Universidade de Aveiro Departamento de Biologia
Ano 2010

Joana Bastos Damásio

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e do Doutor Carlos Barata Martí, Investigador Titular do Consejo Superior de Investigaciones Científicas, Barcelona.

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À minha mãe

Ao meu pai (in memoriam)

o júri

presidente

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palavras-chave

biomarcadores, avaliação dos riscos ambientais, ecossistemas de água doce, invertebrados de água doce, mecanismos toxicológicos de acção, ensaios *in situ*

resumo

Um assunto que requer atenção é a avaliação ecológica da qualidade da água de ecossistemas de água doce. Uma abordagem que surge como promissora é a biomonitorização baseada em biomarcadores, porque pode avaliar a saúde dos organismos e obter sinais de alerta precoce acerca dos riscos ambientais. Até agora, porém, o uso de biomarcadores em espécies de invertebrados, para diagnosticar danos ecológicos nos rios, é escasso. Por essa razão, existe uma necessidade urgente de desenvolver biomarcadores nas principais espécies de macroinvertebrados dos ecossistemas fluviais que são alvo de estudo.

Esta tese tem como objectivo averiguar se as respostas *in situ*, aliadas aos biomarcadores, podem ser um método viável para avaliar os danos ecológicos de contaminantes em ecossistemas de água doce. Numa primeira fase, os biomarcadores foram usados para averiguar os mecanismos fisiológicos de adaptação genética de clones de *Daphnia magna* ao pesticida organofosforado fenitrothion.

Numa segunda fase, os biomarcadores foram usados como ferramentas de diagnóstico de poluição em zonas ribeirinhas. Estes estudos foram realizados com três espécies-chave de macroinvertebrados: *Daphnia magna*, *Corbicula fluminea* e *Hydropsyche exocellata*, nos rios Besós e Llobregat e no Delta do rio Ebro (NE Espanha). Além disso, foram realizados com animais capturados nos rios, ou com ensaios de transplantes, e foram complementados com índices biológicos de macroinvertebrados e análises químicas da água e dos animais. Como os contaminantes químicos têm vários modos toxicológicos de acção e, portanto, afectam várias respostas bioquímicas dos organismos, foram analisados nas três espécies um conjunto de biomarcadores pertencentes a diferentes vias metabólicas.

A abordagem experimental indica que o uso combinado de biomarcadores e outras medidas, tais como índices biológicos e testes *in situ*, contribui para diagnosticar os efeitos prejudiciais de contaminantes nas comunidades ribeirinhas.

keywords

biomarkers, environmental risk assessment, freshwater ecosystems, freshwater invertebrates, mechanisms of toxic action, *in situ* bioassays

abstract

An ecological assessment of water quality in freshwater ecosystems is an issue of major concern. A biomarker based biomonitoring presents a promising approach, because it can be used to assess the health status of organisms and to obtain early-warning signals of environmental risks. Until now, however, the use of biomarkers in invertebrate species to diagnose an ecological impairment in rivers is scarce. Therefore, there is an urgent need to develop biomarkers in key macroinvertebrate species within the river ecosystems that are the object of study.

This thesis aims to ascertaining if *in situ* responses and biomarkers can be a reliable method to diagnose the ecological impairment on freshwater systems due to contaminants. In a first stage, biomarkers were used to find out the physiological mechanisms of genetic adaptation to the organophosphate pesticide fenitrothion in *Daphnia magna* clones.

In a second stage, biomarkers were used as diagnostic tools of pollution in riparian habitats. The latter studies were carried out with three key macroinvertebrate species: *Daphnia magna*, *Corbicula fluminea* and *Hydropsyche exocellata*, in the Besós and Llobregat rivers and in the Ebro Delta (NE Spain). These studies were developed with field collected animals, or with transplants assays, and were complemented with macroinvertebrate biological indices and chemical analyses of river water and animals tissue. As chemical contaminants have multiple toxicological modes of action and hence affect many biochemical responses within the organisms, a battery of biomarkers belonging to different metabolic pathways was analyzed in the three species.

The experimental approach indicates that the combined use of biomarkers with different metrics, such as biological indices and *in situ* tests, improve substantially our ability to diagnose detrimental effects of pollutants in riparian communities.

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General Introduction

1. GENERAL INTRODUCTION

Into the scope

The environment is being continuously loaded with foreign organic chemicals (xenobiotics) released by urban communities and industries. Since the early sixties mankind has become aware of the potential long-term adverse effects of these chemicals in general and their potential risks for aquatic and terrestrial ecosystems in particular. The ultimate sink for many of these contaminants is the aquatic environment, either due to direct discharges or to hydrologic and atmospheric processes (Stegeman and Hahn, 1994).

Ecological assessment of water quality is fundamental to the management of surface waters and to the protection of freshwater ecosystems. The presence of a xenobiotic compound in a segment of an aquatic ecosystem does not indicate, by itself, injurious effects (to aquatic life). Connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects. Many of the compounds and their metabolites, which contaminate aquatic ecosystems, have yet to be identified and their impact on aquatic life has yet to be determined. Therefore, the exposure, fate and effects of chemical contaminants or pollutants in the aquatic ecosystem is an issue of major concern.

1.1. Environmental risk assessment (ERA)

A definition

Environmental risk assessment (ERA) is defined as the procedure by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies (Depledge and Fossi, 1994). ERA has become increasingly important since environmental scientists, as well as people in general, have learned that chemicals which are not toxic to humans can have deleterious effects on natural resources.

The risk assessment process can be divided into a scientifically oriented *risk analysis* and a more politically oriented *risk management*. Risk analysis is the process of estimating

the magnitude and probability of effects, whereas risk management examines solutions to the risks and deals with regulatory measures (Van Leeuwen and Hermens, 1995).

ERA, in its classic form, was mainly focused on the relationship between toxic substances in the environment and the potential hazards of these substances if they exceed certain threshold levels. However, in the last decades, an ERA based only on pollutant levels in the environment is not considered to be reliable. There is a growing awareness that risk assessment should focus on the *effects* of the total mixture of contaminants present in the environment, instead of focusing on chemical data alone (Calabrese, 1991). When a deleterious effect (on populations or ecosystems) becomes clear, the destructive process has often gone too far to be reversed by remedial actions or risk reduction.

A pollutant stress situation triggers a cascade of biological responses

The sequential order of responses to pollutant stress within a biological system, from the molecular to the ecosystem level, is visualized in Fig. 1.1 (adapted from Bayne et al., 1985). Such scenario has triggered the research to establish early-warning signals reflecting the adverse biological responses towards anthropogenic environmental toxins (Bucheli and Fent, 1995).

Effects at higher hierarchical levels are always preceded by *earlier* changes in biological processes, allowing the development of early-warning signals *(biomarkers)* of effects at *later* response levels (higher hierarchical levels) (Bayne et al., 1985).

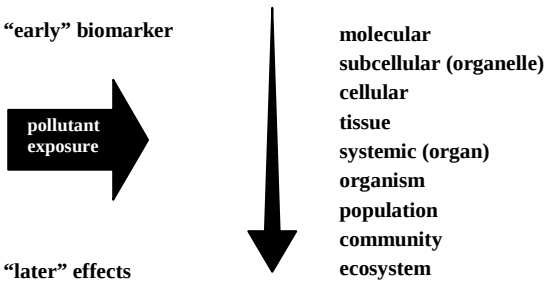

Fig. 1.1. Schematic representation of the sequential order of responses to pollutant stress within a biological system (adapted from Bayne et al., 1985).

1.2. Biomarkers

A definition

The biomarker concept was initially applied to a medical context in the early 70s and, in the early 90s, it became very appealing in environmental studies (McCarthy and Shugart, 1990; Walker, 1992; Depledge and Fossi, 1994; Peakall, 1994). Benford et al. (2000) reported a breadth of definitions; it is apparent from this breadth that any use of the term "biomarker" must also include a definition of what is meant within the context of a specific discussion.

The term "biomarker" is generally used in a broad sense to include almost any measurement that reflects an interaction between a biological system and a potential hazard, whether chemical, physical or biological (WHO, 1993).

One definition of the term can be a change in a biological response (biochemical, cellular, physiological or behavioural changes) that can be measured in tissue or body fluid samples, or at the level of whole organisms, to provide evidence of exposure and/or effects of contaminants (Depledge, 1994; Peakall, 1994).

The levels of "biological responses" that can be considered range from the molecular to community structure and even to the function and structure of ecosystems. While this reflects the fact that pollutants can exert their influence at all levels, it does result in a definition of biomarkers that is too broad; in this dissertation the focus is on *biochemical biomarkers*.

According to the National Research Council (NRC, 1987), World Health Organization (WHO, 1993), biomarkers can be subdivided into *three classes*:

. Biomarkers of *exposure*: covering the identification of an exogenous substance (or its metabolite), the product of an interaction between a xenobiotic and endogenous components (target molecule or cell), or other event within an organism related to exposure. They can be used to confirm and assess the exposure to a toxicant or other stressor.

. Biomarkers of *effect*: including measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognized as associated with an health impairment or disease. They can provide insights into both the causal factors of the

hazard and its ecological consequences and are associated with the toxicants' mechanisms of action.

. Biomarkers of *susceptibility*: indicating the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic, including genetic factors and changes in the receptors which alter the susceptibility of an organism to that exposure. They help to elucidate the variations in the degree of responses to a toxicant exposure observed between different individuals.

Nevertheless, the subdivision of biomarkers in the literature is rather diffuse and misleading; since all biomarkers are biomarkers of exposure and indeed all biomarkers demonstrate an effect of some sort or another (Peaker and Walker, 1994). The responses of biomarkers can be regarded as biological or biochemical effects after a certain toxicant exposure, which makes them theoretically useful as indicators of both exposure and effects (van der Oost et al., 2003).

1.3. Biomarkers overview

Animals have been faced with a continual input of potentially toxic compounds, so-called xenobiotics. Central to the defence against such an enormous and diverse number of xenobiotics there is an impressive array of enzymes and biotransformation pathways involved in the detoxication and removal of xenobiotics, but also those involved in the generation of molecular species, sometimes more toxic than the parent compound. So, the potential sources of toxic molecular species, derived either directly or indirectly from the presence of organic xenobiotics, are the parent compound itself, reactive metabolites and free radical derivatives of the compound and enhanced production of toxic reactive oxygen species (ROS) (Livingstone, 1991).

Biotransformation or metabolism

Biotransformation or metabolism is generally an enzyme-catalyzed conversion of a xenobiotic compound into a more water-soluble form, which can be excreted from the body more easily than its parent compound (Lech and Vodicnik, 1985).

However, the toxicity of a foreign compound may be affected by metabolism, which can be either beneficial (detoxication) or harmful (bioactivation) to an organism (van der

Oost et al., 2003). The second case is the case of the formation of reactive electrophilic intermediates that are more toxic than their parent compound (mainly the phase I biotransformations) and occur, for example, with organophosphorous (OPs) compounds.

The activity of biotransformation enzymes may be induced or inhibited upon exposure to xenobiotics (Bucheli and Fent, 1995). Enzyme induction is an increase in the amount or activity of these enzymes or both. It is generally assumed that *de novo* protein synthesis is the most important enzyme induction process (Stegeman and Hahn, 1994). Inhibition is the opposite of induction. In this case, enzymatic activity is blocked, possibly due to a strong binding or complex formation between the enzyme and the inhibitors (van der Oost et al., 2003).

Classically, xenobiotic biotransformation was earlier divided into two major types of enzymes: Phase I enzymes; Phase II enzymes and cofactors.

Phase I Biotransformation enzymes. The enzymes of phase I metabolism (oxidation, reduction, hydration, hydrolysis) introduce (or modify) a functional group (–OH, –COOH, –SH, $–NO_2$) into the xenobiotic, to which enzymes of phase II metabolism attach a large polar moiety (glutathione, sulphate, glucuronide, amino acid, etc.) (Livingstone, 1991).

Phase I reactions are catalyzed by microsomal monooxygenase (MO) enzymes, also known as the mixed-function oxidase (MFO) system (i.e. cytochrome P450 [cyt P450], cytochrome b5 [cyt b5], NADPH cytochrome P450 reductase [P450 RED]) (van der Oost et al., 2003). The MFO system is the responsible for the metabolic activation of OP compounds to its oxon metabolite, which is the effective toxic form that inhibits the enzyme acetylcholinestase, causing neurotoxic effects (Neal, 1980).

Phase II Biotransformation enzymes. The second phase of metabolism involves a conjugation of the xenobiotic, or its metabolites, with an endogenous ligand, often a more polar group, leading to the formation of less-hydrophobic compounds that are more easily excreted.

The phase II type enzymes *glutathione-S-transferases* (GSTs, EC 2.5.1.18) catalyse the conjugation of glutathione (GSH) with electrophilic substances. GSTs play a critical role in the cellular defence against oxidative damage and peroxidative products of DNA and lipids (van der Oost et al., 2003).

Recently, it has been realized that two additional steps, called Phase 0 and Phase III, are just important as the previously known processes. These phases involve the modulation of the cellular *entry* and *exit*, respectively, of either the unmodified or metabolized compounds, and are carried out by multiple membrane transporters (Szakács et al., 2008). One group of these transporters is involved in the so-called mechanism of multixenobiotic resistance (MXR). MXR is a cellular defence strategy for the protection of the organisms from both endogenous and xenobiotic chemicals, by recognizing and removing them from the cell, thus preventing their accumulation and cytotoxic effects (van der Oost et al., 2003; Szakács et al., 2008). MXR is equivalent to the multidrug resistance (MDR) phenomenon that was first observed in cancer cells (resistant to anti-cancer drugs).

Furthermore, the determination of xenobiotic biotransformation products in fish bile is considered to be a biomarker of Phase III metabolism. E.g. levels of biliary polycyclic aromatic hydrocarbons (PAHs) metabolites are sensitive biomarkers to assess recent exposure of fish to PAHs (van der Oost et al., 2003).

Oxidative stress

Oxidative stress is defined as injurious effects due to cytotoxic reactive oxygen species (ROS), also referred to as reactive oxygen intermediates (ROIs), oxygen free radicals or oxyradicals (Di Giulio et al., 1989). These reduction products of molecular oxygen (O_2) include the superoxide anion radical (O_2^- •), hydrogen peroxide (H_2O_2) and the (highly reactive) hydroxyl radical (•OH). These are extremely potent oxidants that are capable of reacting with critical cellular macromolecules, possibly leading to enzyme inactivation, lipid peroxidation, DNA damage, protein damage and, ultimately, cell death (Winston and Di Giulio, 1991).

Possible anthropogenic-related sources of enhanced ROS and other pro-oxidant free radical production include organic contaminants such as redox cycling compounds (e.g. quinones, nitroaromatics, nitroamines), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins; metals; air contaminants (NO_2, O_3, SO_2); peroxides; UV-radiation; hypoxia and hyperoxia (Di Giulio et al., 1995; Halliwell and Gutteridge, 1999). The xenobiotics that act as redox cycling compounds take part in one-electron transfer reactions, resulting in the generation of the superoxide anion radical (O_2^- •) via so called redox cycle. The O_2^- • may be converted, by various processes, to

other oxyradicals, including H_2O_2, and •OH (Livingstone, 1991). Referring to metals, of particular interest are transition metals like iron (Fe), copper (Cu), chromium (Cr) and vanadium (V) that through the Fenton reaction are able to facilitate the conversion of superoxide anion (O_2^- •) to the highly reactive hydroxyl radical (•OH). Other metals including aluminium (Al), arsenic (As), cadmium (Cd), nickel (Ni), lead (Pb) and mercury (Hg) may also produce oxidative stress indirectly, depleting antioxidant defences (glutathione (GSH) levels) or via metal-induced displacement of redox metal ions (Stohs and Bagghi, 1995).

Defence systems exist in organisms to prevent or reduce the formation of ROS (and organic free radicals), including low molecular weight free radical scavengers (such as reduced glutathione (GSH)) and antioxidant enzymes (Fig. 1.2). The latter include superoxide dismutase (SOD), catalase (CAT), glutathione-dependent peroxidase (GPX) and glutathione reductase (GR) (Livingstone, 2003). A failure of antioxidant defences to detoxify excess ROS production can lead to significant oxidative damage to key cellular molecules, possibly leading to enzyme inactivation, protein damage, DNA damage, lipid peroxidation and, ultimately, cell death (Halliwell and Gutteridge, 1999). Therefore, antioxidant enzymes can be classified not only as biomarkers of exposure, but also as indicators of toxicity. An induction traduces an adaptative reaction to a perturbed environment, on the other hand, an inhibition is predictive of cell damage and reflects the toxicity of bioavailable pollution in a dose-dependent manner (Vasseur and Cossu-Leguille, 2003).

Superoxide dismutase (SOD, EC 1.15.1.1) is a group of metalloenzymes that catalyses the conversion of reactive superoxide anions (O_2^- •) to yield hydrogen peroxide (H_2O_2), which, in itself, is an important ROS as well. H_2O_2 is subsequently detoxified by two types of enzymes: catalase (CAT) and glutathione dependent peroxidase (GPX). Most techniques for the measurement of SOD activity are indirect assays in which an indicating scavenger (cytochrome C) competes with endogenous SOD for $O_2^{-\bullet}$. A unit of SOD activity is defined as the amount that causes 50% inhibition in the reduction of the scavenger under specified conditions (Stegeman et al., 1992).

Catalase (CAT, EC 1.11.1.6) is an hematin-containing enzyme that facilitates the removal of hydrogen peroxide (H_2O_2), which is metabolized to molecular oxygen (O_2) and water

(H_2O). Unlike some peroxidases, that can reduce various lipid peroxides as well as H_2O_2, CAT can only reduce H_2O_2 (Stegeman et al., 1992).

Glutathione peroxidase (GPX, EC 1.11.1.9) detoxifies hydrogen peroxide (H_2O_2) or organic hydroperoxides (produced, e.g., by lipid peroxidation), employing GSH as a cofactor (which is reduced to its oxidized form (GSSG)).
In animals, the principal peroxidase is a selenium (Se) -dependent tetrameric enzyme that catalyses the metabolism of H_2O_2 to H_2O. The other peroxidase reduces organic hydroperoxides to their corresponding alcohols (i.e., ROOH→ROH); this is considered an important mechanism for protecting membranes from damage due to lipid peroxidation.
Glutathione-S-transferase (GST) enzymes can also employ GSH in the reduction of a broad range of organic hydroperoxides, but they cannot reduce H_2O_2. This peroxidatic activity by GST is referred to as "selenium (Se) -independent peroxidase", although GST is not a true peroxidase. However, GST can apparently serve a significant peroxidatic function, particularly in Se-restricted animals (Stegeman et al., 1992).

Glutathione reductase (GR, EC 1.6.4.2) Although perhaps not involved in antioxidant defence in the same way as the enzymes previously described, GR merits attention because of its importance in maintaining GSH/GSSG homeostasis under oxidative stress conditions (Winston and Di Giulio, 1991). GR catalyses the transformation of the oxidized disulfide form of glutathione (GSSG) to the reduced form (GSH), with the concomitant oxidation of NADPH to $NADP^+$. Altered GR may have a relation with altered ratio GSH/GSSG (mentioned below).

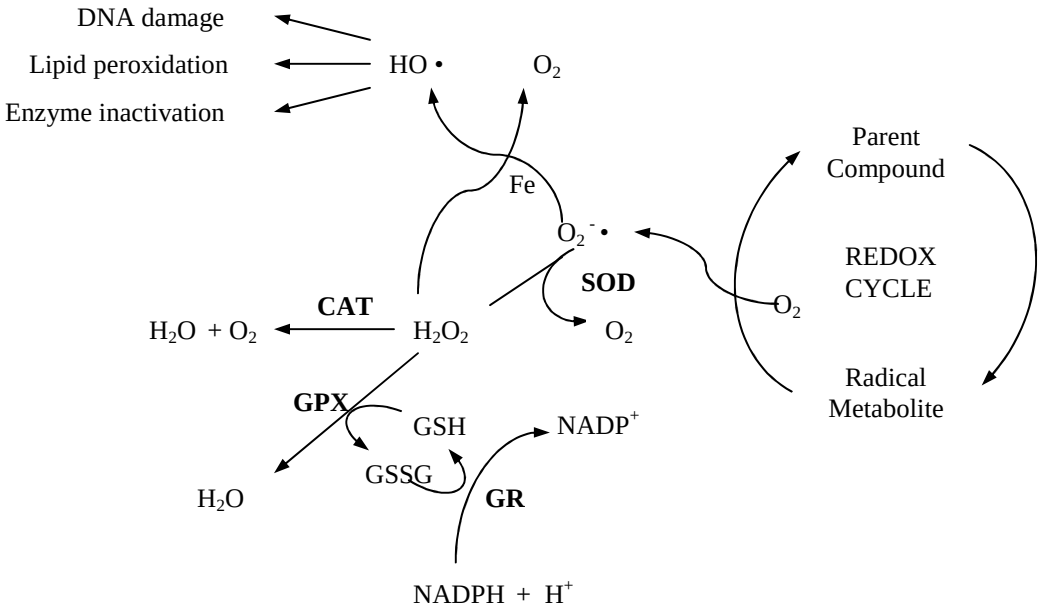

Fig. 1.2. Schematic representation of the detoxification of reactive oxygen species by antioxidant enzymes (adapted from Stegeman et al., 1992). Abbreviations are explained in the text.

Reduced glutathione (GSH)

Reduced glutathione (GSH), a tripeptide consisting of *g*-glutamine, cysteine and glycine, has two roles in detoxifications: as a conjugate of electrophilic intermediates, principally via GST activities (in Phase II metabolism) and as an important antioxidant (Stegeman et al., 1992). In the second case, GSH is consumed due to the direct scavenging of oxyradicals or as a cofactor for glutathione peroxidase (GPX) activity, that involves an oxidation of reduced GSH to its oxidized form (GSSG). So, an alteration in the ratio of reduced and oxidized glutathione (GSH/GSSG), also called redox-status, is an indice of oxidative damage (van der Oost et al., 2003).

To maintain the reduction potential of the cell, GSSG is reduced back to GSH by glutathione reductase (GR) or exported from the cell, like some GSH conjugates, via multidrug resistance associated proteins (Halliwell and Gutteridge, 1999). Another enzyme responsible for maintaining the GSH pool is γ-glutamylcysteine synthetase (γ-GCS, EC 6.3.2.2), the rate-limiting enzyme in the novo synthesis of GSH. Indeed, it is known that redox cycling chemicals can increase or decrease the intracellular GSH pool by inducing conjugation, oxidation or by affecting GR and γ-GCS enzymes (Shi et al., 1994; Brouwner and Brouwner, 1998; Stephensen et al., 2002). Therefore, if intracellular GSH becomes

limiting, competition for available GSH may modulate the activity of GPX and GST enzymes (Brouwner and Brouwner, 1998; Halliwell and Gutteridge, 1999).

Biochemical indices of oxidative stress

Biochemical effects / consequences have been associated with oxidative stress, as lipid peroxidation, DNA damage, protein damage and perturbed redox status.

Lipid peroxidation (LPO). The process of LPO proceeds by a chain reaction and, as in the case of redox cycling, demonstrates the ability of a single radical species to propagate a number of deleterious biochemical reactions (van der Oost et al., 2003). Oxidative damage may be caused directly by peroxides or from more reactive and toxic breakdown products of peroxides such as epoxides, ketones and aldehydes, the most important of which is malondialdehyde (MDA) (Leibovitz and Siegel, 1980). LPO can lead to impaired cellular function and alterations in physicochemical properties of cell membranes, which in turn disrupt vital functions (Rikans and Hornbrook, 1997).

DNA damage. Exposure to xenobiotics may induce structural alterations in DNA, DNA damage and subsequent expression in mutant gene products and diseases. A general and sensitive approach to identify damage involves the detection of DNA strand breaks, that are produced, either directly by the toxic chemical (or its metabolite) or by the processing of structural damage (Shugart et al., 1992a).

Protein damage. Proteins can become modified by a large number of reactions involving ROS. Among these reactions, carbonylation has attracted a great deal of attention due to its irreversible and unrepairable nature. In the process of protein carbonylation, carbonyl groups (CO) are introduced into the side chains of specific amino acids in the active center of the protein, triggering the initial steps in the degradation of the enzyme (Nyström, 2005).

Oxyradical-generating compounds can influence the *redox status* (GSH/GSSG) of cells by imposing a drain on intracellular reducing equivalents, potentially affecting a variety of metabolic processes (mentioned above) (Stegeman et al., 1992).

Neuromuscular parameters

B-esterases are a large group of serine hydrolases that include cholinesterases (ChEs) and carboxylesterases (CbEs) (van der Oost et al., 2005). With respect to neural functions,

enzymes of interest are cholinesterases (ChEs). Several types of ChEs are recognized: firstly (the most important ones) those with a high affinity for acetylcholin (Acetylcholinesterase, AChE); lastly those with affinity for butyrylcholin (Butyrylcholinesterase, BChE) or propionylcholin (Propionylcholinesterase, PrChE), also known as non-specific esterases or pseudocholinesterases (Walker and Thompson, 1991; Sturm et al., 2000).

AChE (EC 3.1.1.7) is responsible for hydrolysing the neurotransmitter acetylcholine into choline and acetic acid, preventing accumulation of acetylcholine (and consequently a prolonged electrical activity) at nerve endings, which is vital for normal functioning of sensory and neuromuscular systems (O'Brien, 1960; van der Oost et al., 2003). Organophosphorous (OPs) and carbamates pesticides inhibit AChE activity by binding to its active site, phosphorylating the enzyme and, consequently, causing neurotoxic effects (O'Brien, 1960).

CbE (EC 3.1.1.1) are important detoxifying enzymes (Yuan and Chambers, 1996) that act as alternative targets for OP inhibition by removing a significant amount of the activated metabolite (oxon) before reaching the main target site, and hence, protecting AChE against poisoning (Chambers and Chambers, 1990; Chambers et al., 1990).

Apart from B-esterases, the A-esterases also are involved in the organism defensive mechanism against natural and synthetic esthers such as OPs. A-esterases are capable of hydrolysing OP/oxons and not being inhibited by them (Aldridge, 1953). Nevertheless, unlike mammals, most vertebrate and arthropod species have little or no A-esterase activity (Hemingway and Karunarate, 1998) and they seem to have affinity only to few OP/oxons compounds (Chambers et al., 1994).

Metabolic state of the organism

Lactate dehydrogenase (LDH, EC 1.1.1.27) catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. It sustains the continued process of glycolysis under conditions of hypoxia (anaerobic metabolism), being particularly important when a considerable amount of additional energy is rapidly required (Diamantino et al., 2001), therefore it can be used to assess the metabolic state of the organism (Moreira et al., 2006).

1.4. Biomarkers in environmental risk assessment

It is virtually impossible to monitor all the contaminants which form a potential threat to the environment. In order to assess the overall quality of the aquatic environment, a biomarker based biomonitoring seems to be a promising approach (McCarthy and Shugart, 1990).

Biomarkers can be used to assess the health status of organisms and to obtain *early-warning* signals of environmental risks (van der Oost et al., 2005). Since many of the biomarkers are short-term indicators of long-term adverse effects, these data may permit intervention before irreversible detrimental effects become inevitable (McCarthy and Shugart, 1990). This early warning can be either a response earlier in time, since the effects at lower levels of biological organization occur more rapidly than those at higher levels, or it can be a response at lower exposure levels than classic toxic endpoints (such as lethality, reproduction or growth impairment).

By screening multiple biomarker responses, important information may be obtained about organism *toxicant exposure* and stress. Biomarkers can provide evidence that organisms have been exposed to toxicants at levels that exceed normal detoxification and repair capacity and that have induced responses within molecular and cellular targets (McCarthy and Shugart, 1990). Also, they can help to understand the toxic action of chemicals, their metabolites and complex mixtures of chemicals.

