacteria, fungi, algae and their secretory products such as extracellular polymeric substances) which have been reported to act as a trap for nutrients or metals (Sekar et al., 2002), could explain the continuous sorption of Cd by biofilms. By using X-ray analysis to observe metals contained in algae collected from heavy metal pollution sites, Lai et al (2003), Chien (2004) and Nakanishi et al (2004) showed that heavy metal elements could be found in diatom species such as *Nitzschia palea, Achnanthidium minutissimum* and *Fragilaria tenera.* According to Khoshmanesh et al (1997), smaller cells of algae have relatively large surface area and more sites for metals binding than those of larger cells. So, the abundance of small diatom species in Joanis station (Figure 6.5b) could accumulate more metal concentration from surrounding water by biofilm in July (Fig 6.3).

Beside the higher species richness and diversity in March, IPS index values obtained from this investigation indicated rather poor to moderate quality status of water for the two stations and reflected the organic overload of the two sampling stations located in an urban area (table 6.1). Though nutrients availability was found along the Riou Mort River, metal pollution was solely detected in Joanis station, downstream the confluence with the Riou-Viou River (Figure 6.1) and seems to characterize diatom assemblages. It was expected that distribution and presence of diatom species in the Riou-Mort River reflects metal levels along the river (at Firmi and Joanis stations). Our results showed marked differences in periphytic diatoms compositions between stations and seasons. Throughout the experiment in July, relative abundances of dominant diatom species at Joanis and Firmi stations was developed stably (Figure 6.4). Planktonic species such as *Cyclotella meneghiniana* (CMEN), *Melosira varians* (MVAR) and

raphids species *Navicula gregaria* (NGRE) presented large proportions in diatom biofilms of Firmi station. Several authors have found these species to be sensitive to metal pollution (Genter et al., 1987; Medley and Clements, 1998; Morin et al., 2006). This is confirmed in table 6.3 by negative correlations of these species to Cd accumulation in biofilm in both seasons and by the low proportion of these species observed in the polluted site (Joanis). To adapt high level of Cd in both water column and natural biofilm, diatom communities in Joanis station in July tend to be dominated by *Eolimna minima* (EOMI) (around 50% of the total community) (Figure 6.4) and other small forms of diatom species such as *Mayamaea atomus* (MAPE), *Achnanthidium saprophila* (ADSA), *Aulacoseira subarctica* (AUSU) (Figure 6.4 and 6.5). *Eolimna minima* (EOMI) with its high proportion are known to be metal resistant species (Gold et al., 2002; Szabo et al., 2005). Additive presence of many diatom small forms in communities compared to diatom communities from reference station (Firmi) (figure 6.5a and b) supports the hypothesis that effects of metallic pollution on diatoms composition are detectable even in overload zone like the Joanis station which receives organic discharges according to wastewater treatment plant activity. Our results are in accordance with observations of Medley and Clements, (1998) who found that communities under metal pollution were dominated by adnate and small species. Similarly, considerable increases of small diatom species under high Cd concentration in laboratory experiment were also observed by Peres (1996). Finally, high proportions of adnate and small species were underscored and were strongly related to Cd presented in table 6.3 as indicator of high Cd level.

Composition of benthic diatom communities in March were varied and differed from those in July (Fig 6.3 and 6.5b). The differences in diatom communities might be attributed to changes in the environment (table 6.1) with metal concentrations in water column lower in March and related to spring seasonal conditions leading to a lower accumulation in biofilms. Seasonal variations of metal concentrations have been suggested to influence periphytic diatom composition (Medley and Clements, 1998). Firmi's diatom communities were characterised by abundances of *Navicula gregaria* (NGRE) and *Surirella brebissonii* (SBRE) quite stable throughout the experiment, and corresponding to a total ratio of 40 %. Unlike diatom composition in July, under lower cadmium concentration, in March, Joanis's communities showed appearance of several big diatom species such as *Nitzschia linearis* (NLIN), *Fragilaria ulna var acus* (FUAC), *Gomphonema olivaceum* (GOLI) (Figure 6.4 and 6.5b); however, small forms of diatom were still well represented. The modifications in diatom structure at Joanis station compared to communities in Firmi station were noticeable with decrease of *Ulnaria ulna* (UULN) species and increase of *Surirella angusta* (SANG) and *Nitzschia palea* (NPAL) species

(Figure 6.4). The gradual increase of SANG and NPAL species, accompanied and relatively correlated with considerable increase of Cd content in biofilm, from its initial to mature stage, could therefore indicators of high level of Cd. Indeed, relative abundances of SANG occuping around 50% of total diatom community with high concentration of metals in biofilm were found in Kakehashi River (Nakanishi et al., 2004). Complex relationships of UULN and several metals according to seasons were recorded when effects of heavy metal were examined on attached diatoms in the Uintah basin of Utah (Rushforth et al., 1981) with a positive correlation of UULN with cadmium, copper and mercury were positive in Spring season, and on the contrary a negative correlation with copper in Winter season. In the present study, this phenomenon was revealed by Pearson correlation for UULN and cadmium between summer and spring seasons (table 6.3). We could suggest that such relations of diatom species to heavy metals may concern seasonal differences.

Heavy metal affects have been observed not only diatoms structure but also the formation of their valves. Morphological aberrations of diatom species under environment stress have been reported by several authors (Ruggiu et al., 1998; Yang and Duthie, 1993; Gold et al., 2003; Cattaneo et al., 2004). They suggested that increasing metals concentration (in water or sediment) could trigger the formation of deformed valves within some diatom genera. In this study, Cd contamination in water column and significant Cd accumulation in natural biofilm at polluted station (Joanis) have lead the the frequent appearance of abnormal forms at this station. The occurrence of deformed valves within *Fragilaria* genus (synonym *Ulnaria* or *Synedra*) have been widely reported, for example by McFarland et al (1997) for *F. capucina*, by Ruggiu et al (1998) for *Synedra tenera*, by Gold et al (2003 a) for *F. capucina var. gracilis* and by Nunes et al (2003) for *F. crotonensis* and *F.capucina var. rumpens*. In addition, our study also presented other abnormal morphology forms belonging centric and raphids diatom species.

In conclusion, our study shows that, cadmium accumulations in natural biofilm were detected despite not acute Cd concentration levels in water column and in an environment characterized by an organic overload. Hence, using biofilm method is suitable for heavy metal monitoring. Due to the different seasons and levels of metal load in an organic overload medium, Cd accumulation in natural biofilms and periphytic diatom communities significantly varied. Season appear as an important factor not to overlook. Metal pollution has caused changes in periphytic diatom composition along the river. Diatom assemblages dominated by small, adnate diatom species and the presence of teratological forms at polluted station, could be indicator of contamination by metals. Positive or negative correlations of dominant diatom taxa to cadmium accumulation in natural biofilm indicates groups of species tolerant or sensitive to metal, which

will be meaningful for biomomitoring in aquatic systems. Such interpretations could be confirmed for validation by further indoor studies concerning metal bioaccumulation in biofilms and their relation with diatom.

Acknowledgements,

This study forms part of the PhD thesis of T. T. Duong. This work was financially supported by CNRS (Centre National de la Recherch Scientifique). The authors would like to thank Henri Bouillard, Cécile Bossy, Véronique Duflo from the GEMA team (UMR 5805 EPOC, Université Bordeaux 1) for their technical assistance and Muriel Bonnet and Maryse Boudigues form the Cemagref laboratory for carrying out nutrients analyses.

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CHAPTER 7

Long term survey of heavy metal pollution, biofilm contamination and diatom community structure in the Riou-Mort watershed, South West France [*]

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Keywords:

Geochemical survey; Cd; Zn; Metal bioaccumulation; Biofilms; Periphytic diatoms; Valve abnormalities

[*] This chapter is submitted as article to Environmental Pollution

7.1 Abstract:

In a metal-polluted stream in the Riou-Mort watershed in SW France, periphytic biofilm was analyzed for diatom cell densities and taxonomic composition, dry weight and metal bio-accumulation (cadmium and zinc). Periphytic diatom communities were affected by the metal but displayed induced tolerance, seen through structural impact (dominance of small, adnate species) as well as morphological abnormalities particularly in the genera *Ulnaria* and *Fragilaria*. Species assemblages were characterized by taxa known to occur in metal-polluted environments, and shifts in the community structure expressed seasonal patterns: high numbers of *Eolimna minima*, *Nitzschia palea* and *Pinnularia parvulissima* were recorded in summer and autumn, whereas the species *Surirella brebissonii*, *Achnanthidium minutissimum*, *Navicula lanceolata* and *Surirella angusta* were dominant in winter and spring. Commonly used indices such as the Shannon diversity index and Specific Pollution sensitivity Index reflected the level of pollution and suggest seasonal periodicity, the lowest diversities being observed in summer.

Capsule:

Periphytic biofilm diatom communities are suitable indicators for the bioassay of elevated levels of metals in contaminated river water.

7.2 Introduction

The Gironde estuary exhibits a polymetallic pollution which has notable consequences on the biota in the coastal zone; the estuary has been classified as “zone D” by the National Observation Network (RNO), i.e. it is forbidden to harvest oysters for consumption, production or purification. The most important source of cadmium is located in the former zinc ore treatment area of Decazeville, in the Riou-Mort watershed, a small tributary of the Lot River (Boutier et al., 1989; Latouche, 1992). Although metal emissions in the source zones have clearly decreased during the last two decades (Audry et al., 2004), Zn and Cd concentrations in water and suspended particulate matter (SPM) still are important (Coynel, 2005). Moreover, decreasing acceptable threshold concentrations for seafood (e.g. Cd: $< 5\ \mu g.g^{-1}$, dry weight; European Community, 2002) and the historically high levels of metal in the Lot River sediments

(Audry et al., 2004), strengthen the potential socio-economic impact of the Cd pollution, (e.g. for oyster production at Marennes-Oléron, ~30 km north of the mouth of the Gironde estuary).

Biomonitoring is a powerful tool for assessing aquatic ecosystem health further to physical and chemical analyses. It involves organisms that are likely to reveal the environmental changes brought about by natural and anthropogenic phenomena. In freshwater environments, periphytic algal assemblages are the main primary producers. As they are composed of a large number of species with various ecological tolerances, and due to their position in the foodweb and their short life cycle, they are powerful ecological indicators. However, few field studies have been carried out to characterize the alterations occurring in periphytic communities owing to long-term metal contamination. Most of the surveys performed one to four samplings per year (Foster, 1982; Lindstrøm and Rørslett, 1991; McFarland et al., 1997; Hill et al., 2000a; Sabater, 2000), but studies on periphyton based on monthly sampling frequencies are rare (Takamura et al., 1990; Nakanishi et al., 2004). In the present experiment, a long-term geochemical and diatom survey was conducted from April 2004 to March 2005 at a highly metal-polluted site on the Riou-Mort River. Our approach aimed to relate benthic community structure to metal exposure. Cadmium and zinc concentrations were measured in water, suspended particulate matter (SPM) and in the biofilm to characterize the geochemical behaviour of Cd and Zn and their impact on bioaccumulation kinetics. Benthic communities are described through diatom assemblages, applying the Index Polluosensitivity Specific (IPS, Coste in Cemagref, 1982) and the Biological Diatom Index (Lenoir and Coste, 1996; nationally standardized in 2000, NF T90-354) commonly used for routine biomonitoring in France. The taxonomic composition of the assemblages, commonly used indices (IPS, Shannon index) and the frequency of morphological aberrations occurring in some diatom species were determined and discussed as a response to metal contamination.

7.3 Material and methods

7.3.1 Study site

The Riou Mort River (watershed area: 155 km^2) drains the Decazeville area known for polymetallic pollution due to former open-cast coal mining and Zn ore treatment. The mean annual discharge of the Riou Mort River at the studied site was ~1.9 $m^3.s^{-1}$ during 2000-2003 and ~0.98 $m^3.s^{-1}$ during the study period (March 2004 to March 2005, data from the DIREN: French regional environment department). The experimental site on the Riou-Mort River is located close

to the outlet of the watershed and downstream from the former ore treatment site, at Joanis stations, receives cadmium- and zinc- enriched water from this pollutant source (Boutier et al., 1989; Latouche, 1992).

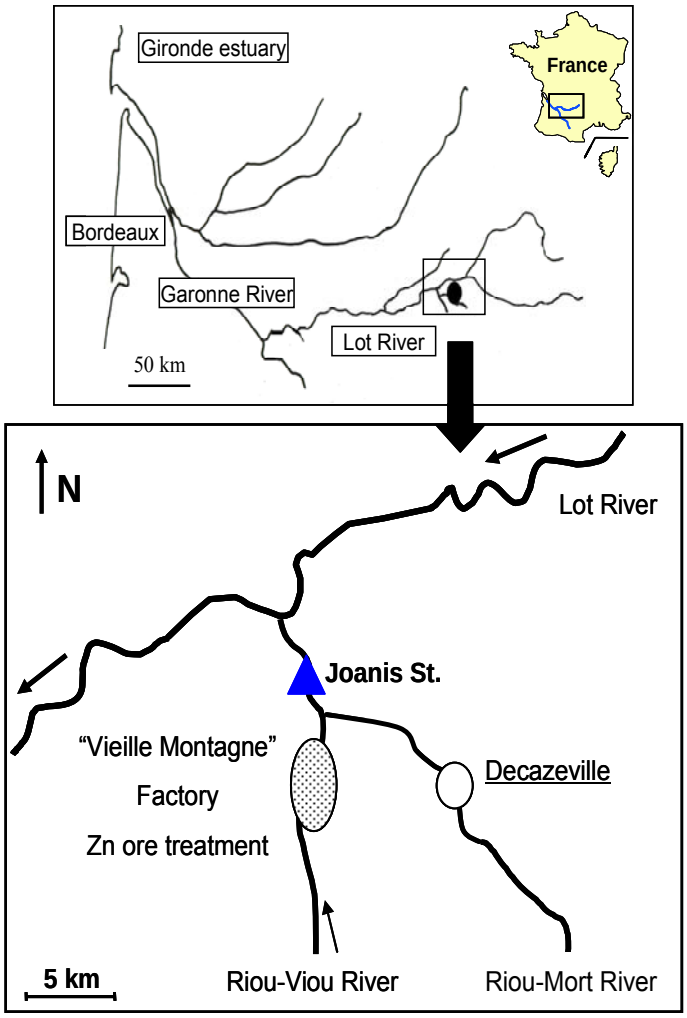

Figure 7.1: Sampling station

7.3.2 Sampling and sample analyses

7.3.2.1 Stream water physicochemical parameters

Temperature, pH, conductivity and dissolved oxygen were measured in the river every 24 days (WTW, Weilheim, Germany), during the 13-month experimental period (March 2004 to March 2005). Two-litre stream water samples were simultaneously collected and brought back to the laboratory for nutrient measurements. Phosphate, silica, ammonium, nitrite and nitrate

concentrations were determined according to French and international standards (NF T90-023, NF T90-007, NF EN ISO 11732 and NF EN ISO 13395, respectively).

Water and SPM for trace metal analysis were sampled every 24 days using clean techniques. All materials in contact with the water samples were made of polypropylene (PP), carefully decontaminated as previously detailed in Audry et al. (2004). The dissolved phase was sampled using an acid pre-cleaned PP bottle (1 L), previously rinsed with the river water of the site. Water samples were filtered immediately through 0.2 µm Nucleopore polycarbonate filters in a glove box (laboratory van). Filtrates were collected in an acid-washed PP bottle after thoroughly rinsing it with an aliquot of the filtrate, acidified (0.1% ultrapure HNO_3, Merck, Darmstadt, Germany) and stored in the dark at 4°C until analysis (Blanc et al., 1999). Particulate matter for trace element analyses was retrieved by pumping up to 200 L of river water (1m from the bank at 0.3 m depth) using an all PP-pump with PP-tubing followed by centrifugation (Westfalia, Oelde, Germany; 12,000 g). This technique is considered a practicable and reliable method for SPM sampling in all hydrological situations (Lapaquellerie et al., 1996; Schäfer and Blanc, 2002). Daily cumulative 1-liter samples of river water consisting of 8 subsamples taken at regular time intervals (every three hours) were obtained using an automated sampling system (SIGMA 900P, American Sigma, Hach, Loveland, Colorado). The samples were recovered every 24 days and filtered through pre-weighed 0.7 µm glass fiber filters (Whatman GF/F) to obtain daily SPM concentrations (Schäfer et al., 2002; Coynel et al., 2004). During low and/or stable hydrological conditions, water aliquots and SPM of up to six daily samples were then cumulated and were considered representative of up to a six day-period whereas samples representing particular hydrological events (e.g. floods, high SPM concentrations) were analyzed separately. Cumulative or isolated samples were filtered in the laboratory using 0.2 µm Sartorius® polycarbonate filters. Filtrates were collected in 60 cc polypropylene bottles, previously decontaminated and thoroughly rinsed with the filtrate, acidified (HNO_3 suprapur grade; 1:1000) and stored at 4°C until analysis (Schäfer et al., 2002). Representative sub-samples (30 mg of dry, powdered and homogenized material) were digested in closed Teflon reactors (Savillex®) using 750 µL HCl (12 M, suprapur), 250 µL HNO_3 (14 M, suprapur) and 2 mL HF (22 M, suprapur). The reactors were then heated to 110°C for 2 h. After complete cooling, the solutions were evaporated to dryness and then brought to 10 mL using 150 µL HNO_3 (suprapur) and ultrapure (Milli-Q®) water (Blanc et al., 1999; Audry et al., 2005).