One of the most compelling reasons for using biomarkers is that they can give information on the *biological effects* of pollutants rather than a mere quantification of their environmental levels (Shugart et al., 1992b)

The use of biomonitoring methods has *advantages over chemical monitoring* (van der Oost et al., 2005). Firstly, these methods measure effects in which the bioavailability of the compounds of interest is integrated with the concentration of the compounds and their intrinsic toxicity. Secondly, most biological measurements form the only way of integrating the effects into a large number of individual and interactive processes. Another limitation of chemical monitoring is that it does not account for the effect of chemical specification in the environment, kinetics and sorption of chemicals to sediment, accumulation through food chains and modes of toxic action which are not readily measured as short-term (7-21 day) effects on survival, growth or reproduction. Moreover,

biomarkers can provide evidence of exposure to compounds that do not bioaccumulate or are rapidly metabolized and eliminated; in many cases the metabolites are more toxic than their parent compound (McCarthy and Shugart, 1990).

Furthermore, biomarker measurements integrate the toxic exposure from different *routes of exposure* (water, food, sediment) over time and geographically over the spatial range of the sentinel species. The former can be particularly useful in the case of variations in contaminant concentrations over time (e.g. resulting from intermittent releases of effluents) that cannot be accounted without repeated chemical analysis. Referring to the latter, mobile organisms integrate the exposure over their spatial range, and sessile animals, such as clams or mussels, provide a good spatial resolution, and may aid in the identification of "hot spots" of contamination. So, the selection of mobile or sessile species as test organisms will depend on the objectives of the study (Shugart et al., 1992b).

Since biomarkers can be applied both in the laboratory and in the field, they may provide an important linkage between laboratory toxicity and field assessment. For field samples, biomarkers can provide an integrated measure of the total contaminants that are biologically available in a real exposure scenario (Shugart et al., 1992b; van der Oost et al., 2005).

1.4.1. Studies on aquatic invertebrates

The historical development of the biomarker approach had a strong link with medicine and vertebrate biology (NRC, 1987), thus most of the field studies and applications in the aquatic environment have been focused on fish. However, biomarker measurements are equally feasible on aquatic invertebrates.

Aquatic invertebrates are commonly used in biological monitoring programs, but their use in biomarker studies has been limited. For example, the number of scientific publications (WOK, ISI Web of Knowledge, www.isiknowledge.com) published annually, since 1980, containing the keyword "biomarker", "environment" and "aquatic or marine or water" is of about 3561. From these ones, 1600 were about fish and 1041 publications included the term "invertebrate or Daphnia or insect or crustacean or amphipod or Chironomus or mussel or clam or oyster". It's worth noting that less than 400 were conducted in species different than bivalves. This means that, despite the large variability

of filogenetically and hence physiologically distinct groups of aquatic invertebrates, only a few of them have been considered in biomarker studies.

Aquatic invertebrates offer distinct advantages for biomonitoring, including (a) their ubiquitous occurrence; (b) their huge species richness, which offers a spectrum of environmental responses; (c) their basic sedentary nature, which facilitates spatial analysis of pollution effects; (d) the long life cycles of some species, which can be used to trace pollution effects over longer periods; (e) their compatibility with inexpensive sampling equipment; (f) the well described taxonomy for genera and families; (g) the sensitivities of many common species, which have been established for different types of pollution; and (h) the suitability of many species for experimental studies of pollution effects (Bonada et al., 2006).

In biomarker studies, aquatic invertebrates have further advantages. As invertebrate populations are often numerous, samples can readily be taken for analyses without a significant impact on the population dynamics. Also, the application of biomarkers in invertebrate species allows the linkage between biomarkers responses and adverse effects on populations and communities. Currently, increasing knowledge of the biochemistry and physiology of invertebrates permits a reasonable interpretation of biomarker responses (Deplege and Fossi, 1994; van der Oost et al., 2005).

1.4.2. Studies on freshwater benthic invertebrates

The benthic invertebrate community is considered an appropriate endpoint because it is highly susceptible and has a scale appropriate to the site. The high susceptibility is due to the association of these organisms with the sediment, which is the repository of most of the contaminants. In addition, the organisms that dominate this community are sensitive to a variety of contaminants. Because these organisms are sedentary, the scale of the community is highly appropriate to the scale of the site (Jones et al., 1999).

Traditionally, biomonitoring of freshwaters has been based on benthic macroinvertebrates using indexes to quantify changes in community composition (Rosenberg and Resh, 1993). These indexes are based on pollution tolerance of macroinvertebrate families and involve identification of macroinvertebrates to the family level and calculation of scores. Although this is a robust, simple, and rapid method of

bioassessment, it is unspecific for diagnostic purposes of specific pollutants. Furthermore, these indexes can respond to disturbances other than those they were developed to detect, making diagnosis more difficult. Macroinvertebrate indexes also may lack precision because identification of macroinvertebrates to family level only could decrease sensitivity and increase failure to detect impacts. Furthermore, community-level effect measures are insensitive to sublethal levels of stress (Maltby, 1999), so impacts from low levels of contaminants may be missed. Additionally, these indexes depend on the type of river sampled, the method of sampling, type of marginal area, and the individual carrying out the sampling (Armitage et. al, 1983). Because of the lack of standardization, results within and between sites may vary considerably, making the scoring method inconsistent when used to assess impacts of contamination on macroinvertebrate communities (McWilliam and Baird, 2002a).

Biomarker studies in macroinvertebrate species used to determine biological indexes of river water quality are scarce and, for some species, inexistent, but they seem to be appropriate to detect specific effects of environmental contaminants and, therefore, significant for the environmental risk assessment process.

1.4.3. Biomarkers to study mechanisms of toxic action

It is known that different species exhibit different levels of tolerance to xenobiotics. In some cases, this may be due to differences in the xenobiotic metabolism or detoxification process. Biotransformation reactions can have a significant effect on the *in vivo* disposition as well as the toxicity of the xenobiotics, therefore, their characterization is an important step toward the evaluation of the potential risks to the wild populations in a field situation (Escartín and Porte, 1996).

Differences in the biotransformation and detoxification processes can be reflected in varying abilities of the species to survive in highly polluted environments (Lagadic et al., 1994). This leads us to the problem of the development of resistance to pesticides.

Over 600 species of arthropods have developed resistance to all major classes of pesticides to which they are repeatedly exposed (Lagadic et al., 1994). According to the National Research Council (NRC, 1987) classification of biomarkers, resistance represents a marker of susceptibility to the effects of a toxicant exposure. The development of

resistance depends on the genetic variability already present in a population or arising during the period of the selection.

Biomarker studies related to the target of the pesticide and its mechanisms of toxic action offer the possibility of detecting the initial stages of resistance in a population and the mechanisms involved in the development of resistance (Lagadic et al., 1994; Hyne and Maher, 2003). This is important to understand the capability of wild populations to adapt and hence to avoid extinction under long term exposure to pesticides.

1.4.4. Biomarkers in In situ bioassays

In situ bioassays have certain advantages over more traditional methods of water-quality assessment, such as whole effluent toxicity tests and biological surveys of the benthic macroinvertebrate community. Key advantages include the integration of physical, chemical, and biological processes, which cannot be reproduced in the laboratory, into the test, and the elimination of artefacts associated with laboratory testing, such as the collection and storage of samples (Chappie and Burton, 1997), in other words, their greater relevance to the natural situation, especially with respect to the contamination scenario. Additionally, in situ bioassays can also give a rapid indication of water quality, because effects measured at the individual level will often be manifested more rapidly (hours to days) than the resulting changes in community structure (months to years) measured during macroinvertebrate sampling (Maltby, 1994).

An *In situ* bioassay could be accomplished in two ways: the use of a suitable species already existing in the habitat to be examined or the exposure of experimental animals to the area to be evaluated. The earlier case, that is to say, the use of local / autochthonous species, may be limited to the availability of animals of the same species across reference and contaminated sites and may be subject to large variations, due to seasonal and inter population variability across sites. For example, animals of different ages or at different stages of gonadal maturity may show different biological responses and pollutant accumulation patterns (Viarengo et al., 2007).

However, the use of caged organisms of the same population across reference and contaminated sites allow minimizing inter-population variability (Crane et al., 1996; Maltby et al., 2000). Using caged organisms in biomonitoring studies makes it easier to

standardize the results and to compare control organisms to animals collected from potentially polluted sites (Viarengo et al., 2007).

Caged bioassays can be performed either with animals reared in the laboratory, like *Daphnia magna*, or collected in the field in a clean site. The need for ecologically relevant methods in environmental risk assessment has led to the development of new bioassays with caged keystone species, to determine pollutant effects in situ. Namely, the effects on survival, on growth, on post-exposure feeding inhibition and on biochemical responses (Barata et al., 2007 ; Faria et al., 2006, 2007; Lopes et al., 2007; McWilliam and Baird, 2002a, 2002b ; Moreira-Santos et al., 2005; Pereira et al., 1999, 2000; Pestana et al., 2009).

1.5. Study sites

The studies within this thesis were conducted in the Llobregat river, the Besós river and the Ebro Delta (NE Spain).

Llobregat and Besós rivers

The Llobregat and Besós river basins belong to the Spanish region of Catalonia (NE Spain). Their characteristics are common to other Mediterranean rivers. They are relatively small, with a variable water flow, being low in Summer and maximum in Spring and Autumn. Their waters are over exploited by the agricultural, industrial and domestic activities of many urban vicinity areas, including Barcelona city, thus it is not surprising to find strong anthropogenic impacts on these river communities (Prat and Munné, 2000). In many occasions, the water flow is very low or even null and these rivers are transformed in urban and industrial effluents with a very poor water quality (Prat and Munné, 2000).

To improve water quality, many waste water treatment plants (WWTP) have been implemented in these rivers, more than 70 in both Llobregat and Besós basins in 20 years (Prat and Rieradevall, 2006). However, these measures may be insufficient for the recovery of stream communities if the stream lacks a natural flow, due to the climate or the high demand of the water resources, as most of the water carried by these streams is the effluent from WWTP and few or any dilution from the natural stream exists (Prat and Munné, 2000). In this situation (Mediterranean rivers) water quality is poor, and the measures that are effective for wet countries, such as the building of WWTP, fail to recover river water biological quality (Prat and Munné, 2000).

It's worth noting that small rivers, like these ones, are ecologically important systems that provide diverse habitats for aquatic biota, however, because of the above stated, like their physical size and often relatively low flows, they are particularly susceptible to human pressure and activities.

Ebro Delta

Ebro Delta is located at the end of the largest river in Spain, the Ebro river. The Ebro Delta (NE Spain) is a 320 km^2 wetland area of international importance for conservation. It holds the ''Reserva del Delta del Ebro'' (areas protected for their plants and animal species by Directive 79/409 of the European Commission) and natural aquatic resources. The Ebro Delta is of capital importance for fish and mussel production and waterbird feeding and reproduction.

However, the Ebro Delta is not only devoted to nature conservation. This area supports a well developed rice farming activity, with 21600 ha of rice fields producing 113500 tons of rice per year (Mañosa et al, 2001). The rice fields and the remaining natural areas are also the final recipients of most of the pollutants produced in agricultural activities, or in the well developed industrial Ebro basin. Thus the Ebro Delta receives human generated pollutants by two main different ways. First, the river carries industrial and agricultural wastes, which end up on the delta or into the sea. Secondly, the crops in the delta receive a large annual input of pesticides to fight agricultural pests. All these chemicals may accumulate on water, sediments, soils, plants and animals, may have direct toxicological effects in some non-target organisms, or may produce a reduction of food supply or refuge for others (Cooper, 1991; Mañosa et al., 2001).

1.6. Study species

The studies within this thesis were conducted with the species: *Daphnia magna*, *Hydropsyche exocellata* and *Corbicula fluminea*.

Daphnia magna Strauss, 1820 (Crustacea, Cladocera), commonly known as water flea, is among the most sensitive studied organism to toxic chemicals. It is widely used in aquatic risk assessment and it is broadly distributed within freshwater habitats (Baird and Barata, 1998). It is of easy culture in laboratory, producing a high number of individuals in

a relatively short time. It can reproduce asexually by ameiotic parthenogenesis (giving rise to a population of only females), thus genetic and environmental factors affecting tolerance to toxic chemicals can be easily separated (Baird and Barata, 1998), being also suitable for studies on biochemical mechanisms. It's a grazer and an important component of the zooplankton, which controls the phytoplankton biomass and species composition, and also serves as prey items for predatory zooplankton. Thus, it provides an important link between different trophic levels in freshwater communities (Mc willliam and Baird, 2002a).

Hydropsyche exocellata Dufour, 1841 (Insecta, Trichoptera), commonly known as caddisfly, is widely distributed in freshwater ecosystems, both in pristine and with anthropogenic pressure, being particularly resistant to pollution (Bonada et al., 2004). This specie has a variable life cycle with two or several generations per year (Bonada, 2003) and therefore specimens from the last instar can be found throughout the year. Almost all part of its life cycle (about one year) is in the form of benthic larva that lives in water attached to stones at the bottom of the river, constructs cases with substrate materials and feeds collecting algae and suspended particles, thus having a key role transferring energy from producers to invertebrate and vertebrate predators (Barata et al 2005; Resh, 1992). The adult stage (winged) lasts only few weeks and feed very little, thus essentially all the nutrients required for pupation and reproduction must be accumulated by the larva (Hyne and Maher, 2003). Caddisfly larvae have been regarded as an appropriate group to assess water quality (Resh, 1992) and feature an important role in the list of the species used for the macroinvertebrate biotic indices.

Corbicula fluminea Müller, 1774 (Mollusca, Bivalvia) commonly known as Asiatic clam, is originate from Asia and is now widely distributed in American and European freshwater habitats, being first recorded in the Iberian Peninsula in 1980 (Pérez-Quintero, 2008). Its invasive success relies on its natural characteristics like rapid growth, earlier sexual maturation (within the first three to six months), short life span (ranging from one to five years), high fecundity, extensive dispersal capacities and its association with human activities (Sousa et al., 2008). This specie is also useful as a sentinel specie for environmental risk assessment (Doherty, 1990; Vidal et al., 2002) due to several characteristics. Namely, it's a major component of benthic communities and is now widely

distributed; it may be found in pristine and polluted environments; it may be maintained in the laboratory and may be transplanted into the field using caging procedures; it has a great filtration capacity allowing the uptake of large amounts of pollutants; the size of the adults makes possible the dissection and separation of their main organs for posterior analysis (Sousa et al., 2008).

1.7. Thesis aims and structure

The main aim of this thesis is ascertaining if *in situ* responses and biomarkers can be a reliable method to diagnose ecological impairment on freshwater systems due to contaminants. To address this, the thesis is structured in seven chapters. In this first chapter, the basic principles and concepts supporting this work are drawn. In chapters two to six the work is detailed in the form of five Manuscripts that were published or will be later on submitted to relevant SCI journals. Finally, the major achievements of the thesis are drawn in the Concluding Remarks chapter (chapter 7). Thus, the thesis includes studies that use biomarkers for the assessment of exposure and ecological effects of environmental contaminants to various aquatic key macroinvertebrate species.

It follows a brief explanation of the steps taken and the rationale.

Chapter 2 – Biochemical mechanisms of resistance in *Daphnia magna* exposed to the insecticide fenitrothion.

Organophosphorous (OPs) insecticides have been widely used in agriculture, though they are toxic to non-target organisms at low concentrations and also non-target organisms living near areas sprayed with OPs have shown a development of resistance to these compounds. The objective of this chapter was to investigate the underlying biochemical mechanisms involved in the development of resistance to OPs in non-target aquatic invertebrates, since it is crucial to understand the capability of wild populations to adapt and hence to avoid extinction under long term exposure to OPs. To achieve our goal, toxicity bioassays and biochemical responses of *Daphnia magna* clones with different sensibilities to the OP fenitrothion were accessed.

Chapter 3 – Combined use of *Daphnia magna* in situ bioassays, biomarkers and biological indices to diagnose and identify environmental pressures on invertebrate communities in two Mediterranean urbanized and industrialized rivers (NE Spain).

A major challenge in environmental risk assessment is to identify indicators which can be used to diagnose the causal agents of adverse change. Traditionally, biomonitoring of freshwaters has been based on biological indices of benthic macroinvertebrates. The objective of this chapter was to address if the use of toxicological and biochemical responses may complement the existing water quality monitoring procedures (biological indices) in identifying the causal agents affecting aquatic invertebrates exposed to multiple environmental factors. Furthermore, the usefulness of in situ bioassays was assessed. To achieve our goal, in situ and biochemical responses of *Daphnia magna* transplanted along two Mediterranean rivers were assessed and compared with biological indices.

Chapter 4 – Identifying major pesticides affecting bivalve species exposed to agricultural pollution using multi-biomarker and multivariate methods.

The exposure of non-target organisms to pollutants associated to agriculture is a major concern and makes necessary the developing of effective monitoring tools. Moreover, in real field situations, aquatic organisms are currently being exposed to multiple chemical and environmental stressors with different mechanisms of toxicity, each contributing to a final overall adverse effect. For that reason, there is a need to identify the contaminants that might be hazardous in the field. The objective of this chapter was to identify the major pesticides that may cause detrimental effects for bivalve species affected by agricultural pollution. To achieve our goal, biochemical responses in *Corbicula fluminea* transplanted in drainage channels of an agricultural field were assessed and complemented with chemical analyses of river water and animals tissue.

Chapter 5 – Evaluation of side -effects of glyphosate mediated control of giant reed (*Arundo donax*) on the structure and function of a nearby Mediterranean river ecosystem.

Glyphosate is a broad-spectrum herbicide that has been used to control a wide range of weeds and invasive species. The objective of this chapter was to evaluate the effect of the application of glyphosate to control giant reed (*Arundo donax*) in a river restoration project. Specifically, the objective was to evaluate the effects on the structure and function

of a nearby river ecosystem. To achieve our goal, in situ and biochemical responses of transplanted *Daphnia magna* and biochemical responses of field collected *Hydropsyche exocellata* were assessed and complemented with biological indices and glyphosate environmental fate in the surrounding water.

Chapter 6 – Biochemical responses of benthic macroinvertebrate species as a tool to diagnose the water quality of polluted rivers.

Biological indexes of benthic macroinvertebrate species are currently used world wide to measure river water quality with ecological criteria. These indexes assign a global ecological status of the biotic community, but, can they detect specific effects of water pollutants? Can they detect small changes in water quality? The objective of this chapter was to answer these questions and to address if biochemical responses may complement the existing water quality monitoring procedures. To achieve our goal, biochemical responses in a keystone macroinvertebrate benthic species, *Hydropsyche exocellata*, were assessed and compared with biological indexes across a polluted gradient in an industrialized Mediterranean river.

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Chapter 2

Biochemical mechanisms of resistance in *Daphnia magna* exposed to the insecticide fenitrothion

2. Biochemical mechanisms of resistance in *Daphnia magna* exposed to the insecticide fenitrothion

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ABSTRACT

Resistance to fenitrothion and enzyme activities associated with the toxicity and metabolism of organophosphorous insecticides were measured in three genetically unique *Daphnia magna* clones collected from rice fields of Delta del Ebro (NE Spain) during the growing season and a lab sensitive clone. The studied clones showed up to six fold differences in resistance to fenitrothion. The lack of correlation between in vitro sensitivity of acetylcholinesterase (AChE) to fenitrooxon and resistance to fenitrothion indicated that insensitivity of AChE to the most active oxon metabolite was not involved in the observed differences in resistance. Inhibition of mixed- function oxidases (MFOs) by piperonyl butoxide (PBO) increased the tolerance to fenitrothion by almost 20-fold in all clones without altering their relative ranking of resistance. Conversely, when exposed to fenitrooxon, the studied clones showed similar levels of tolerance, thus indicating that clonal differences in the conversion of fenitrothion to fenitrooxon by MFOs were involved in the observed resistance patterns. Despite that resistant clones showed over 1.5 higher activities of carboxilesterase (CbE) than sensitive ones, toxicity tests with 2-(O-cresyl)-4H-1,3,2-benzodioxaphosphorin-2 oxide, which is a specific inhibitor of these enzymes, evidenced that this system only contributed marginally to the observed clonal differences in tolerance. Glutathione-S-transferases activity (GST) varied across clones but not under exposure to fenitrothion, and was only related with tolerance levels in the field clones. In summary, our results indicate that MFO mediated differences on the bio-activation of the phosphorotionate OP pesticide to its active oxon metabolite contributed mostly in explaining the observed moderate levels of resistance, whereas the activities of CbE and GST had only a marginal role.

KEYWORDS: Resistance, Insecticide, Daphnia, Metabolism, Fenitrothion

INTRODUCTION

Organophosphorous insecticides (OPs) have been widely used in agriculture. Their initial success was mainly based on their high toxicity, high biological specificity and rapid environmental degradation (Eto, 1974). Unfortunately, their high biochemical specificity has resulted in the development of resistance in many insect and mite pests (Roush and Mckenzie, 1987; Taylor and Feyereisen, 1996). Furthermore, most OPs are also toxic to non-target organisms (Day and Scott, 1990) and some appear to have relatively high environmental persistence (Eto, 1974). Thus, populations of non-target organisms living near areas sprayed with OPs could become adapted by developing increased resistance to these compounds. Investigating the underlying biochemical mechanisms involved in the acquisition of resistance to OPs in non-target organisms is crucial to understand the capability of wild populations to adapt and hence to avoid extinction under long term exposure to OPs.

The present work focuses on *Daphnia magna* clones sampled from Ebro Delta (NE Spain), an area devoted to intensive agricultural activities, principally rice culture, that poses important environmental threats derived from the extensive use of pesticides such as the insecticide fenitrothion (Escartín and Porte, 1996). Therefore, the responses to fenitrothion of these *D. magna* clones can be a model system to study the biochemical processes involved in the development of population resistance of non-target aquatic invertebrates to pesticides, as reported in a previous study (Barata et al., 2001).

D. magna is very sensitive to OPs (Guilhermino et al., 1996; Barata et al., 2001, 2004), and inhabits small waterbodies in and around Delta del Ebro agricultural fields receiving OP treatments (Menéndez and Comín, 1986). Moreover, *D. magna* can reproduce asexually by ameiotic parthenogenesis, thus genetic and environmental factors affecting tolerance to organophosphorus insecticides can be easily separated (Baird and Barata, 1998) and hence the biochemical processes responsible for differences in tolerance among *Daphnia* populations may give insight into evolutionary responses.

Organophosphorous insecticides inhibit type ‘‘B’’ esterases, including cholinesterases (ChEs) and carboxylesterases (CbE), by binding to the active site and phosphorylating the enzyme (Escartín and Porte, 1996; Barata et al., 2004). Fenitrothion like other phosphorothionate compounds, must be metabolically activated to their oxon

metabolites to be effective inhibitors of acetylcholinesterase (AChE) and then to cause neurotoxic effects due to the accumulation of the neurotransmitter acetylcholine in cholinergic synapses of both vertebrates and invertebrate organisms. This bio-activation is performed mostly by the mixed-function oxygenase (MFO) system, a large cytochrome P450 isoenzyme superfamily that catalyzes both activation (desulfuration) and detoxication (dearylation) reactions (Neal, 1980). In *Daphnia*, fenitrothion is primarily metabolised through oxidation of P=S to P=O to form fenitrooxon, and dearylated to form 3-methyl-4-nitrophenol. Demethylation of fenitrothion and its oxon probably by GST and conjugation of liberated phenolic groups with sulfate have also been described (Takimoto et al., 1987). These metabolites are less toxic than the parental insecticide with exception of the oxon metabolite, which is much more toxic (Forsyth and Chambers, 1989). Oxon metabolites are able to irreversibly inhibit AChE, leading to enhanced levels of the neurotransmitter acetylcholine and subsequent disruption of nervous function, a situation that may lead to death of the organism (Chambers and Carr, 1995).

Additionally, a number of A and B esterases are able to hydrolyze and sequestrate the oxon metabolites, respectively (Aldridge, 1953). Nevertheless, unlike mammals, most vertebrate and arthropod species have little or no A esterase activity (Hemingway and Karunaratne, 1998). In arthropods, B esterases such as CbEs are important detoxifying enzymes (Yuan and Chambers, 1996) that act as alternative targets for OP inhibition by removing a significant amount of the activated metabolite (oxon) before reaching the main target site, and hence, protecting AChE against poisoning (Chambers and Chambers, 1990; Chambers et al., 1990). More specifically, Barata et al. (2004) studying *D. magna* responses to malathion and chlorpyriophos co-administered with specific inhibitors of CbEs, reported that inhibition of these enzymes by 80–90% increased the toxicity of these OPs by twofold.

In fish and mammals, differences in resistance to OPs have been related to differences in AChE and CbE sensitivity, MFO mediated transformation and A-esterase-mediated hydrolysis (Chambers and Carr, 1995). In arthropod pest organisms (including insect and mite species) resistance to OPs is usually related to three biochemical mechanisms: decrease uptake or/and increased levels MFO mediated detoxication enzymes (Morton, 1993; Taylor and Feyereisen, 1996), mutations that decrease the sensitivity of the AChE (Roush and McKenzie, 1987; Morton, 1993) or enhanced production of detoxication

esterases, such as CbE and glutathione transferases (Morton, 1993). In contrast, there are few studies about the biochemical mechanisms involved in resistance to OPs in aquatic invertebrates. Barata et al. (2001) reported that *D. magna* clones from an area exposed to OPs were able to develop resistance to ethyl-parathion, and that the most likely explanation for that was clonal differences in the OP metabolism and/or detoxification mechanisms rather than differences in AChE sensitivities.

The aim of this study was to investigate whether genetically- based differences in sensitivity to fenitrothion among *D. magna* clones were due to differences in (i) acetylcholinesterase activities or sensitivities, (ii) the protective role of CbEs, (iii) the rate of fenitrothion metabolism. These objectives were addressed by: (1) determining and hence selecting *D. magna* field clones with different tolerances to fenitrothion; (2) assessing biochemical and lethal responses after in vitro and in vivo exposures to fenitrothion and to its oxon metabolite in the absence and presence of the specific inhibitor of CbEs, 2-(*O*-cresyl)-4H-1,3,2-benzodioxaphosphorin- 2 oxide (CBDP) and of the MFOs specific inhibitor piperonyl butoxide (PBO). Toxicity bioassays with and without specific inhibitors coupled with enzyme assays were used to achieve our goals since according to previous authors this is the best and cost effective approach to study the physiological mechanisms of tolerance to OP insecticides (Yuan and Chambers, 1996; Ahmed andWilkins, 2002).

MATERIAL AND METHODS

Chemicals

Chemicals used for toxicity bioassays were fenitrooxon (97% purity) from Dr. Ehrenstorfer GmbH (Augsburg, Germany); CBDP (97% purity) kindly provided by OksanaLockridge (Nebraska Medical Center, Omaha, NE); fenitrothion (99% purity) and PBO (99% purity) from Aldrich Chemicals (Gillingham, UK). Acetylthiocoline iodine, 5,5- dithiobis-2-nitrobezoic acid (DTNB), a-naphtyl acetate, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), bovine serum albumin and Bradford reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental organisms and culture conditions

One laboratory clone of *D. magna* Straus (Barata et al., 2004), designated as S1 and three field clones designated as S2, R1 and R2 were chosen based on their acute sensitivity

to fenitrothion. Field clones were selected as the most sensitive (S2) and resistant (R1, R2) clones among 60 clones originated from resting eggs collected from rice field sediments of Delta del Ebro River (NE Spain), an area regularly exposed to fenitrothion and other organophosphate pesticide runoffs (Escartín and Porte, 1996). The field clones can be considered genetically different since originated from different resting eggs, which in *D. magna* are produced by sexual reproduction (Barata et al., 2000). Although the exact origin of the selected laboratory clone is not known, previous studies indicate that this clone can be considered sensitive to OPs (Barata et al., 2004). Thus differences in resistance to fenitrothion among these field and laboratory clones can inform us about how much genetic variation in tolerance exists among *D. magna* populations.

For each clone, two 1 l bulk cultures of five animals were maintained in ASTM hard synthetic water (APHA-AWWA- WEF, 1995) as described by Barata et al. (2004). Animals were fed daily with *Chorella vulgaris* Beijerinck (1×10^6 cells ml $^{-1}$; corresponding to 3.6 mg Carbon l^{-1}). The culture medium was changed every other day and neonates were removed within 24 h. To ensure that clonal differences in resistance were not due to physiological acclimation, field clones were acclimated to the standard laboratory conditions for more than 10 generations. To minimize maternal effects, all experiments started with $\leq$ 24 h – old neonates, originated from third to sixth brood females (Barata et al., 2004).