Dissolved and particulate metals were measured using ICP-MS (X7, THERMO) with external calibration. Indium was used as internal standard and after each batch of five samples, a calibration blank and one calibration standard were measured to check potential sensitivity

variations or memory effects. The analytical methods employed were continuously quality checked by analysis of certified reference sediments (PACS-1, BCR 320, SL-1) and river waters (SLRS-3, SLRS-4). Accuracy was within 5% of the certified values and the analytical error (relative standard deviation) was generally better than 5% for concentrations ten times higher than detection limits.

7.3.2.2 Biofilm characteristics

Biofilms were grown on glass slides (total surface area reaching 300 cm^2) used as artificial substrates. After a 24-day immersion, the slides were removed from the water and scraped into a standardized volume of 200 mL mineral water. Three replicate samples per sampling date were collected and separated into aliquots. Another aliquot of 20 mL was used for particulate matter analysis: biofilm dry weights (DW) were determined following the European standard NF EN 872. After filtration through pre-weighed glass fibre filters (Sartorius, Göttingen, Germany), samples were dried at 105°C for 1 hour. The amount of material retained (i.e. biofilm DW, as expressed in $mg \cdot cm^{-2}$ glass substrate) was determined by re-weighing.

Aliquots of 20 mL were used for metal measurements (cadmium and zinc). Biofilm samples were filtered with an aspiration pump, through a pre-weighed metal-free filter paper (0.45 µm membrane, Millipore) to obtain the dry weight (DW) of each sample after drying at 60°C for 48 hours in incubation tubes. Then, the filters were digested using nitric acid (3 mL of HNO_3 65%) in a pressurized container at 100° C for 3 hours (hot block CAL 3300, Environmental Express, USA). Digestates were then diluted with ultra-pure water (Milli Q, Bedford, MA, USA), and Cd and Zn concentrations measured by flame atomic absorption spectrometry (Varian AA20), with a detection limit of 15 $\mu gCd.L^{-1}$ and 10 $\mu gZn.L^{-1}$. The validity of the method was checked periodically by parallel analysis of certified biological reference materials (Tort 2 – lobster hepatopancreas and Dolt 2: dogfish liver from NRCC – CNRC, Ottawa, Canada). Reference samples were measured in triplicate. Values for Cd and Zn were consistently within the certified ranges for each metal and each standard reference (data not shown).

Finally, 5 mL were preserved in a formalin solution for quantitative counting and diatom identifications to the species level. Enumeration was done in each sample using a Nageotte counting chamber: the total number of cells counted in 10 fields (1.25µL each, 0.5mm depth) using light microscopy at 400x magnification (photomicroscope Leica DMRB, Wetzlar, Germany) was then recorded as cells per unit area of sampled substrate (number of diatom $cells.cm^{-2}$). Samples assigned to taxonomic analysis of diatom assemblages were prepared

according to ANSP (Academy of Natural Sciences of Philadelphia) protocols (Charles et al., 2002), *i.e.* digestion in boiling hydrogen peroxide (30% H_2O_2) and hydrochloric acid (35%) followed by three cycles of centrifugation of the sample and pellet rinsing with distilled water. After the last treatment, the pellet was once again resuspended in distilled water, and this solution was pipetted onto coverslips which were mounted onto slides after air drying, using the high refractive index (1.74) medium Naphrax© (Brunel Microscopes Ltd, UK). Diatom counts were conducted at a magnification of 1.000x; individual fields were scanned until at least 400 valves had been identified using specialized literature (Krammer and Lange-Bertalot, 1986 - 1991) from which theoretical biovolumes of each species were also extracted. Diatom species diversity was calculated using the Shannon index $H'=-\Sigma p_i \cdot \log_2 p_i$ (where p_i: relative abundances of species i, Shannon and Weaver, 1963). Individual deformities (cells with abnormal general shape and or diatoms with deformed valve wall ornamentation) were observed and their frequency determined.

7.3.4. Statistical analyses

Major differences in physicochemical parameters between sampling dates were investigated using Principal Components Analysis (PCA) performed with PC-ORD software (McCune and Mefford, 1999). Accumulation rates of metals in the biofilm as well as correlations between DW and diatom densities were determined by linear regression on the raw data set and tested for statistical significance. Regression least-squares assumptions (homoscedasticity and normality of the error term) were checked using the residual term (Levene test and Chi^2 and Kolmogorov-Smirnov goodness-of-fit tests). Commonly used indices (IPS, Shannon index) were calculated using OMNIDIA software (Lecointe et al., 1993). Statistical significance of metal contents in biofilm and particulate matter was examined using analysis of variance. For all statistical results, a probability of $p<0.05$ was considered significant. Values are mean ± standard error (SE).

7.4 Results

7.4.1 Field colonization conditions

7.4.1.1 Physical and chemical characteristics

The sampling site is next to a permanent station of the regional environment department (DIREN), measuring water levels for discharge observation. The hydrological evaluation of the experimental period is based on the data available at http://hydro.rnde.tm.fr/. The mean water discharge for this period is 1.5 ± 2.0 $m^3.s^{-1}$, and the study period is quite representative of the situation observed for several years. Typical, short flood events occurred in winter, when discharge reached 54 $m^3.s^{-1}$ in mid-March. The physical and chemical parameters measured in the sampled waters are shown in table 7.1. During this study, water had a uniform pH (around 7.7 ± 0.3) and silica levels were found at concentrations sufficient for diatom development. Temperature showed strong seasonal variations, ranging from about 5 °C in march to almost 25 °C in july. Accordingly, dissolved oxygen level was minimum during the summer. Nutrient values differed greatly among sampling dates. Located downstream of the urban area of Decazeville, the Joanis site is strongly impacted by quite high levels of organic and domestic contamination (Lemaire et al., 2006): orthophosphates as well as high levels of ionic forms of nitrogen (nitrates, nitrites and ammonia; Table 7.1). Average conductivity values were quite high (1,130 ± 320µS.cm^{-1}) during almost the whole observation period and drastically decreased to 414 µS.cm^{-1} in May.

7.4.1.2. Dissolved and particulate metal concentrations

The average dissolved Cd and Zn concentrations in the samples obtained every 24 days and filtered immediately after sampling ranged from 5.2 to 49.2 µg·L^{-1} (average 26.2 µg.L^{-1}) and from 259 to 2,965 µg.L^{-1} (average 1,458 µg.L^{-1}), respectively (Table 7.1). The dissolved Cd and Zn concentrations measured in samples retrieved by the automatic sampling system and filtered after several days of storage in the sampling containers ranged from 0.223 to 54.3 µg.L^{-1} (average 22.2 µg.L^{-1}) and from 28 to 3,490 µg.L^{-1} (average 1,280 µg.L^{-1}; data not shown), respectively. Average particulate metal concentrations in SPM sampled manually and automatically ranged from 170 to 1,160 mg.kg^{-1} (average 560 mg.kg^{-1}) for Cd and from 4,750 to 26,540 mg.kg^{-1} (average 13,120 mg.kg^{-1}) for Zn. No clear differences in particulate metal concentrations obtained from the two sample types were observed.

7.4.1.3 Seasonal variability

We applied the PCA method to a data matrix of 13 months observed on 14 variables. These presentations based on stream physicochemical parameters (Figure 7.2) revealed great differences between four groups of sampling dates and underlined the seasonal gradient.

	pH	Temp (^{0}C)	Cond ($\mu S.cm^{-1}$)	O_2 ($mg.L^{-1}$)	NO_3 ($mg.L^{-1}$)	NO_2 ($mg.L^{-1}$)	NH_4 ($mg.L^{-1}$)	PO_4 ($mg.L^{-1}$)	Si ($mg.L^{-1}$)	Discharge ($m^3.s^{-1}$)	Dissolved Cd ($\mu g.L^{-1}$)	Particulate Cd ($\mu g.L^{-1}$)	Dissolved Zn ($\mu g.L^{-1}$)	Particulate Zn ($mg.kg^{-1}$)
04/15/04	8.2	12.4	867	9.3	15.6	0.4	1.0	0.4	8.0	1.4	8.4	474	371	8910
05/13/04	7.8	14.2	414	8.9	9.5	0.2	0.6	0.3	9.0	4.2	5.2	170	259	4750
06/02/04	7.9	17.7	780	8.8	8.6	0.5	1.0	0.8	10.0	0.9	13.5	460	510	13850
07/21/04	7.5	24.8	1220	n.m.	36.0	1.3	3.4	2.9	12.5	0.1	24.4	1020	1963	15790
08/17/04	7.5	20.9	1210	n.m.	7.3	0.5	1.9	1.2	13.0	4.7	22.7	1160	1138	26540
09/07/04	7.3	21.9	1280	5.7	71.3	1.6	3.3	2.3	12.0	4.9	28.5	558	1292	15790
10/06/04	7.5	19.3	1220	4.8	n.m.	n.m.	n.m.	n.m.	n.m.	0.1	47.7	515	2965	15020
10/27/04	7.0	13.2	1110	8.3	6.8	1.6	2.2	1.5	11.0	0.1	32.6	579	2628	14080
11/24/04	7.8	9.3	1200	7.2	41.4	1.2	6.3	2.9	8.5	0.2	49.2	560	2525	14330
12/15/04	7.6	7.0	1240	9.0	38.6	1.2	7.6	2.7	12.0	0.2	49.2	560	2525	14330
01/11/05	7.8	6.7	862	9.6	22.1	0.5	3.7	1.6	9.5	0.2	35.2	456	1985	7860
02/02/05	7.8	6.1	1230	8.9	18.5	0.4	2.2	1.3	8.0	0.5	18.1	359	805	9290
03/03/05	8.0	4.5	1600	9.6	25.5	0.75	2.9	0.9	10.5	0.5	13.7	464	695	9670

Table 7.1: Physicochemical parameters measured in stream water at each sampling date, metal concentrations are mean values for previous 24 days (n.m: not measured).

The results of this analysis showed that the first two principal components mainly account for more than 60 % of the total variability. For this reason, due to the number of variables, the projection in a plane spanned by the first two principal components explains the structure of the data with a good clarification power. It is worth noting that the same tendencies were observed in plane 1-3. Axis 1 separated winter and spring samples on the right-half plane (positive values) and summer and autumn water on the left-half plane (negative values). Separation along Axis 2 expressed the differences between water collected in spring and summer on the one hand, and those sampled in autumn and winter on the other hand. PCA discriminated sampling dates according to the most important seasonal characteristics. Summer samples were correlated with high temperatures and strong particulate metal contamination levels while winter samples were characterized by low temperatures and the highest dissolved oxygen concentrations as well as elevated turbidity. Autumn samples exhibited the highest levels of dissolved metals and nutrients, with nitrate concentrations reaching 71 $mg.L^{-1}$ in September 2004 (Table 7.1).

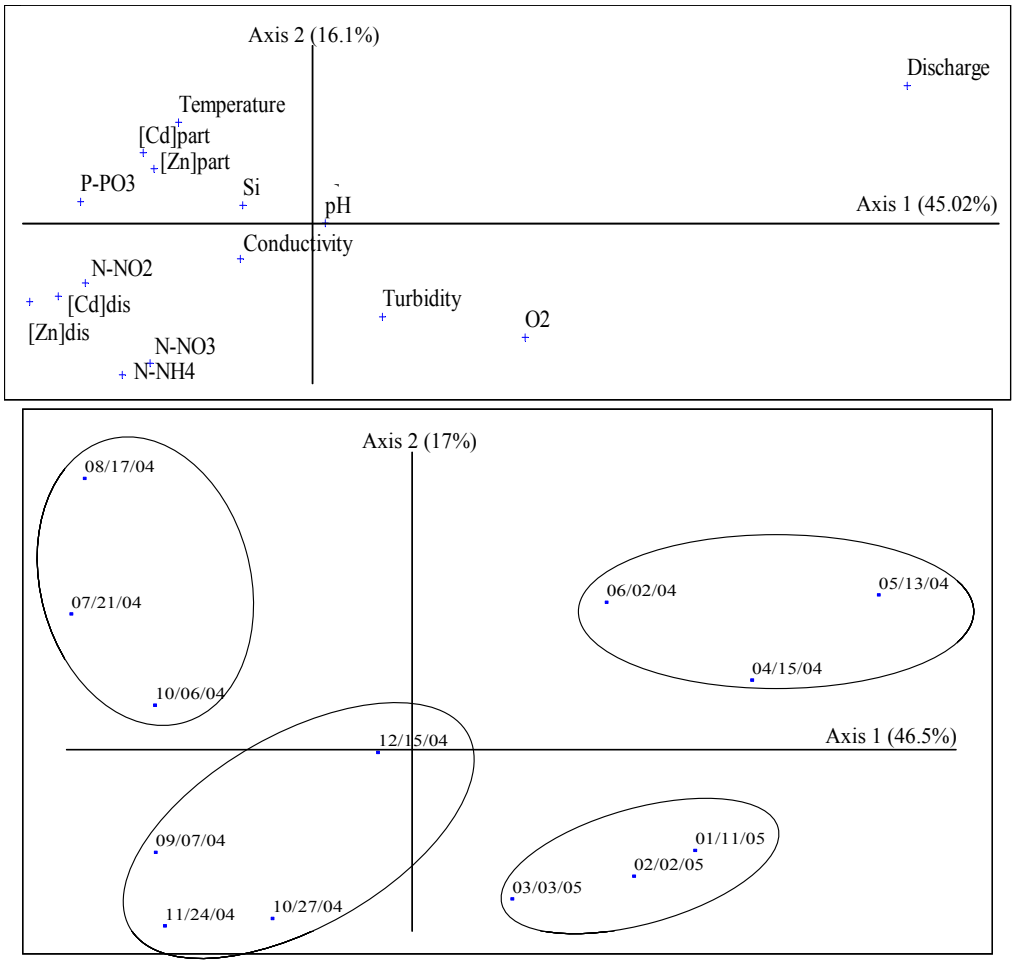

Figure 7.2: Principal Components Analysis (PCA) of physicochemical stream characteristics

7.4.2 Biofilm characteristics and structure of diatom assemblages

7.4.2.1 Trace metal concentrations in the biofilm.

The concentration of the Cd and Zn that had accumulated in biofilm are presented in Table 7.2. They varied considerably throughout the experiment. Zn was very high in August ($23{,}750 \pm 2{,}470$ µg.g^{-1}) whereas Cd was elevated during summer from July to August, October and December. The highest values of Cd content in biofilm were observed in August with an average of around $1{,}809 \pm 200$ µg.g^{-1}. There were strong relationships between metal concentrations in biofilm and those in SPM (Fig. 7.3). In fact, biofilm Cd and Zn concentrations showed significant linear regressions (Cd: $R^2 = 0.7$, $F_{cal} = 26.8$, $p<0.05$ and Zn: $R^2 = 0.46$, $F_{cal} = 9.4$, $p<0.05$) with the respective concentrations in SPM.

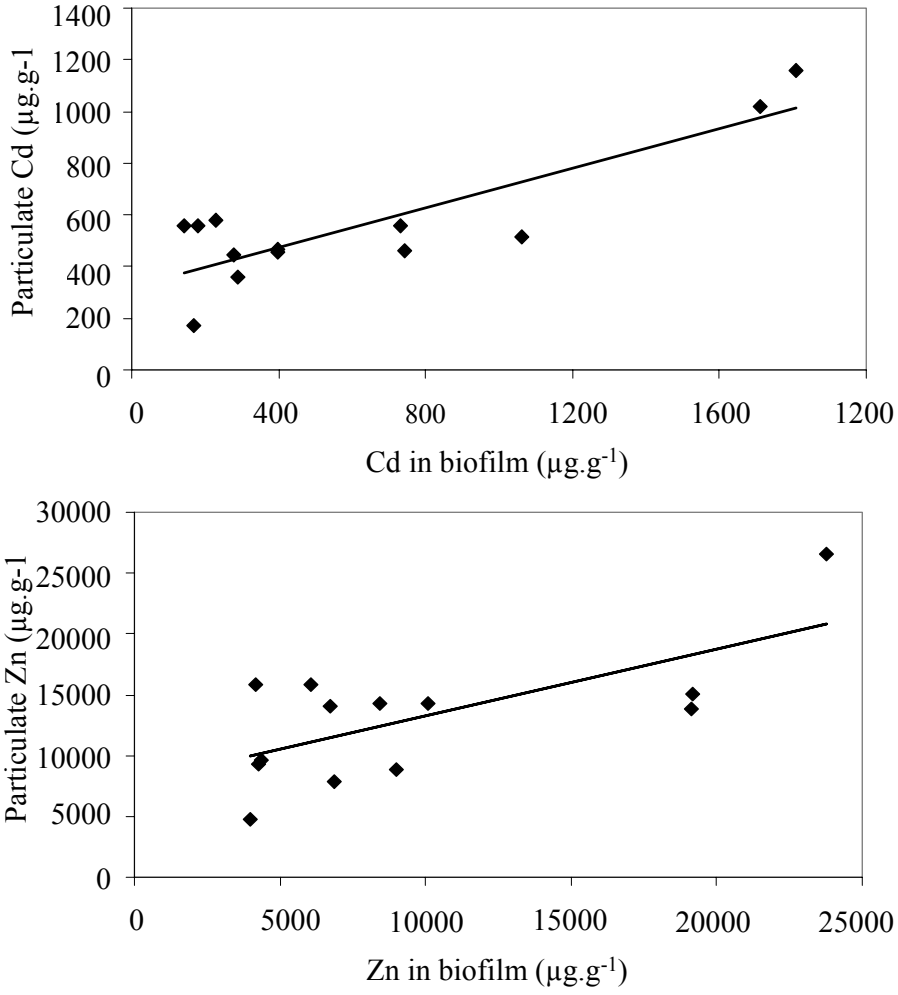

Figure 7.3: Relationships between metal concentrations in the water particular phase and in the biofilm.

	Dry weight ($mg.cm^{-2}$)	Density (Cells. cm^{-2})	Cd ($\mu g.g^{-1}$, DW)	Zn ($\mu g.g^{-1}$, DW)	Species richness (S)	Diversity Index (H')	ISP	Deformed forms (‰)
04/15/04	0.97 ± 0.11	13800 ± 520	278 ± 34	9007 ± 274	66 ± 1	4.63 ± 0.06	12.4 ± 0.2	14.6 ± 7.4
05/13/04	2.68 ± 0.20	11860 ± 590	170 ± 2	3958 ± 41	67 ± 3	4.57 ± 0.32	13.2 ± 0.2	12.5 ± 8.1
06/02/04	0.25 ± 0.03	11920 ± 500	743 ± 31	19149 ± 1014	62 ± 2	4.63 ± 0.11	10.5 ± 0.2	32.4 ± 8.0
07/21/04	2.72 ± 0.33	19870 ± 390	1711 ± 65	4171± 181	46 ± 5	3.51 ± 0.47	9.2 ± 2.4	11.8 ± 6.8
08/17/04	0.12 ± 0.01	3650 ± 1550	1809 ± 200	23750 ± 2469	48 ± 4	3.81 ± 0.34	9.0 ± 2.2	10.6 ± 4.6
09/07/04	2.24 ± 0.15	15600 ± 500	143 ± 8	6070 ± 225	66 ± 2	4.79 ± 0.10	10.9 ± 0.8	6.1 ± 2.6
10/06/04	0.36 ± 0.00	14380 ± 750	1062 ± 542	19166 ± 70	58 ± 6	4.15 ±0.41	7.8 ± 2.0	3.7 ± 0.8
10/27/04	3.63 ± 0.62	26250 ± 2520	228 ± 4	6714 ± 290	67 ± 1	4.66 ± 0.04	10.2 ± 1.4	5.8 ± 3.1
11/24/04	0.91 ± 0.30	5760 ± 100	180 ± 39	10062 ± 379	61 ± 1	4.27 ± 0.20	8.4 ± 2.2	5.1 ± 2.3
12/15/04	3.73 ± 0.78	27450 ± 1430	729 ± 20	8446 ± 108	60 ± 8	4.57 ± 0.36	9.1 ± 2.4	7.7 ± 3.3
01/11/05	0.37 ± 0.03	2240 ± 410	399 ± 89	6885 ± 1235	77 ± 2	5.17 ± 0.04	12.0 ± 1.9	4.2 ± 2.2
02/02/05	1.09 ± 0.08	6174 ± 340	287 ± 5	4239 ± 67	68 ± 5	4.96 ± 0.14	12.5 ± 1.7	1.8 ± 1.1
03/03/05	3.75 ± 0.28	4171 ± 80	399 ± 2	4348 ± 4				9.7 ± 1.6

Table 7.2: Biofilm and diatom assemblage characteristics (mean value ± SE)

7.4.2.2. Species composition and standing crop

The results of the quantitative measurements of periphytic biomass, as expressed by dry weight and cell density, are shown in table 7.2 and reveal a positive correlation between mean dry weights and mean densities ($p < 0.05$). Average cell densities during this 13 months experiment were around 12,200 ± 2,000 cell.cm^{-2}. The significant peaks that were observed for cell densities (see 7.4.2.3 Seasonal variations) were concomitant with peaks in biofilm dry weights. Diatom assemblages were characterized by an association of Naviculaceae, Nizschiaceae and Araphideae, the most abundant species being *Eolimna minima* (Grunow) Lange-Bertalot (16.4% of the total annual standing crop), *Nitzschia palea* (Kützing) W. Smith (7.0%) and *Pinnularia parvulissima* Krammer (6.8%). During the study, 255 species were identified; the most abundant and frequent are presented with their individual biovolumes in table 7.3. Various valve abnormalities affecting general cell shape and/or valve ornamentation were observed in a total of 28 species representing 17 genera, with frequencies reaching 3.2% in June 2004. Malformations were more often observed in genera such as *Ulnaria* (53% of the teratologies enumerated), *Fragilaria* (23%) and some Raphids (22%) than in others (e.g. abnormal valves of *Cocconeis pediculus* Ehrenberg, *C. placentula* Ehrenberg and *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot represented together less than 2%).

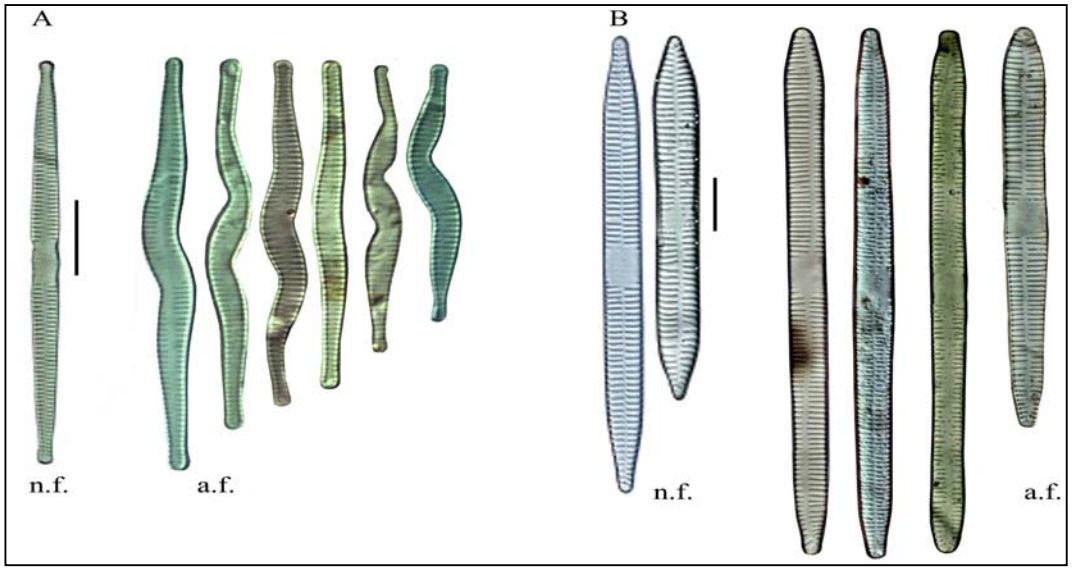

Figure 7.4: Morphological variants of *Fragilaria capucina* var. *gracilis* (A) and *Ulnaria ulna* (B) originated from Joanis (n.f: normal forms, a.f.: abnormal forms, scale bar: 10µm)

	ADMI	CMEN	EOMI	NGRE	NLAN	NPAL	PPVS	SANG	SBKU
Biovolume (μm^3)	76	1240	88	485	1230	391	2650	1820	2650
04/15/04	4.1	0.2	3.5	12.7	18.0	2.3	0.0	1.0	12.4
05/13/04	6.8	0.0	3.7	20.3	15.3	1.9	0.0	0.3	3.8
06/02/04	8.7	0.2	14.7	7.9	10.8	6.9	0.1	1.6	3.5
06/30/04	0.9	12.6	50.2	1.1	1.0	5.9	0.5	0.7	0.4
07/21/04	0.5	18.7	36.3	2.0	1.0	9.0	4.0	0.5	0.3
08/17/04	2.0	21.3	16.4	2.4	2.4	5.3	8.0	0.3	0.8
09/07/04	1.0	4.0	20.8	1.0	0.7	11.9	34.7	0.1	0.7
10/06/04	1.7	10.9	20.4	2.2	1.2	6.3	9.3	1.0	0.6
10/27/04	1.6	6.2	15.4	0.8	1.0	13.1	18.6	1.7	0.7
11/24/04	1.2	3.0	10.0	0.6	1.4	7.1	16.0	20.9	0.3
12/15/04	6.4	3.7	14.1	2.3	1.0	4.8	2.7	3.2	0.3
01/11/05	8.4	4.1	12.4	4.0	0.9	5.6	0.5	1.6	0.7
02/02/05	5.0	3.8	7.5	7.7	1.6	3.8	0.2	6.7	1.6

Table 7.3: Average relative abundances (%) of the most diatom species at each sampling date. (*Achnanthidium minutissimum*: (ADMI); *Cyclotella meneghiniana*: (CMEN); *Eolimna minima*: (EOMI); *Navicula gregaria*: (NLAN); *Nitzschia palea*: (NPAL); *Pinnularia parvulissima*: (PPVS); *Surirella angusta*: (SANG); *Surirella brebissonii var. kuetzingii*: (SBKU).

7.4.2.3 Seasonal variations

Seasonal variations of the periphytic biomass and diatom cell densities are reported in table 7.2. Dry weights and cell densities displayed a similar seasonal pattern with significant peaks in July, September, October and December 2004. Calculations of indices also exhibited seasonal variations. For example, IPS values were generally from 8 to 10, except for spring values which reached up to 13. Species diversity was generally lowest in summer, when only 40-50 taxa were enumerated. The highest Shannon index values were observed in winter and peaked to 5.17 in February 2005. We also observed seasonal cycles of the principal species: *E. minima*, *N. palea*, *P. parvulissima* and *Cyclotella meneghiniana* Kützing were dominant in summer and autumn. The species *Surirella brebissonii* Krammer and Lange-Bertalot var. *brebissonii*, *Achnanthidium minutissimum* (Kützing) Czarnecki, *Navicula lanceolata* (Agardh) Ehrenberg and *Surirella angusta* Kützing reached peak abundances in winter and spring.

7.5 Discussion

7.5.1 Geochemical scenario

The average dissolved metal concentrations in the cumulated samples were ~30% lower than average values in the manually retrieved and immediately filtered samples. This may partly be due to sorption onto the container walls and/or onto SPM in the samples during the storage period (up to 24 days) in the automatic sampling system. However, these samples integrate 8 sub-samples per day over 5-6 days including possible diurnal variations due to urban and/or industrial activities in the watershed. Therefore, these samples may be more representative of real conditions integrated by the biofilms, than the samples taken by hand during the daytime every 24 days. However, given the generally very high dissolved Cd and Zn concentration levels compared to uncontaminated sites (data not shown), the observed differences between the different sample types are not relevant for the interpretation of the biofilm response to metal exposure (see below).

The observed relatively high dissolved and particulate Cd and Zn concentration levels are typical for the Riou Mort River, i.e. similar to the concentration ranges observed during our permanent observation (since 2000) at the same site. Indeed, these levels are 2-3 orders of magnitude higher than those measured in the Riou Mort River tributaries (e.g. the Riou Viou, Enne and Banel Rivers) upstream from the former mining and ore treatment area (Coynel, 2005). An important part of the metal load in the Riou Mort River has been attributed to sulphide oxidation of Zn ore treatment residues (Audry et al., 2005). However, hydrological variations may induce complex responses in dissolved metal concentrations due to dilution effects, leaching of diverse industrial residues and changing interactions between groundwater and surface water (Coynel, 2005). During the period studied, dissolved metal concentrations tended to increase with decreasing discharge. In contrast, SPM were mainly derived from erosion processes and transported during short floods. Therefore, particulate metal concentrations depend on the particle type, source and to a lesser extent on sorption processes, i.e. exchanges between the dissolved and the particulate phases (Coynel, 2005). During the period studied, particulate metal fluxes were relatively low due to the atypically dry autumn and winter season 2004/2005 (DIREN; data not shown).

7.5.2 Metal bioaccumulation in biofilms

The present study shows that high levels of Cd and Zn accumulated in biofim at Joanis station of the Riou Mort River. High levels of metals of this area have been documented in several studies (Andres et al., 1999; Audry et al., 2005). This result suggested that metals can be detected reliably by measuring metals content in biofilm. High levels of trace elements have been reported to be accumulated by natural biofilm in acute metal pollution conditions (Ivorra et al., 1999) and it has been suggested that biofilm could serve as a biological monitor for anthropogenic waste (Newman et al., 1985; Hill et al., 2000b). The metals contained in biofilm were used to provide an indication of both the biological availability of metals and their ambient concentrations over relatively long periods (Foster, 1982; Behra et al., 2002). Our results revealed a significant correlation between metal concentration in biofilm and SPM in contrast to several reports concluding that metal concentrations in water samples reflected metal concentration in biofilm (Ivorra et al., 1999; Behra et al., 2002). The reason may be that the metals accumulated in biofilm are contained into two fractions i.e. the biotic (algae, bacteria) and abiotic (silt, particulate matter). High positive correlations were found between intracellular metal content in biofilm and the exchangeable sediment fraction for Cd, Cu and Zn metals (Holding et al., 2003). Metal levels in periphyton generally parallel those found in surface sediments at the same sites (Ramelow et al., 1992). Accumulation of metals has been suggested to be depend on natural variation of their concentration (Meylan et al., 2003). Further studies should be performed to better understand metal accumulation processes within the periphyton both in short and long-term metal exposure.

7.5.3 Diatom community responses to heavy metal contamination

7.5.3.1 Characterization of the community structure

We established that biofilm dry weights and diatom densities are correlated. This means that, instead of counting the number of cells per unit area, which is too elaborate and time consuming for routine biomonitoring programs, further shifts in standing crop at Joanis may be assessed through DW measurements. Interpretation of DW and density data in the present study is however quite difficult, because they are highly dependent on nutrient and toxicant concentrations as well as on natural disturbances. For example, high discharge may scour the substrate and possibly lead to erroneous estimates of the number of organisms present (Ghosh and Gaur, 1998). It cannot be excluded that the size of the standing crop is influenced by grazing from invertebrates and fish, which can complicate interpretation.

IPS values indicated a moderate to poor quality status of the waters sampled. Water quality assessed by the IPS index probably reflects the nutrient contamination level rather than metal concentrations, although it is also known to be sensitive to micropollutants in some cases. The low values of the SPI index may not be considered as a specific indication of the metal pollution level, there is a need to combine these data with further information. According to species richness or Shannon index values, the best time for sampling diatoms is summer, as winter samples as well as those collected in Spring and Autumn may contain too many species with less defined dominance (John, 2000). Micropollutant alterations are probably best estimated in summer i.e. under extreme conditions, typically exhibiting lowest discharges and highest metal concentrations. Low average biovolume (1,200 μm^3 per cell), inferred from individual biovolumes and relative abundances, and expressed the dominance of species like *E. minima* or *A. minutissimum*. Although several authors have reported such small, adnate species to present the highest abundances in metal-polluted environments (Medley and Clements, 1998; Cattaneo et al., 2004), very few studies have attempted to link community biovolume to metal contamination. Size reduction of the global community (compared to the non-exposed communities found upstream; Morin et al., 2006) appeared to be an excellent specific indicator of metal contamination, more compatible with routine biomonitoring than measurements of individuals, which necessitates an additional counting effort.