Toxicity bioassays

Exposures to fenitrothion and its oxon were conducted by using three types of bioassays:

(1) Acute toxicity bioassays, with and without specific inhibitors for CbE (CBDP) and for MFO (PBO) to allow assessing the role that CbE and MFO play in determining sensitivity to fenitrothion. Lethal responses were obtained after 48 h of exposure of 20 neonates in 200 ml ASTM hard water to 4–5 concentrations of fenitrothion or fenitrooxon in the absence of food. The degree of synergism of the CbE inhibitor and the degree of antagonism of the MFO inhibitor (i.e. the LC_{50} ratio of fenitrothion alone versus fenitrothion co-administered with CbE or MFO inhibitors, respectively) were investigated by exposing *D. magna* clones to fenitrothion with the highest dose of synergist/antagonist that had no significant effect on AChE activity in control

treatments (7 µg l^{-1} for CBDP, 400 µg l^{-1} for PBO). Additionally, the studied clones were exposed to fenitrooxon with and without PBO and CBDP to address if the observed differences in tolerance were due to genetic differences in MFO bio-activation metabolism of the parental insecticide and/or CbE mediated oxon detoxification. At the end of experiments, alive and immobile animals were counted to determine lethal concentration effects.

(2) In vivo AChE, CbE and GST assays. Enzymatic responses were obtained after 24 h exposure of 30 neonates in 150 ml ASTM hard water to fenitrothion with and without CbE inhibitors (CBDP at 7 µg l^{-1}), in the absence of food. Clones were exposed to about ½ of their LC_{50} for fenitrothion, corresponding to 0.25 µg l^{-1} for S1 and S2 clones, and 2 µg l^{-1} for R1 and R2 clones. At the end of experiments, alive animals were pooled in an eppendorf and immediately frozen at -80 ºC until further enzyme analysis.

In all bioassays, acetone (HPLC grade <1 ml l^{-1}) was used as a carrier. Preliminary experiments showed that acetone at this concentration did not affect survival neither B-esterase activity (Barata et al., 2004). Experiments were performed in triplicate following established OECD protocols (Barata et al., 2001).

(3) In vitro AChE inhibition assays with fenitrooxon. Inhibition responses, expressed as IC_{50}, were obtained after 90 min incubation, at 20 ºC, of *Daphnia* homogenates to 4 concentrations of fenitrooxon. Neonates used were isolated as described in Barata et al. (2001) and to each homogenate sample (30 neonates/500 µl buffer) was added 7 µl of fenitrooxon, or 7 µl of ethanol for controls. The activity of AChE was determined immediately after the end of the incubation period.

Enzyme preparation

Neonates were homogenized in ice-cold 100 mM phosphate buffer (30 neonates/500 µl buffer), pH 7.4, containing 100 mM KCl and 1 mM EDTA. Homogenates were centrifuged at 10000*g* for 10 min and the supernatants were immediately used as enzyme sources for AChE, CbE and GST assays.

Enzyme assays

Biochemical measurements were carried out on Cecil 9200 (Cecil instruments, Cambridge, England) and Bio-Tek EL312e microplate reader (Bio-Tek instruments, Vermont, USA) spectrophotometers. AChE was determined by a modification of the Ellman method (Ellman et al., 1961) adapted to microplate (Barata et al., 2001). AChE activity was measured in the presence of 0.33 mM acetylthiocholine and 0.17 mM DTNB, and the increase of absorbance was measured at 405 nm. A previous study showed that the contribution of other esterases on the hydrolysis of acetylthiocholine was negligible, thus all enzymatic activity will be referred to AChE (Barata et al., 2001). CbE activity was measured by the UV method of Mastropaolo and Yourno (1981) in the presence of 0.25 mM anaphtyl acetate, and the formation of naphthol monitored by the increase in absorbance at 235 nm. GST activity towards CDNB was measured as described by Habig et al. (1974). The reaction contained 100 mM phosphate buffer (pH 7.4), 1 mM CDNB and 1 mM GSH. The formation of *S*-(2,4-dinitrophenyl)–glutathione conjugate was evaluated by monitoring the increase in absorbance at 340 nm. Homogenate proteins were measured by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Data analysis

Lethality responses in acute exposures to fenitrothion and in vitro AChE sensitivity to fenitrooxon were expressed as 48 h-LC_{50} and IC_{50} values, respectively, and determined from probit analysis (Finney, 1971). Prior to analysis, absolute AChE rates were converted to proportions relative to controls. ANOVA based procedures (*t*-tests, one way ANOVA and ANCOVA) were used to compare enzymatic responses of the studied clones and when required data were log transformed to meet ANOVA assumptions of normality and variance homocedasticity (Zar, 1996).

RESULTS

Acute toxicity assays

Results depicted in Table 2.1 denoted over sixfold differences in tolerance to fenitrothion between resistant (R2) and sensitive (S2) clones (RR values). Co-administration of specific inhibitors of CbE and MFOs altered substantially the toxicity of fenitrothion but only affected marginally the resistance range of the studied clones. More specifically, CbE inhibition increased fenitrothion toxicity (SR in Table 2.1) from 1.6 to 1.8 times in S1, S2 and R1 clones and 2.6 times in clone R2, thus diminishing to fourfold the degree of resistance between R2 and S2 (RR in Table 2.1). On the contrary, PBO decreased fenitrothion sensitivity by 16.5–19.9-fold (AR in Table 2.1) with apparently no effects on the degree of resistance among clones (RR in Table 2.1). Under exposure to fenitrooxon with or without PBO, the four studied clones showed equivalent levels of resistance (RR, AR in Table 2.2). Co-administration of a specific inhibitor of CbE (CBDP) altered substantially the toxicity of fenitrooxon by twofold (SR ratio in Table 2.2) but did not affect the resistance range of the studied clones.

Table 2.1. Toxicity of fenitrothion with and without CBDP (CbE inhibitor) and PBO (MFO inhibitor) to *D. magna* clones

Population	Fenitrothion			Fenitrothion + CBDP				Fenitrothion + PBO			
	LC_{50} (95% CI)	Slope (SE)	RR[a]	LC_{50} (95% CI)	Slope (SE)	SR[b]	RR[a]	LC_{50} (95% CI)	Slope (SE)	AR[c]	RR[a]
S1	0.93 (0.27-2.00)	5.28 (1.65)	1.27	0.53 (0.41-0.68)	7.73 (0.39)	1.77	1.18	18.56 (9.04-38.23)	3.41 (0.13)	19.87	1.36
S2	0.73 (0.34-1.58)	5.41 (0.70)	1.00	0.44 (0.29-0.66)	6.99 (1.07)	1.66	1.00	13.68 (5.12-37.44)	3.60 (0.35)	18.69	1.00
R1	4.82 (3.46-6.71)	6.95 (0.44)	6.59	2.94 (1.62-5.35)	4.54 (0.44)	1.64	6.66	83.47 (37.21-160.00)	4.90 (0.68)	17.30	6.10
R2	4.98 (3.10-7.97)	5.97 (0.59)	6.81	1.94 (1.52-2.47)	8.01 (0.38)	2.56	4.41	82.11 (42.11-160.00)	4.45 (0.20)	16.47	6.00

LC_{50} values in $\mu g\ l^{-1}$ and reported as 48h-LC_{50}.
[a] RR, resistance ratio: LC_{50}/LC_{50} of the most sensitive clone.
[b] SR, synergism ratio: LC_{50} of fenitrothion alone/LC_{50} of fenitrothion+CBDP.
[c] AR, antagonism ratio: LC_{50} of fenitrothion+PBO/LC_{50} of fenitrothion alone.

Table 2.2. Toxicity of fenitrooxon with and without CBDP (CbE inhibitor) and PBO (MFO inhibitor) to *D. magna* clones

Population	Fenitrooxon			Fenitrooxon+ CBDP				Fenitrooxon + PBO			
	LC_{50} (95% CI)	Slope (SE)	RR[a]	LC_{50} (95% CI)	Slope (SE)	SR[b]	RR[a]	LC_{50} (95% CI)	Slope (SE)	AR[b]	RR[a]
S1	0.74 (0.41-1.32)	5.35 (0.58)	1.01	0.41 (0.22-0.76)	5.74 (0.72)	1.80	1.14	0.73 (0.50-1.07)	5.40 (0.30)	0.98	1.03
S2	0.74 (0.41-1.32)	5.35 (0.58)	1.01	0.36 (0.17-0.74)	5.38 (0.83)	2.05	1.00	0.73 (0.50-1.07)	5.40 (0.30)	0.98	1.03
R1	0.73 (0.22-2.00)	5.38 (1.74)	1.00	0.41 (0.15-1.00)	5.69 (1.91)	1.78	1.14	0.71 (0.43-1.16)	5.49 (0.51)	0.97	1.00
R2	1.13 0.40-2.00)	7.73 (0.64)	1.55	0.52 (0.21-0.78)	5.70 (1.31)	2.26	1.39	1.13 (0.40-2.00)	7.73 (0.64)	0.00	1.60

LC_{50} values in $\mu g\ l^{-1}$ and reported as 48h-LC_{50}.
SR, synergism ratio: LC_{50} of fenitrooxon + CBDP/LC_{50} of fenitrooxon alone.
[a] RR, resistance ratio: LC_{50}/LC_{50} of the most sensitive clone (R1).
[b] AR, antagonism ratio: LC_{50} of fenitrooxon+PBO/LC_{50} of fenitrooxon alone.

In vitro incubations

Enzymatic assays denoted that basal AChE activities for clones S1, S2, R1 and R2 (Table 2.3) were no significantly different ($P < 0.05$, $F_{3,43} = 2.88$) and showed only marginal differences in sensitivity to in vitro incubations to fenitrooxon (Fig. 2.1, Table 2.4). Despite of showing similar slopes (Fig. 2.1, ANCOVA analyses, $F_{3,24} = 0.28$), AChE activities of the sensitive clone (S1) were less sensitive than those of resistant clones (R1, R2; based on ANCOVA analyses on elevations, $F_{3,27} = 3.25$; Fig. 2.1). Additionally, clones R1 and R2 presented significantly ($P < 0.05$, $F_{3,44} = 5.6$) higher CbE activities than clones S1 and S2 (Table 2.3).

Table 2.3. AChE and CbE activities of *D. magna* clones in control treatments (basal activity). S.E.M. are shown in parentheses

Population	AChE (mol.min^{-1}.mg^{-1} prot)	CbE (mol.min^{-1}.mg^{-1} prot)
S1	5.04 (0.51)	190.45 (6.90)
S2	5.26 (0.23)	186.21 (6.75)
R1	5.58 (0.26)	235.13 (19.94)
R2	5.12 (0.35)	242.61 (14.18)

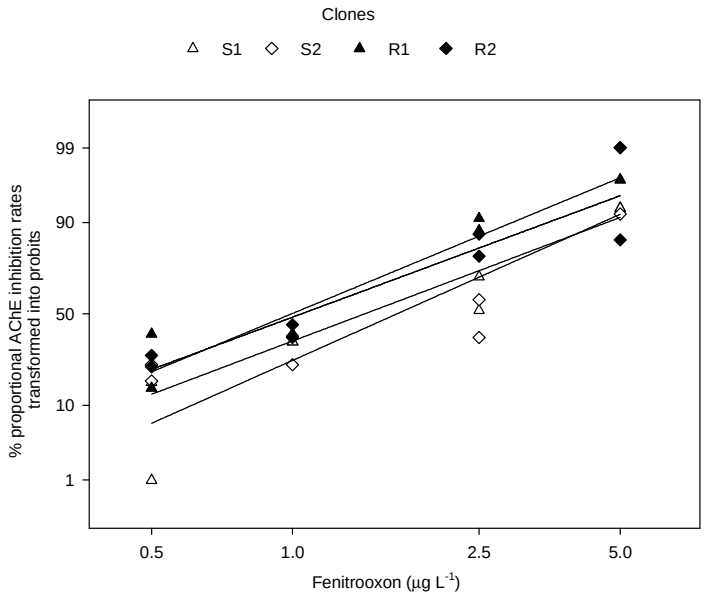

Fig. 2.1. AChE activities as a function of fenitrooxon concentration of the studied clones during in vitro 90 min incubations. Exposure concentrations are expressed in a $\log_e$ scale. Absolute AChE rates were converted to proportions relative to controls, transformed into probits and fitted to the linear models depicted in the graph.

Table 2.4. AChE inhibition levels (IC_{50}) of *D. magna* clones to in vitro incubations with fenitrooxon

Population	IC_{50} (95% CI) (µg L^{-1})	Slope
S1	1.87 (1.30-2.89)	0.27
S2	1.56 (1.05-2.32)	0.20
R1	1.07 (0.82-1.32)	0.22
R2	1.11 (0.83-1.40)	0.19

In vivo exposures

In vivo exposures to CBDP, ½ LC_{50} of fenitrothion and a mixture of both compounds denoted similar inhibition patterns of B esterases among clones (Fig. 2.2). In all four clones activities of CbEs showed a greater degree of inhibition by fenitrothion and CBDP than those of AChE, and co-administration of both inhibitors increased significantly AChE inhibition ($P < 0.05$ based on ANOVA tests).

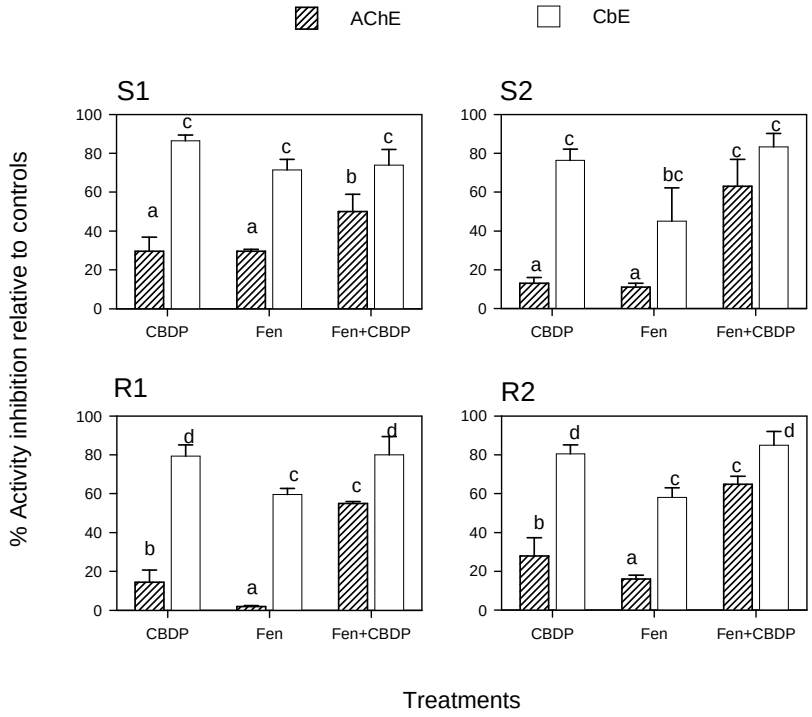

Fig. 2.2. AChE and CbE inhibition responses of *D. magna* clones exposed in vivo to fenitrothion with and without CBDP (CbE inhibitor). The exposure concentrations of fenitrothion were 0.25 µg l^{-1} for the sensitive clones and 2 µg l^{-1} for the resistant ones. Distinct letters indicate significant differences ($P < 0.05$) among enzymatic activities across treatments following ANOVA and post-hoc Tuke's multiple comparison tests. Error bars indicate S.E.M.

Provided the quite large variability in GST activities across trials, only those of the laboratory clone S1 were significantly ($P < 0.05$, $F_{3,24} = 8.6$, Fig. 2.3) higher than those of clones S2, R1, R2. Notice, however, that although not significant, GST activities of R1 and R2 were higher than those of clone S2. In vivo exposures to fenitrothion did not affect significantly GST activity relative to controls ($P > 0.05$, based on *t*-test performed for each clone; Fig. 2.3).

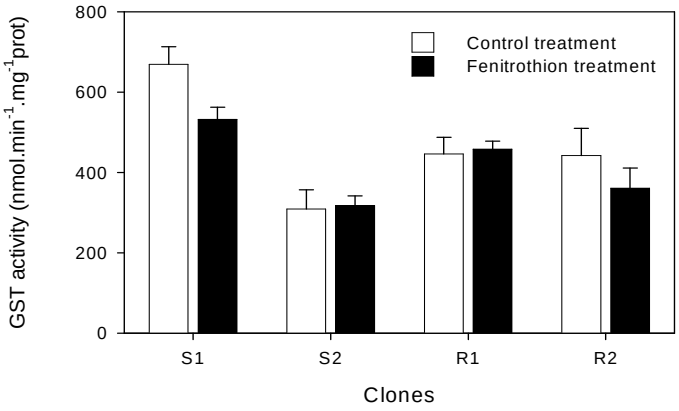

Fig. 2.3. GST activities of *D. magna* clones exposed in vivo to fenitrothion. The exposure concentrations of fenitrothion were 0.25 µg l^{-1} for the sensitive clones and 2 µg l^{-1} for the resistant ones. Error bars indicate S.E.M.

DISCUSSION

The results reported in this study revealed over sixfold differences in resistance to fenitrothion among the studied *D. magna* ephippia clones. Ephippia egg banks collected from rice fields heavily impacted by fenitrothion include clones as sensitive as the laboratory clone, thus indicating substantial genetic variability in resistance. The observed sixfold differences in resistance to fenitrothion among sensitive and resistant strains were similar to those reported previously for other *D. magna* populations historically exposed to ethyl parathion by Barata et al. (2001) and can be considered rather small compared to the over 100 -fold differences observed between sensitive and resistant populations of insects and mite species exposed to OPs (Roush and Mckenzie, 1987; Dunley et al., 1991; Morton, 1993). This may indicate that the selective pressures to develop resistance to fenitrothion have not been maximal for these populations (Roush and McKenzie, 1987), or alternatively

that *D. magna* is unable to develop elevated levels of resistance to OP pesticides. Indeed more than 100× resistance are usually given by mutations in AChE, while less than 100-fold resistance are usually related with genetic differences in the metabolism of organophosphorous insecticides by the MFO or other systems (i.e. CbEs, GST; see for example, Morton, 1993; Taylor and Feyereisen, 1996; Roush and Mckenzie, 1987). Notice, however, that for other contaminants such as cadmium, Baird et al. (1990) reported over 100-fold differences in resistance among sensitive and resistant *D. magna* clones. Contrary to metals, the environmental persistence of OPs in the field is considered to be low (i.e. hours for fenitrothion in Delta del Ebre rice fields (Barcelo et al., 1991), thus it is unlikely for *Daphnia* individuals to have been exposed to high doses over long periods and hence selective pressures to be maximal.

The three main types of mechanisms of resistance to OPs in arthropod pest species (i.e. insects) include reduced uptake, enhanced metabolism by MFOs, CbE, or glutathione-S-transferases and decrease sensitivity of the target site AChE (Oppenoorth, 1985). In this study the lack of relation between in vitro sensitivity of AChE to fenitrooxon and tolerance to fenitrothion indicated that differences in AChE sensivity was not the underlying mechanism explaining the observed differences in tolerance. Additionally, inhibition of MFOs by PBO increased tolerance to fenitrothion by almost 20-fold in all clones without altering their relative ranking in resistance terms, thus suggesting that MFO desulfuration to fenitrooxon rather than dearylation (oxidative cleavage) to 3-methyl-4-nitrophenol contributed to a greater extent to fenitrothion toxicity. These results agree with those reported by Ankley and Collyard (1995) on the amphipod *Hyalella azteca* and the chironomid *Chironomus riparius* exposed to different phosphorotionate OPs and PBO, but disagree with reported synergism effects of PBO co-administrated with OPs in most insects (Yuan and Chambers, 1996; Ahmed and Wilkins, 2002). Notice, however, that the former authors used lower doses of PBO relative to the OPs than in this and Ankley and Collyard (1995) studies, which may have affected differently desulfuration/dealyration MFO activities. Nevertheless, results (data not shown) conducted with the laboratory clone (clone S1) exposed simultaneously to much lower concentrations of PBO and fenitrothion showed always antagonistic toxicity effects. Indeed with increasing PBO concentrations (10, 25, 50, 100, 200, 400 and 1000 g l^{-1}) antagonistic ratios were 1, 1.2, 1.5, 2, 5, 20 and 50, respectively. Therefore, it was feasible to conclude that contrary to most insects, MFO

mediated bio-activation rather than detoxification contributed to a greater extent to fenitrothion toxicity in *Daphnia*. Furthermore, when exposed to fenitrooxon, the studied clones showed similar levels of resistance with and without the presence of PBO, thus suggesting that clonal differences in desulfuration rates (MFO mediated bio-activation due to conversion of fenitrothion to fenitroxon) rather than in dearylation rates (MFO mediated detoxification; oxidative cleavage of fenitrothion to 3- methyl-4-nitrophenol) were likely to be the mechanisms of resistance. These results disagree with most cases of MFO mediated resistance that result from an increase in detoxification of parental insecticides (Scott, 1999). Indeed only in few cases that include methyl parathion and malathion resistance in *Heliothis virescens* and *Culex tarsalis*, respectively, decrease production of oxon metabolites in resistant strains has been presumed to contribute to the decrease susceptibility.

Results obtained for fenitrooxon toxicity also indicated that non-MFO mediated metabolism of fenitrooxon by esterases or GSTs were unlikely to be involved in *Daphnia* resistance. In particular, when fenitrooxon was co-administered with CBDP, toxicity to the former pesticide increased around twofold but the relative ranking of clones in resistance was maintained. Nonetheless, interpretation of the results obtained with in vivo exposures to fenitrooxon should be considered with caution since according to Takimoto et al. (1987) only 0.8–2% of the fenitrothion in *Daphnia pulex* was fenitrooxon, thus oxon enzymatic detoxification systems (i.e. CbE, GST) were probably overwhelmed by an excess of substrate. Indeed high activities of detoxification CbE and GSTs have been reported in fenitrothion resistant strains of insects (Hama, 1984; Rose and Wallbank, 1986; Shiotsuki et al., 1988; Collins et al., 1992; Conyers et al., 1998; Rossiter et al., 2001). The results obtained in this study showed similar CbE enzymatic properties among clones in terms of sensitivity to synergists and protection against fenitrothion or fenitrooxon but about 1.5–2-fold higher activities in the resistant clones. Thus, according to Barata et al. (2004) resistant clones may show a greater ability to sequester fenitrooxon molecules. However, toxicity test with specific inhibitors of CbE evidenced that these system only contributed marginally to the observed clonal differences in tolerance to fenitrothion. In particular, inhibition of CbE only decreased the relative tolerance of the R2 versus the most sensitive clone S2 but not that of R1, thus suggesting that other mechanisms may also be involved in fenitrothion tolerance (Ahmed and Wilkins, 2002).

GST activity varied across clones but not under exposure to fenitrothion neither was correlated with tolerance levels. Indeed the lab sensitive clone (S1) followed by the two resistant clones (R1, R2) presented 2- and 1.5-fold greater activities than the sensitive clone S2, respectively. Studying enzyme activities in profenofos resistant tobacco budworms, Harold and Ottea (1997) found that GST were associated with resistance, although correlations were dependent upon location of field collection. Therefore, excluding out the clone with a different origin (clone S1), our results denoted a positive relation between fenitrothion resistance and GST activity. The previous argument, however, have to be considered with caution since according to Bond and Bradley (1997) a heat-inducible high molecular mass protein complex with GST rather than an increase enzyme activity was related with resistance to malathion in *D. magna*. Indeed the results of Takimoto et al. (1987) indicated that most of the fenitrooxon present in *Daphnia* was demethylated probably by GST isoforms. Furthermore, according to Escartín and Porte (1996) metabolization of fenitrothion by purified microsomes of the crayfish *Procambarys clarkii* was faster in GSH- than in NADPH-supplemented system, thus indicating a greater importance of GST system versus the MFO system as detoxification pathway. Unfortunately, little is known about the GST isoenzymes involved in organophosphorous metabolism in *Daphnia* (Baldwin and LeBlanc, 1996).

Although not studied, differences in fenitrothion uptake kinetics and hence on its bioaccumulation may also have contributed to the observed genetic differences in tolerance. Previous studies on different crustaceans indicate that uptake kinetics of fenitrothion showed a rapid increase followed by a steady state or a sharp decrease, depending of the metabolization rates of each species (Takimoto et al., 1987; Escartín and Porte, 1996). Indeed in crustaceans and fish species low bio-concentration factors for fenitrothion and other OPs have been attributed to a combination of their low environmental persistence (Barcelo et al., 1991) and their relatively rapid biotransformation (Landis, 1991; Escartín and Porte, 1996; Kitamura et al., 2000). Therefore, it is plausible to hypothesize that metabolization rather than pesticide uptake may explain the observed differences in *Daphnia* resistance to OPs.

In summary, our results although not conclusive indicate that MFO mediated detoxification (dearylation metabolism), and AChE insensitivity did not affect significantly the observed clonal differences in resistance to fenitrothion. Conversely, MFO mediated

differences on the bio-activation of the phosphorotionate OP to its active oxon metabolite contributed mostly in explaining the observed moderate levels of resistance. Furthermore, the observed positive correlation between higher activities of CbE and GST and tolerance to fenitrothion in most clones, may also suggest that these enzymes are likely to be involved only marginally on its metabolism and consequently on its resistance. Future work, thus, should be focused in studying with greater detail the metabolism of fenitrothion across sensitive and resistant populations of *D. magna*, for example by using the radiolabelling and chromatographic methods of Takimoto et al. (1987) and Escartín and Porte, 1996.

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Chapter 3

Combined use of *Daphnia magna* in situ bioassays, biomarkers and biological indices to diagnose and identify environmental pressures on invertebrate communities in two Mediterranean urbanized and industrialized rivers (NE Spain)

3. Combined use of *Daphnia magna* in situ bioassays, biomarkers and biological indices to diagnose and identify environmental pressures on invertebrate communities in two Mediterranean urbanized and industrialized rivers (NE Spain)

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ABSTRACT

Environmental factors affecting aquatic invertebrate communities were assessed using *Daphnia magna* in situ bioassays and biological indices based on community assemblages of benthic macroinvertebrates. Investigations were carried out in two heavily industrialized and urbanized river basins from the NE of Spain (Llobregat and Besós). Measures of energy consumption (i.e. algal grazing), and of specific biochemical responses (biomarkers) were conducted on individuals transplanted upstream and downstream from effluent discharges of sewage treatment plants. In both rivers there was a clear deterioration of the ecological water quality parameters and benthic communities towards downstream reaches. In all but one of the 19 locations studied, transplanted organisms were affected in at least one of the five measured responses. In three of them, significant effects were detected in most of the traits considered. Principal Component and Partial Least Square Projections to Latent Structures regression analyses indicated that the measured responses in *D. magna* in situ bioassays and those of macroinvertebrate assemblages were affected by distinct environmental factors. From up to 20 environmental variables considered, seven of them including habitat degradation, suspended solids, nitrogenous and conductivity related parameters affected macroinvertebrate assemblages. On the other hand, levels of organophosphorus compounds and polycyclic aromatic hydrocarbons were high enough to trigger the responses of *D. magna* in situ bioassays. These results emphasize the importance of combining biological indices with biomarkers and more generalized and ecologically relevant (grazing) in situ responses to identify ecological effects of effluent discharges from sewage treatment plants in surface waters.

KEYWORDS: *Daphnia*, Feeding, Biomarker, In situ, Benthic macroinvertebrate, River, Water quality

INTRODUCTION

Identifying indicators which can be used to diagnose causal agents of adverse change in ecological systems is a major challenge in environmental risk assessment (Baird and Burton, 2001). Traditionally, biomonitoring of freshwaters has been based on measures of community structure, focussing on assemblages of benthic macroinvertebrates and calculating biotic indices (Rosenberg and Resh, 1993). Although such diagnostic measures have been described for communities impacted by organic pollution, acidification, flow modification or habitat degradation (Bonada et al., 2006), there are no reliable indicators of impairment caused by contaminants (Baird and Burton, 2001). Furthermore, these indices can respond to pressures other than those they were developed to detect, making diagnosis of the actual pressure more difficult.

Recently, the development of new bioassays with caged, single species has allowed determining toxic effects of pollutants in situ (Maltby et al., 2000, 2002). A key advantage of in situ bioassays over whole effluent toxicity tests and biological surveys of benthic macroinvertebrate communities is their greater relevance to the natural situation, especially with respect to the contamination scenario. Additionally, in situ bioassays are able to detect effects in caged individuals more rapidly (hours to days) than the time taken to observe changes in community structure (months to years) measured during macroinvertebrate sampling (Maltby et al., 2002). A set of in situ and cost effective bioassays based on feeding and biochemical responses of invertebrate species have permitted detecting lethal and sublethal responses that are biologically linked with key ecological processes such as detritus processing and algal grazing rates, and of specific toxicological mechanisms. Nevertheless, it is important to consider that most of these studies have been focused to detect major point contaminant impacts such as the effects of pesticides after application or rainfall run-offs (Mc William and Baird, 2002b; Schulz, 2003; Barata et al., 2007), of metals from mine drainage (Mc William and Baird, 2002b), and of industrial effluent discharges (Maltby et al., 2000). Therefore, further studies are needed on the usefulness of in situ bioassay responses in detecting subtle effects on aquatic organisms exposed chronically to multiple environmental factors or/and to low levels of contaminants (Maltby et al., 2002).

Studies conducted by Mc William and Baird (2002a, 2002b) demonstrated that in situ post-exposure feeding depression in *Daphnia magna* was a sensitive, robust and ecologically relevant endpoint to diagnose sublethal effects of toxic effluents rich in metals and agrochemicals. Recently, combining biomarker and toxicological responses into the post-exposure feeding *D. magna* in situ bioassay, Barata et al. (2007) were able to identify exposure to and effects associated with specific pesticides in the field. The main objective of this study was to address if the use of toxicological and biochemical responses in *D. magna* in situ bioassays may complement existing ecological water quality monitoring procedures in identifying causal agents affecting aquatic biota inhabiting rivers contaminated by multiple environmental factors. In particular, we assessed and compared toxicological and biochemical responses of caged *D. magna* with biological indices calculated from taxa assemblages of benthic macroinvertebrates across sites and river basins differing in their ecological quality status. *D. magna* in situ bioassay responses included effects on survival, post-exposure feeding inhibition and the activities of acetylcholinesterase (AChE), catalase and glutathione S-transferase (GST). Multivariate techniques were then used to relate measured contaminant levels and general physicochemical water parameters with the above-mentioned biological responses to identify the likely main causal agents behind those responses. This study was conducted in the Llobregat and Besós river basins (NE Spain), which supply water to the city of Barcelona and are good examples of intensively used Mediterranean stream systems, receiving extensive urban, agricultural and industrial wastewater discharges. Previous studies have investigated the presence and effects of micropollutants, endocrine disruptors, metals and of persistent organic pollutants in fish and invertebrates from these rivers (Solé et al., 2000; Teixido et al., 2001; Fernandes et al., 2002; Barata et al., 2005a). Effects on carps (*Cyprinus carpio*), crayfish (*Procambarus clarkii*) and caddisfly (*Hydropsyche exocellata*) included alteration of normal levels of vitellogenin, cytochrome P450, phase II and antioxidant enzymes. However, there are no data either on the bioavailability of other contaminants or on their toxicological effects on in situ exposed organisms.

MATERIAL AND METHODS

Study sites

The Llobregat and Besós river basins (NE Spain) include an area of 6400km^2 and main channel flows of 0.1–12m^3/s (Prat et al., 1984). Like other Mediterranean systems, the natural resources of Llobregat and Besós river basins have been greatly affected by human activities such as agriculture, urbanization, a salinity increase as a result of mining activities and an intensive water use for human consumption (supplying water to many urban areas including Barcelona city), which together have severely deteriorated the ecological status of the main rivers and tributaries since 1970s (Prat et al., 1984; Prat and Munné , 2000; Barata et al., 2005a). During 1990s the construction of sewage treatment plants (STPs) and of salt collectors substantially improved the chemical and biological quality of water, thereby allowing the survival of fish and invertebrate species in middle and lowland reaches (Prat and Munné, 2000; Barata et al., 2005a). The ecology of Llobregat and Besós rivers has been extensively studied (e.g. Prat et al., 1984) and since 1994 a surveillance monitoring program is being carried out in those rivers to determine general physicochemical parameters, contaminant residues in water, biological measures (benthic macroinvertebrates) as well as measurements of the quality of the riparian habitat (http://ecostrimed.net/; http://mediambient.gencat.net/aca/). The present study has been performed in collaboration with the surveillance program using the same sampling sites at the same time. Deployment sites comprised eleven and eight points along the Llobregat and Besós river systems, respectively (Fig. 3.1). Stations were selected to include clean upstream (sites L1, L2, L3, L4, L5, B1, B2, B3), polluted middle (site L6, L8, B5, B6) and downstream reaches (sites L9, L10, L11, B4, B7, B8).

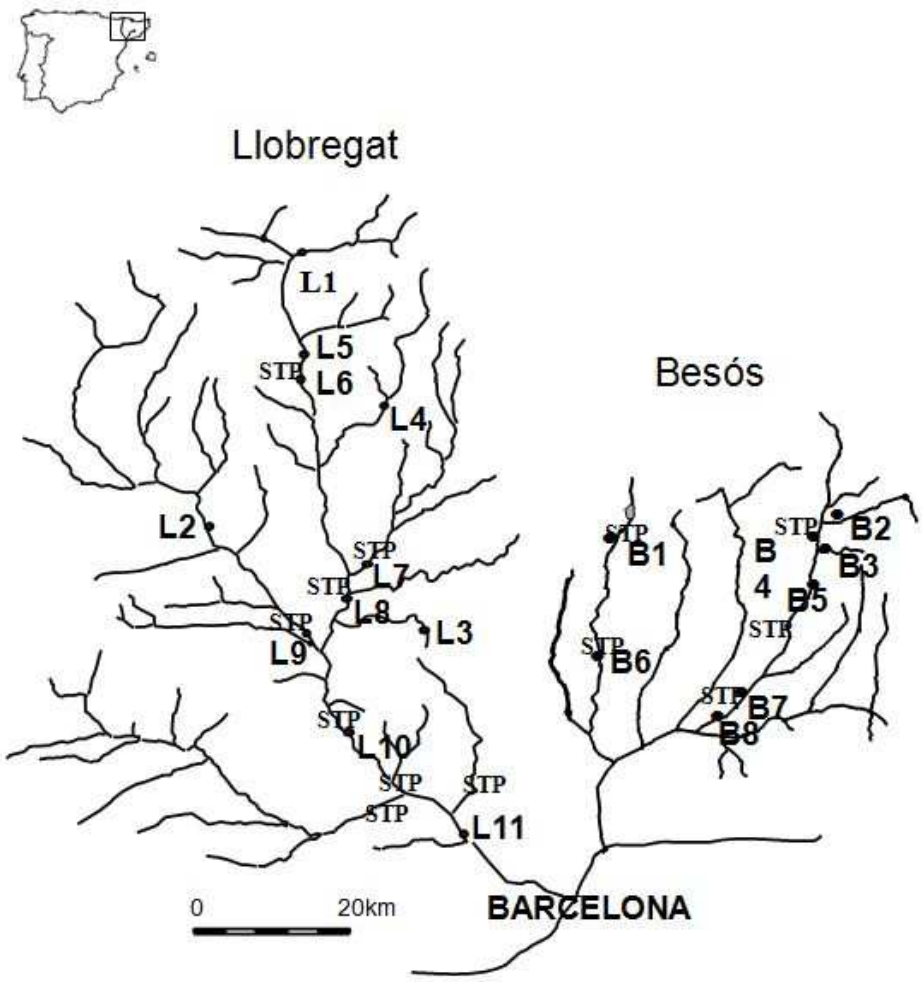

Fig. 3.1. Map of the sampling sites across Llobregat and Besós river basins, which also includes effluent discharges of sewage treatment plants (STP).

Environmental measurements

A set of environmental variables were measured on each deployment occasion. Water physicochemical parameters including flow rate (l/s), temperature (T; ºC), pH, conductivity (µS/cm), sulphates (SO_4, mg/l), chlorides (Cl, mg/l), dissolved oxygen (O_2, mg/l), suspended solids (SS, mg/l), N-ammonium (NH_4, mg/l), N-nitrites (NO_2, mg/l), N-nitrates (NO_3, mg/l), P-phosphates (PO_4, mg/l) were obtained following well-established methods for procedures measuring water physicochemical parameters [see, ASTM (1998) and Barata et al. (2005a)]. Briefly, water flow rates were determined with a Mini Air probe (Technika-Schiltknecht) in those places where it was possible to establish a cross section (transect) and measure water flow at different depths across the section. T, pH, conductivity and O_2 were measured in situ using a WTW Multi 340i handheld meter, whereas SS, NH_4, NO_2, NO_3, PO_4, SO_4 and Cl were measured in the lab following ASTM

Methods (ASTM, 1998). Limits of detection for the above mentioned parameters were 20 µS/cm for conductivity, 0.4 mg/l for SO_4, 1 mg/l for Cl, 0.2 mg/l for O_2, 0.1 mg/l for SS, 0.01mg/l for NH_4, NO_2 NO_3 and PO_4. From up to 191 contaminants that are regularly analyzed by the local stage agency only 22 occurred above detection levels in the studied locations. These included the metals Cu and Zn, the triazines (TRZ) atrazine, simazine, terbutilazine, terbutrin; the polycyclic aromatic hydrocarbons (PAH) pyrene, fluoranthene, fluorene, phenantrene; the organochlorate pesticides (OCL) α,β,δ,γ hexachlorocyclohexanes (HCH) metolachlor, alachlor; the organophosphorous compounds (OP) diazinon, chlorpyrifos and four alkylphenol ethoxilates (APE) (three nonylphenol ethoxilates (NPEO) and *t*-octylphenol). Water samples were stored in glass bottles and refrigerated to 4 ºC immediately after sampling. Samples for metal analysis were acidified with nitric acid (pH <1). The metals Cu and Zn were analyzed from filtered water samples following Barata et al. (2005a) using a PerkinElmer model Elan 6000 inductively coupled plasma mass spectrometer (ICP-MS). Calibration standards and a reagent blank were analyzed with every 10 samples to monitor signal drift. In every instance, the signal typically changed by 3–5% throughout an analytical run. Additionally, rhenium was used as an internal standard to correct for any non-spectral interferences. Organic contaminant residues were extracted from water using a liquid–liquid extraction procedure carried out according tomethod 625 of the U.S. Environmental Protection Agency (EPA, 1984; Teixido et al., 2001). One litre sample was added with surrogate standards (nitrobenzene-d5, 2-fluorobiphenyl and 4-terphenyl-d14) and extracted twice with dichloromethane (150 and 100ml) by stirring for 10 min. The organic extracts were combined and dichloromethane volume reduced under reduced pressure, using first a round bottom flask and thereafter a conic flask, until a volume of 0.5 ml was reached. The concentrate was transferred to a 1 ml conic vial, washing the flask with isooctane, and dried under N_2 stream until a final volume of 100 µl was obtained. Analyses of TRZ, PAH, OP, APE and OCL were conducted by gas chromatograph coupled to a mass spectrometer detector.

Chromatographic analysis was carried out in a HP 6890 Gas Chromatograph (Agilent Technologies) equipped with a HP E2M 1.5 rotatory vane pump, coupled to a MSD 5973N Mass spectrometer (Agilent Technologies) using a 5% phenyl methyl siloxane column HP-5MS (30m×0.25mm×0.25 µm) with a 5m pre-column. Helium at a flow rate of 0.7 ml/min was used as a carrier gas, temperature of interface was set at 280 ºC and temperature of

injector at 280 ºC. The chromatographic conditions were as follows: 90 ºC for 2 min, to 25 ºC/min up to 150 ºC, to 3 ºC/min up to 200 ºC, 8 ºC/min up to 310 ºC (10 min). Detection was performed with an electrospray ionization source (70 eV). Identification was accomplished by comparing the retention time and the GC–MS signals of the target compounds in the samples with those of standards analyzed under the same conditions. Internal standards were used to monitor the extraction efficiencies and for quantification. All analyses were performed according to ISO 9000 norm in the certified laboratory of the Agencia Catalana del Aigua (Barcelona, Spain). The detection limits achieved (LODs) were below 1 µg/l for Cu and Zn, 1 ng/l for OCL, 4 ng/l for TRZ and PAH, 10 ng/l for OP and 1.2 ng/l for APE.

Biological conditions

A detailed investigation of benthic macroinvertebrates, riparian vegetation and habitat quality was also conducted to establish the ecological quality of the studied sites and to assess the extent to which habitat quality affected the occurrence of benthic macroinvertebrate taxa (Jáimez-Cuéllar et al., 2002). The quality of riparian vegetation was measured using the Riparian habitat Ecological Quality Index (QBR, Munne et al., 2003), which is based on four components: total riparian vegetation cover, cover structure, cover quality and channel alterations. This index scores from 0 (highly disturbed) to 100 (natural). Additionally, the Fluvial Habitat Index (IHF), developed for Mediterranean areas (Jáimez-Cuéllar et al., 2002), was used to assess the in-stream habitat quality. This index evaluates a river's physical characteristics related to habitat heterogeneity produced by different hydrological conditions, substrate classes and autochthonous (aquatic vegetation) and allochthonous (leaves and coarse debris) sources. It scores between 0 and 100.

Benthic macroinvertebrates were obtained by sampling all available habitats with a kick net with opening size of 250 µm. Specimens were then preserved in formalin (5%), identified to the family level and used to determine the biological quality of water (Jáimez-Cuéllar et al., 2002). The previous authors have established five water quality levels for the Iberian Monitoring Working Party Biological Index (IBMWP) that include very clean waters, waters with signals of stress, contaminated waters, very and extremely contaminated waters for IBMWP scores >100, 61–100, 36–60, 16–35 and <15, respectively. The Iberian Average Score per taxon (IASPT), which is derived from

IBMWP and is considered less biased by sampling effort, ranges from 0 (no scoring macro invertebrates present) to 10 (all scoring macroinvertebrates, which are pollution sensitive, are present).

D. magna in situ bioassays

Exposure regime

D. magna in situ bioassays were conducted as described by Barata et al. (2007) using the same test chambers and procedures of Mc William and Baird (2002b) with only minor modifications that included 9–10 test chambers to allow collection of animals for biomarker determination and to increase the number of replicates for post-exposure feeding rate measurements. Chambers were constructed from clear polyvinyl chloride cylindrical piping (13 cm long, 5 cm external diameter). Each chamber had two rectangular windows (7cm×3.5 cm) cut into either side of the cage, covered with 150- µm nylon mesh. Pipe ends were sealed with polypropylene caps. Groups of 4–5 chambers were placed inside a 13-mm^2 wire-mesh cylinder that was positioned in the stream perpendicular to flow. Deployments were conducted during spring (April) and summer (July) of 2004 and 2006 in the Llobregat and Besós river basins, respectively. Within each month two to three deployments were conducted simultaneously in three to four locations that always included at least one reference site.

The procedure for the *D. magna* in situ bioassays was briefly as follows. Juveniles were transported to field sites in groups of 10 in 175 ml glass jars filled with American Society for Testing Materials (ASTM) hard water (Mc William and Baird, 2002b). All field sites were less than 100 km drive from the lab. At each site 5–7 chambers, each containing 10 individuals, and 4 chambers containing 20 individuals, were placed inside a 13-mm^2 wire-mesh cylinder that was positioned in the stream perpendicular to flow. In each deployment, a lab manipulation control treatment in which the animals were transported to the studied sites in the beginning and in the end of the test in closed 500 ml jars and then back to lab, but never exposed to the field conditions, was also included.

Post-exposure responses

After 24 h, animals were retrieved from the chambers. Surviving animals from each of the four chambers holding groups of 20 individuals were pooled in four eppendorf tubes and immediately frozen in liquid N_2 and kept at –80 ºC until further enzyme analysis. Within 1 h of exposure five surviving juveniles from chambers holding groups of 10 animals were placed into 60 ml screw-capped glass jars containing 50 ml of ASTM hard water, with *Chlorella vulgaris* (Beijerink, strain CCAP C211/12) at a concentration of 5×10^5 cells/ml, and allowed to feed for 4 h (Mc William and Baird, 2002b). Three jars containing no animals were used to establish initial algal densities. Post-feeding experiments were conducted in darkness to avoid algal growth and under relatively constant temperature conditions (20±2 ºC) provided by a thermostatted chamber transported inside the sampling vehicle. Individual feeding rates (cells individual^{-1} h^{-1}) were determined as the change in cell density during 4 h according to the method given by McWilliam and Baird (2002b). Cell density was estimated from absorbance measurements at λ = 650 nm in a dual-beam spectrophotometer (Uvikon 941) using standard calibration curves based on at least 20 data points, with an $r^2 > 0.98$.

Enzyme assays

Juveniles were homogenized at 4 ºC in 1:4 wet weight/buffer volume ratio in 100 mM phosphate buffer, pH 7.4 containing 100 mM KCl and 1mM EDTA. Homogenates were centrifuged at 10,000×g for 10 min and the supernatants were immediately used as enzyme sources. Biochemical measurements were carried out on Uvikon 941 Plus dual-beam and Spectra-max Plus microplate reader spectrophotometers. Assays were run at least in duplicate. AChE was determined by a modification of the Ellman method adapted for microplate (Barata et al., 2007). AChE activity was measured in the presence of 1 mM acetylthiocholine and 0.1 mM 5,5 dithiobis-2-dinitrobenzoic acid (DTNB), and the increase of absorbance was measured at 405 nm. Catalase activity was measured by the decrease in absorbance at 240 nm due to H_2O_2 consumption (extinction coefficient 40 M^{-1} cm^{-1}) according to Aebi (1974). The reaction volume was 1 ml and contained 50 mM phosphate buffer, pH 6.5, 50 mM H_2O_2 (Ni et al., 1990). GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was measured as described by Habig et al. (1974). The reaction mixture contained 100 mM phosphate buffer (pH 7.5), 1 mM CDNB and 1mM of reduced

glutathione. The formation of S-2,4 dinitro phenyl glutathione conjugate was evaluated by monitoring the increase in absorbance at 340 nm. Proteins were measured by the method of Bradford (1976) using serum albumin as standard.

Data analysis

Within each deployment date, post-exposure feeding rates and enzymatic activities at reference and contaminated sites were compared with the lab manipulation control by one way ANOVA followed by post-hoc Dunnet's multiple comparison test (Zar, 1996). Prior to analysis data were log transformed to meet ANOVA assumptions of normality and variance homoscedasticity. To explore cause–effect relationships between the studied environmental variables and parameters and the biological responses, Principal Component Analysis (PCA) and Partial Least Square Projections to Latent Structures regression (PLS) methods were used (Vandeginste and Massart, 1998). Prior to analyses biological responses of *D. magna* in situ bioassays were converted to responses relative to manipulated lab controls. To reduce the number of environmental variables and avoid an excessive occurrence of undetected contaminant levels across stations (i.e. zero values), contaminant residues in water (Ci) were changed to toxic units (TU) following the procedures of Liess and Von der Ohe (2005), and Barata et al. (2007) (Eq. (1)). TU for contaminants within the same family were summed.

$$TU = \frac{Ci}{LC50_{i\ (D.\ magna)}} \qquad (1)$$

Where $LC50_{i\ (D.\ magna)}$ is the 48 h acute median lethal concentration reported for the measured compounds (from Tomlin, 2000; Liess and Von der Ohe, 2005; Barata et al., 2007, PAN Pesticides database: www.pesticideinfo.org; ECOTOX EPA database//cfpub.epa.gov/ecotox/). LC50 (µg/l) were set to 50 for Cu, 1000 for Zn, atrazine, and simazine, 5000 for terbutilazine, 8000 for terbutrin, 50 for pyrene, 120 for fluoranthene, 212 for phenanthrene, 1200 for fluorene, 25 for lindane, 680 for α,β,δ,γ hexachlorocyclohexane isomers, 7000 for metolachlor and alachlor, 1 for diazinon, 0.5 for chlorpyrifos, 180 for NPEO and 90 for octylphenol. TU were based on acute rather than sublethal endpoints determined for *Daphnia* since for most of the compounds analyzed it was the only toxicity information available. Due to the great disparity of reported data, for

those chemicals with more than one reported LC50, the lowest value was used unless otherwise specified. Furthermore, previous studies have shown that for most taxa, relative sensitivities of benthic macroinvertebrates across contaminants is proportional to those reported for *D. magna* (Liess and Von der Ohe, 2005), thus the TU approach may be used for both taxa. Other potential environmental parameters including water flow, O_2, T, conductivity, NH_4, NO_2, NO_3, PO_4, SO_4, Cl and quality indices QBR, FHI were also considered since previous studies have shown that these parameters may affect the studied *Daphnia* and macroinvertebrate responses (Mc William and Baird, 2002b; Munne et al., 2003).

PCA was conducted using a data matrix of 28 samples (rows) and 27 variables (columns). Since variables were very different (physico-chemical, quality indices and behavioral and biochemical responses) and they were not measured using the same scale units, the data was auto-scaled prior to analysis (each element was subtracted by its column mean and divided by the standard deviation of its column). PLS analysis was performed considering 20 environmental parameters as X block independent variables, and considering each one of the following: IBMWP, IASPT, species richness, feeding, catalase, AChE and GST as Y block dependent variables. For *y*-dependent variables of the *D. magna* in situ bioassay, habitat quality parameters (FHI and QBR) were excluded from the X block since they only describe the physical characteristics of the fluvial habitat and hence are unrelated to *D. magna* in situ bioassay responses. PCA and PLS analyses were conducted using the Matlab 6.0 software (MathWorks, Natick, MA).

RESULTS

Environmental water parameters

For the sake of clarity physicochemical water parameters were averaged across sites, rivers and seasons (Table 3.1). In general, water flow decreased in summer and flow rates, nutrient load, conductivity and SS increased substantially from upper to downstream reaches. The quite large observed variations of T, water flow and O_2 levels across sites within upstream, middle and downstream reaches were related to seasonality of Mediterranean weather (temperate and rainy springs and hot and dry summers).

Selected contaminant residue levels for upstream, middle and downstream sites are summarized in Table 3.1. Cu and Zn levels did not vary from upstream to downstream reaches, whereas most organic contaminants increased towards downstream sites (Table 3.1). Organic contaminants except TRZ, diazinon, alachlor, metalachlor and octylphenols showed relatively low environmental levels, which in most occasions were close to their respective detection limits. Among the studied organic microcontaminants TRZ, diazinon, alachlor, metalachlor and octylphenols showed relatively high environmental levels. Mean TRZ residue levels in middle and downstream stations were higher than 300 ng/l, those of OP (mainly diazinon) increased from 50 to over 400 ng/l from upper to downstream sites and those of APE varied from undetected levels in most upper and middle stations to over 190 ng/l in downstream sites (Table 3.1). Alachlor and metalachlor residue levels in water were undetectable in most sites except in site L10, reaching levels near 0.5 µg/l. It is interesting to notice that most middle and lower stations were located downstream effluent discharges of STPs (Fig. 3.1) and hence it is likely that most of their organic contaminant residues came from those plants.

Biological conditions of the riparian habitat

The quality of the riparian habitat (FHI, QBR) and that of the riparian macroinvertebrate community (taxon richness, IBMWP, IAST) decreased substantially from upper to middle sites (Table 3.2) reaching relatively low values in downstream sites. Exceptions included the upstream site L2 which was located in a private fishing and camping resource and hence showed its riparian habitat moderately altered and site B1 during Spring, which showed a relatively low taxon richness and low IBMWP scores, probably due to episodic or accidental effluent discharges coming from an upstream STP.

Table 3.1. Mean ± standard deviation (S.D), minimum or LOD (Min), and maximum (Max) of physicochemical water quality parameters at upstream, middle and downstream stations in Llobregat and Besós river basins.

Station	Uppstream				Middle				Downstream			
	Mean	Sd	Min	Max	Mean	sd	Min	Max	Mean	sd	Min	Max
Flow	555	1102	0	3788	2552	3409	12	10938	10504	15697	178	43590
T	15	5	8	23	17	5	10	24	21	5	14	27
SS	4.4	5.3	0.5	20.3	27.5	29.1	3.0	87.5	35.1	31.8	3.3	109.0
pH	8.4	0.3	7.4	8.9	8.3	0.2	7.8	8.6	8.2	0.4	7.5	8.7
Cond	503	147	226	757	1257	663	521	2500	1584	315	1208	2198
O_2	9.8	1.7	4.9	11.7	8.9	1.7	5.4	11.0	8.6	1.7	5.9	11.6
NH_4	0.09	0.13	<0.01	0.41	0.28	0.34	<0.01	1.15	4.79	9.80	0.13	34.18
NO_2	0.05	0.16	<0.01	0.60	0.47	1.21	<0.01	3.88	0.24	0.35	0.01	1.20
NO3	0.54	0.63	0.02	2.44	3.00	2.97	0.01	8.67	2.92	2.06	0.18	6.97
PO_4	0.03	0.04	<0.01	0.14	0.39	0.56	<0.01	1.80	1.28	1.77	0.11	5.88
SO_4	72.9	83.4	21	345	171.8	84.7	90	345	173.0	27.6	144	235
Cl	22	20	5	75	200	173	21	621	300	94	151	421
Cu	2	1	<1	4	2	2	<1	5	3	2	1	5
Zn	24	12	6	37	19	63	9	26	30	6	21	37
Pyrene	5		<4	5					5	<4	<4	7
Fluorene	5		<4	5	4	1	<4	5	6	1	5	8
Phenanthrene	5	1	<4	6	5		<4	5	22	35	5	85
Fluoranthene	5		<4	5	5		<4	5	20	25	5	49
Atrazine	9	6	4	19	13	16	<4	40	78	86	4	181
Simazine					20	19	6	33	53	69	<4	131
Terbutrin					8	5	4	14	29	20	5	57
Tertbutylazina	6	2	4	7	280	361	24	535	293	747	6	2140
Diazinon	40	17	28	52	70	62	<10	124	439	1053	13	2826
Chlorpyrifos	11		<10	11					89		<10	89
HCH	1	1	<1	3	1	1	<1	3	4	4	<1	12
Lindane	2	1	<1	5	2	1	<1	3	4	4	<1	12
Alachlor	6	1	5	7	10	6	6	14	174		<4	174
metolachlor	9	4	7	12					309		<4	309
NPEO					4.5		<1.2	4.5	28.2	39.9	1.4	96.0
t-Octylfenol	40.0		<1.2	40.0	40.0		<1.2	40.2	152.3	169.7	19.0	448

Measured parameters included: water flow (flow), temperature (T) and conductivity (Cond) reported in l/s, ^{o}C and µS/cm, respectively, oxygen (O_2), suspended solids (SS), nutrients (NH_4, NO_3, NO_2, PO_4), SO_4, Cl reported in mg/l, metal levels in µg/L and organic contaminants in ng/l. Contaminant abbreviations are explained in the text.

Table 3.2. Diversity and biotic indices for benthic macroinvertebrate assemblages and for the riparian habitat sampled across the Llobregat and Besós river basins.