7.5.3.2 Sensitivities of algal species

The majority of the commonly occurring species in the Riou Mort River are quite cosmopolitan in distribution and their ecological preferences in this experiment were consistent with those described in the literature. Indeed, most of them had already been recorded in metal-contaminated sites. High relative abundances of *E. minima* (in July 2004 this species represented around 50% of the total community) were in accordance with several indications of its tolerance to heavy metals found in the literature (Peres et al., 1997; Gold, 2002; Feurtet-Mazel et al., 2003; Szabó et al., 2005). This study also confirmed the resistance of *N. palea* (Peres et al., 1997; Medley and Clements, 1998; Lai et al., 2003; Whitton, 2003) as well as *S. angusta* (Takamura et al., 1989; Gold, 2002; Feurtet-Mazel et al., 2003). The species *P. parvulissima* was found to be tolerant to heavy metals. Many studies have already revealed the presence of the genera *Pinnularia* (Admiraal et al., 1999; Gold, 2002; Hirst et al., 2002; Gomez and Licursi, 2003) exposed to metal contamination. Although the status of *A. minutissimum* is still a matter of debate, Sabater (2000) and Blanck et al. (2003) reporting that it is quite sensitive to metal exposure, many authors have found this species tolerant to heavy metals (Ivorra et al., 2000;

Gold et al., 2002; Feurtet-Mazel et al., 2003; Nunes et al., 2003; Cattaneo et al., 2004; Guasch et al., 2004; Nakanishi et al., 2004; Szabó et al., 2005). The presence of *C. meneghiniana* in Summer and Autumn was found quite surprising, since species from the genera *Cyclotella* have been described as metal-sensitive by several authors (Ruggiu et al., 1998; Shehata et al. 1999; van Dam and Mertens, 1990); it was probably linked to high nutrient availability at this period, which favoured this species' development. Although most of these taxa are found in uncontaminated sites as well, their joint presence and co-dominance may be considered as an indicator of metal pollutions.

7.5.3.3 Interest of valve abnormalities for biomonitoring studies

Many teratological valves were evidenced during this study. The most difficult types of abnormalities to assess were those involving changes in the sculpting of the valve surface, particularly in small diatom species. This may be attributed to the limited resolution of the photomicroscopes used here, but it may also signify that small species are less susceptible to morphological deformations. Work on the valve abnormalities has established that both the incidence of abnormal light and the concentration of salts in the growth medium can produce teratological results, which appear identical to the observer. However, several observations have reported extreme changes in valve morphology associated with heavy metal pollution in freshwater environments (e.g. Yang and Duthie, 1993; McFarland et al., 1997; Peres, 2000; Nunes et al., 2003; Szabó et al., 2005; Morin et al., 2006), in seawater (Thomas et al., 1980; Dickman, 1998) and in sediment cores (Ruggiu et al., 1998; Cattaneo et al., 2004). In our case, deformities were undoubtedly related to the acute toxicity of Cd and Zn, the amounts of abnormal diatoms being significantly higher than the "background levels" of deformities found in the upstream Riou-Mort (less than 0.5%). Dickman (1998) and Stevenson and Balhs (1999) have suggested that morphological aberrations are good indicators of heavy metal contaminations. Here, we quantified many abnormal diatoms, but there are two major limitations for their use in routine biomonitoring. Firstly, the observation and enumeration of abnormal valves assumes a good knowledge of diatom taxonomy and hence requires experienced operators. Secondly, deformed cells are found in very low abundances suggesting that a better estimate would be obtained by increasing the counting effort and by performing abnormality enumerations separately from the taxonomic identifications. This study demonstrates that the rarely reported effects of heavy metals on the morphology of diatoms need to be studied more thoroughly and that diatom morphology may provide an efficient indicator.

7.6 Conclusion

Substantial evidence has been gathered to justify the use of biofilms, and particularly diatoms, as indicators of heavy metal contaminations. This study has:

- provided an overall view of metal contamination in Joanis water and biofilms;
- underlined the distribution patterns of diatoms as a consequence of metal contamination at Joanis;
- attempted to produce a basis for further studies on biofilms and diatoms as indicators of metal pollution in this watershed or elsewhere;
- confirmed the tolerance to heavy metals of several periphytic species.

Acknowledgements

The authors would like to thank Henri Bouillard, Cécile Bossy, Véronique Duflo, Naïg Girardot, Gilbert Lavaux and Jean-Pierre Lissalde from the GEMA team for their technical assistance in the field and Muriel Bonnet and Maryse Boudigues from the Cemagref laboratory who carried out the water sample analyses.

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CHAPTER 8

Experimental toxicity and bioaccumulation of cadmium in freshwater periphytic diatom in relation with the organic matrix of the biofilm*

8.1 Abstract

A study was undertaken to examine cadmium accumulation in freshwater biofilm, its effects on biofilm development and on diatom community structure. A suspension biofilm colonized in the Riou-Mort River (South West France) was introduced in three artificial stream systems in laboratory to develop on clean substrates. Young and mature biofilms were exposed to a Cd concentration of 100 $\mu g.L^{-1}$. Metal accumulation (total and intracellular metal contentin biofilm), dry weight and ash-free dry mass, diatom density and diatom composition were analyzed. Both total and intracellular Cd accumulated by the biofilm throughout the experiment increased with duration of metal exposure. Lower Cd content was found in mature biofilm. Inhibition of dry weight and biomass by Cd exposure was more effective in young than in mature biofilm. Diatom communities in young biofilm exposed to Cd increased their tolerance to Cd by a highly significant development of tolerant *Nitzschia palea* species. In contrast, Cd had less effect in mature biofilm and taxonomic composition of diatom communities did not significantly differ after Cd exposure from those before Cd contamination. These results indicate that the role of the biofilm as a protective layer may limit Cd accumulation into its architecture and protect diatom communities developed in it from the effects of metals.

Keywords: biofilm, Cd accumulation, diatom communities, artificial stream

* This chapter is prepared as an article to be submitted under reference T.T. Duong, S. Morin, M. Coste [2], O. Herlory[3], A. Feurtet-Mazel, A. Boudou

8.2. Introduction

Diatoms have been commonly used as indicators of a variety of environmental conditions because of their high diversity, sensitivity to changes in water quality and their fundamental role in the food webs (Dixit et al., 1992; Stevenson and Pan, 1999). By investigating diatoms in polluted rivers, it has been found that diatoms can be used as the bioindicators of several types of pollution such as eutrophication (Kelly, 1998; Kelly, 2003), organic pollution (Sládecek, 1984; Watanabe et al., 1986; Descy and Coste, 1996; Rott et al., 1998) and metallic pollution (Gold et al., 2003; Lai et al., 2003; Cunningham et al., 2005, Morin et al., 2006). The relationship between diatom composition and the level of chemical contamination of water quality have been subjected to extensive monitoring studies for several years (Say and Whitton, 1980; Foster, 1982; Medley and Clements, 1998, Sabater, 2000). It has been long recognized that metal contamination can be toxic to diatoms. *In situ* studies conducted at sites exhibiting high level of metal and microcosm experiments have demonstrated that metals caused a decrease in productivity, diversity and changes in species composition of diatom communities (Takamura et al., 1989; Hill et al., 1997; Sabater, 2000). Trace metals have also been shown to decrease photosynthetic rate, and the uptake of various nutrients ions. Changes in morphology and increase in cell size are the main manifestations of high concentration of metals (Fisher et al, 1981; McFarland et al., 1997; Dickman, 1998; Cattaneo et al., 2004, Morin et al., 2006). Diatom communities are subjected to multiple anthropogenic inputs into aquatic environments through wastewater disposal as well as natural disturbance such as flood and temperature changes. A large number of factors like temperature, light (Patrick, 1971; Hill et al., 1995), current velocity (Ghosh and Gaur, 1998; Stevenson, 1996; Abe et al., 2000), nutrients availability (Rosemond et al., 2000), dissolved chemicals, organic or inorganic substances (Genter, 1996; Hoagland et al., 1996) are known to influence on structural characteristics of diatom communities during its development. In aquatic systems, interaction between these factors often occur and lead to difficulties of interpretation about the effects of one separate factor on structure and function of diatom communities.

On the other hand, succession of organisms often occurs on hard substrates in an aquatic system with the development of an organic matrix and bacteria followed by a transition from small adnate diatoms and then by other algae (green algae or Cynanobacteria), these organisms are associated in a mucopolysaccharide matrix, forming biofilm (Stevenson et al., 1996). Periphytic biofilm may achieve a high structural complexity with a complex function (Sekar et al., 2002) and can form a protective layer thereby reducing the exposure of solid surface to the

external environment and decreasing toxicity of contaminants (Ivorra et al., 2000; Gold et al., 2003). Periphytic biofilms have a remarkable ability to accumulate heavy metals and other contaminants from their environment (Newman et al., 1985; Clement, 1991; Ramelow et al., 1992, Berha et al., 2002; Holding et al., 2003). Accumulation of contaminants can be toxic to biofilms themselves, leading to decreased primary production and altered community composition (Sabater, 2000), and other consumers too in the food webs. A laboratory experiment was therefore performed aiming to examine cadmium accumulation and effects on freshwater biofilm as well as on structure of diatom communities. The role of the biofilm as a protective layer in response to cadmium exposure and metal accumulation for diatom communities is discussed.

8. 3. Material and methods

8.3.1 Field sampling

The Riou Mort stream, a small tributary of the Lot River in the industrial basin of Decazeville (South West France, 44°N/2°E) carries seepage from its confluence with the Riou-Viou stream which origins from a former zinc factory, presenting high level of metals (Audry et al., 2004, Morin et al., 2006). Periphytic biofilm were obtained from the Riou Mort stream, at a Decazeville site located upstream from the junction with the Riou-Viou stream (Figure 8.1).

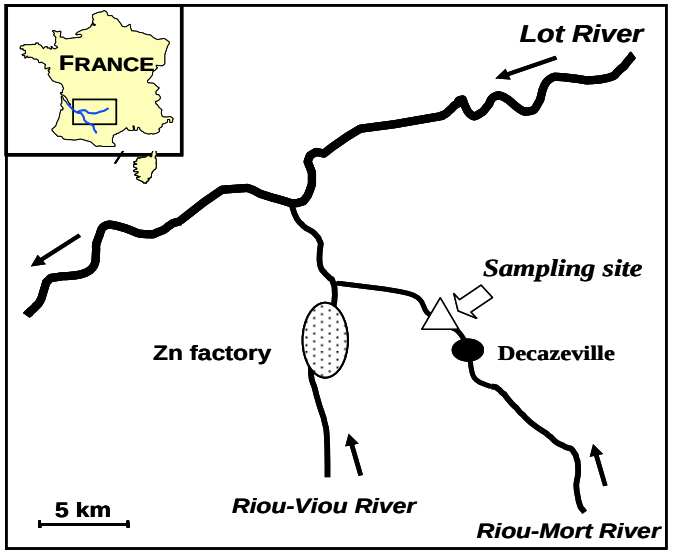

Figure 8.1: Location of the Riou-Mort stream and sampling site, South West France

The site was characterized by low metal concentrations and high nutrients (Morin et al., 2006). Plastic racks equipped with floaters, containing 6 separate and vertical glass substrates (6 x 15 cm, 300 cm² surface for both side), as artificial substrates for algal attachment, were immersed in the stream, parallel to the current, at a depth of 15-20 cm below the surface, and tied to the bank with a rope. The sampling was carried out in spring and glass substrates were left in the stream to allow biofilm's growth for five weeks prior to sampling. On the day of sampling, the racks were removed from the stream and biofilms colonized on glass substrates were scraped with a blade then rinsed and diluted to a volume of 2L with mineral water. This biofilm suspension was transferred into a polyethylene bottle and transported to a laboratory in a cool box (4^0C).

8.3.2 Laboratory experiment

Artificial stream

The set up was designed to allow the exposure of natural periphytic communities to cadmium under stable conditions. The effects of cadmium on biofilm, structure of diatoms communities were investigated using three experimental systems. Each system consisting of three small artificial streams (6 L volume each) corresponding to three replicates connected in parallel to a tank (Figure 8.2).

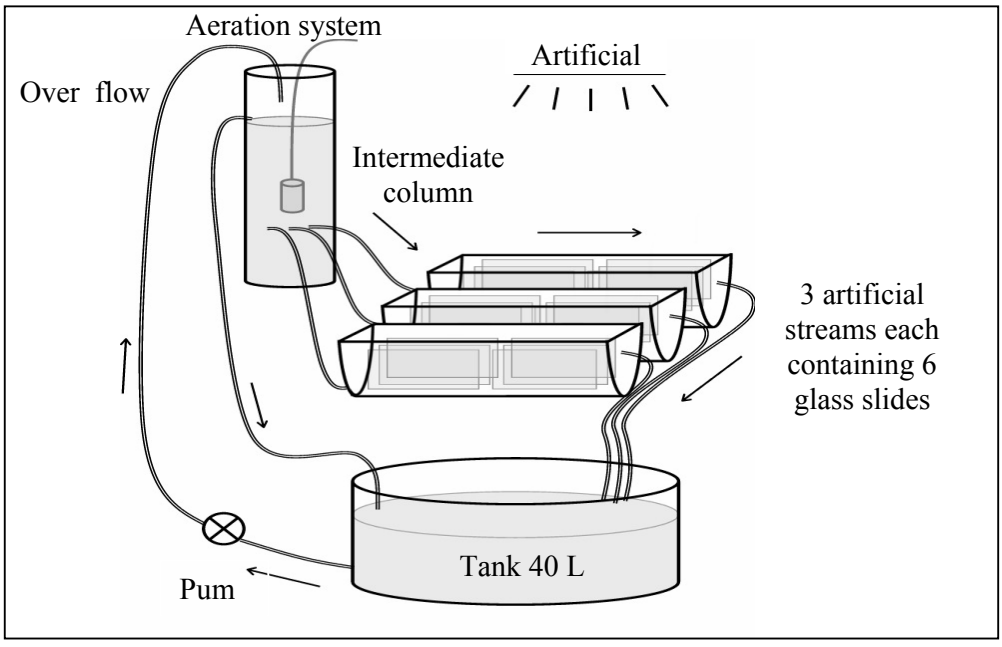

Figure 8.2: Schematic representation of an experimental system consisting of three artificial streams, each containing 6 glass substrates. Arrows indicate flow direction.

An external pump allowed consecutive circulation of water through each system at a velocity of 0.4 cm/s. Artificial stream systems were made with PVC tubes (60 cm length and 6cm radius), each containing six clean glass substrates (6x 15,300 cm² surface) for periphyton colonization. Glass substrates were placed vertical, side by side in the three small artificial stream systems, parallel to the current about 0.5 cm below water surface, at a light intensity of approximately 70 $\mu mol.s^{-1}.m^2$ (10h light:14h dark regime) in a thermo-regulate room at a temperature of about 15^0C. The suspension biofilm sample collected in the field was divided into three equal fractions; each fraction was introduced and incubated in each artificial stream system. Biofilms were incubated by supplying 40L of modified of Woods Hole culture medium (WC). This medium was kept without ethylenediaminetetraacetic acid (EDTA) (Gold et al., 2003 b). Before the start of the experiment, the systems were equilibrated overnight, then the experimental concentration of cadmium was added. During the experimental course, pH, temperature, conductivity and dissolved oxygen concentration of the water column for each experimental system were determined daily at the end of the light cycle. Nutrient concentrations (phosphates, nitrates, and silica) sampled in each system were measured weekly in laboratory according to French and international standards (NF T90-023 and NF EN ISO 13395, respectively). Depending on the results of the analyses, additions of culture medium were realized to compensate decrease of nutrients concentrations relative to algal uptake.

Cadmium exposure

To examine the effects of Cd exposure on young and mature periphytic biofilms, contamination of water column of experimental systems was made as followed: (i) Experiment unit 1 (EU_1) was left free of cadmium concentration (reference); (ii) EU_2 was contaminated with Cd concentration of 100 $\mu g.L^{-1}$ during six weeks and suspension biofilm was introduced in the artificial stream system; (iii) EU_3 was left uncontaminated during the first two weeks of the experiment to allow development of reference biofilm on substrates, and then contaminated with Cd concentration of 100 $\mu g.L^{-1}$ during four weeks. Cadmium chloride ($CdCl_2$) 10 mg. L^{-1} (Merck, Darmstadt, Germany) was used as stock solution to add in the system experiment to obtain a final nominal concentration of 100 $\mu g.L^{-1}$. Cadmium concentration was measured daily during the first and the third week of contamination and twice per week during the rest of the experiment. During the course of the experiment, cadmium was added into each system to maintain a relatively stable cadmium concentration close to the nominal level.