Site	Season-Month	Taxon richness	IBMWP	IASPT	FHI	QBR
Llobregat						
L1	April	21	120	5.7	88	100
	July	41	214	5.2	98	100
L2	April	28	139	5	68	65
	July	37	169	4.6	69	65
L3	April	38	213	5.6	82	100
	July	43	243	5.7	88	100
L4	April	30	174	5.8	76	100
	July	29	172	5.9	86	100
L5	April	31	175	5.6	81	100
	July	27	137	5.1	91	100
L6	April	7	31	4.4	66	60
	July	19	83	4.4	84	60
L7	April	7	25	3.6	52	35
	July	9	40	4.4	64	35
L8	April	6	19	3.2	69	50
	July	11	46	4.2	73	50
L9	April	8	24	3	72	0
	July	13	50	3.8	57	0
L10	April	8	25	3.1	50	65
	July	8	24	3.0	76	65
L11	April	4	11	2.8	63	0
	July	4	10	2.5	46	0
Besós						
B1	April	10	49	4.9	64	85
	July	25	104	4.2	77	85
B2	April	20	117	5.9	70	100
B3	July	29	162	5.6	82	95
B4	April	10	37	3.7	67	5
	July	6	17	3.2	66	5
B5	April	10	35	3.5	67	25
	July	10	41	3.7	69	25
B6	April	4	11	2.8	49	0
	July	10	32	3.4	35	0
B7	April	4	11	2.8	61	5
	July	8	37	4.1	49	5
B8	April	7	23	3.3	46	5
	July	5	21	4.2	41	5

Site numbers are defined in Fig. 3.1. IBMWP, Iberian Monitoring Working Party; IASTP, Iberian Average Score per taxon; FHI, Fluvial Habitat Index; QBR, Riparian Habitat Ecological Quality Index

Responses of Daphnia in situ bioassays

The mean percentage of animals recovered (dead and alive) from the chambers after field exposures were always higher than 90%. Mean survival rates of *D. magna* recovered from in situ chambers were high in most field sites (>95%). In 13 out of 36 deployments, post-exposure feeding rates of transplanted *Daphnia* individuals differed significantly ($p < 0.05$) from that of manipulated lab controls. Interestingly, almost half of the observed effects on feeding were located in exposures to upstream stations (6 out of 13) with the lowest values detected in station L5. Effects on feeding also varied across deploying dates with July being the month with the highest effects (10 out of 13). It is also interesting to note that in all deployments, feeding rates were relatively constant in the manipulated lab control with values ranging between 5.5 and 7.8×10^5 cells individual^{-1} h^{-1}.

Mean±S.E.M. (N= 40) catalase, GST and AChE activities of manipulated lab controls varied little across deployments being 0.391±0.026 mmol/min/mg protein, 480.9±21.6 and 2.85±0.07 nmol/min/mg protein, respectively. In 7 out of 36 deployments, transplanted individuals had higher catalase activities than manipulated lab controls, in 3 of them showed lower activities and July accounted for most of the observed effects (7 cases in Fig. 3.2). GST was significantly affected in seven deployments showing higher activities than manipulated lab controls in four of them and lower activities in the remaining three (Fig. 3.2). For both enzyme activities (catalase and GST) the occurrence of observed significant effects was greater (12 out of 17) in middle and downstream sites. AChE activities were significantly inhibited in five deployments from middle and downstream reaches mostly in July (Fig. 3.2).

Causal relationships between environmental parameters and biological effects

From the 204 residues of Cu, Zn, TRZ, PAH, OCL, OP, APE detected in water samples, 139 had a toxicity of ≥0.0001 TU (Table 3.3). For the sake of clarity it was assumed that contaminant residues with toxicity thresholds lower than 0.0001 TU could not be considered potentially toxic to *Daphnia* or to most invertebrate taxa (Liess and Von der Ohe, 2005; Barata et al., 2007). In all 36 deployments, combined joint estimated toxicity of the contaminant residues measured in water in middle and downstream reaches were on average 1.2 and 2 fold more toxic than that of upstream stations (Table 3.3). On average the contaminant contributing the most to the combined joint toxicity was Cu

followed in decreasing order by OP, Zn, OCL, APE, TRZ and PAH. Indeed, the estimated toxicity for residue levels of Cu, OP and Zn was over two orders of magnitude greater than that of TRZ and PAH.

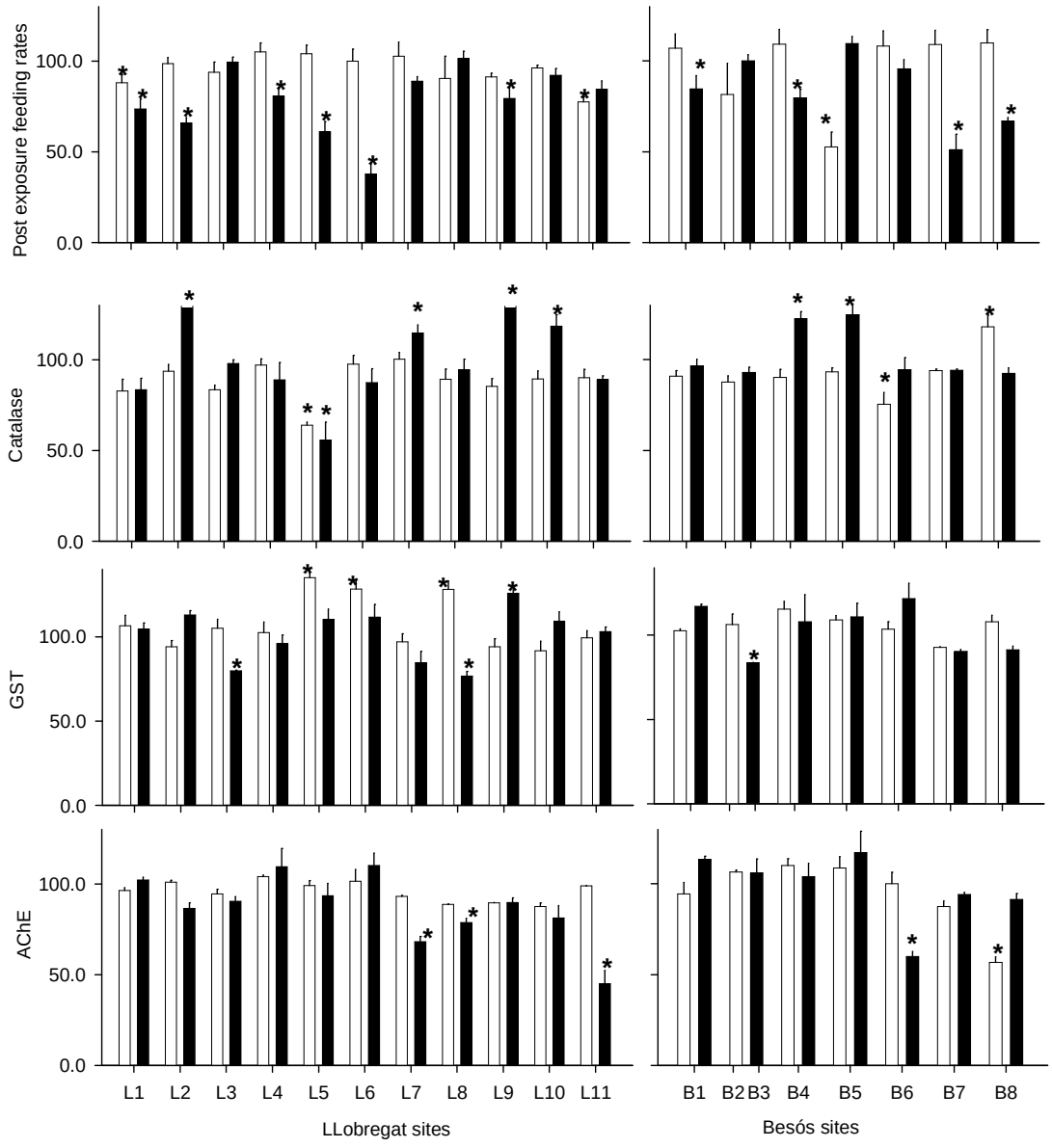

Fig. 3.2. Mean post-exposure feeding rates (cells individual^{-1} h^{-1}) and activities (nmol min^{-1} mg protein^{-1}) of catalase (CAT), glutathione S-transferase (GST) and acetylcholinesterase (AChE) of *D. magna* juveniles after in situ exposures across the studied sites during Spring (white bars) and Summer (black bars). Results are depicted as proportional responses (%) relative to manipulated laboratory controls. * significant ($p< 0.05$) differences relative to manipulated laboratory controls following ANOVA and Dunnet's multiple comparison tests. Error bars indicate standard errors (N = 4-6).

Table 3.3. Expected mean toxicities (averaged across seasons) depicted as toxic units (TU) of the measured contaminant residues (grouped by family) to *D. magna* at the studied sites.

	Cu	Zn	TRZ	PAH	OCL	OP	APE	Σ TU
Llobregat								
L1	0.0800	0.0110	-	-	0.0001	0.0003	-	0.0914
L2	0.0300	0.0189	-	-	0.0001	0.0059	-	0.0550
L3								
L4	0.0300	0.0189	-	-	0.0001	0.0059	-	0.0548
L5	0.0300	0.0240	-	0.0001	0.0004	0.0245	0.0001	0.0790
L6	0.0300	0.0240	-	-	0.0001	0.0003	-	0.0544
L7								
L8	0.0900	0.0160	-	0.00005	0.0003	0.0003	-	0.1066
L9	0.0300	0.0008	-	0.0002	0.0001	0.0058	-	0.0368
L10	0.1034	0.0270	0.00035	0.0001	0.0143	0.0003	0.0005	0.1460
L11	0.0620	0.0245	-	0.0001	0.0001	0.0889	0.0003	0.1761
Besós								
B1								
B2	0.0029	0.0008	-	-	-	0.0003	-	0.0040
B3	0.0029	0.0008	-	-	-	0.0003	-	0.0040
B4	0.0044	0.0179	0.00035	0.00005	0.0001	0.0166	0.0004	0.0399
B5								
B6	0.0029	0.032	-	0.0002	0.0002	0.0785	0.0004	0.1143
B7	0.0029	0.0008	-	0.0002	-	0.0003	0.0051	0.0093
B8	0.01245	0.0365	0.0014	0.0004	0.0005	0.064	0.0028	0.1180

Cu, Zn, TRZ, PAH, OCL, OP, APEs are copper, zinc, triazine, polycyclic aromatic hydrocarbons, organochlorine, organophosphorous and alkylphenol compounds, respectively. - Cells with TU $_{D.\ magna} \leq$ 0.0001; empty cells are missing values.

Bi-plots of variable loadings and site scores from PCA are depicted in Fig. 3.3. The first component accounted for 32.4% of data variance and had high negative loadings for community and habitat quality parameters (IBMWP, IASTP, taxon richness, FHI, QBR), AChE activities in *D. magna* in situ bioassays, and high positive loadings for levels of nutrients (NH_4, NO_2, NO_3, PO_4), conductivity, Cl, SO_4 and estimated toxicity of OP. As a result, this first component discriminated upstream from middle and downstream sites of both rivers and described well their water quality. The second axis (PC2), which explained 12.7% of data variance, differentiated middle and downstream reaches of Llobregat and Besós sites mainly due the contribution of flow rates, SS, O_2, and the estimate toxicity of

Cu (high positive 2nd PC loadings). It is interesting to point out that in both PC1 and PC2, the estimated toxicity parameters of the studied organic microcontaminants and most of the *D. magna* in situ bioassay responses (feeding, catalase and GST) had moderate contributions. Therefore, a further characterization of the main sources that modulated the observed biological responses was conducted using a PLS regression analysis (Table 3.4). The obtained PLS models explained 69, 50, 76, 64 and 55%, of the total variances of IBMWP, feeding rate, AChE, catalase and GST responses, respectively. Models for IASTP and taxon richness are not depicted since they were almost identical to those of IBMWP. For brevity in Table 3.4, only the PLS regression coefficients and VIP scores, which measure the relative contributions of the environmental variables in explaining the variance of the selected biological responses, are shown. According to Table 3.4 results, the quality of the benthic macroinvertebrate communities in the Llobregat and Besós river (IBMWP scores) were directly related to the quality of the riparian habitat (FHI, QBR) and varied negatively with the levels of Cl, SO_4, SS and NO_3. Alternatively, measured responses in *D. magna* in situ bioassays responded differently to the studied environmental parameters. Post-exposure feeding responses were negatively correlated to the estimated toxicity units of PAH, T and SS but positively related to the levels of NO_3 and NO_2. The estimated toxicity of OP was the most important variable explaining the observed variation of AChE. Catalase activity was positively related with T, conductivity, Cl levels and TRZ toxic units and negatively with O_2 and toxic units of OP and PAH; whereas GST responses were positively associated with conductivity and negatively by T, NO_3 and toxic units of APE and PAH.

DISCUSSION

Environmental water parameters

Physicochemical water parameters and contaminant concentration indicated a clear decrease of water quality from upper to downstream reaches in the Llobregat and Besós river systems. These results are characteristic of Mediterranean regions, where intensive water resource use is frequently linked to the lack of water flow due to climatic constraints, and rivers can receive effluents from cities, industries and agriculture with little dilution. In this situation water quality is poor, and measures that are effective for wet countries, such as the building of wastewater treatment plants fail to restore the biological quality of river water (Prat and Munné, 2000).

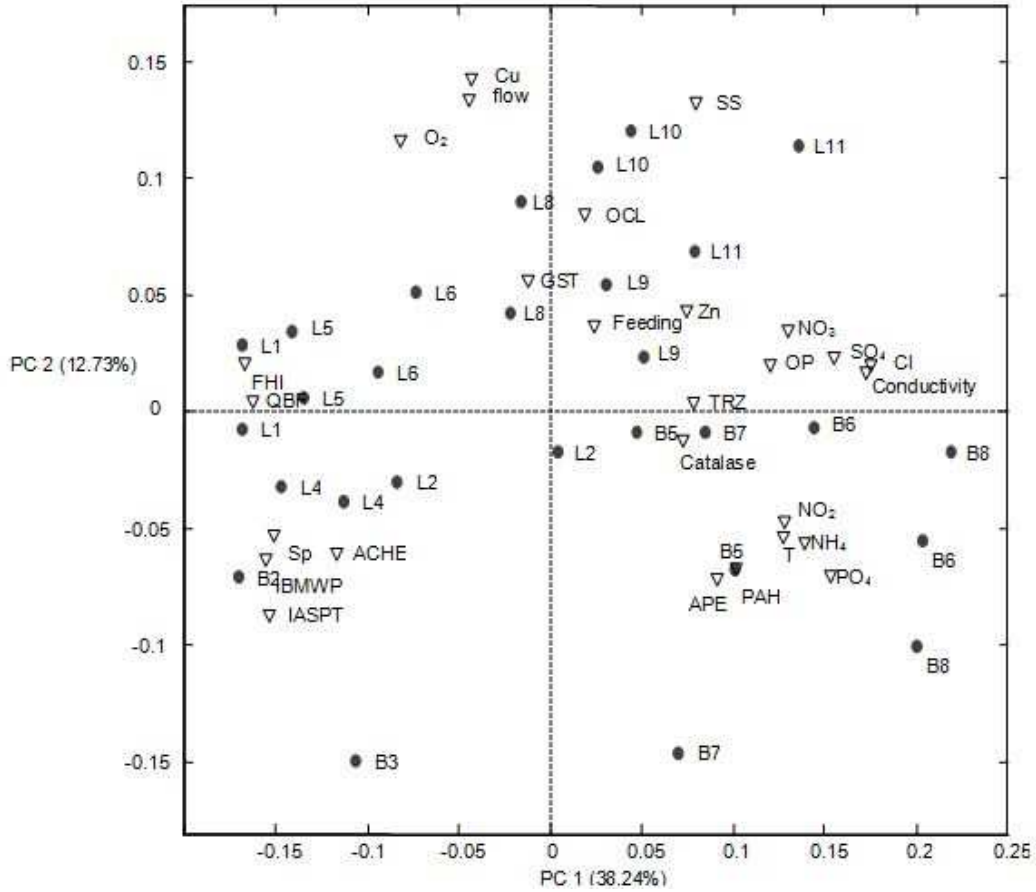

Fig. 3.3. Bi-plot of the variable loadings (triangles) and site scores (black circles) of the fist two Principal Components obtained for the studied biological and environmental variables across 19 sites within Llobregat and Besós river basins during April and July 2004, 2006. Abbreviations are explained in the text. The first and second component explained 50.97 % of variance of data.

Measured physicochemical water parameters including conductivity, SS and nutrient load along the studied sites (Table 3.1), were high compared to levels usually found in undisturbed rivers within Europe (Chapman and Kimstach, 1996). In particular, conductivity, SS, PO_4 and NH_4 levels observed in downstream reaches exceeded 1000 µS/cm, 10 mg/l, 0.3 and 0.2 mg/l, respectively, thus denoting high organic and saline pollution. It is worth noting that the relatively high (>1000 µS/cm) conductivity levels found in most studied middle and downstream locations from Llobregat are likely to be related to salinization caused by mining activities located upstream in the Cardener river (Casas et al., 2003), whereas those of Besós river are associated to industrial effluent discharges (Prat and Munné , 2000).

Table 3.4. Regression vector standardized PLS partial coefficients (Coeff) and VIP scores (Scores) of the environmental variables for the regression models obtained for the community index (IBMWP) and the responses of *D. magna* in situ bioassays (Feeding rates, AChE, catalase and GST) across 19 sites within Llobregat and Besós river basins.

	IBMWP		Feed		AChE		Catalase		GST	
	Coeff	Scores	Coeff	Scores	Coeff	Scores	Coeff	Scores	Coeff	Scores
Flow										
T			-0.23	1.95			0.34	2.72	-0.57	2.94
SS	-0.17	1.64	-0.25	1.23	-0.09	1.23				
Cond	-0.1	1.73			-0.06	1.03	0.29	2	0.62	2.15
O_2							-0.28	1.84		
NH_4										
NO_2			0.19	1.25			-0.23	1.22		
NO_3	-0.17	2.09	0.34	2.96	0.27	1.19			-0.56	1.53
PO_4										
SO_4	-0.09	1.39								
Cl	-0.17	2.55			-0.14	1.13	0.15	1.43		
Cu										
Zn										
TRZ			0.14	1.91			0.33	1.45		
PAH			-0.4	3.69			-0.3	1.33	-0.15	1.11
OCL										
OP					-0.56	4.32	-0.28	1.31		
APE									-0.15	1.64
FHI	0.12	1.79	na	na	na	na	na	na	na	na
QBR	0.14	2	na	na	na	na	na	na	na	na

Abbreviations are explained in Table 3.1. For clarity only the coefficients having a VIP score greater than 1 are depicted, na, not applicable.

Additionally, the riparian habitat quality (QBR, FHI Table 3.2) of middle and downstream locations were deteriorated substantially due to anthropogenic effects on the nearby

riparian forest and the use of dams and wells to canalize the river (Prat and Munné , 2000; Munne et al., 2003).

Dissolved levels of Cu and Zn found across the studied stations were relatively low and constant between upstream and downstream reaches, thus indicating that urban and industrial STPs contributed little to these trace metal levels (Puig et al., 1999; Fernandez-Turiel et al., 2003). Conversely, most organic residue levels increased dramatically towards downstream locations, in particular in those sites located near effluent discharges from STPs, thus supporting previous studies which indicate that effluent discharges are the major source of organic pollution in these rivers (Teixido et al., 2001). In particular pesticides with a relatively high environmental persistence (TRZ, OCL such as lindane, alachlor; Rivera et al., 1986) or/and broadly used to control pests in agriculture (OP such as diazinon and chlorpyrifos; Barata et al., 2007) increased dramatically from upper to downstream sites.

Evaluation of biological effects and causal relationships

According to Prat and Munné (2000), the IBMWP, IASTP scores and taxon richness values obtained for the benthic macroinvertebrate community inhabiting the studied sites indicate a good ecological status for upper reaches except for site B1 in April and a poor ecological state for middle and downstream reaches (Table 3.2). PCA and PLS analysis showed that there was a clear geographic water deterioration trend from upstream to downstream affecting the quality of benthic macroinvertebrate communities (Fig. 3.3), with levels of conductivity related compounds (CL and SO4), habitat deterioration and NO_3 contributing negatively to their quality (Table 3.4).

D. magna in situ bioassay responses depicted in Fig. 3.2 did not always follow the observed detrimental effects on benthic communities. Post-exposure feeding rates were mainly affected negatively by PAHs and positively by nitrogen sources (NO_3, NO_2), although T, SS, and TRZ also contributed marginally to the observed responses. There is reported evidence that PAHs inhibit the feeding rates of *Daphnia* at lower concentrations than those impairing survival (Barata and Baird, 2000). Furthermore, environmental variables such as UV radiation, which was likely to be maximal in summer in the studied rivers owing to longer days and reduced flow rates, and hence water column depths, may increase the toxicity of PAHs several orders of magnitude (Hatch and Burton, 1999;

Nikkila et al., 1999). Therefore, the estimated lethal toxicity of PAHs measured in the studied river sites (Table 3.3), although low (<0.001 TU) may affect feeding rates of *Daphnia* in field situations where UV radiation is high. In our study, feeding rates were mostly affected in summer and in upstream locations characterized by their low content of suspended solids, and hence with relative transparent waters and high UV transmittance. Therefore, the combination of elevated levels of UV radiation and low PAH concentrations might explain the observed feeding rate inhibition responses.

Among the other reported environmental variables affecting feeding, it is interesting to consider the apparently positive effects of the main river nitrogenous sources, which may be related with the presence of toxic cyanobacteria. It is generally known that cyanobacteria impair *Daphnia* feeding (DeMott, 1991) and previous studies suggested that in Llobregat river the occurrence of cyanobacteria was directly related to limiting concentrations of nitrogen relatively to phosphorus (Aboal et al., 2002; Vilalta et al., 2001). Therefore, it is plausible to consider that the presence of cyanobacterial blooms under limiting N conditions could have contributed to the observed feeding responses. Negative effects of T may be related to the negative contribution of sites L5 and L6 on feeding, which received cold water from the hypolimnion of La Baells water reservoir (Fig. 3.2). For levels of SS and TRZ, however, the reported values in this present study (Table 3.1) were in most deployments within the reported range with no effects on post-exposure *Daphnia* feeding rates (Dodson et al., 1999; Mc William and Baird, 2002b; Barata et al., 2007). Therefore, it is plausible to consider that other non-measured environmental factors could affect the observed behavioral responses.

According to Barata et al. (2004, 2005b, 2007) the studied enzymatic activities can provide us information of particular compounds causing toxic effects. For OP and carbamate pesticides, AChE constitutes one of the best prognostic biomarkers to monitor biological effects in exposed biota (Barata et al., 2004). Many different contaminants (i.e. hydrocarbon quinones, nitro-aromatics, organochlorine compounds, polycyclic aromatic hydrocarbons, metals) and environmental factors (UV radiation, O_2) may affect free radical processes and hence may alter free radical scavenging systems such as antioxidant and GST enzyme activities (Livingstone, 2001). In *Daphnia*, AChE and antioxidant enzyme responses have been used successfully in laboratory as prognostic and specific indices of toxicity effects for many compounds (Barata et al., 2004, 2005b). In this study PLS

regression results showed that inhibition patterns of AChE of *D. magna* individuals deployed across the Llobregat and Besós river basins could be explained by the measured OP levels in water, which according to previous results should inhibit AChE activities (Barata et al., 2004, 2007). The other environmental factors affecting AChE (SS, conductivity, levels of CL and of NO_3) contributed only marginally to the observed variation (Table 3.4).

For the other two studied enzymes, observed inhibition and induction of activity patterns precluded the identification of a single causal relationship. For example, higher catalase and/or GST activities indicating enhanced antioxidant and phase II detoxification metabolism, respectively, were found in the upstream (L2, L5), middle (L7, B4, B5) and downstream (B8, L9, L10) stations. Alternatively, lower activities were also observed in upstream (L5, B3), middle (L6, L8) and downstream (B6) reaches. PLS analysis showed that the variation of both responses was affected negatively by conductivity per se or/and its related major ions (Cl). Barata et al. (2005a) studying antioxidant enzyme responses in the trichopteran *Hydropsyche exocellata* collected across the Llobregat river also found a positive relationship between conductivity and catalase and GST activities thus suggesting that salinization may be an environmental factor of concern.

The contributing effect of other environmental parameters such as T, O_2, NO_2 or NO_3 and residue levels of TRZ, PAH, OP and APE, on the activities of catalase and GST have to be considered with caution since they may have specific site effects. For example, the effect of temperature was likely to be related to the quite low catalase and high GST activities found in transplanted *D. magna* in site L5 (Fig. 3.2), which also received cold water from the hypolimnion of La Baells reservoir (Prat et al., 1984). In sites L8 and L9, the presence of contaminant residues coming from nearby upstream effluents of STPs from Navarcles and Manresa cities, respectively, may explain the observed high GST detoxification levels. Of special interest are deployed individuals from station L9 that showed enhanced levels in both antioxidant (catalase) and detoxification (GST) enzyme activities, thus suggesting the occurrence of biologically active compounds. In this site, there is evidence for the presence of relatively high amounts of estrogens that may be disrupting the sexual endocrine systemof fish populations (Solé et al., 2000; Fernandes et al., 2002).

CONCLUSIONS

In summary, our results lend positive support to the use of biological indices in combination with *D. magna* in situ bioassays to assess effects and identify environmentally detrimental factors in a better way within a complex multi-stressed river systems in the field, thus contributing to a more realistic assessment of ecological risks. Furthermore, the observed effects of OP in *D. magna* in situ bioassays across the studied rivers were also reported in the Ebro river (Barata et al., 2007), thus indicating that these highly active substances should be controlled and if possible their use minimized in the near future. Contaminant residues of PAHs should also be considered since they affected negatively three out of the four *D. magna* in situ bioassay responses measured. Nevertheless, it is important to consider that, while the approach used in this study drives us closer towards the "in situ environmental hazard identification evaluation", issues arising from other confounding factors influencing the responses of macroinvertebrate and *D.magna* in situ bioassays still should be considered with caution in the interpretation of such findings as conclusive diagnostic proofs of individual factors causing effects.

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Chapter 4

Identifying major pesticides affecting bivalve species exposed to agricultural pollution using multi-biomarker and multivariate methods

4. Identifying major pesticides affecting bivalve species exposed to agricultural pollution using multi-biomarker and multivariate methods

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ABSTRACT

The aim of this investigation was to identify major pesticides that may cause detrimental effects in bivalve species affected by agricultural pollution. Investigations were carried out using freshwater clams (*Corbicula fluminea*) transplanted in the main drainage channels that collect the effluents coming from agriculture fields in the Ebro Delta (NE Spain) during the main growing season of rice (from May to August). Environmental hazards were assessed by measuring simultaneous up 46 contaminant levels and 9 biomarker responses. Measured biological responses showed marked differences across sites and months. Antioxidant and esterase enzyme responses were in most cases inhibited. Lipid peroxidation levels increased steadily from May in upstream stations to August in drainage channels. Principal Component (PCA) and Partial Least Squares to Latent Structure regression (PLS) analyses allowed the identification of endosulfan, propanil, and phenylureas as being the chemical contaminants causing the most adverse effects in the studied species.

KEYWORDS: Pesticide, Clam, *Corbicula fluminea*, Biomarker, Oxidative stress, Cholinesterase

INTRODUCTION

The simultaneous use of wetlands and bays for agriculture and mariculture production, respectively, is a common phenomenon (Feldman et al. 2000). Under this scenario shellfish species inhabiting or cultured in the bays may become increasingly threatened by exposure to pollutants associated to agriculture (Feldman et al. 2000). Among the available techniques to measure effects, the integrated use of chemical analyses and biochemical and cellular responses to pollutants is a sound procedure for detecting impact of anthropogenic contaminants in freshwater and marine systems. Moreover, since in real field situations aquatic organisms are currently being exposed to multiple chemical and environmental stressors with different mechanisms of toxicity, each contributing to a final overall adverse effect, the use of a large set of biochemical responses may allow us to identify contaminants that might be hazardous in the field. This approach has been successfully used in studying bivalve marine mollusks (Livingstone 2001) and include biochemical responses that are related to the metabolism and toxicity modes of action of contaminants.

Most pesticides currently used in developed countries have low environmental persistence, do not accumulate in great amounts in organisms, and suffer a strong dilution in coastal areas (Santos et al. 2000). Therefore, coastal bivalve species are usually exposed to low concentrations of pesticides when compared to freshwater organisms (Terrado et al. 2007). Furthermore, other contaminants with greater potential to bioaccumulate, naturally occurring stressors and additional physiological factors may also affect the measured bivalve responses (Vidal et al. 2002a, b). Accordingly, it is difficult to establish specific pesticide–effect relationships in field collected coastal bivalve mollusks. A plausible alternative, however, is to use transplanted bivalve species deployed close to the major pesticide sources in the studied coastal locations. The use of transplanted freshwater bivalve species can be particularly useful to identify potential environmental pollutants in coastal locations affected by contaminants coming from freshwater sources. This is particularly important in confined coastal areas (bays) affected by pesticides coming from nearby rice fields or rivers (Barata et al. 2007).

This study is intended to assess environmental hazards of pesticides used for rice farming in Delta del Ebro (NE, Spain). This is an area of 320 km^2 in the NW

Mediterranean sea whose natural aquatic resources receive large amounts of pesticides due to the cultivation of rice (Terrado et al. 2007). The coexistence of intense mariculture activities in Delta del Ebro bays makes necessary the developing of effective monitoring tools to assess exposure of nontarget organisms to operationally used insecticides. Most studies conducted in this area in bivalve mollusks have been limited to the assessment of levels and effects of persistent organic pollutants and organophosphorous pesticides a decade ago (Escartín and Porte 1997; Morcillo et al. 1999; Solé et al. 2000; Porte et al. 2001). At present time, however, despite that the use of organophosphate insecticides have been reduced drastically, herbicides are used in large amounts (Terrado et al. 2007; Kuster et al. 2008), and episodic massive mortalities of shellfish cultured species still occur in Delta del Ebro associated bays (Ramón et al. 2005). Indeed, recently, using feeding and biomarker responses in transplanted *Daphnia magna*, Barata et al. (2007) found a good relationship between major herbicide residue levels in water and the measured toxic effects. Within this context, this study was designed to integrate chemical analyses and biochemical effects of major pesticides and of other contaminant residues present in Delta del Ebro channels and bays in transplanted Asiatic clams (*Corbicula fluminea*). This study was conducted during the major usage of pesticides associated with rice farming. In this area rice farming involves the use of over 20,000 tons per year of up to 25 agrochemicals to treat pests with triazines, phenylureas, anilines, and organophosphates being the compounds of broader use (Mañosa et al. 2001; Kuster et al. 2008).

Asiatic clams have been used extensively as sentinel species to monitor residue levels and biological effects of pollutants (Doherty 1990; Vidal et al. 2002a). In Ebro river, the Asiatic clam is specially abundant in its lower part including the freshwater habitats of its delta, thus constitute an excellent organism to be used to monitor pesticide effects in rice fields.

Contaminant analyses included the assessment of (a) residue levels in whole clams of organochlorine pesticide residues, alkylphenols, polycyclic aromatic hydrocarbons, and metals due to their close association with agricultural and industrial activities occurring upstream in Ebro river and their great potential to accumulate in organisms (Lacorte et al. 2006); (b) residue levels in water of major herbicides and insecticides found in Delta del Ebro during the main growing season of rice (Kuster et al. 2008). Herbicide and organophosphorous levels were not analyzed in bivalve species due to their low bio-

concentration potential (Santos et al. 2000). Biomarkers included the phase II glutatione S-transferase activity (GST), antioxidant enzymes involved in detoxifying reactive oxygen species such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and markers of oxidative tissue damage (lipid peroxidation) (Halliwell and Gutteridge 1999). Finally the activity of B esterases was also measured to diagnose exposure to organophosphorous pesticides among other chemicals (Mora et al. 1999).

MATERIAL AND METHODS

Study site

This study was conducted in the Delta del Ebro, which is located at the end of the largest river in Spain ‘‘The Ebro river’’ (Fig. 4.1a). It holds 21,600 Ha of rice fields producing 113,500 Tons of rice per year (Fig. 4.1b). It has a triangular shape going some 20 km into the sea (Fig. 4.1b), with one coastal split in the north and one in the south that close two shallow bays of capital importance for fish and mussel production (Mañosa et al. 2001). Two main channels, one on each side of the river, bring the water from Xerta, some 30 km upstream to the rice cultivation system. From these two channels, water is carried to and collected from the rice fields by a network of irrigation and drainage ditches, respectively. Three big drainage channels in either site of the hemi-delta collect all water and drive it straight to the sea.

Transplants were conducted at four sites of coordinates 40º55′26.6″ N, 0º29′36.3″ E for c1; 40º37′54.4″ N, 0º38′50.6″ E for c2; 40º38′49.6″ N, 0º42′26.2″ E for c3; 40º38′59.3″ N, 0º45′35.6″ E for c4. Locations c2, c3, c4 (Fig. 4.1c) are situated at the end of three of the main drainage channels that collect and transport the water from the south hemi-delta rice fields into one of the sea lagoons. An additional site upstream of the Delta del Ebro just before the collection of water into the main channel that irrigates the rice fields were also included as surrogate control (site c1, Fig. 4.1b). By selecting the main drainage channels instead of individual rice fields it was possible to detect and evaluate the effects of most pesticide residues that are applied in the rice fields. Although organisms at location c1 are likely to be exposed to Ebro river contaminants, it is an ideal reference site for assessing Ebro Delta rice field mediated effects on clams due to its situation, upstream the delta (Barata et al. 2007).

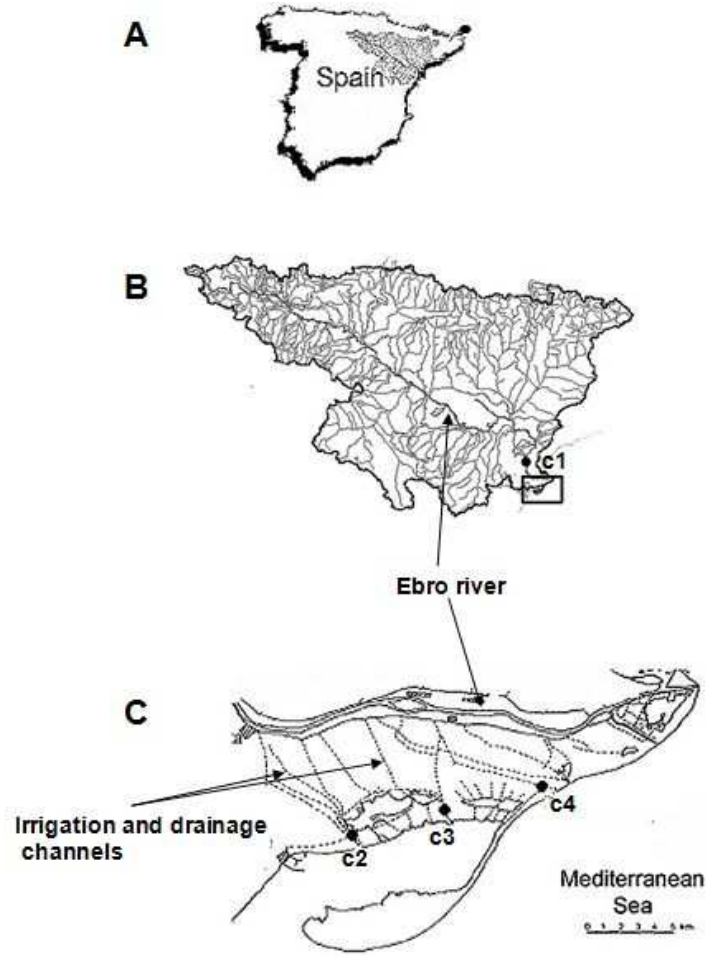

Fig. 4.1. Studied sites showing the location of the Ebro river basin (**a**), that of Delta del Ebro with site c1 (**b**) and that of sites c2, c3, c4 in the south hemi-delta (**c**).

Experimental animals and transplants

From 150 to 100 clams of size (mean ± 1 SD) 1.78 ± 0.12 cm were transplanted in specially designed stained steel cylindrical cages (20 × 50 cm) with a mesh of 0.5 cm that were suspended 1 m above the river/channel bottom. Deployments lasted 15 days and were initiated each month with clams collected from the river bed near site c1 and subsequently transplanted at the four sites. Just after collection, gills and digestive glands of 30 clams were dissected and frozen in liquid nitrogen for biochemical determinations. Due to sample constrains tissue pools of three individuals were used. Additional pools of 50 whole clams were also collected at each sampling and frozen at -20 ºC to determine metals and organochlorine residue levels.

Physicochemical water parameters

At each site at the start and the end of the exposure period or when collecting clams from the field, water temperature, conductivity or salinity and oxygen levels were measured using a WTW Multi 340i handheld meter. At the end of exposures, water samples (1 l) were also collected and stored at 4 ºC to determine pesticide residue levels of the 17 most abundant compounds in water (Kuster et al. 2008).

Metallic and persistent organic pollutants in soft tissues of clams were determined in freeze dried and homogenized whole samples. Levels of Zn, Cu, Cd, Hg, As were determined in acid digested samples by Perkin Elmer model Elan 6000 inductively coupled plasma mass spectrometer (ICP-MS) following Barata et al. (2005) methods. Samples of similar weight of a certified reference material of dogfish muscle (NRCDORM-2; LGC Promochem, Middlesex, UK) were digested during each analytical run. Detection and quantification limits were calculated from blank measurements and varied across metals being these values 0.003–2 and 0.003–3 µg/g, respectively.

Organic contaminants were extracted and analyzed using a pressurized liquid extraction (PLE) system and GC/MS analysis following Lacorte et al. (2006). GC/MS analysis was performed with a gas chromatograph Thermo Electron (San Jose, CA USA) model Trace 2000 coupled to a mass spectrometer from Thermo Electron. The mass spectrometer was operated in the electron impact ionization mode with an ionizing energy of 70 eV. Compound separation was achieved using a capillary column HP-5MS of 30 m × 0.25 mm i.d. with 0.25 µm film thickness from J&W Scientific (Folsom, CA USA). Acquisition was achieved in time scheduled Selected Ion Monitoring (SIM) mode to increase sensitivity and selectivity. Identification and internal standard ion quantification were carried out automatically by the Xcalibur software. The quantification of blanks within each batch of analyzed samples was used in order to calculate the limits of detection (LOD).

Biochemical determinations

Most biochemical determinations have been described and characterized previously (Faria et al. 2009). Hereafter we only provide a brief description. Clam digestive glands and gills were homogenized in a 1:5 weight: volume proportion in ice cold phosphate buffer 100 mM, pH 7.4 at 4 ºC, containing 100 mM KCl and 1 mM EDTA. Homogenates

were further centrifuged at 10,000×g for 30 min and supernatants were immediately used for biochemical determinations. Supernatant proteins were measured by the Bradford method (Bradford, 1976) using bovine serum albumin as standard. In the digestive gland the following biomarkers were measured: the activities of SOD, CAT, Total (GPX Tot) and Se dependent glutathione peroxidase (GPX Se) and GST toward 1-chloro-2,4-dinitrobenzene; and lipid peroxidation levels (LPO). Esterase activities were determined in the gills according to Mora et al. (1999). Acetylthiocoline iodide (2 mM), propionylthyocoline chloride (2 mM) and S-phenyl thioacetate (3 mM) were used as substrates for acetyl-(AChE), propionylesterase (PChE) and carboxylesterase (CbE) enzymes, respectively. The final results for enzymatic activities were normalized by protein content whereas those of LPO by wet weight.

Data analyses

Data were log transformed to meet the assumptions of normality and variance homocedasticity (Zar 1996); and analyzed using two or one way ANOVA followed by post hoc Tukey's multiple comparison tests.

For the exploration of the relationships between the studied environmental variables and the biological responses, Principal Component Analysis (PCA) and Partial Least Square To Latent Structure regression (PLS) methods were used (Wold et al. 2001). PCA was used to investigate the existing relationships between variables. PLS is a regression extension of PCA, which is used to correlate the information between two blocks of variables by a linear multivariate model. PLS models can be expressed in terms of traditional regression coefficients, b, of the multilinear model (i.e., $y = X\ b$ model). However, information about the correlation structure among variables and responses may be lost by direct inspection of these regression coefficients b, and for interpretative purposes it is better to study either PLS individual weights w, or other measures from them derived, like the variable influence (importance) on projection, vip, parameters. This parameter is a weighted sum of squares of PLS weights taking into account the amount of explained Y variance and it summarizes the information content of all latent and X variables (Wold et al. 2001).

To reduce the number of environmental variables and avoid an excessive occurrence of undetected or very low contaminant levels across stations, the number of organic

compounds measured in water (Kuster et al. 2008) and organisms (see methods) were reduced from 46 to only 11 categories: organophosphates (OPs; fenitrothion and diazinon), triazines (atrazine and simazine), phenylureas (isoproturon and diazinon), propanil, acidic herbicides (MCPA, bentazone, 2,4-D, mecoprop), molinate, polycyclic aromatic hydrocarbons (PAHs, 16 compounds), alkylphenols (APEs, nonyl and octylphenol), DDTs (6 compounds), endosulfan (ENDO, 3 compounds), hexachlorobenzene (HCB). Furthermore, below detection limit values were converted to LOD/2.

PCA was conducted using a data matrix of 16 samples (rows) and 27 variables (columns) (see Appendix in Electronic Supporting Information). SOD was excluded from the analyses provided that no significant differences were observed across samples. Since variables were very different and they were not measured using the same scale units, the data was auto-scaled prior to analysis (each element was subtracted by its column mean and divided by the standard deviation of its column). PLS analysis were performed considering 19 environmental parameters as *X* block independent variables, and considering as *y* block dependent variables each one of the following: GST, CAT, GPX Se, GPX Tot, CbE, AChE, PChE, LPO. The number of PCA and PLS components was finally selected according to cross-validation leaving one out prediction errors criteria (Wold et al. 2001). Multivariate analyses were conducted using the Matlab 6.0 software (MathWorks, Natick, Massachusetts).

RESULTS AND DISCUSSION

Water quality and contaminant levels

Water quality parameters and contaminant levels were averaged across months (Table 4.1). The full set of data is depicted in the Appendix (Electronic Supporting Information). Physicochemical water parameters measured in the upstream, drainage channel sites and at the bay were directly related with the geological and hydrological dynamics of the delta and Ebro river. High conductivity values were measured in the drainage channels indicating an increasing salinization toward the east end of the delta (from c2 to c4). Most freshwater habitats of the delta are at intertidal level, thus the water of drainage channels is pumped into the sea when required and water can reach moderate to relatively high conductivity levels depending on the influx of sea water and the outflux

Table 4.1. Mean, minimum or LOD, and maximum of environmental parameters (n = 40-60) and contaminant levels measured in water, transplanted clams (*C. fluminea*) at the four studied sites during May to August 2005.

	c 1			c 2			c 3			c 4		
	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Water												
Oxygen	84.7	67.5	105.5	79.1	65.0	94.1	74.5	72.0	77.0	76.8	63.0	94.0
T	22.7	20.4	24.7	23.3	20.9	25.5	23.3	20.9	25.5	23.3	20.9	25.5
Cond / Sal	939	769	1091	1524	1375	1887	2045	1413	2980	3364	2675	4250
Pesticides in water (ng/l)												
Ac Herb	403.9	364.1	439.2	34913.9	3009.8	71034.9	43136.8	10401.6	84504.5	72372.6	18331.0	128824.8
Triazines	697.1	522.7	935.1	400.2	167.1	826.8	304.5	179.4	440.6	271.4	97.8	408.8
Phenylureas	19.7	5.5	33.6	14.5	5.6	23.5	18.1	<1.6	27.3	11.9	<1.6	19.1
Propanil	1.5	<0.4	1.5	387.5	1.8	1344.6	1756.8	13.0	4680.3	375.2	2.5	1195.2
Molinate	<2.7	<2.7	<2.7	317.9	108.7	613.3	271.2	113.3	621.9	256.0	134.1	485.2
OPs	0.6	0.3	0.8	143.2	0.3	398.2	267.5	0.3	588.4	70.6	<0.1	207.1
Metals in animals (µg/g)												
Zn	185	167	216	159	147	179	184	158	214	170	149	202
Cu	70	48	89	53	38	83	74	49	120	61	47	81
Cd	0.7	0.5	0.9	0.4	0.3	0.6	0.7	0.6	0.8	0.6	0.5	0.7
Hg	3.0	1.8	3.9	1.9	1.2	3.3	2.6	1.6	4.7	2.2	1.6	2.9
As	6.1	5.7	6.5	6.2	5.4	7.4	7.6	7.3	7.8	7.3	6.4	8.0
Organic contaminants in animals (ng/g)												
PAHs	131.63	57.45	244.22	129.36	62.16	231.24	87.59	64.50	120.50	109.70	51.75	171.07
APEs	1221	745	1868	940	304	1898	839	656	950	697	435	1150
DDTs	1012	467	1426	579	302	855	677	356	1242	707	319	1151
ENDO	8	<1.92	9	378	<1.92	569	48	<1.92	88	25	<1.92	37
HCB	26	6	44	16	<1.03	39	8	<1.03	15	8	<1.03	11

Oxygen, temperature (*T*), conductivity (Cond), pesticides in water, metals and organic contaminants in soft tissues are reported as % saturation, ºC, µS/cm, ng/l, µg/g d.w., ng/g d.w., respectively. The full data set is in the Appendix of the Electronic Supporting Information.

of freshwater from the river Ebro (Mañosa et al. 2001). The moderated saturation levels of oxygen in some drainage channel sites were directly related to the low water exchange regimen of drainage channels and associated bay (Mañosa et al. 2001).

There were distinct contaminant patterns between residues of pesticides in water and those of contaminants measured in organisms (Table 4.1). Pesticide residue groups in water depicted in Table 4.1 support previous studies evidencing that acidic herbicides (bentazone, MCPA), propanil, and molinate were used in great amounts for weed control in the Ebro delta (Mañosa et al. 2001), reaching quite high levels in drainage channels (Terrado et al. 2007). Organophosphorous pesticides (mainly fenitrothion, Kuster et al. 2008), despite of the low environmental persistence in the natural conditions occurring in the delta, were present in drainage channels, reaching levels near 0.6 µg/l in water. Other compounds including triazines and phenylureas accounted for only 1–2% of the total herbicides in the Delta del Ebro (Kuster et al. 2008). On the other hand, triazine pesticide residues reached greater levels in Ebro river than in delta channels, thus supporting previous results that also indicated that these herbicides were also used upstream in the lower part of the Ebro river (Terrado et al. 2007). Contaminant concentrations of most compounds in transplanted clams did not vary more than two fold across sites (Table 4.1). The exception being the high endosulfan levels detected in clams transplanted in site c2 during May and June.

In several species of freshwater mussels and in the Asiatic Clam (*Corbicula* sp.) from polluted rivers of North America and Spain, levels as high as 9, 7, 7, 100, and 1,000 µg/g dw of Cd, As, Hg, Zn, Cu, respectively; and of 50, 100, 400 ng/g ww of DDT, endosulfan, PAHs, respectively, have been reported (Doherty 1990; Oros and Ross 2005; Angelo et al. 2007; Carrasco et al. 2008). Accordingly in this study measured contaminant levels in Asiatic clams could be considered moderate or high when compared to other freshwater locations.

Biological responses

Percentage mortality in transplanted clams across deployments was low in c1 (Mean ± SD; 4.5 ± 2.4%) but increased marginally ($P > 0.05$, Kruskal–Wallis test) in drainage channel sites (c2, 9.3 ± 6.1%; c3, 13.0 ± 10.4%; c4, 20.6 ± 16.8%). Enzymatic activities and levels of lipid peroxidation can be used as fast and specific biological responses to

identify compounds causing toxic effects in individuals (Porte et al. 2001). The response of most of the studied biomarkers varied across sites or/and months (Table 4.2). Eight out of nine biomarkers showed significant differences among sites within and/or across months (Fig. 4.2, Site, Month, and Interaction effects in Table 4.2). The activities or levels of GST, antioxidant enzymes (CAT, GPX) and of lipid peroxidation were quite constant in clams deployed at c1, but showed different patterns in clams transplanted in drainage channels across the studied months (Fig. 4.2). For example, GST activities were lower in clams transplanted at c2, c3, c4 from May to July, those of CAT at c3 and c4 during June and those of GPX Tot at sites c2, c3, c4 in June (Fig. 4.2). Conversely, lipid peroxidation levels increased in clams transplanted at stations c2, c3, c4 during May and except at station c4, also in June. B esterase activities of transplanted clams also varied among sites and months showing the greatest inhibition effects during July and August (Fig. 4.2).

PCA and PLS

PCA defined five interpretable components that explained 83.4% of data variance. Bi-plots of variable loadings and site scores from PCA are depicted in Fig. 4.3. The first component accounted for 32.8% of data variance, had high positive loadings for levels of LPO, PChE, AChE, phenylureas, propanil, organophosphorous, and organochlorine pesticides; and negative loadings for most antioxidant enzyme responses, Hg, Cu and temperature (T). As a result, this first component discriminated samples from May and June from those of July and August. The second axis (PC2), which explained 21.6% of data variance, differentiated the reference site c1 from those located in drainage channels mainly due the positive contribution of triazines, APEs, oxygen levels and the negative loadings of acidic herbicides, molinate, As and conductivity. A further characterization of the main sources that modulate the observed biological responses was conducted using a PLS regression analysis. Only PLS models that explained more than 50%, of the total variances of the studied biological responses of transplanted clams are depicted in Table 4.3. The number of PLS components selected was two. For brevity in Table 4.3, the PLS regression coefficients and Vip scores, which measure the relative contributions of the environmental variables in explaining the variance of the selected biological responses, were depicted. Higher Vip scores show what variables are more important (influential) in the regression model. The threshold Vip value used in Table 4.3 was the unity. Vip scores

below one were not shown in this table because they were not considered important for the corresponding latent variable (Wold et al. 1993).

Table 4.2. Two way ANOVA results for biomarker responses of transplanted clams across sites and months.

	Factor	df	*F*	*P*
GST	Site	3	1.32	0.28
	Month	3	18.54	<0.01
	Interaction	9	3.01	<0.01
	Error	63		
SOD	Site	3	0.57	0.63
	Month	3	1.83	0.15
	Interaction	9	0.67	0.73
	Error	63		
CAT	Site	3	8.69	<0.01
	Month	3	8.00	<0.01
	Interaction	9	3.18	<0.01
	Error	63		
GPX Se	Site	3	1.50	0.22
	Month	3	7.33	<0.01
	Interaction	9	1.06	0.41
	Error	63		
GPX Tot	Site	3	1.08	0.37
	Month	3	15.41	<0.01
	Interaction	9	1.58	0.14
	Error	63		
AChE	Site	3	2.15	0.10
	Month	3	35.15	<0.01
	Interaction	9	2.47	0.02
	Error	64		
PChE	Site	3	0.43	0.74
	Month	3	62.93	<0.01
	Interaction	9	6.17	<0.01
	Error	64		
CbE	Site	3	4.25	0.01
	Month	3	21.64	<0.01
	Interaction	9	4.89	<0.01
	Error	64		
LPO	Site	3	17.97	<0.01
	Month	3	52.56	<0.01
	Interaction	9	18.84	<0.01
	Error	62		

df, degrees of freedom; *F*, Fisher's coefficients, and significance levels (*P*) are shown. For further information see text.

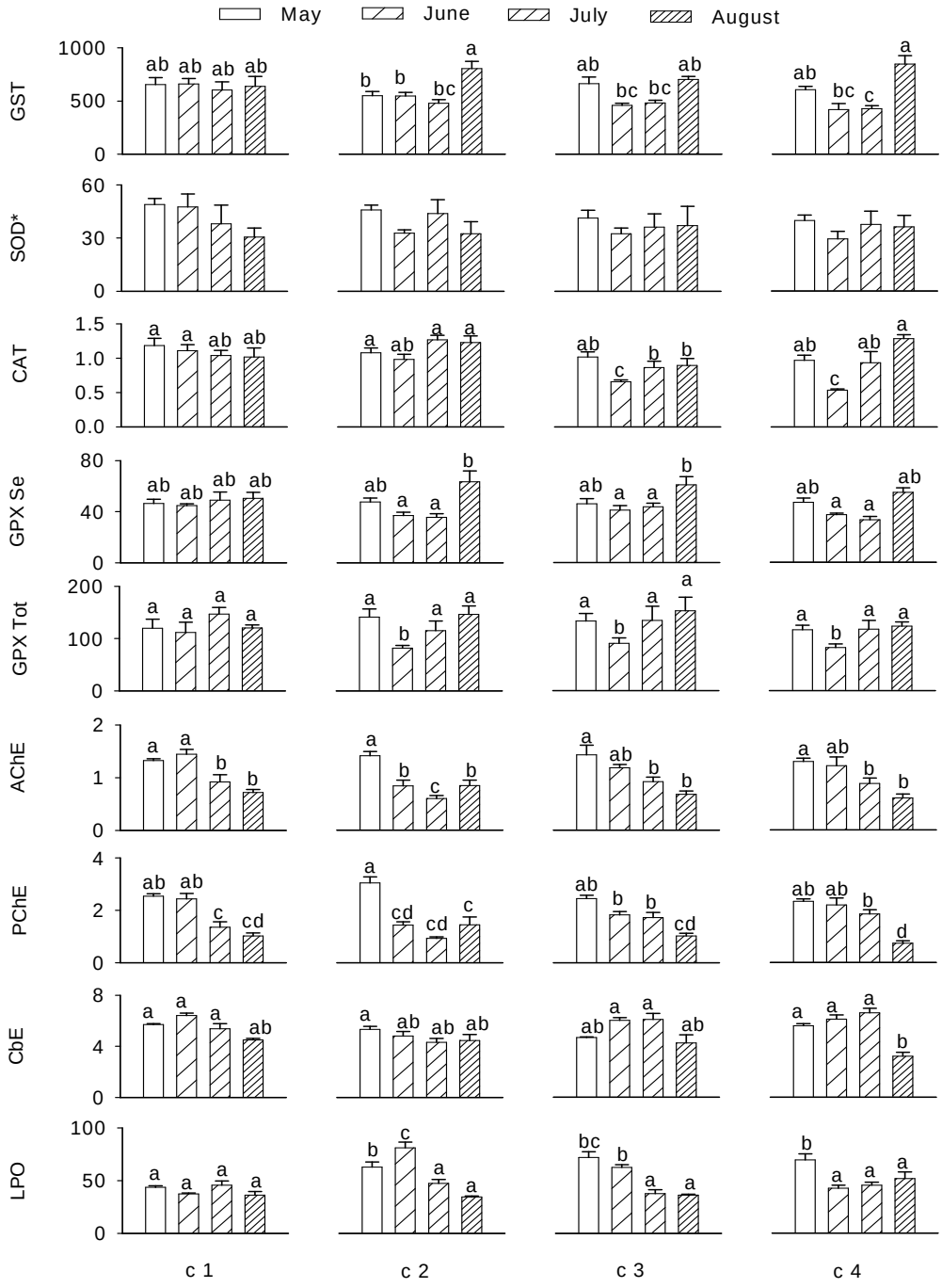

Fig. 4.2. Levels and activities (Mean ± SE, n = 5) of biomarkers in transplanted Asiatic clams (*Corbicula fuminea*) across the studied sites and months in Ebro Delta. *Different letters* indicate significant ($P < 0.05$) differences across sites and months following ANOVA and Tukey's post hoc tests. Units for SOD, CAT and LPO were U/mg protein, mmol/min/mg protein and nmol MDA/g w.w., respectively. The other enzyme activities were expressed in nmol/min/mg prot. * no letters shown due to the lack of significant ($P < 0.05$) differences.

According to these PLS parameters in Table 4.3, the environmental parameters that affected most biological responses and had higher effects (high Vip scores) were endosulfan, DDTs, HCB, APEs, Cu and Hg concentration levels in organisms and the concentrations of the acidic herbicides, propanil, phenylureas, and temperature in water. PLS results also revealed the presence of additive or antagonistic effects among environmental parameters (equal or different signs of the regression coefficients, respectively). For example GST activities were negatively correlated to endosulfan, acidic herbicides and temperature and were positively correlated with Hg and PAHs. CAT activities only showed negative relationships. GPX Tot and GPX Se activities despite of being affected by different factors shared in common their negative relationship with levels of acidic herbicides and endosulfan. AChE and PChE activities showed a different pattern, being negatively correlated to temperature, Cu and Hg and positively affected by the rest of parameters (Table 4.3). Levels of LPO were positively correlated to the concentrations of the major pesticides including phenylureas, propanil, and endosulfan with negative contributions of Hg and Cu.