Biofilm sampling

After a colonization period of 1, 2, 4 and 6 weeks, glass substrates colonized by biofilm were randomly removed from EU_1, EU_2 and EU_3. After each time of sampling, the artificial stream systems were reset by new glass substrates to maintain identical flow conditions. Biofilms were scraped by using a blade, washed and then diluted in 100 mL of mineral water. The obtained biofilm samples were then divided into five fractions for various analyses. The first fraction (5 mL) was preserved with formalin solution (Formaldehyde 37%, Prolabo, France) for diatom identification. The second fraction (20 mL) was filtered through a GF/C filter using an aspiration pump for Chlorophyll a measurement (French standard NF T 90-117). The third fraction (20 mL) was drying and weighing at 500 ^{0}C for one hour in a muffle furnace (Solax Technology Ltd, China) for AFDM (ash free dry mass) analyses following European standard NF EN 872 and results were expressed in mg AFDM.cm^{-2}. To measure intracellular metal (non-exchangeable) content in biofilm, the fourth fraction (20 mL) of sample was washed with EDTA (ethylenediaminetetra-acetic acid) 4mM at pH = 8, for 10 minutes to remove cadmium adsorbed onto the surface of algal cells and most of inorganic complexes embedded in the biofilm. The remaining of the sample (20 mL) was used to determine the total amount of metal accumulated in biofilm (Behra et al., 2002; Meylan et al., 2004). Each sample was filtered with an aspiration pump through a tared metal free paper (0.45 µm membrane, Millipore) to obtain the dry weight after 60 ^{0}C for 48 hours in incubation tube.

Cd analyses

Cd concentrations in water samples were analysed through three replicates, filtered (0.20 µm Millex® Millipore) and were mineralized with nitric acid (HNO_3 2%) before measurements. Dried biofilm samples were first digested with nitric acid (3 mL HNO_3, Merck, Darmstadt, Germany) in a pressurized medium at 100° C for 3 hours (hot block CAL 3300). The digestates were diluted with 20 mL ultra pure water (Milli Q, Bedford, MA, USA). Cd concentrations in biofilm and filtered water samples were measured by atomic absorption spectrophotometry (Varian AA 400) equipped with a model GTA graphic tube atomizer and auto-samples. 10 µL of sub-biofilm and sub-filtered water samples were taken and mixed before atomization with 4 µL of 'palladium-$Mg(NO_3)_2$ mixture. The detection limit was 0.1 µL Cd.L^{-1} (LD: mean ± 3 SD of 10 reagent blanks). The validity of the method was checked periodically with certified biological reference materials (Tort 2-lobster hepatopancreas and Dolt 2: dogfish liver from NRCC-CNRC, Ottawa, Canada); values were consistently within the certified ranges (data not shown).

Analysis diatom communities

Diatom densities in formalin-preserved sample (100µL) were estimated using Nageotte counting chamber (Marienfeld, Germany), by counting the total number of diatoms in 30 fields (1.25 µL each, 0.5m depth), using light microscope (Olympus BX 50) at 200x magnification. Data are reported as cells per unit area of artificial substrate (cells /cm ^{2}). For identification to the species level, diatoms were cleaned with hydrogen peroxide (30 %) and hydrochloric acid (35 %) to remove organic material and dissolve calcium carbonates. Cleaned frustules were mounted in a microscope glass slide in a high refractive index medium (Brunel Microscopes Ltd, UK; RI=1.74). A minimum of 400 diatom valves or frustules were identified and counted in each slide by using a Leitz DMRD light microscope at 1000 X magnification. The flora of Krammer and Lange-Bertalot (1986-1991) were used as references for identification. Relative abundances of the species (percentage of the total abundance) and the species richness were estimated and diversity was calculated using the Shannon-Weaver index (H$^{'}$) (Shannon and Weaver, 1949).

8.3.3 Data treatment

Differences in dry weight (DW), AFDM, diatom density and Cd accumulation in biofilm between experimental system, colonization time and Cd contamination duration were analyzed using one-way ANOVA after testing normality and homogeneity of variance. *Post hoc* Turkey tests were applied to get statistical significance of differences. One-way ANOVA were performed using STATISTICA software (version 5.1, 97 editions).

8.4 Results

8.4.1 Physicochemical characteristics of the water column

Physical and chemical parameters measured in the artificial systems are shown in table 8.1. During the experiment period, the pH scarcely varied between the three artificial systems. A slight increase in pH was measured in each system ranging from 7 at the beginning to 7.9 at the end of the experiment. Water temperature ranged from 17.4 to 19.1^{0}C and dissolved oxygen ranged from 8.2 to 9.2 mg. L^{-1}. Both water temperature and dissolved oxygen in the three systems were similar and rather constant during the course of the experiment respectively (18.6 ± 0.2 ^{0}C and 8.7 ± 0.1 mg.L^{-1} for EU_1; 18 ± 0.3 ^{0}C and 8.4 ± 0.2 mg.L^{-1} for EU_2 and 18.7 ± 0.1 ^{0}C and 8.7 ± 0.1 mg. L^{-1} for EU_3). The average conductivity gradually increased from 143 to 202 µS/cm in EU_1, from 135 to 181 µS/cm in EU_2 and 145 to 200 µS/cm in EU_3.

	Reference (EU_1)					Contaminated at W_0 (EU_2)					Contaminated at the end of W_2 (EU_3)				
	W_0	W_1	W_2	W_4	W_6	W_0	W_1	W_2	W_4	W_6	W_0	W_1	W_2	W_4	W_6
pH	7.1	7.4	7.3	7.7	7.9	7	7.4	7.2	7.8	7.4	7	7.4	7.3	7.7	7.6
Temp (^{0}C)	18	18.8	18.9	18.8	18.7	17.8	18	18.2	17.4	18.1	18.3	19	19.1	18.7	18.7
O_2 (mg.L^{-1})	8.5	8.7	8.5	9.2	8.8	8.3	8.3	8.2	9.2	8.2	8.5	8.7	8.5	9.3	8.6
Conductivity (µS/cm)	143	153	163	183	202	135	139	155	181	181	145	149	159	179	200
PO_4 (mg.L^{-1})	2.9	2.5	2.3	1.7	0.9	2.9	2.4	2.2	1.9	2.2	2.9	2.4	2.2	1.9	2.2
NO_3 (mg.L^{-1})	33.5	27.5	25.7	22.2	19	32.6	27.6	28.0	31.6	37.2	32.6	26.7	26.1	29.1	33
SiO_2 (mg.L^{-1})	3	1	0.5	1	0.5	2.5	2	2	1	2.5	2.5	1.5	0.5	1	2.5
Dissolved Cd (mg.L^{-1})	< dl	< dl	< dl	< dl	< dl	70.4	106	92.5	101.1	110.5	< dl	< dl	78	99	97

Table 8.1: Physicochemical parameters and nutrients concentrations measured during the 6-weeks experimental period in the water column of the 3 experimental systems (d.l.: detection limit: 0.1µg.L^{-1}). EU_1 (Reference); EU_2 (Cd contaminated during 6 weeks; [Cd] = 100 µg.L^{-1}) and EU_3 (contaminated at the end of week 2; [Cd] = 100 µg.L^{-1})

In all systems, at the beginning of the experiment (W_0), phosphates and nitrates concentrations were around 2.9 $mg.L^{-1}$; 33 $mg.L^{-1}$ respectively, then gradually decreased. Continuous additions of culture medium resulted in restoration of the initial concentrations of phosphates and nitrates in EU_2 and EU_3 at the end of week 3 and week 5, but yet a continuous decrease was observed at EU_1. Silica concentrations decreased in three systems also but sharp decline was found in EU_1 and EU_3 (before Cd exposure). By frequent measurements of Cd concentrations, variation of actual Cd concentrations were observed during the first week of contamination, then were appropriate with the nominal concentration. The actual concentrations of cadmium in EU_1 were below 0.1µg.L, and the average cadmium concentrations in the EU_2 and EU_3 were 96.1±7 $\mu g.L^{-1}$ and 91 ± 6 $\mu g.L^{-1}$ respectively.

8.4.2 Dry weight and biomass of biofilm

Dry weight (DW) and AFDM showed the same pattern during the experiment course (Figure 8.3a and b). Average DW ranged from 0.014 ± 0.002 $mg.cm^{-2}$ to 0.08 ± 0.003 $mg.cm^{-2}$ in EU_1 system. Lower values were found in EU_2 system with mean values ranging from 0.008 ± 0.002 $mg.cm^{-2}$ to 0.0355 ± 0.007 $mg.cm^{-2}$. Average DW ranged from 0.02 ± 0.001 $mg.cm^{-2}$ to 0.06 ± 0.007 $mg.cm^{-2}$ in EU_3 system. Mean values of AFDM in EU_1, EU_2 and EU_3 systems ranged from 0.001 ± 0.004 $mg.cm^{-2}$ to 0.056 ± 0.0017 $mg.cm^{-2}$; 0.008 ± 0.002 $mg.cm^{-2}$ to 0.032 ± 0.002 $mg.cm^{-2}$ and 0.013 ± 0.005 $mg.cm^{-2}$ to 0.04 ± 0.005 $mg.cm^{-2}$ respectively. DW and AFDM in EU_1 increased following a linear model. On the other hand, EU_2 and EU_3 systems showed a similar increase in DW and AFDM from week 1 to week 2 but stabilised at week 4 and week 6. After 2 weeks of colonization, DW and AFDM were significantly higher in EU_1 and EU_3 (before Cd exposure) than in EU_2 system, and after 2 weeks of Cd exposure, DW and AFDM in EU_3 system did not significantly differ from EU_1 system (week 4). At the end of the experiment, the mean values of DW and AFDM in EU_1 were significant higher than those measured in EU_2 and EU_3 systems at week 6 ($p < 0.05$). There was no difference between AFDM of EU_2 and EU_3 systems after 4 and 6 weeks of colonization.

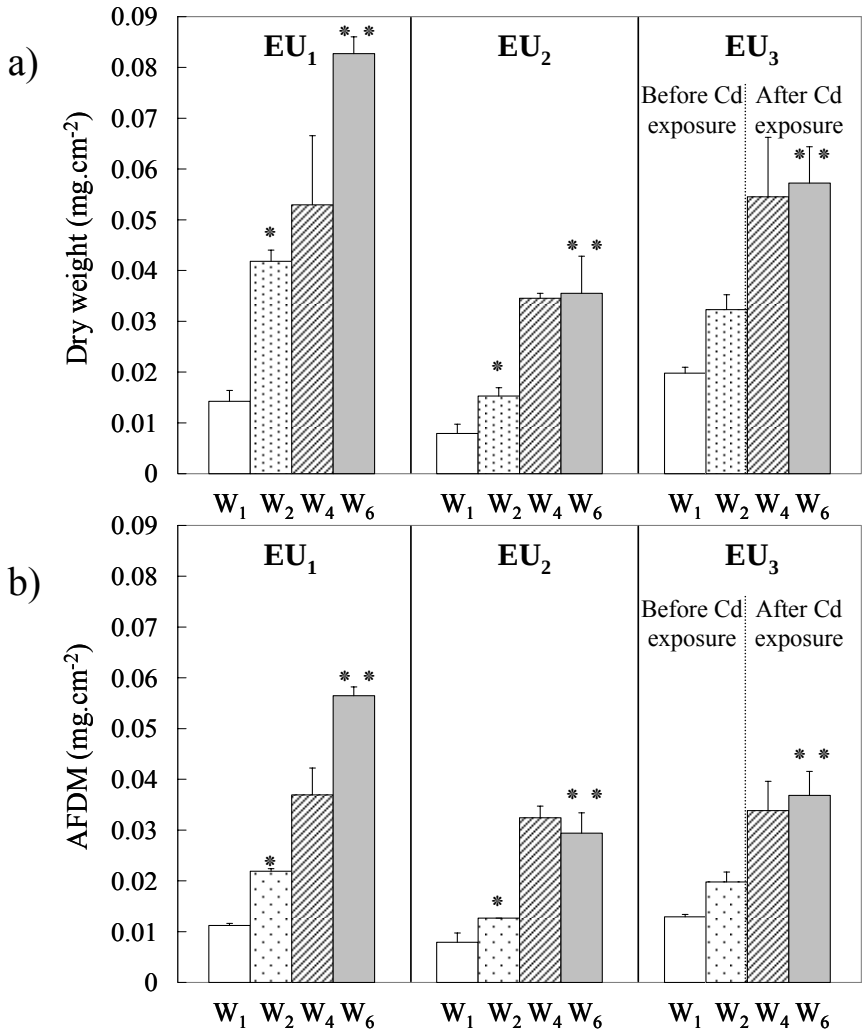

Figure 8.3a and b: Dry weight (DW) (a) and ash free dry mass (AFDM) (b) of biofilm in the experimental systems (EU_1; EU_2 and EU_3) during 6 weeks of colonization (mean value and n = 3). *: significant difference ($p < 0.05$) with EU_1 at week 2 and **: significant difference ($p < 0.05$) with EU_1 at week 6.

8.4.3 Cd accumulation

Cd adsorbed on abiotic or biotic material and intracellular (non-exchangeable) were obtained by measuring Cd content in non-washed biofilm and washed biofilm with EDTA (ethylenediaminetetraacetic acid), a strong metal complexing ligand. Both total and non-exchangeable Cd accumulated by biofilms (per unit dry weight, DW) are presented in figure 8.4a and b. In all cases, there was a trend that Cd accumulation in biofilms increased with time exposure, non-exchangeable Cd roughly corresponding to a third of total Cd content in biofilm.

In the reference system (EU_1), measurement of Cd in biofilm was low around 3.7 ± 0.8 µg.g^{-1}DW for total Cd and 0.57 ± 0.1 µg.g^{-1}DW for non-exchangeable Cd. In EU_2 system, average values of total and non- exchangeable were around 2935 ± 427 µg.g^{-1}DW and 1026 ± 394 µg.g^{-1}DW respectively, and the highest values of Cd accumulation were observed after 4 weeks of colonization. EU_3 system had a similar Cd content in its biofilm to those determined in the reference system (EU_1) at week 1 and week 2 (before Cd exposure).

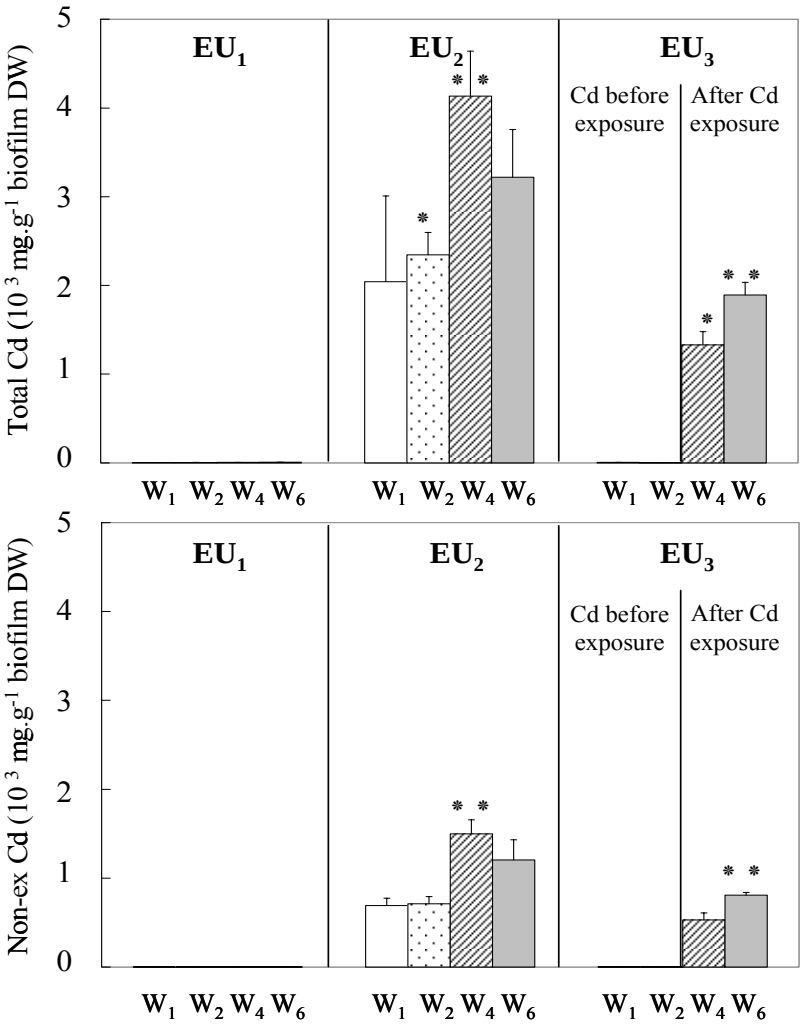

Figure 8.4a and b: Total Cd content in biofilm (a) and intracellular Cd content in biofilm (b) in the experimental systems (EU_1; EU_2 and EU_3) during 6 weeks of colonisation (mean value and n = 3). *: $p < 0.05$ (significant differences after 2 weeks of Cd exposure) and **: $p < 0.05$ (significant differences after 4 weeks of Cd exposure).