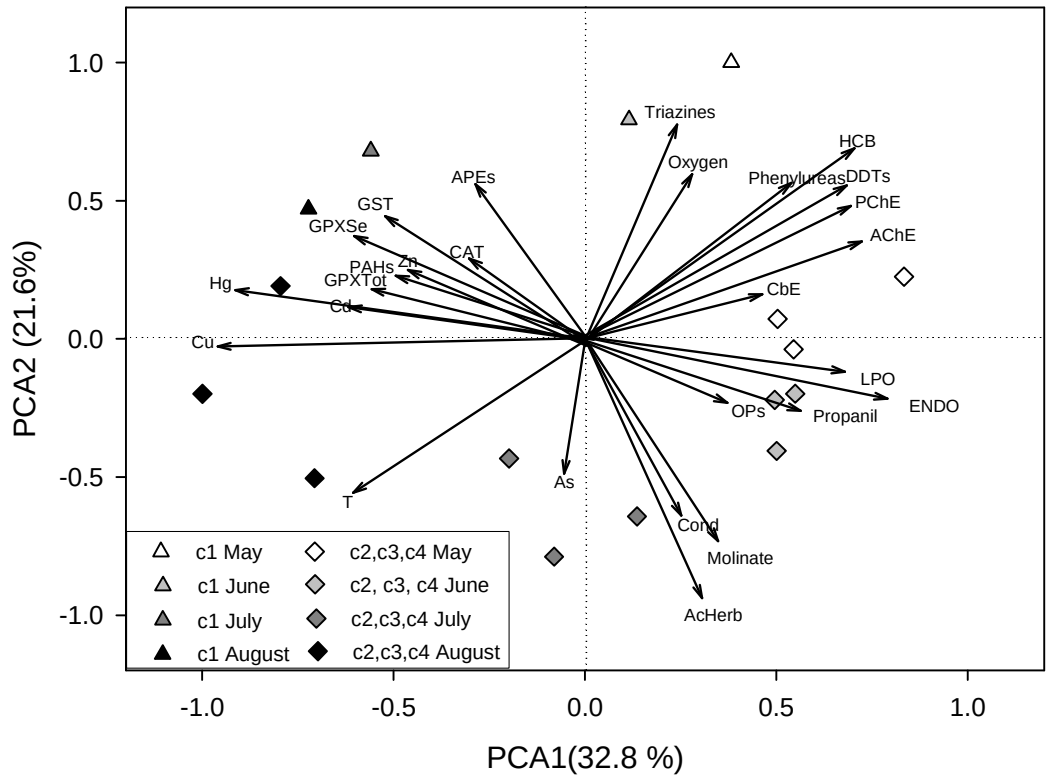

Fig. 4.3. Variable loadings (*arrows*) and site scores of the fist two Principal Components obtained for the studied biological and environmental variables across the studied sites and months in Ebro Delta. Abbreviations are explained in the text. Explained variance of each PCA component is also depicted

Table 4.3. Regression vector standardized PLS partial coefficients (reg) and vip scores (Vip) of the environmental variables for the regression models obtained for the prediction of the activities and levels of the studied biomarkers in transplanted clams across sites and months in Ebro Delta channels and bays.

	GST		CAT		GPX Tot		GPX Se		AChE		PChE		LPO	
	Reg	Vip	Reg	Vip	Reg	Vip	Reg	Vip	Reg	Vip	Reg	Vip	Reg	Vip
T	-0.41	3.48					-0.27	2.05	-0.19	2.55	-0.16	2.13		
Cond			-0.23	2.36										
Ac Herb	-0.32	2.93	-0.23	2.27	-0.23	1.51	-0.20	1.69						
Triazines					-0.21	1.12								
Phenylureas									0.11	1.49	0.12	1.48	0.24	1.79
Propanil			-0.25	2.69					0.11	1.03	0.09	1.02	0.14	1.52
OPs					0.39	3.65								
Zn					0.18	1.23								
Cu					0.18	1.78	0.17	1.87	-0.09	1.64	-0.10	1.89	-0.10	1.99
Cd			-0.22	1.42	0.14	1.10								
Hg	0.09	1.10			0.09	1.16	0.16	1.95	-0.05	1.08	-0.07	1.40	-0.14	2.25
As			-0.17	1.29										
PAHs	0.28	2.00					0.24	2.14						
APEs			-0.22	1.46										
DDTs			-0.23	1.70	-0.22	1.62			0.21	3.06	0.19	2.54		
ENDO	-0.22	1.85			-0.14	1.35	-0.16	1.76			0.06	1.26	0.18	2.34
HCB									0.20	2.86	0.19	2.59		
PLS VAR(%)	74.0		74.4		79.2		73.2		80.1		74.7		65.4	

Abbreviations are given in Table 4.1 and explained in the text. For clarity only the coefficients having vip scores greater than 1 are given. The percentage of variance of the *y* variable explained by PLS models (VAR) is shown.

Environmental hazard assessment

In this study a biological interpretation of site and monthly differences in the studied biological responses with those of physicochemical parameters may allow identifying potential hazardous factors to clams. Inhibition of B esterases are considered specific markers of exposure to organophosphorous and carbamate compounds and in *C. fluminea* have been well characterized and used to monitor pesticide usage in the field (Mora et al. 1999). The activities of cholinesterases measured in gills of clams decreased to a greater extent from May to August than were affected by site. PCA and PLS results indicated that water temperature, Cu, and Hg were inversely related with PChE and AChE activities. Conversely, bio-accumulated organochlorine pesticides and levels of some pesticides in water were positively related with activities of PChE and AChE (Table 4.3). This apparent contradiction was related in part to the fact that clams deployed at the upstream site (c1) had the highest levels of organochlorine pesticides and showed high activities of PChE and AChE. Indeed previous studies have shown that cholinesterase activities in this and other bivalve species are quite insensitive to pesticide exposure and varied across seasons (Escartín and Porte 1997; Mora et al. 1999; Binelli et al. 2007). Therefore, the results reported here may suggest that temperature or/and other environmental factors rather than measured contaminant levels modulated cholinesterases in the studied bivalve species. Many different contaminants (i.e., hydrocarbon quinones, nitro-aromatics, organochlorine compounds, polycyclic aromatic hydrocarbons, metals) and environmental factors (UV radiation, oxygen) may affect free radical processes and hence may alter free radical scavenging systems such as antioxidant and GST enzyme activities in bivalves (Livingstone 2001). Depending on the duration and intensity of chemical disturbance, the response of antioxidant enzymes can be increased, decreased, or be biphasic to pro-oxidant challenges (Livingstone 2001). In this study, observed distinct inhibition and induction antioxidant enzyme activity patterns in clams precluded an easy identification of contaminant–effect relationships. With the aid of PCA and PLS analyses, however, it was possible to identify major contaminants affecting measured biomarkers. The most important ones were acidic herbicides, propanil, alkylphenol, and endosulfan levels that were negatively related with the activities of biotransformation (GST) or antioxidant (CAT, GPX Tot, GPX Se) enzymes; those of organophosphorous pesticides, PAHs, and metals that were positively related with either GST or antioxidant enzyme activities; and

those of phenylureas, propanil, and endosulfan that were positively related with lipid peroxidation levels. It is also important to consider that temperature and conductivity also affected negatively the observed responses probably by increasing the metabolism, respiration rates, and hence reactive oxygen species (Vidal et al. 2002b).

Disparity of results evidenced that antioxidant enzyme responses to pollution are transient and can be positively or negatively related to the studied chemical disturbances. In *M. galloprovincialis* from the Barcelona harbour, Solé et al. (1994) found significant positive correlation between accumulation of PAHs and antioxidant enzyme activities. Field and lab studies also demonstrated that metals can increase or decrease the activities of antioxidant enzymes in freshwater zebra mussels (Faria et al. 2009). Peña-Llopis et al. (2002) found that impaired glutathione redox status was associated with decreased survival in organophosphate -poisoned marine bivalves. Matozzo et al. (2004) showed that nonylphenol inhibited the activities of SOD but not of CAT in clams (*Tapes philippinarum*). In fish, there is evidence that phenylureas, acidic herbicides like MCPA, propanil, and endosulfan cause oxidative stress (Fatima et al. 2007; Moraes et al. 2007). Therefore, it is reasonable to assume that the measured high levels of the abovementioned contaminants were likely to cause oxidative stress in transplanted clams.

Failure of antioxidant defenses to remove exogenous reactive oxygen species produced by pollutants either by being inhibited by those compounds or overwhelmed by excess of free radicals will disrupt the balance between antioxidant/pro-oxidant system within the organisms leading to oxidative damage. This is a toxicity phenomenon widely demonstrated in field conditions, which implies the increase in the levels of lipid peroxidation of affected organisms (Livingstone 2001). In this study only few of the contaminants or environmental factors that affected antioxidant responses were related positively with lipid peroxidation levels. These include endosulfan, propanil, and phenylureas. According to PLS results, the first two compounds were causing oxidative stress probably by inhibiting antioxidant enzymes, whereas phenylureas by producing an excess of reactive oxygen species and hence overwhelmed antioxidant defenses.

CONCLUSION

The results obtained showed that it was possible to identify potentially hazardous pollutants related with detrimental effects on transplanted bivalve species using appropriate multivariate PCA and PLS analyses on biological and environmental data; PLS analysis is specially suited for these cases where environmental variables are highly correlated (i.e., high co-correlations among the concentrations of the different pesticides applied simultaneously). Under these difficult cases, PLS methods are specially adequate and recommended compared to other classical multilinear regression methods even if they use variable selection methods (Wold et al. 2001). In PLS, apart from the improved precision in the estimation of the regression coefficients and on the predicted response values, the parameters of the models can also be used (as in this work) for the interpretation of the causal (hidden) relationships among the two blocks of variables (biological versus environmental data). Despite that multivariate PLS analyses have been widely used in biology (Wold et al. 1993), their use in environmental risk or hazard assessment is limited (Damásio et al. 2008).

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Appendix. Mean vales for biological and physico-chemical parameters measured in clams across sites and months. Abreviations and units are depicted in Table 4.1.

		CAT	SOD	GST	GPX Tot	GPX Se	LPO	CbE	AChE	PChE	Oxygen	T	Cond	Ac Herb	Triazines	Phenylureas
c1	May	1.18	49.06	654.75	119.76	46.38	43.73	5.70	1.32	2.54	105.5	20.4	817.5	408.3	935.1	33.6
c1	June	1.11	47.62	660.76	111.87	44.63	37.32	6.40	1.44	2.43	67.5	22.5	769.0	439.2	633.6	20.1
c1	July	1.04	38.12	604.45	146.59	49.08	45.76	5.38	0.92	1.36	79.5	24.7	1091.0	370.59	697.1	19.73
c1	August	1.01	30.61	638.85	120.72	50.45	36.41	4.50	0.72	1.03	86.3	23.0	1077.0	364.1	522.7	5.5
c2	May	1.08	45.85	547.77	141.06	47.60	62.95	5.32	1.41	3.05	94.1	20.9	1886.5	9158.7	294.3	20.3
c2	June	0.98	32.71	545.01	81.28	36.92	81.14	4.80	0.84	1.44	74.2	23.0	1375.5	56452.1	826.8	23.5
c2	July	1.27	43.83	477.32	114.74	35.58	47.53	4.31	0.60	0.93	65.0	25.5	1458.0	71034.9	167.1	8.5
c2	August	1.22	32.32	804.83	145.89	63.35	34.65	4.45	0.85	1.45	83.2	24.0	1374.5	3009.8	312.8	5.6
c3	May	1.01	41.25	661.70	133.09	45.97	72.01	4.67	1.43	2.44	72.0	20.9	2980.0	10998.7	440.6	27.3
c3	June	0.65	32.49	457.67	90.50	41.10	62.40	6.00	1.18	2.08	77.0	23.0	2001.0	84504.5	386.7	19.1
c3	July	0.86	36.07	478.97	134.29	43.63	37.69	6.08	0.92	1.72	74.2	25.5	1785.0	66642.6	211.4	7.9
c3	August	0.89	37.07	700.38	172.64	61.09	26.19	4.24	0.68	1.02	74.8	24.0	1412.5	10401.6	179.4	<1.6
c4	May	0.96	39.81	604.11	115.71	47.16	69.56	5.59	1.30	2.33	94.0	20.9	3070.0	18331.0	312.9	19.1
c4	June	0.53	29.50	416.76	82.35	37.41	42.50	6.11	1.22	2.20	83.6	23.0	4250.0	128824.8	408.8	<1.6
c4	July	0.93	37.58	427.39	117.30	33.45	45.29	6.60	0.88	1.85	66.7	25.5	3460.0	117740.9	97.8	4.6
c4	August	1.28	36.32	845.25	123.07	54.94	51.90	3.21	0.61	0.74	63.0	24.0	2675.0	24593.6	266.1	<1.6

Appendix. Cont.

		Propanil	Molinate	OP	Zn	Cu	Cd	Hg	As	PAH	APE	DDT	ENDO	HCB
c1	May	1.5	<2.7	0.6	166.0	47.0	0.5	1.8	6.4	57.4	745.4	1426.3	9	38
c1	June	1.0	<2.7	0.3	173.1	53.7	0.6	2.4	6.6	109.6	929.1	1419.2	7	44
c1	July	1.27	<2.7	0.6	215.2	88.9	0.8	3.8	6.2	115.3	1868.1	733.3	<2	15
c1	August	<0.4	<2.7	0.8	181.4	88.0	0.6	3.9	5.8	244.2	1340.1	467.5	<2	6
c2	May	1344.6	613.3	398.2	151.6	37.9	0.4	1.2	5.6	119.4	560.9	854.7	530	39
c2	June	197.4	413.3	0.3	156.2	45.0	0.4	1.5	7.6	104.7	996.5	813.1	569	6
c2	July	6.2	136.4	173.7	145.7	44.8	0.3	1.2	5.7	62.2	304.1	346.9	35	3
c2	August	1.8	108.7	0.7	177.6	83.0	0.6	3.2	6.7	231.2	1897.8	302.5	<2	<1
c3	May	4680.3	621.9	588.4	157.2	48.4	0.6	1.5	7.5	64.5	890.9	750.0	40	8
c3	June	2220.1	227.7	0.4	171.4	50.7	0.6	1.7	7.8	93.3	950.1	1242.5	88	15
c3	July	13.0	121.9	481.1	212.8	77.2	0.7	2.3	7.9	72.0	656.1	356.1	16	2
c3	August	113.8	113.3	0.3	192.6	119.1	0.8	4.7	7.6	120.5	859.5	361.4	<2	<1
c4	May	1195.2	485.2	207.1	201.3	47.2	0.5	1.5	7.4	171.1	711.9	1013.7	37	11
c4	June	300.6	210.3	0.1	147.8	46.9	0.5	2.1	6.6	51.7	1149.6	1151.4	25	10
c4	July	2.7	194.6	4.2	175.8	66.8	0.7	2.2	8.2	107.4	434.7	345.5	13	1
c4	August	2.5	134.1	0.6	151.9	80.6	0.6	2.9	7.6	108.5	490.8	318.9	<2	<1

Chapter 5

Evaluation of side-effects of glyphosate mediated control of giant reed (*Arundo donax*) on the structure and function of a nearby Mediterranean river ecosystem

5. Evaluation of side-effects of glyphosate mediated control of giant reed (*Arundo donax*) on the structure and function of a nearby Mediterranean river ecosystem

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ABSTRACT

The aim of this study was to evaluate the effect of the application of the herbicide Herbolex (Aragonesas Agro, S.A., Madrid, Spain) to control giant reed (*Arundo don*ax), which has glyphosate as active ingredient, on the structure and function of a nearby river ecosystem. Specifically, we assessed glyphosate environmental fate in the surrounding water and its effects on transplanted *Daphnia magna*, field collected caddisfly (*Hydropsyche exocellata*) and on benthic macroinvertebrate structure assemblages. Investigations were conducted in the industrialized and urbanized Mediterranean river Llobregat (NE Spain) before and after a terrestrial spray of glyphosate. Four locations were selected to include an upstream site and three affected ones. Measured glyphosate levels in river water following herbicide application were quite high (20–60 μg/l) with peak values of 137 μg/l after three days. After 12 days of its application, leaching of glyphosate from sprayed riverbanks was quite high in pore water (20–85 μg/l) but not in the river. Closely linked with the measured poor habitat and water physico-chemical conditions, macroinvertebrate communities were dominated by taxa tolerant to pollution and herbicide application did not affect the abundance or number of taxa in any location. Nevertheless, significant specific toxic effects on transplanted *D. magna* and field collected *H. exocellata* were observed. Effects included *D. magna* feeding inhibition and oxidative stress related responses such as increased antioxidant enzyme activities related with the metabolism of glutathione and increased levels of lipid peroxidation. These results emphasize the importance of combined chemical, ecological and specific biological responses to identify ecological effects of pesticides in the field.

KEYWORDS: Glyphosate, Aminomethylphosphonic acid (AMPA), Herbicide, Macroinvertebrates, Biomarkers

INTRODUCTION

The degradation of riparian vegetation constitutes one of the major causes affecting the biological quality of surface waters within industrialized countries (Prat and Munné, 2000; Damásio et al., 2008) and one of the main causes of river degradation is the presence of invasive alien species, which poses a significant threat to the ecological integrity of river ecosystems. Alien species are often cited as the second most pressing threat (after direct habitat destruction) to global biodiversity (Mooney and Hobbs, 2000; Van Wilgen et al., 2007. Giant reed (*Arundo don*ax) is an invasive plant for riparian habitats (Spencer et al., 2008) and can be considered a primer riparian management problem. As river restoration has become a priority for water authorities and river managers in many countries (Bernhardt et al., 2005; Palmer et al., 2005; Yoshimura et al., 2005; Woolsey et al., 2007), several methods for controlling this plant have been attempted and among them is chemical control with non-specific herbicides. Glyphosate is a broad-spectrum systemic herbicide that has been used to control a wide range of weeds; during the past four decades it has also been applied to control exotic or invasive species. Many commercial herbicides have been formulated using glyphosate (isopropyl amine salt) as active ingredient (Mozdzer et al., 2008; Tjelmeland et al., 2008; Papchenkova et al., 2009). However, the published data on glyphosate toxicity is predominantly related to acute toxicity (Tsui and Chu, 2003, 2004). Even though there are also some studies on the chronic influence of glyphosate at low sub-lethal concentrations in *Daphnia* (Papchenkova, 2007) or its effects on maternal exposure in rats (Daurich et al., 2001), their environmental hazards to invertebrate river species have not been assessed in depth (Alistock et al., 2001). The most important processes of dissipation that may be involved after application of glyphosate are complexation in water with ions, sorption to sediment, suspended particles in water and soil, photodegradation in water, uptake by plants and biodegradation (Daurich et al., 2001). Glyphosate is intended to be used in many Mediterranean rivers to eliminate foreign riparian vegetation and hence to reestablish autochthonous riparian vegetation. Recently the combined use of macroinvertebrate biotic indices and a large set of biomarker responses of field collected or transplanted invertebrate species allowed discriminating ecological effects of pollutants from those caused by organic pollution, flow modification or habitat degradation (Barata et al., 2005, 2007; Damásio et al., 2008). The aim of this

study was to evaluate the effect of the application of the herbicide Herbolex (Aragonesas Agro, S.A., Madrid, Spain) to control giant reed (*A. donax*), which has glyphosate as active ingredient, on the structure and function of a nearby river ecosystem. Accordingly, our specific objectives included the assessment of glyphosate environmental fate in the surrounding water and the study of its effects on transplanted *Daphnia magna*, on field collected caddisfly (*Hydropsyche exocellata*) and on benthic macroinvertebrate structure and function.

MATERIAL AND METHODS

Study site and sampling dates

The study was conducted in the Llobregat river basin (Catalonia, NE Spain), which supply water to the city of Barcelona and is a good example of an intensively used Mediterranean stream system, being impacted by urban, agricultural and industrial activities (Prat and Ward, 1994; Prat and Rieradevall, 2006; Damásio et al., 2008). On behalf of river restoration project to control the giant reed, glyphosate was applied in the riparian vegetation across a restricted area in the mid section of the Llobregat river basin (Fig. 5.1). In order to evaluate the side-effects of this application, three locations were selected: L1 and L2 situated in the river Llobregat, up and downstream of a small sewage outflow, respectively, while a third location was placed in the Gavarresa stream (G), prior to its confluence with the Llobregat main channel. Llobregat is a middle mountain Mediterranean river type with a relatively high discharge while Gavarresa river type is lowland Mediterranean with more variable and lower discharge. The ecology of the Llobregat river has been extensively studied (e.g. Prat et al., 1984; Prat et al., 2002; Prat and Rieradevall, 2006; Damásio et al., 2008) and since 1994 a surveillance monitoring program is being carried out in this river supported by regional government and the Catalan water agency (http://ecostrimed.net/; http://mediambient.gencat.net/aca/).

According to previous studies the selected three sites are characterized by showing a moderate ecological quality due to degraded riparian vegetation and poor water quality, specially due to salt discharge from an upstream mine (Prat and Ward, 1994; Damásio et al., 2008). Terrestrial application of herbicide (2,1 kg glyphosate/Ha) was conducted in an

area of 0.5 Ha of riparian forest by the enterprise on June 8th, 2007, at these locations. Three months before spraying of leafs, stems were cut and reeds sprouted up to 1 m high.

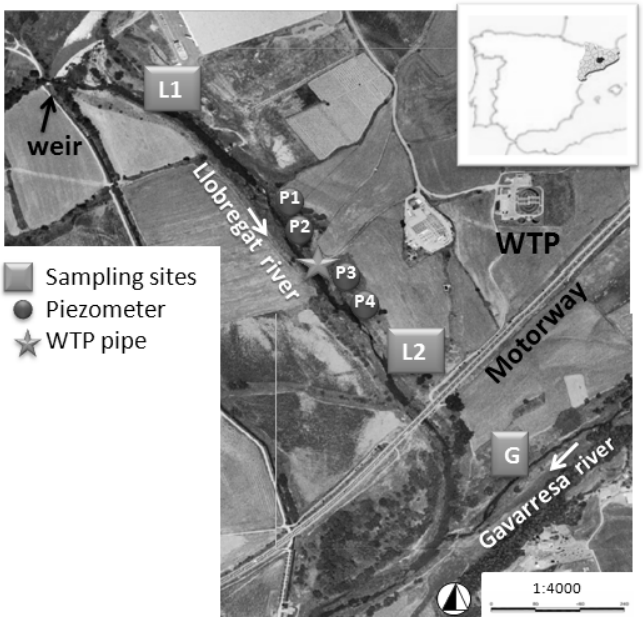

Fig. 5.1. Study site in the Llobregat and Gavarresa junction in Catalonia (NE, Spain). Boxes are sampling sites: L1, L2 and G. Piezometer stations in spots from P1 to P4, two located upstream of the water treatment plant (WTP) pipe and two downstream.

According to the remediation procedure scheme depicted above, our monitoring program included six samplings (Fig. 5.2): time 1, five months before pesticide application on 13th January 2007; time 2, two months before, on 24th April 2007; time 3, just on herbicide application, on 8th June 2007; and two, three and twelve days after (time 4, 5 and 6). Both glyphosate and its major metabolite (AMPA) were determined from river water samples collected from the studied sites at times 3, 4, 5 and 6. In addition, contaminant levels in the pore water of the sprayed riverbanks were also measured using four piezometers deployed across the studied riverbank site (Fig. 5.1). PVC piezometers were 1 m long, completely porous and water extraction was made by manual suction. The structure of the benthic macroinvertebrate assemblages was assessed at times 1, 2, 3, 5 and 6. Transplants with *Daphnia magna* were deployed at times 3 and 6, whereas *Hydropsyche exocellata* samples were collected at times 3 and 5. The previous sampling schedule, summarized in Fig. 5.2, was selected to increase recent historical data of the studied communities before treatment (time 1–2), and to include the periods of exposure to (time 3–5) and post-exposure (time 6) to the herbicide. Due to experimental constrains it was not

possible to deploy *D. magna* organism just prior to herbicide application; thus transplants conducted at time 6 can be considered as no exposure to the herbicide.

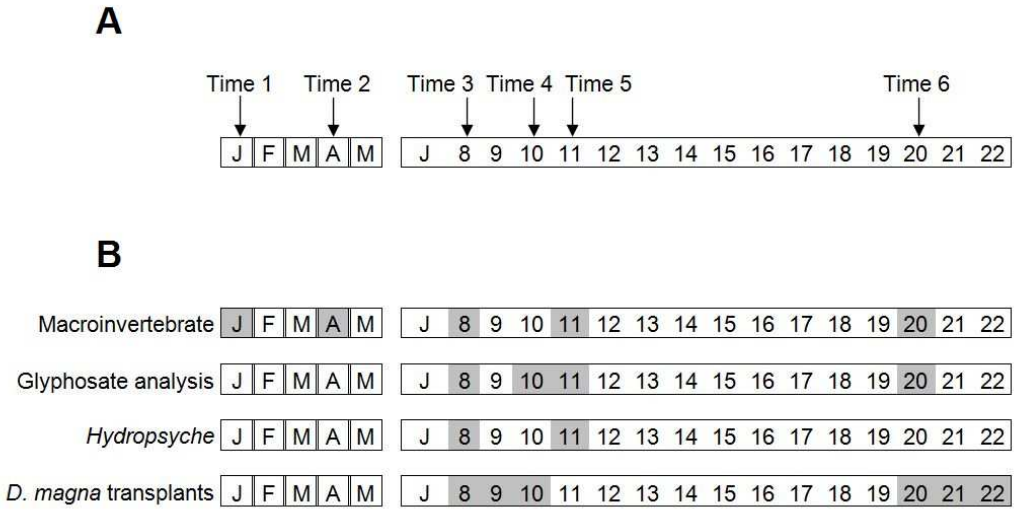

Fig. 5.2. Sampling scheme diagram. Months of sampling periods, time 1-6, and the exact days of sampling in June are depicted (A). Gray boxes indicate the sampling periods used for macroinvertebrates, glyphosate analyses, *Hydropsyche* samples and *Daphnia magna* transplants (B). J, F, M, A, M and J are January, February, March, April, May and June, respectively.

Environmental measurements

A set of environmental variables was measured on each sampling or deployment period. Discharge was determined using a Mini Air flow meter (Technika-Schiltknecht) and making a cross river section (transect) and measuring water flow at different depths across it. Water physico-chemical parameters, such as temperature, pH, conductivity and dissolved oxygen, were measured in situ by using a WTW Multi 340i handheld meter, whereas total suspended solids, TOC, DOC, anions, cations, NH_4, NO_2, NO_3, PO_4, SO_4 and Cl were measured in the lab following ASTM Standard Methods (ASTM, 1995). An additional 500 ml of water was stored in clean amber bottles at 4 °C until analysis.