However, Cd addition led to a significantly higher Cd at the week 4 and week 6 than those in the biofilm of EU1 system ($p < 0.05$) without reaching EU_2 values. Cd accumulation at the different

stages of biofilm developement varied significantly (Figure 8.4 a and b). After 2 and 4 weeks of Cd exposure, total Cd content in the young biofilm of EU_2 system was significantly higher compared to Cd accumulated in mature biofilm of EU_3 ($p < 0.05$). For non-exchangeable Cd content in biofilm, a similar trend was recorded, although, a slight but non-significant elevation of Cd content in biofilm of EU_2 system to EU_3 system after 2 weeks of Cd exposure.

8.4.4 Quantitative and qualitative criteria of diatom assemblages

Diatom density colonised on substrates is one of the variables used to describe the response of diatom communities to Cd contamination (Figure 8.5). Glass substrates in EU_1 and EU_3 systems (before Cd exposure) were covered by a thin layer on the first week and then became denser with appearance of patchy colonies on the second week.

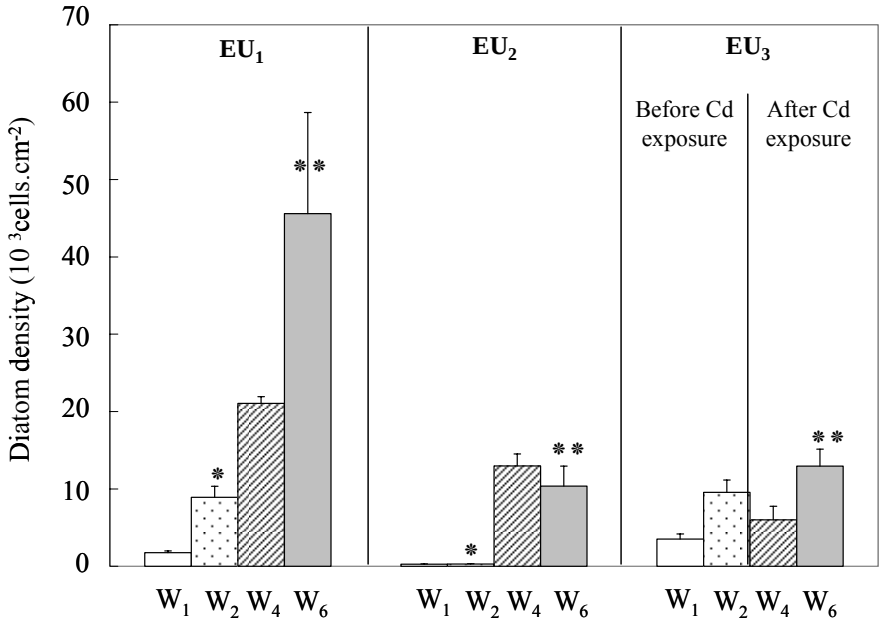

Figure 8.5: Diatom density (mean value and n = 3) developed on glass substrates in three artificial systems (EU_1; EU_2 and EU_3) during period of Cd contamination). *: significant difference ($p < 0.05$) with EU_1 at week 2 and **: significant difference ($p < 0.05$) with EU_1 at week 6.

Diatom density did not differ significantly between EU_1 and EU_3 system and ranged from 1776 ± 216 cells.cm^{-2} (W_1) to 8914 ± 1432 (W_2) and from 3522 ± 652 cells.cm^{-2} (W_1) to 9568 ± 1575 cells.cm^{-2} (W_2) respectively. Afterwards, diatom density in EU_1 system increased significantly and reached maximum values at the end the experiment (45,589 ± 13,073 cells.cm^{-2}). In contrast,

Cd contamination in EU_3 system led to a slight decrease in diatom density at the week 4 (6005 ± 1768 cells.cm^{-2}) and then densities increased on the last week of the experiment (12,968 ± 2189 cells.cm^{-2}) but this value was always significantly lower than in EU_1 system. In contaminated system (EU_2), very thin layer covering the glass substrates was observed, with very low densities during the first two weeks of the experiment (around 300 ± 27 cells.cm^{-2}). The highest mean value was recorded after 4 weeks of colonization; however, it was significantly lower from those in reference system ($p < 0.05$). At the end of the experiment, diatom density in the EU_2 system slightly decreased around 10,368 ± 2599 cells.cm^{-2}.

Global qualitative characterization of diatom is given by their species richness (S) and diversity index (H') (Figure 8.6).

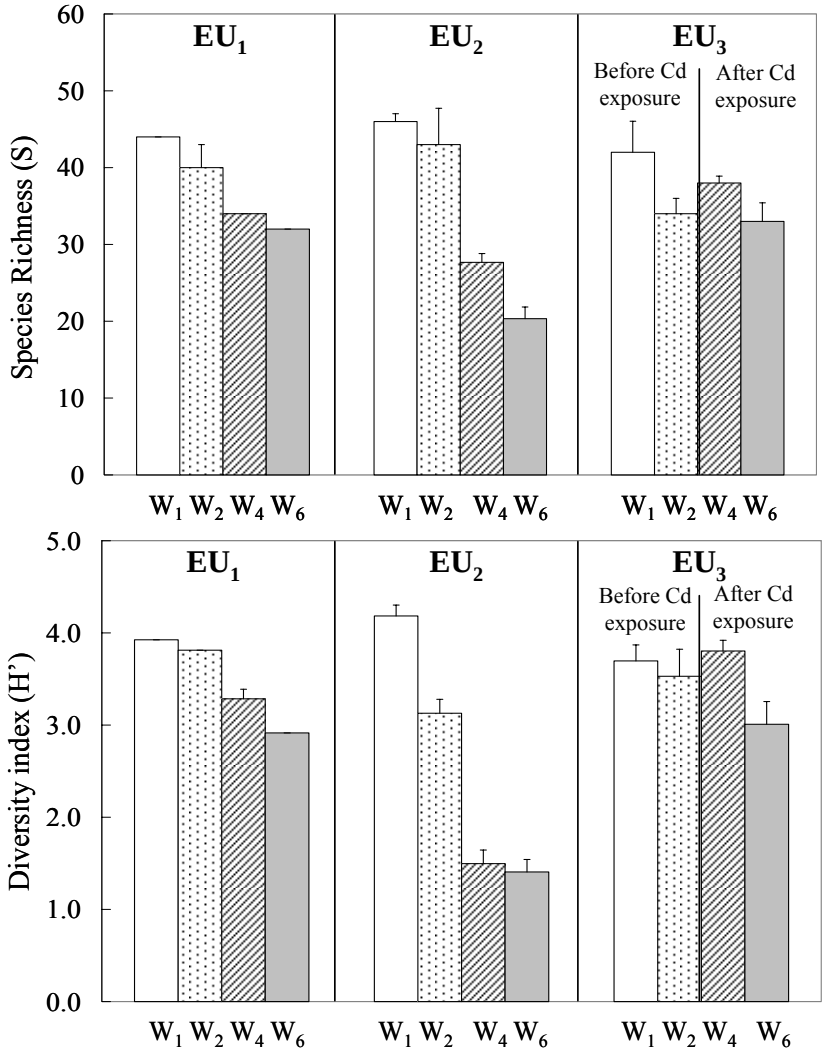

Figure 8.6: Diatom species richness and diversity index (mean value and n = 3) in EU_1; EU_2 and EU_3 experimental systems during 6 weeks of colonization.

According to one-way ANOVA, no significant difference were observed in species richness (S) and diversity index ($H^{'}$) between the three systems after one week of colonization, with species richness around 44 taxa and a diversity of 3.9 in each experiment unit for a total of 176 identified species. There was a trend of decrease in both species richness and diversity index in EU_1 and EU_2 system until the experiment ended; however, significant higher values were recorded in reference system (EU_1) than those in contaminated system (EU_2). On the other hand, Cd addition in EU_3 system caused a slight but non-significant, increase of species richness and diversity index with values not significantly different with those obtained in EU_1 and before Cd contamination at week 1. Nevertheless, longer Cd exposure caused slight diminution in species richness and diversity index in week 6 (after 4 weeks of contamination).

Among 176 identified species, 7 dominant diatom species with mean relative abundances > 9% were described as characteristic of each diatom community developed throughout the experiment (Figure 8.7). After 1 week of colonization, diatom composition in the three systems was similar with the presence of *Navicula gregaria*, *Navicula lanceolata, Nitzschia palea* and *Nitzschia dissipata*. Higher proportion of these species was observed in EU_1 and EU_3 than in EU_2 system. At week 2, the dominant species and their proportions were still similar in EU_1 and EU_3 (before Cd contamination) (Figure 8.7 and 8.8), whereas they differed from contaminated system EU_2 characterized by a significant increase of *Nitzschia palea* species from 10 % (W_1) to 52% (W_2) and a less important decrease of *Navicula lanceolata* from 15% (W_1) to 8% (W_2) and *Nitzschia dissipata* from 6% (W_1) to 2% (W_2). After 4 and 6 weeks of colonisation, the species composition in EU_1 system differed from that noted at week 1 and 2 with the proliferation of *Navicula (dicta) seminulum* and the decrease of *Navicula gregaria* and *Navicula lanceolata*. For EU_2 system, long Cd exposure caused a significant increase of massive proportion of *Nitzschia palea* relative abundances during the last 4 weeks of experiment (from 52% at week 2 to 81% at week 4 and week 6). In EU_3 system, after two weeks of Cd contamination (at week 4), diatom assemblages did not differ significantly from those of week 1 and 2 (before Cd exposure) except for *Nitzschia acidoclinata* which explodes at week 6 (Figure 8.7 and 8.8).

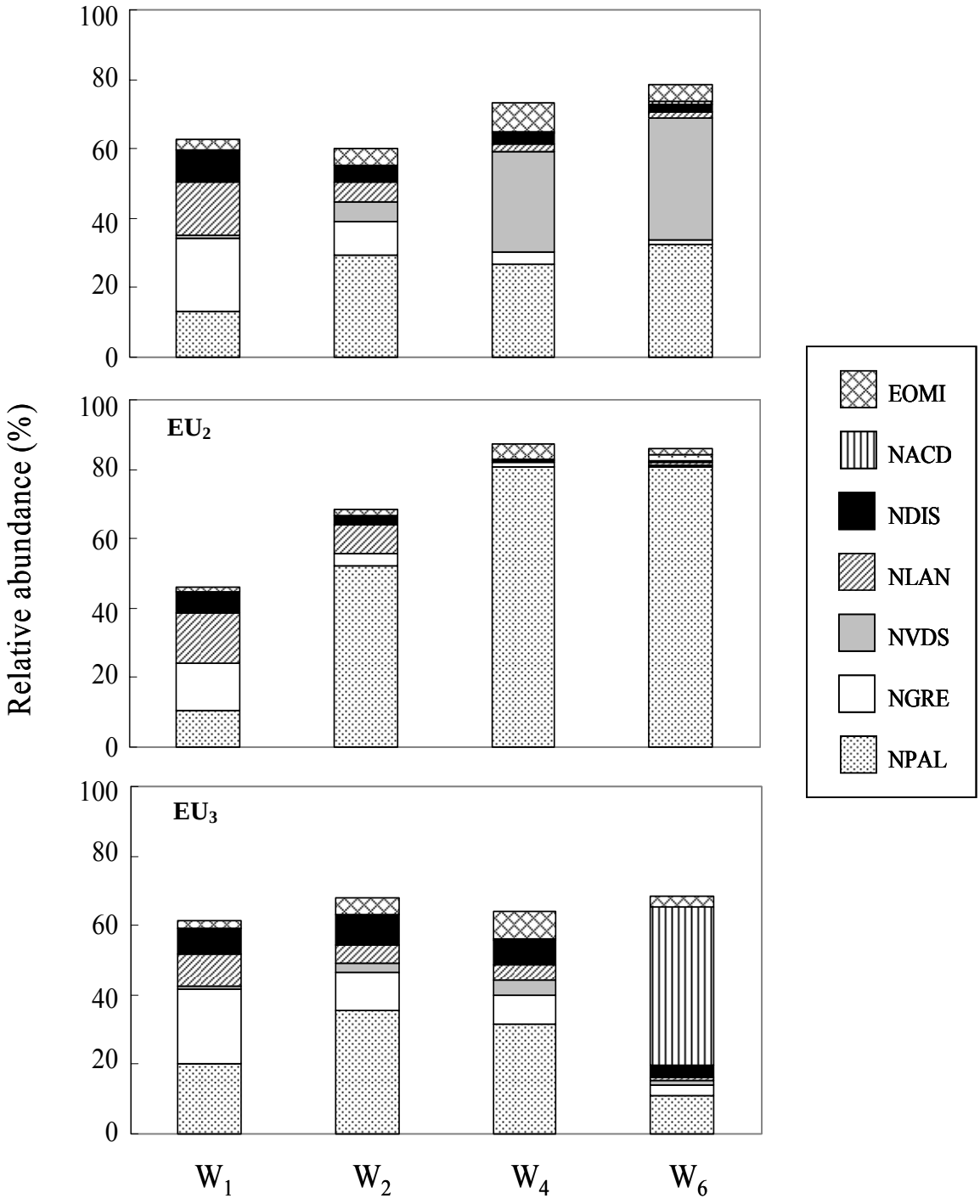

Figure 8.7: Relative abundances (mean value, n = 3) of the 7 major diatom species (> 9%) within diatom communities collected in the three experimental systems (EU_1, EU_2 and EU_3) during 6 weeks of colonization. (*Eolimna minima* (EOMI); *Nitzschia acidoclinata* (NACD); *Nitzschia dissipata* (NDIS); *Navicula lanceolata* (NLAN); *Navicula (dicta) seminulum*; (NVDS); *Navicula gregaria* (NVDS); *Nitzschia palea* (NPAL)

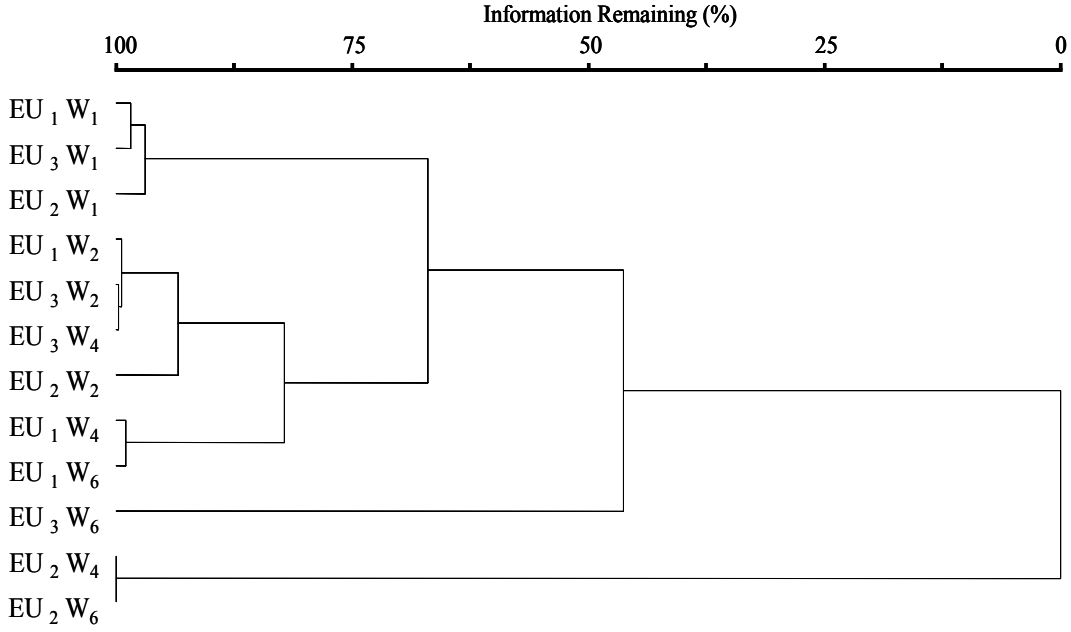

Figure 8.8: Dendrogram produced by Ward's method showing the similarity in taxonomic composition of diatom communities in EU_1, EU_2 and EU_3 experimental system during 6 weeks of colonization.

In addition to changes in species composition to Cd exposure, the response of diatoms communities to metal contamination is characterised by the appearance of diatoms deformities. Mean values of abnormal forms proportion in diatom communities of the three experimental systems are shown in table 8.2.

Experiment unit	W_1	W_2	W_4	W_6
Reference (EU_1)	2 ± 1.1	3 ± 0.6	2.6 ± 0.6	10 ± 0.8
Contaminated (EU_2)	3 ± 2.3	15 ± 2.6	9 ± 2.3	33 ± 2.8
Contaminated (EU_3)	3 ± 2.4	2.3 ± 1.4	13 ± 0.6	29 ± 2.1

Table 8.2: Mean proportion (‰) of species presenting abnormal forms (± standard errors) in the three experimental systems: EU_1 (Reference); EU_2 (Cd contaminated during 6 weeks; [Cd] = 100 $\mu g.L^{-1}$) and EU_3 (contaminated at [Cd] = 100 $\mu g.L^{-1}$ at the end of week 2).

Abnormal forms increasingly occurred with Cd exposure duration in EU_2 system and varied from 3‰ (W_1) to 33‰ (W_6). In EU_3 system, before Cd contamination, abnormal forms frequencies were low, around 2.8 ‰, and then (after Cd contamination) significantly increased

and reached their maximum values during the last week of the experiment (29 ‰). In reference system (EU_1) the frequency of diatom deformities stayed low with around 2.5 ‰ ± 0.3 from week1 to week 4, but a slight increase in abnormal forms abundances was observed at week 6.Among the 26 diatom species which were identified as abnormal forms, teratological forms of *Nitzschia palea* species were the most frequent observed in biofilm samples. Other species were concerned by deformities such as *Fragilaria capucina, Eolimna minima, Fragilaria vaucheriae* and *Fragilaria gracilis*.

8.5 Discussion

8.5.1 Cd accumulation within biofilm matrix

Our results show that cadmium accumulation by biofilm differed between experimental systems and temporally within systems (Figure 8.4a and b). In general, increases in Cd concentrations in biofilm were related to duration of metal exposure in the medium. In addition, both total and non-exchangeable Cd content in biofilms displayed similar pattern with duration of Cd contamination. The cadmium content in biofilm was higher in EU_2 and in EU_3 (after Cd exposure) than in reference system (EU_1), demonstrating that biofilm was able to accumulate considerable amounts of cadmium as mentioned by Ramelow et al (1992) and Holding et al (2003). As shown by several authors such as Pistocchi et al (1997), Barranguet et al (2000), Decho (2000) and Hill et al (2000), biofilms have a large number of metal binding sites located in either organic matrix produced by algae, bateria and fungi) at the surface of cells or in the organic particles trapped by the biofilm. These substances can play an important role in the sorption of metals from water column. Metal accumulation by microalgae has been generally described as consisting of two phases: rapid sorption onto the surface cell wall (biosorptive process) and a slow active phase (Garnham et a., 1992; Torres et al., 1998; Hill et al., 2000). Therefore, the linear increase in Cd accumulation per dry weight unit (both total and non-exchangeable) occurred till week 4 for EU_2 system, and week 6 for EU_3 system, that is after 4 week of Cd exposure. The increasing Cd content in biofilm simultaneous with increasing biofilm biomass underline a significant contribution of surface cells to metal sorption (Hill et al., 2000). As can be observed from figure 8.4a and b, equilibrium in Cd accumulation was reached at week 6 in EU_2 system. This could be related to the saturation of the aforementioned binding sites of the biofilm leading to reduce metal absorption capacity (Garnham et al., 1992). Similar pattern have been obtained for Cd sorption by natural biofilm (Duong et al., in preparation) or by

monoculture of diatom *Phaeodactylum tricornutum* Bohlin (Torres et al., 1998) and *Chorella vulgaris* (Ruangsomboon and Wongrat, 2006).

Regarding the EDTA capacity to trap external Cd (Figure 8.4b), it permits to quantify the amounts of non- exchangable Cd in the biofilm with 35% (in EU_2) and 41% (in EU_3) of the total Cd content within biofilm. This result suggests that most of Cd was adsorbed onto the surface of cell walls of algal and bacterial which mainly compose the biofilm. Comparison of Cd content in biofilm after 2 and 4 weeks of Cd contamination between two systems EU_2 and EU_3 indicates that difference in biofilm's thickness could explain the observed difference in Cd accumulation (Figure 8.4a and b). Indeed, young biofilms in EU_2 system had larger area exposed to Cd which led to higher level of Cd content (both total and non-exchangeable) compared to lower level in mature biofilm in EU_3 system. This seems to confirm that the thickness of biofilm could influence significantly metal sorption into biofilm itself and is in agreement with previous observation of Rose and Cushing (1970) and Ivorra et al (2000), who suggested that biofilm matrix restricted Cd and Zn penetration into deeper layer of the biofilm overall in slow current condition (Hill et al., 2000) . This limitation has been considered as pH local (Ivorra et al., 2000) and partly attributed to the binding to algal and bacterial polysaccharide exudates (Pistocchi et al., 1997). Further experiment with different contaminated concentrations should be considered during the development of young biofilm to state whether the non-exchangeable Cd fraction is proportional to Cd concentration available in water column or depend on a limited incorporation process.

8.5.2 Effects of Cd exposure on biofilm and diatom communities

It was foreseen that exposure of biofilm to Cd resulted in the inhibition of dry weight and biomass of biofilm (Figure 8.3a and b) in comparison with the reference system (EU_1) as soon as the first week of Cd exposure. Unlike response of young biofilms contaminated in EU_2 system, mature biofilm's development in delay contaminant (EU_3) system was not inhibited after two weeks of contamination and seemed to be resistant to Cd toxicity. It could be related probably to the protective role of the biofilm formed before exposure in relation with the tri-dimensional architecture design of diatom assemblages which constitutes an important part of the biofilm (Admiraal et al., 1999; Barranguet et al., 2000; Guasch et al., 2003). This hypothesis could explain the similarity observed between dry weight and biomass of biofilm in EU_1 and EU_3 at week 4 (after 2 weeks of Cd exposure). However, the follow-up of Cd exposure caused slow development of mature biofilm biomass. In agreement with our results, the same observations

have been reported by other authors on the inhibitory effects of metals on algal growth capacity (Prasad and Prasad, 1982; Lasheen et al., 1989; Payne and Price, 1999; Nayar et al., 2003).

If we focus on our study on the diatom assemblages, it has been elsewhere described that metal affects not only specific growth rate but also structure of diatom assemblages (Foster, 1982; Ivorra et al., 2000; Sabater, 2000; Gold et al., 2003). Thus, Cd caused a general slow development of diatom cells in both EU2 and EU_3 contaminated systems (Figure 8.5). Low diatom densities were reported after 2 weeks of Cd exposure in EU_2. During the last four weeks, an increase in diatom densities occurred; however, densities remained lower than in the reference (EU_1) after 6 weeks of colonization, with communities characterized by a proliferation of *Nitzschia palea* (Figure 8.7). Specific growth rate perturbation was also observed within mature diatom communities after Cd exposure (EU_3). As reported by Gold et al (2003), metal contamination had strong effect on the density of diatom communities, possibly corresponding to a reduction in the rate of cell division of diatom species as demonstrated by Rivkin (1979). Inhibition of diatom growth accompanied by the development of a few species developed in contaminated system (EU_2) led to marked decrease in species richness and diversity index (Figure 8.6), which have been found in metal pollution rivers (Genter and Lehman, 2000; Sabater, 2000). Nevertheless, minor changes of diatom communities in EU3 system in species richness and diversity index were noticeable after Cd exposure (Figure 8.6), when the abundances of species previous developed before Cd exposure were still being presented (Figure 8.7). Mature diatom communities can therefore maintain a good diversity of diatom assemblages by limiting the Cd effects through the thickness of biofilm.

Throughout the experiment, Cd exposure seems to play a role in determining Cd-tolerant communities (Figure 8.7) with the development of more resistant species and exclusion of sensitive ones. The significant development of *Nitzschia palea* in EU2 under Cd exposure in the whole exposed assemblage seems to provide favor and tolerance of this species to Cd contamination. This coincides with the observations of Gold et al. 2003b in low Cd contamination, those obtained by Sabater (2000) in metal polluted studied site and during high Zn level contamination experiment (Loez et al., 1995; Medley and Clements, 1998). On the other hand, effects of metal stress on the composition of mature diatom assemblage in EU_3 system were less visible than those in EU_2 system and related to the role of the protective layer of the biofilm (Ivorra et al, 2000; Gold et al., 2003b, which protect species such as *Nitzschia dissipata,* being considered as sensitive to Cd still present within the developed communities after Cd contamination in EU_3. However, longer Cd exposure could lead to some delay modifications in the composition of diatom assemblages by reducing percentage of main sensitive species and

increasing the proportion of *Nitzschia acidoclinata* which might be resistant to metallic contaminant.

The influence of metal on diatom structure of the biofilm becomes much more evident when diatom deformities are discussed within communities. In our study, we observed the increase of abnormal valves frequencies (table 8.2) with duration time of Cd exposure in both systems EU_2 and EU_3 (after Cd contamination). The effects of metal stress caused morphological changes of diatom valves in our experiment, and are in agreement with some observations of several authors such as McFarland et al. (1997), Dickman (1998), Gold et al. (2003b) and Cattaneo et al. (2004), demonstrating that deformities of diatom valves could be good indicator in assessment of aquatic ecosystem's health. It has been suggested that abnormalities may resulted in the damage of normal membrane function reducing silicic uptake and amino acid synthesis caused by metal (Fisher and Jones, 1981). The occurrence of some deformities valves in reference system (EU_1) during the last week of the experiment is not in relation with the metal exposure, but could be caused by nutrients limitation (William et al., 1980) in the cultural medium conditions. Frustules malformations, commonly found in cultures (Estes and Dute, 1994; Bates, 1998) and the size reduction at each cell asexual division may lead nevertheless to alteration of the morphological characteristic of the frustules, but do not occur as often as in contaminated conditions.

In conclusion, the whole biofilm matrix and diatom assemblages structure are clearly affected by Cd contamination in *indoor* experimental conditions. A better knowledge of the multiple composition of the biofilm would bring new data and put forward new development taking into account the bacterial fraction as well as periphytic diatom assemblages versus their behaviors front of Cd contamination.

Acknowledgments:

The authors wish to thank Herry Bouillard and Bruno Etcheverria from GEMA for their technical assistance of the experiment system and Muriel Bonnet and Maryse Boudigues who carried out the nutrient analyses. This study was supported by grants from BDI fellowship (CNRS).

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General conclusions

Periphytic diatoms are an essential component of the biofilm that covers the upper surfaces of substrates in wetlands, streams and rivers. They integrate all of the influential biotic and abiotic parameters in their habitats and provide a continuous record of environmental quality. In the present study, periphytic diatom communities were examined in relation to their responses to short term or long term exposure to urban or metallic pollution and changes of environmental conditions. As it is clearly showed in the results presented in this thesis, periphytic diatom communities are valuable indicators for both organic and metallic pollution.

In the first part of this thesis, the dynamic of benthic diatom communities in the three rivers (Red-Nhue and Tolich in Vietnam) submitted to urban pollution were studied through various criteria such as diatom density, relative abundance of diatom species and diatom indices for the first time. They showed that effects of pollution on development of biofilm and structure of diatom communities appear as soon as the second week of colonization. Growth inhibition of both biofilm and diatom communities in Tolich River mainly related to an increase of organic load rather than low level of metal concentration in water. Communities exposed in the heavy and moderate polluted sites included species which are known as saprophylous taxa or tolerant to organic pollution. Our results support idea that water quality may be diagnosed in the environment after a short period of periphytic diatom exposition.

Looking at the relationship between diatom communities from Red - Nhue and Tolich Rivers and water quality by using multivariable analysis Canonical Correspondences Analysis (CCA), we have highlighted that diatoms composition was highly correlated with environmental variables. The Tolich River with high concentrations of ammonia and phosphates, TOC, TIC, BOD, COD and minimal dissolved oxygen level, was represented by cosmopolitant dominant polysaprobic taxa *Eolinima minima, Nitzschia umbonata, Nitzschia palea*. Changes in communities composition differed according to the gradient of pollution along the Nhue River. Downstream Nhue River, which is supposed to illustrate an increasing pollution, is characterized by resistant taxa like *Nitzschia palea, Aulacoseira granulate, Gomphonema parvulum*. And in Red River, major taxa found either sensitive to organic pollution such as *Encyonema leei, Achnanthidium minutissimum*, *Sellaphora rectangularis* or tolerant taxa to organic pollution such as *Cymbella tumida, Nitzschia recta* or halophylous taxa. Two diatom indices (IPS and DAIPo)

were successfully applied in Vietnam for the first time to estimate water quality of the three rivers. The results concluded that water was heavily polluted in Tolich River, polluted in Nhue River and moderately polluted in Red River. These indices still need to be enriched by new tropical taxa with probable endemic status often encounted and collected during experiements but never identified before. They are currently in the process of determination and of name assigment, and are preseted in plate 1a and b. Such quality indices can be proposed as appropriate and transferable tools to study ecological status rivers in Vietnam.

However, water quality is not always constant but may highly vary, depending on the ocurrence and extent of pollutants. Thus, responses of periphytic diatom communities to water quality changes have been investigated by transferring early diatom communities colonized on glass substrates from a non-polluted river to less polluted or polluted rivers and conversely. Responses and adaptability of periphytic diatom communities to environmental changes varied but greatly depended on sites environments. In transfer diatom communities, from comparatively unpolluted site to moderate site resistant replaced characteristic species of comparatively unpolluted site, while dominant taxa typical of this site considerably increased after transferring from heavy polluted site to comparatively unpolluted site. Transfer experiments have led to a replacement of species to adapt periphytic biofilm to new environmental condition after 2 to 4 weeks of exposition. These changes in taxonomic composition are recoded by the shifts in IPS and DAIPo indices, which prove to be sensitive to water quality changes. Recovery or replacement of diatoms communities biofilm highlights their potentialities to integrate environmental variation over long periods of time, and their possible role of implement to check both restoration and pollution status of aquatic system.

In the second part of this study, investigation were pursued in France and concened an hydrosystem mainly reported by its polymetallic pollution. Study conducted on diatoms communities in natural biofilm in the Riou-Mort watershed (South West France) which is affected by historic polymetallic pollution have contributed to increase the knowledge concerning the use of diatoms as indicator of metallic pollution. Relationships between cadmium accumulation levels in biofilms after a colonization period on artificial substrates (20 days), and the structural characteristics of periphytic diatom communities have been examined in the industrial basin of Decazeville (SW France). Cd accumulation and dynamics of benthic diatom communities along metallic pollution gradient in natural biofilms varied considerably according to sites and seasons. Diatom composition in polluted site was presented by small forms and resistant taxa. High correlation between some diatom species and Cd level in biofilm was

observed in two seasons. The frequency of diatom abnormal forms found in polluted site was related to the high level of metals accumulated in biofilm or to high metal concentrations in this area.

The effects of the industrial basin of Decazeville (SW France) were clearly seen with high concentration of metals Cd and Zn in both biofilms and suspended particulate matters and specific distribution patterns of diatoms as a consequence of metal contamination at Joanis station during a 1-year experiment conducted in the Riou-Mort watershed (South West France). Metal accumulation in the biofilm was significantly correlated with concentrations measured in suspended particulate matters. The results also suggested that calculating the total biovolume of the diatom community could serve as an additional criterion to assess metal pollution. Further, frequency of abnormal forms found in polluted sites was confirmed and supported by the theory mentioned above.

Toxicity and accumulation of cadmium in freshwater diatom biofilm studied in experiment channels were in adequacy with the results obtained from the field surveys. The results showed that Cd exposure promoted abnormal diatom valves development, diminished biofilm biomass as well as diatom densities. However, diatom communities increased their tolerance to Cd by significant development of tolerant species like *Nitzschia palea* which seem to increase their accumulation of Cd during exposure. Laboratory experiment attested the protective role of organic matrix constituting the periphytic diatom biofilm against toxicity of Cd which limits penetration of Cd into biofilm, and finally reinforces our opinion about diatoms as valuable indicator for assessing metal pollution.

The opportunity to work into different hydrosystems allowed us to confront and apply different methodologies to study the impact of pollutants on periphytic diatom communities in laboratory experiment and in outdoor *in situ* study. Both aspects are complementary and bring valuable information. Laboratory investigations can be of great interest in Vietnam to deepen urban pollution effects on diatoms and by taking into account new criteria as biovolumes, morphometry, and concentration of contaminant in organisms. Endemic species till need to be identified and further investigation is needed to increase our knowledge of the ecological requirement of these species by increasing the number of samples and sites from upstream to downstream of the whole river catchments. All these results may contribute to improve the use of diatom indices for water quality assessment in Vietnam.

In the complex natural system of industrial basin of Decazeville (SW France), sources of metallic pollution have been well documented. However, considerable nutrients levels found in this area could also simultaneously affect the distribution and structure of periphytic diatom communities. It can cause some difficulties to identify the influences of metal on distribution and structure of periphytic diatom communities. Because of this limitation, further experiments in microcosms with both metal and nutrients addition should be conducted to examine how metal and nutrient influence together and modify the composition of diatom communities within biofilm. In addition, experiment with monospecific diatom species isolated from field could be of valuable interest to compare of the effects of metal pollution on both monospecific and natural diatom biofilm. Moreover, further investigations should be performed to get additional information concerning the biovailability of the metal and sublethal effects on microorganisms communities.

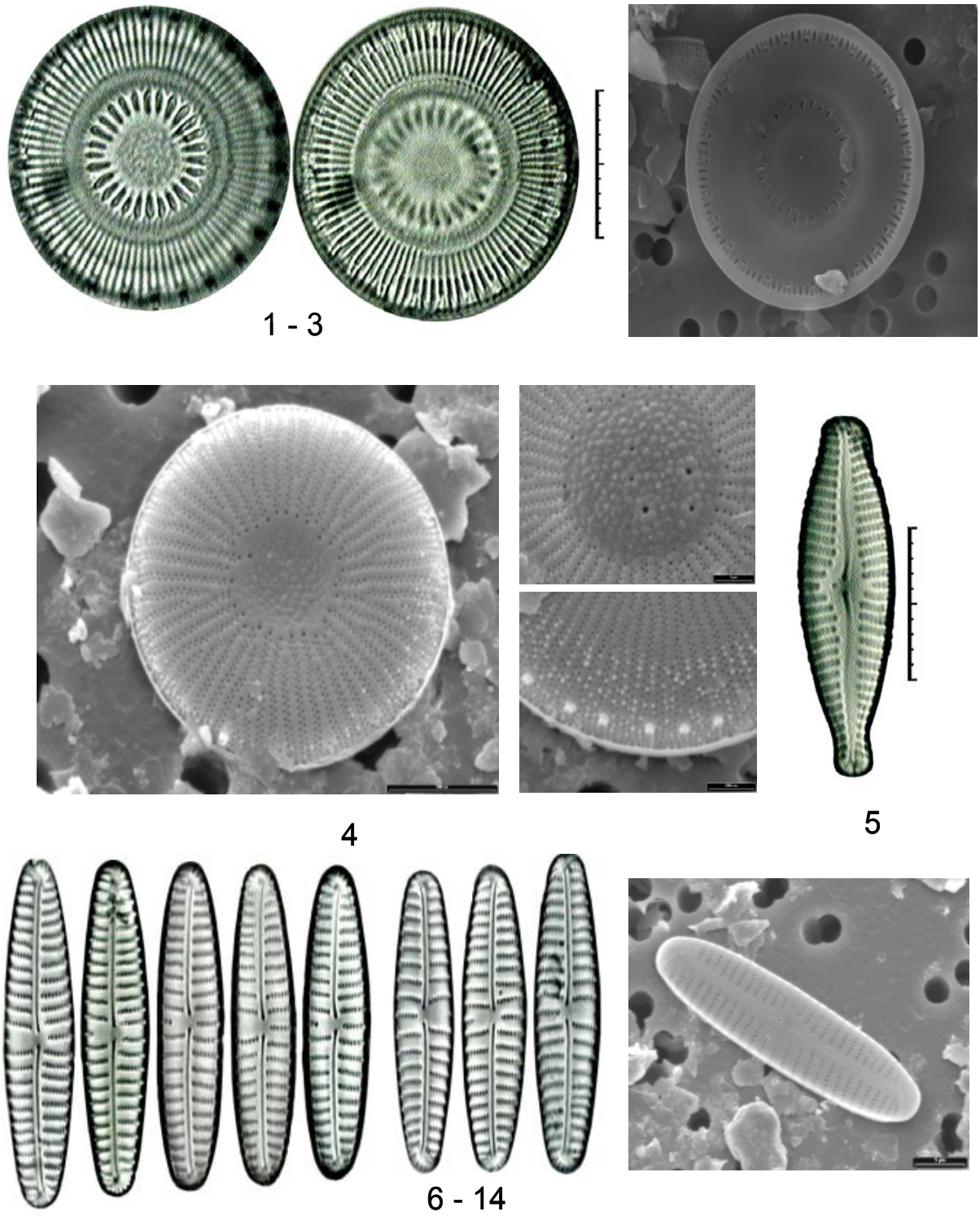

Plate 1a: Endemic diatom taxa from Red, Nhue and Tolich River. Fig *1-3: Cyclotella sterocostata* Lin Xie & Cai; Fig *4: Cyclotella fottii* Hustedt; Fig 5: *Gomphonema chubichuensis* Jüttner and Cox; Fig 6-14: *Encyonopsis leei* Krammer, Bar scale = 10µm

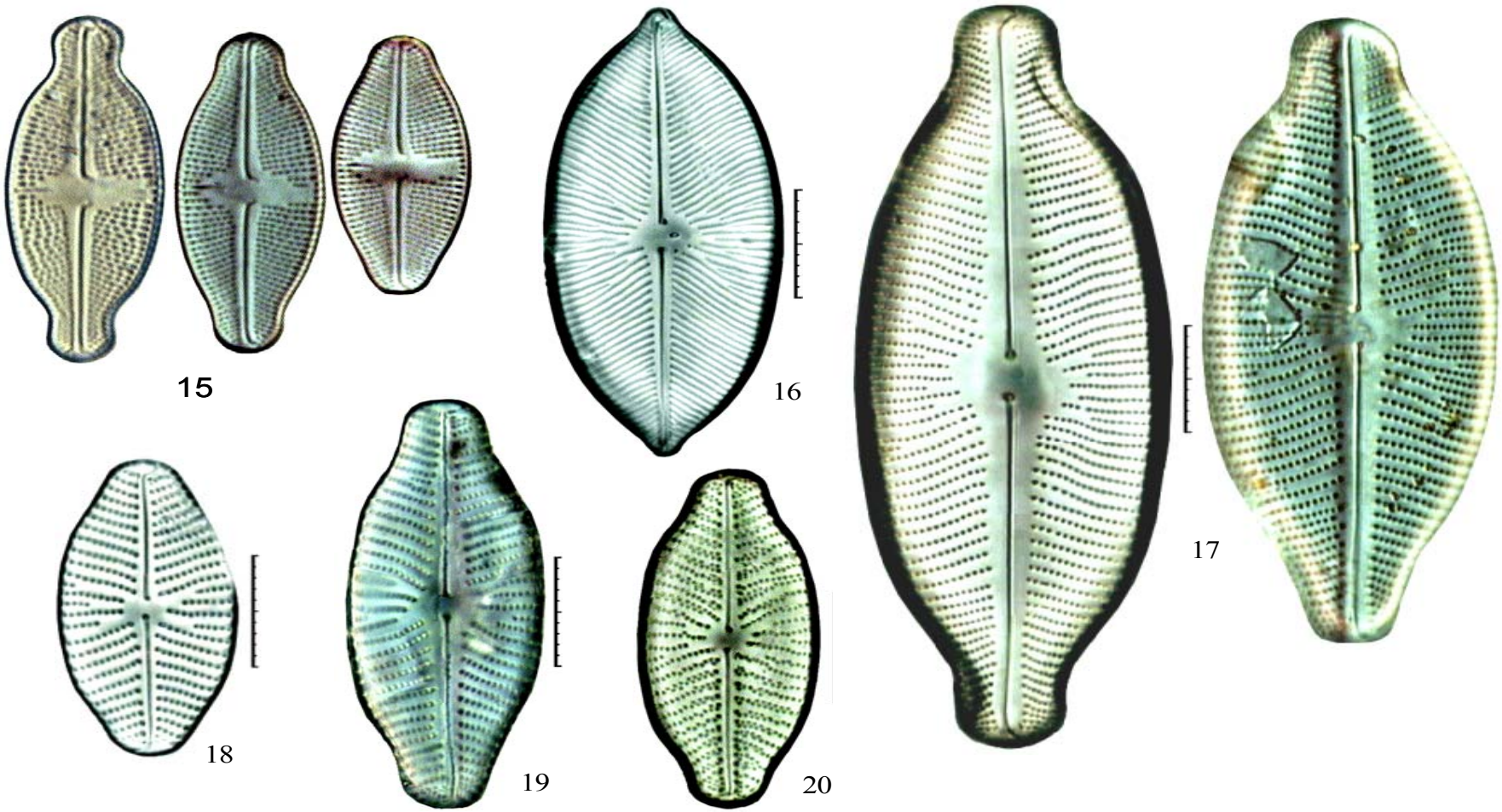

Plate 1b: Some unidentified diatom taxa (untill now) Fig 15*: Luticola aff seposita* and Fig 16 *Placoneis sp aff. diversipuntata*; Fig 17-20 *Placoneis plurisp.* from Red and Nhue Rivers bar scale =10µm

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ANNEX

ADDITIONAL PUBLICATIONS

ADDITIONAL PUBLICATIONS

Dynamics of benthic diatom colonization in a cadmium/zinc-polluted river (Riou-Mort, France)*

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Periphytic diatom communities were sampled from glass substrates immersed along a gradient of organic and metallic pollution. We investigated the influence of nutrients and a combination of nutrients and metals on biofilms and diatom communities settling on the glass over three weeks. Biofilm was characterized through organic biomass, chlorophyll *a* concentrations and metal content; structure of diatom assemblages was assessed by studying densities, mean biovolumes and taxonomic composition. Exposure to organic pollutants resulted in an increase of biomass (dry weight, chlorophyll *a* concentrations and diatom densities) and diatom community structure was similar to that at an unpolluted site relative to nutrient concentrations. *Cyclotella meneghiniana* was dominant and the species *Nitzschia palea*, *Navicula gregaria* and *Melosira varians* were well-represented. Downstream of the metal-contamination source, biofilm biomass, as well as chlorophyll *a* concentrations, decreased as cadmium and zinc content got higher (up to 60µgCd/g dry weight and 1400µgZn/g dry weight). Concurrently, the size distribution of diatoms, changing from larger to smaller individuals, reflected changes in the taxonomic composition of the assemblages where *Eolimna minima* was found in high proportions. Statistically significant amounts of abnormal frustules were also enumerated in the metal-polluted environment ($p<0.05$).

* Article is accepted by Archive für Hydrobiology (in press)

Anomalies morphologiques en conditions de stress métallique*

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La littérature évoque la possibilité d'utiliser les anomalies morphologiques des diatomées comme indicateur du niveau de contamination métallique. Dans le cadre du suivi des communautés périphytiques du bassin industriel de Decazeville, nous avons caractérisé et quantifié les formes anormales de diatomées, en relation avec l'exposition aux métaux. Les abondances relatives des formes anormales sont mieux corrélées aux concentrations en cadmium qu'en zinc, et traduisent plutôt l'exposition des communautés périphytiques aux métaux dosés dans le biofilm que dans l'eau.

* Article is accepted by Diatomania (In press)

Impact des pollutions urbaines de l'agglomération d'Hanoi sur les communautés de diatomée benthiques des rivieres Red, Nhue et Tolich (Vietnam)*

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Une étude préliminaire avait souligne l'intérêt floristique de l'hydrosystème nord du Vietnam. Il a paru souhaitable de poursuive des investigations sur la contamination essentiellement organique de ces milieux et d'en appréhender les principaux effets sur les communautés diatomique récoltée a l'aide de substrats artificiel (lame de verre) et naturels (macrophytes).

Un suivi portant sur 6 stations échelonnées sur les 3 cours d'eau portant sur une période de 4 mois a permis d'évaluer l'impact des la pollution essentiellement organiques. Une microflore plutôt halophile (*Nitzschia kurzii* Rabenhorst, *Entomoneis spp.*, *Seminavis strigosa* (Hustedt) Danielidis et Mann, Bacillaria paxillifer (O.F. Müller) Hendey) a été observée dans la rivière Red (secteur amont) bien que la conductivité des eaux ne dépasser pas 300 $\mu S.cm^{-1}$. La Tolich héberge une flore de milieux fortement pollués (*Nitzschia umbonata* (Herenbeg) Lange-Bertalot avec des teneurs en sel ammoniacaux élevés alors que la rivière Nhue présent une microflore intermediare avec une abondance du genre *Craticula* (*C. perrotettii* Grunow) et des formes planctoniques électives des milieux eutrophes (*Aulacoseira grannulata* (Ehrenberg) Simonsen ou plus ou moins endémiques *Cyclotella fortii* Hustedt et C. asterocostata Lin, Xie et Cai.

Un fonctionnement hydrographique complexe (systèmes d'écluses, crus susceptibles d'inverser le courant de la Tolich) et une forte turbidité des eaux ont rendu les approches quantitative difficiles en particulier les évaluations des densités et biodiversité. La confrontation des assemblages des lames de verre a ceux récoltes sur macrophytes confirme les mêmes tendances en ce qui concerne l'estimation de la qualité des eaux obtenue a l'aide de deux indices diatomiques (européen et japonais). Les cinétiques de colonisation avant et après translocation devraient compléter utilement ces premières observations et apporter des indications utiles sur la capacité de récupération de ces hydrosystème. Un essai d'estimation des pollutions métalliques a été tente en s'appuyant sur l'abondance de formes anomales plus nombreuses sur les secteurs aval.

* Communication at Actes du 23 ème Colloque de l'Association des Diatomites de Langue Française 13-16 Septembre 2004 Orléans.

Effets à court terme des paramètres physico-chimiques sur les assemblages de diatomées dans trois rivières du Vietnam parallèlement a une étude prolongée de leur cinétique de colonisation *

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Les effets à court terme de la pollution de l'eau on été testes sur trois rivières du Vietnam (le Red, la Nhue et la Tolich), en transférant des communautés de diatomées benthique sur substrats artificiels precolonisé d'un site très pollue (TL), a un site peu polluée puis de référence (Red) et vice versa. Le développement, ainsi que les changements de composition, de densité et de diversité spécifique après le transfert ont été mesurés.

Les densités se sont rapidement réduites de 10,995 ± 1379 à 2192 ± 123 cellules/cm^2 après transfert d'un site Red à un site TL et ce des la première semaine. A l'inverse, les densités son été trouvées 10 fois supérieures une semaine après le transfert d'un site à site Red.

Apres transfert, les communautés diatomiques se sont modifiées dans le sens des communautés locales. *Navicula recens* et *Bacillaria paxillifera* abondants dans le site R se sont raréfies lors du transfert des biofilm dans le site fortement polluée TL. A l'inverse les espèces caractéristique des sites fortement polluée comme *Nitzschia palea*, *Cyclotella meneghniana* ou *Nitzschia umbonata* sont devenues plus abondantes. De la même façon, les espèces très abondantes dans le site TL se raréfient rapidement après transfert dans un site Red alors que Navicula recens, Gyrosigma scalproides et *Bacillaria paxillifera* augmenttaient.

* Communication at Actes du 24 ème Colloque de l'Association des Diatomites de Langue Française 6-8 Septembre 2004 Bordeaux

Cadmium toxicity to diatom communities assessed in freshwater microcosm *

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In situ diatom surveys conducted on the river Riou-Mort, a cadmium and zinc-polluted tributary of the river Lot (South West France), have revealed shifts in biofilm settlement and diatom community structure. The present experimental study is aimed at determining dissolved cadmium toxicity to biofilms and diatom assemblages.

Diatom communities sampled from the field (at a clean site on the river Riou-Mort) were inoculated in experimental units consisting of replicate artificial streams, supplied with water contaminated by different levels of dissolved cadmium (0, 10 and 100µgCd/L), at a temperature of 15°C in order to prevent the development of filamentous algae. Glass substrates immersed in the artificial streams were sampled after a 1-, 2-, 4- and 6-week exposure. The response of biofilm characteristics to metal contamination was described through dry weight, ash-free dry matter, chlorophyll *a* and cadmium content. Diatom communities were assessed by quantitative countings and qualitative identifications down to the species level. Teratological valves of diatoms were also enumerated.

There was a positive correlation between cadmium accumulation in the biofilm and dissolved cadmium concentrations and duration of exposure. Biofilm settlement was affected by high cadmium concentrations. Dry weight and ash-free dry weight were similar in the biofilms grown under 0 and 10 µgCd/L, and were significantly higher than those measured in the samples exposed to a 100µg/L contamination. We also observed for all stages of settlement a reduction of diatom densities correlated to the highest cadmium contamination, compared to control units and low cadmium concentrations.

* Communication at 19th International Diatom Symposium Listwanka, Irkustk, Russia, 28 August-3 September, 2006.