Herbicide determination

Herbolex is a mixture containing a concentration of glyphosate isopropyl amine salts of 486 and 200 g/l of surfactant compounds. Their polar nature and water solubility make extraction difficult. Chemical analyses were restricted to glyphosate and its major metabolite aminomethylphosphonic acid (AMPA). Our approach adapted a standardized method (Stalikas et al., 2000) to the specific characteristics of our two environmental water matrices (Llobregat and Gavarresa) that showed a relatively high conductivity due to the

presence of salt. Half a liter of water was concentrated to 1 ml using a rotor evaporator. To remove precipitated salts water extracts were treated with ethyl acetate at 50 °C and hot filtered with a hydrophobic filter. Once ethyl acetate was evaporated, samples were derivatized using trifluoroacetic anhydride and trifluoroethanol at 95 °C during 30 min. After the reaction, excess of reactives were evaporated and 400 µl of ethyl acetate was added prior to its analysis with a gas chromatograph coupled to a mass spectrometer from Shimadzu (Japan) model GCMS-QP2010. The mass spectrometer was operated in the negative chemical ionization mode. Compound separation was achieved using a capillary column VF-5 MS of 30 m × 0.25 mm i.d. with 0.25 µm film thickness from Varian Inc. (CA, USA). Acquisition was achieved in time scheduled Selected Ion Monitoring (SIM) mode to increase sensitivity and selectivity. Ions SIM were 245, 351 and 370 UMA. Identification and internal standard ion quantification were carried out automatically by the GCMS solutions software in version 2.5. Quality assurance included three concurrent replicate samples at each sampling day and location, the use of blanks (only ethyl acetate) and standard reference materials (SRM's). Both blanks and SRM's were prepared and analyzed within each batch of samples, both with pure water and river water matrices. SMR's included glyphosate at 98.0% and aminomethylphosponic acid 99.0% from Dr. Ehrenstorfer GmbH, at 0.1, 1, 10 and 100 µg/l.

Biological conditions

Biological responses focused on functional traits (in situ post-exposure feeding *D. magn*a, sensu Mc William and Baird, 2002), specific responses (biomarkers) and community level effects (changes in benthic macroinvertebrate assemblages). Benthic macroinvertebrates, riparian vegetation and habitat quality at the studied sites were studied in order to establish the ecological status of the sites using the Guadalmed protocol (Jáimez-Cuéllar et al., 2002) and to assess the extent to which benthic macroinvertebrate assemblages were affected by herbicide treatment. Benthic macroinvertebrates were obtained quantitatively by sampling all available habitats with a kick net of 250 µm during 8 min, specimens were then preserved in formalin (5%), identified to the family level and used to determine the biological quality of water (Prat and Munné, 2000; Prat et al., 2002).

Biomarker analysis

Biomarkers were determined in transplanted *D. magna* and in field collected *H. exocellata*. The former bioassay has been already used in this river system to characterize toxicological effects in situ (Damásio et al., 2008). *H. exocellata* is a tolerant species widely distributed within Llobregat and other disturbed Mediterranean rivers, whose biomarker responses have been previously characterized and applied in the same river system (Barata et al., 2005).

H. exocellata biomarkers were determined at times 3 and 5 on larvae collected at the studied sites, frozen with liquid N_2 and preserved at −80 °C. *D. magna* deployments were conducted only at site L1 and G and were initiated at times 3 and 6 and lasted two days.

Provided that the mechanism of toxicity of glyphosate is largely unknown in most aquatic invertebrate species (Lee and Steinert, 2003; Conners and Black, 2004; Contardo-Jara et al., 2009), up to 12 different markers were used to include the major detoxification and toxicological pathways of contaminants. These included: phase II gluthatione S-transferase activity (GST) that catalyzes the conjugation of glutathione (GSH) with various electrophilic substances, and plays a role in preventing oxidative damage by conjugating breakdown products of lipid peroxides to GSH (Ketterer et al., 1983); glutathione (GSH) levels, glutathione reductase (GR), which aids in maintenance of GSH levels recycling oxidized glutathione (Regoli and Principato, 1995; Canesi et al., 1999); antioxidant enzymes involved in detoxifying reactive oxygen species such as superoxide dismutase (SOD EC 1.15.1.1 converts to H_2O_2), catalase (CAT EC 1.11.1.6 – reduces H_2O_2 to water), glutathione peroxidase (GPX EC 1.11.1.9 – detoxifies H_2O_2 or organic hydroperoxides) and markers of oxidative tissue damage (lipid peroxidation, DNA strand breaks and the GSH/GSSG ratio) (Halliwell and Gutteridge, 1999; Peña-Llopis et al., 2003). Finally the activity of B esterases was also measured to diagnose exposure to organophosphorous pesticides among other chemicals (Barata et al., 2004).

In situ bioassays

Exposure regime

In situ *D. magna* deployments were conducted as described by Damásio et al. (2008). In each deployment, a lab control treatment with animals maintained in the lab and never exposed to the field was also included as a surrogate control.

Briefly the procedure for the in situ bioassays was as follows. Four day old juveniles were transported to field sites in groups of 10 in 175 ml glass jars filled with American Society for Testing Materials (ASTM) hard water (ASTM, 1995; Mc William and Baird, 2002). At each site 5–7 chambers, each containing 10 individuals, and 4 chambers containing 20 individuals, were placed inside a 13 mm^2 wire-mesh cylinder that was positioned in the stream perpendicular to flow.

Post-exposure responses

After 48 h, animals were retrieved from the chambers. Surviving animals from those chambers holding groups of 20 individuals were pooled in an eppendorf and immediately frozen in liquid N_2 and kept at −80 °C until further enzyme analysis.

Shortly after exposure (within 1 h) five surviving juveniles from those chambers holding groups of 10 animals were placed into 60 ml screw-capped glass jars containing 50 ml of ASTM hard water, with *Chlorella vulgaris* (Beijerink, strain CCAP C211/12) at a concentration of 5×10^5 cells/ml, and allowed to feed for 4 h (Mc William and Baird, 2002). Three jars containing no animals were used to establish initial algal densities. Biomarker and post-exposure feeding rates were also measured in animals maintained in the lab during the deployments and transported to the field sites to include a surrogate lab control. Post-feeding experiments were conducted in darkness to avoid algal growth and under constant temperature conditions (20±2 °C) provided by a thermostatized chamber. Individual feeding rates (cells $animal^{-1}$ h^{-1}) were determined as the change in cell density during 4 h according to the method given by Mc William and Baird (2002). Cell density was estimated from absorbance measurements at λ=650 nm in a dual-beam spectrophotometer (Uvikon 941) using standard calibration curves based on at least 20 data points, with an $r^2>0.98$.

Biochemical determinations

Most biochemical determinations have been described previously (Barata et al., 2004; Damásio et al., 2008; Faria et al., 2009); hereafter we only provide a brief description. Samples were homogenized in ice-cold 100 mM phosphate buffer (PBS), pH 7.4, containing 100 mM KCl and 1 mM EDTA. For *D. magna*, groups of juveniles were homogenized in 500 µl buffer. Homogenates were centrifuged at 10,000 g for 10 min and

the supernatants were immediately used for biochemical determinations. For *H. exocellata* heads were separated from the body, homogenized in the proportion 1 head:200 µl PBS, centrifuged at 10,000 g for 30 min and supernatants used for B esterases determination. All the other biomarkers were determined in the body. Bodies were homogenized in 1:8 proportion wet weight: PBS buffer volume and centrifuged at 10,000 g for 30 min. A total of 12 biomarkers were examined for *H. exocellata*, but due to sample constraint *D. magna* biomarkers were restricted to GST, CAT and B esterases: acetylcholinesterase (AChE) and carboxylesterase (CbE). CAT measurements and CbE were carried out using a spectrophotometer Cecil-CE 9200 (Cambridge, England), whereas all the rest of the biomarkers were determined using a Multi-Detection Micro-plate Reader, BioTek® (Vermont, USA). Assays were run at least in duplicate. CAT, GST, SOD, GPX and GR activities were measured, respectively, according to Aebi (1974), Habig et al. (1974), Mc Cord and Fridovich (1969), Lawrence and Burk (1976) and Carlberg and Mannervik (1985). GSH levels were quantified according to Kamencic (2000). Glutathione redox status (GSH/GSSG) was determined by measuring total glutathione (TG) content (GSH+GSSG) and GSSG, according to Peña-Llopis et al. (2003) and was calculated as number of molecules: GSH/GSSG=(TG–GSSG)/(GSSG/2). Lactate dehydrogenase (LDH) activity was determined according to Diamantino et al. (2001). Lipid peroxidation (LPO) was determined according to Esterbauer et al. (1991) using the Malondialdehyde (MDA) assay. DNA strand breaks were quantified according to Lafontaine (2000) using the DNA alkaline precipitation assay. AChE activity was determined by a modification of the Ellman method adapted to microplate (Barata et al., 2004). CbE activity was determined by the UV method of Mastropaolo and Yourno (1981). Proteins were measured by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Data analysis

Basic statistical analysis was performed with environmental and herbicide data with mean and standard error calculation. The structure and composition of benthic macroinvertebrate assemblages were characterized using a broad range of metrics adapted to river types and generated with the MAQBIR software (Munné and Prat, 2009). *D. magna* and *H. exocellata* responses across sites and deployments or sampling dates were compared by two way ANOVA followed by post-hoc Tuckey's multiple comparison test

(Zar, 1996). Within each deployment *Daphnia* responses were transformed to proportions relative to the lab controls to account for inter-trail differences in the studied parameters (Damásio et al., 2008). Prior to analyses *H. exocellata* and *D. magna* data was log and arcsine transformed, respectively, to meet ANOVA assumptions of normality and variance homoscedasticity.

RESULTS

Physico-chemical water characteristics

In general, the area has not very good conditions for invertebrate fauna: relatively low water flows, high temperatures and conductivity of water and high nutrient content (Table 5.1). Water discharge is variable (Fig. 5.3), and it shows the effects of water extraction and flow regulation due to dams and weirs upstream of the study site for electricity generation purposes. Later in summer, the Gavarresa system tends to be more similar to a pond with low flow conditions, high conductivity values or high oxygen during daytime because of the amount of *Cladophora* and other algae present in the stream. In addition, conductivity values rarely decrease from 1000 µS/cm in both rivers and sometimes even reach values of more than 3000 µS/cm. This and other chemical parameters are presented in Table 5.1. The presence of a sewage discharge coming from a water treatment plant (WTP) poses some differences among stations L1 and L2, especially in conductivity and nutrient parameters although no temporal trend is distinguishable in these sites.

Table 5.1. Mean chemical composition (±SE) of river water on study sites during the sampling period (*n*=5).

Parameter	L1	L2	G
Ox (mg/L)	9.1 (± 0.9)	9.1(±1.1)	12.8(±1.3)
Cond (µS/cm)	1504.0 (± 174.6)	1699.4(±199.7)	3001.2(±294.1)
Cl^- (ppm)	379.1(±34.4)	398.5(±43.0)	672.8(±124.5)
SO_4^-(ppm)	135.0(±18.0)	130.4(±15.0)	596.3(±90.7)
NO_3^-(ppm)	1.8(±0.5)	3.1(±0.1)	4.1(±2.2)
PO_4^{3-}(ppm)	0.3(±0.1)	0.4(±0.1)	0.6(±0.1)
TOC (ppm)	2.3(±0.4)	3.3(±0.8)	6.2(±1.8)
DOC (ppm)	3.4(±1.2)	3.2(±0.7)	4.1(±0.7)

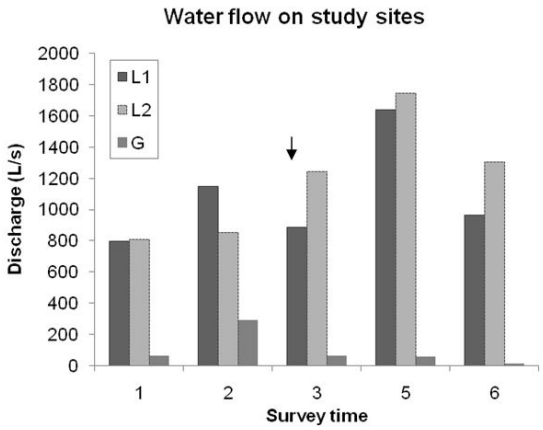

Fig. 5.3. Discharge evolution on the study sites between January and June 2007. The black arrow indicates herbicide treatment.

Detection of glyphosate in river water

The standard curves for glyphosate for the Llobregat and Gavarresa rivers had a correlation (R2) of 0.977 and 0.999, respectively. The limit of detection in the studied rivers for glyphosate and AMPA was 3 μg/l. Levels of glyphosate in surface and pore water are depicted in Table 5.2. Chemical analyses of water only evidenced the presence of Glyphosate with no traces of AMPA. Glyphosate concentrations in surface water were only detected during application (time 3), two (time 4) and three days after (time 5) reaching a maximum of 137 μg/l at G station, levels decrease to 20–60 μg/l in the Llobregat river channel after 3 days of application, and finally undetected levels were found on day 12. In the riverbank pore water, glyphosate levels were undetected until day 12 (time 6), when in piezometers situated farther away from the river reached levels of 28–89 μg/l.

Table 5.2. Glyphosate (mean concentration with standard error) and AMPA for study samples.

Station and sampling scheme	Glyphosate (µg/l)	AMPA (µg/l)
Time 3		
L1	21.4 (±0.9)	<3
L2	31.0 (±0.2)	<3
G	3.6 (±0.0)	<3
P1	<3	<3
P2	<3	<3
P3	<3	<3
P4	<3	<3
Time 4		
L1	55.0 (±10.9)	<3
G	7.9 (±0.3)	<3
Time 5		
L1	40.6 (±26.8)	<3
L2	11.1 (±5.8)	<3
G	139.6 (±27.9)	<3
P1	<3	<3
P2	<3	<3
P3	<3	<3
P4	<3	<3
Time 6		
L1	<3	<3
L2	<3	<3
G	<3	<3
P1	<3	<3
P2	<3	<3
P3	89.8	<3
P4	26.8	<3

Invertebrate community composition

Herbicide application did not affect the abundance or number of taxa of macroinvertebrates in any location (Fig. 5.4). Closely linked with poor chemical conditions macroinvertebrate communities were dominated by taxa tolerant to pollution (Fig. 5.5). Provided the small differences in water quality, the studied communities were quite similar among all the studied sites. One-third of the individuals belonged to Baetidae (Ephemeroptera) and Chironomidae (Diptera) families. Oligochaeta was also abundant, especially downstream of sewage discharge. The most abundant Trichoptera family was Hydropsychidae, and most of them were from the species *H. exocellata*. To characterize the ecological status taking into consideration the tolerance to pollutants and the diversity of the community, the results from several biotic indices are presented. These indices are

used currently for the water management authorities and have been integrated with other European methodologies (Munné and Prat, 2009). Values are presented as the standard quality ratio (EQR), related to the reference condition for each river type. Both the Iberian Biological Monitoring Working Party (IBMWP) and the quantitative multimetric iMMi-T (Fig. 5.6) (Munné and Prat., 2009) showed that no appreciable patterns of change over time existed, with the studied communities always situated within the deficient category.

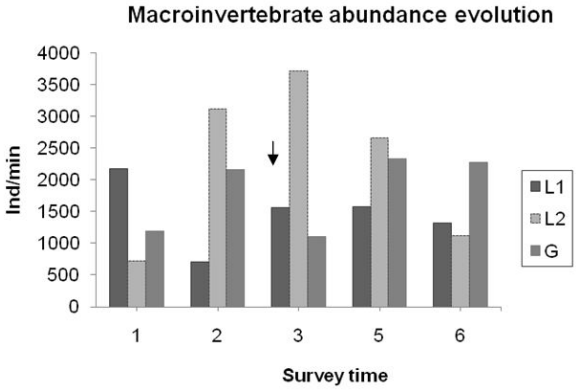

Fig. 5.4. Evolution of the macroinvertebrate abundance during the sampling period. The black arrow indicates the beginning of herbicide treatment.

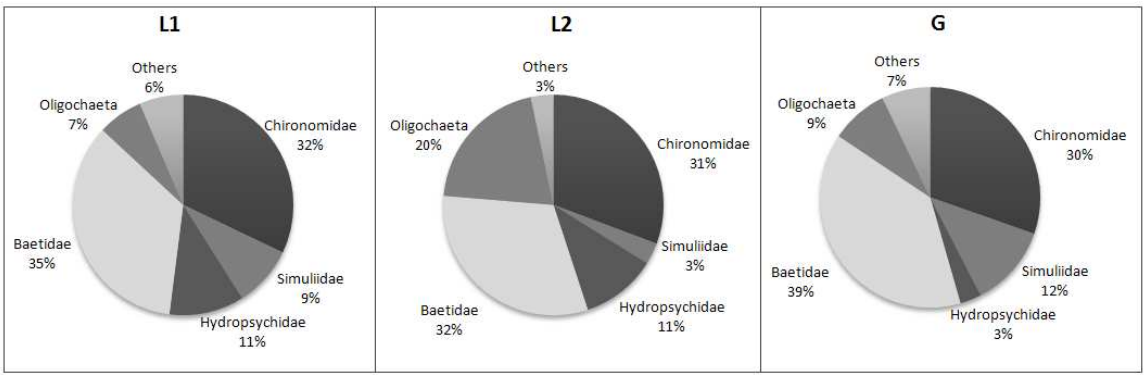

Fig. 5.5. Mean proportion of the most common macroinvertebrate taxa at the study sites from January to June 2007.

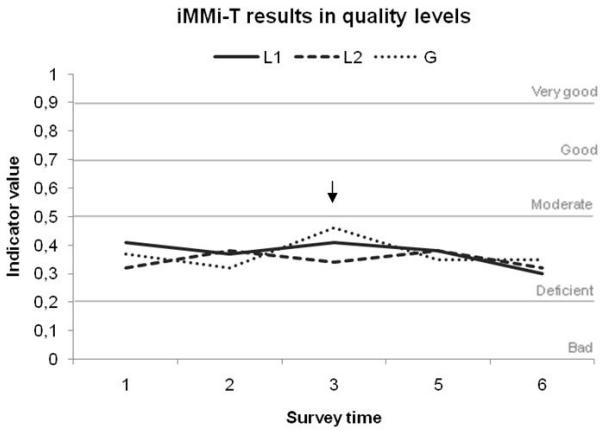

Fig. 5.6. Results of the iMMi-T index for macroinvertebrate samples at the study sites from January to June 2007 (quality levels of iMMi-T index in grey). The black arrow indicates herbicide treatment.

Individual toxic effects

H. exocellata responses of individuals collected at times 3 and 5 varied across sites, exposure period and biomarkers (Fig. 5.7). In 7 out of the 12 analyzed biomarkers, there were significant effects among sites within and across sampling periods (Table 5.4).

General *H. exocellata* response patterns to pesticide application included increased activities of GST and GPX enzymes in Llobregat sites and a strong decrease in the ratio GSH/GSSG in all locations (Tables 5.3, 5.4 and Fig. 5.7). Site specific responses included decreased activities of LDH and of B esterases in L1, the opposite behavior in site L2, decreased activities of GST, GPX, and increased levels of lipid peroxidation in site G1.

Table 5.3. Proportional *Daphnia magna* response values (MEAN and SE) relative to lab controls across sites and deployments. Different letters indicate significant differences following ANOVA and Tuckey's post-hoc tests. Absolute mean values of lab controls were, Feed: 5.35 - 6.13 × 10^5 cells/ind h, GST: 428 - 380 nmol/min/mg prot, CbE: 254 -322 nmol/min/mg prot, CAT: 0.41 - 0.37 mmol/min/mg prot, ChE 2.44 - 5.48 nmol/min/mg prot.

Site	Deployments	Feed		GST		CbE		CAT		ChE	
L1	Time 3	0.46	0.32a	1.30	0.01a	1.03	0.01a	1.04	0.11a	1.04	0.06a
	Time 6	0.9	0.15b	1.14	0.02b	1.12	0.05b	1.09	0.04a	0.98	0.06a
G	Time 3	0.75	0.17a	1.13	0.01b	1.02	0.01a	0.78	0.09b	1.01	0.08a
	Time 6	1.01	0.09b	0.99	0.02a	1.02	0.01a	0.87	0.02b	0.93	0.03a

Table 5.4. Two way ANOVA results for *Daphnia magna* and *Hydropsyche* responses across sites and deployments or sampling periods (time). Only *F* coefficients, degrees of feedom (*df*) and probablility levels (*P*) are shown.

Element	Factors	*df*	*F*	*P*	Element	Factors	*df*	*F*	*P*
Daphnia magna					*Daphnia magna*				
Feed	Stations	1.17	4.83	0.04	CbE	Stations	1.12	13.19	0
	Time	1.17	15.51	0		Time	1.12	8.17	0.01
	Interaction	1.17	1.1	0.31		Interaction	1.12	9.04	0.01
Daphnia magna					*Daphnia magna*				
GST	Stations	1.12	0.83	0.38	CAT	Stations	1.12	39.57	0
	Time	1.12	0.5	0.49		Time	1.12	3.84	0.07
	Interaction	1.12	304.2	0		Interaction	1.12	0.3	0.6
Daphnia magna					*Hydropsyche*				
ChE	Stations	1.12	1.67	0.22	GST	Stations	2.30	12.7	<0.01
	Time	1.12	5.88	0.03		Time	1.30	3.7	0.07
	Interaction	1.12	0.09	0.77		Interaction	2.30	4.1	0.03
Hydropsyche					*Hydropsyche*				
SOD	Stations	2.30	1.2	0.31	CAT	Stations	2.30	5.1	0.01
	Time	1.30	0.1	0.76		Time	1.30	0.7	0.41
	Interaction	2.30	1.6	0.21		Interaction	2.30	0.3	0.74
Hydropsyche					*Hydropsyche*				
GPX	Stations	2.30	5.6	0.01	GR	Stations	2.30	8.1	<0.01
	Time	1.30	0.6	0.46		Time	1.30	4.3	0.05
	Interaction	2.30	5.6	0.01		Interaction	2.30	1.5	0.23
Hydropsyche					*Hydropsyche*				
GSH	Stations	2.30	12.7	<0.01	GSH/GSSG	Stations	2.30	1.2	0.32
	Time	1.30	0.8	0.38		Time	1.30	12.6	<0.01
	Interaction	2.30	1.3	0.29		Interaction	2.30	0.4	0.68
Hydropsyche					*Hydropsyche*				
LDH	Stations	2.30	44.7	<0.01	ChE	Stations	2.30	1.4	0.27
	Time	1.30	0.2	0.65		Time	1.30	0.8	0.38
	Interaction	2.30	14.1	<0.01		Interaction	2.30	3.8	0.03
Hydropsyche					*Hydropsyche*				
CbE	Stations	2.30	1.4	0.27	LPO	Stations	2.30	36.9	<0.01
	Time	1.30	1.1	0.3		Time	1.30	1.4	0.24
	Interaction	2.30	3.5	0.04		Interaction	2.30	4.7	0.02
Hydropsyche									
DNA	Stations	2.30	1.6	0.21					
	Time	1.30	0.9	0.36					
	Interaction	2.30	0.5	0.6					

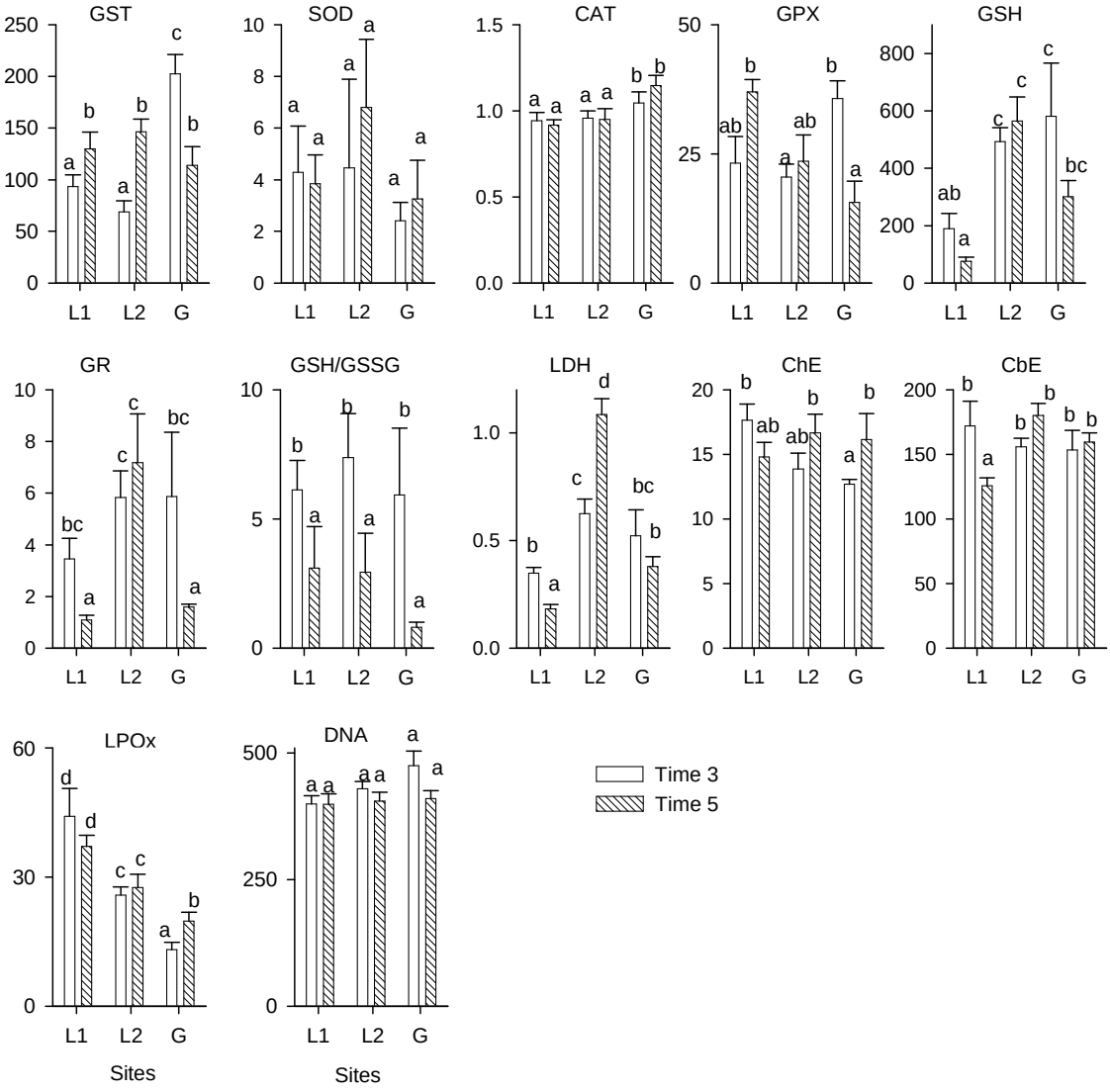

Fig. 5.7. *Hydropsyche* biomarker responses across sites (L1, L2, G) and samplings (Time 3 and 5). Biomarkers units are nmol/min/mg protein for GST, GPX, GR, CbE, ChE; µmol/min/mg prot for LDH; nmol/g ww for GSH; nmol MDA eq/g ww for LPO and µg DNA/g ww for DNA breaks. Different letters denoted significant differences following ANOVA and poshoc tests. Error bars are SE.

Mortality in transplanted *D. magna* individuals was negligible (<5%) but proportional responses relative to lab controls evidenced significant effects of exposure period within or across sites in 4 out of the 5 traits studied (Tables 5.2 and 5.3). Effects included a strong inhibition of post-exposure feeding rates in sites L1, G and enhanced activities of GST and CbE in site L1 in daphnids exposed during the first two days of herbicide application (Tables 5.2 and 5.3).

DISCUSSION

Due to its high adsorption tendency in soil (Kd values up to 900 l/kg) and its fast degradation by microorganisms, glyphosate and/or its formulations are generally regarded as having low potential to contaminate surface waters or groundwater (Giesy et al., 2000; Borggaard and Gimsing, 2008). However, leaching of glyphosate and its degradation product AMPA (aminomethylphosphonic acid) up to 1 m depth has been observed in laboratory and field studies, suggesting a potential risk for the aquatic environment (Landry et al., 2005). Moreover, glyphosate and AMPA are present in surface waters worldwide in considerable concentrations, e.g. up to 2.2 μg/l in US rivers (Kolpin et al., 2006). The limit value for single pesticides in groundwater in Europe is 0.1 μg/l and for the sum of pesticides 0.5 μg/l (Council of the European Communities (CEC), 1991). In the present study measured glyphosate levels in river water following herbicide application were quite high (20–60 μg/l) with peak values of 137 μg/l. Furthermore, after 12 days of its application leaching of glyphosate from sprayed riverbanks was quite high in pore water (20–85 μg/l) but not in the river. Therefore, it is plausible to conclude that glyphosate, sprayed to riverbanks to control giant reed contaminated the nearby streams, may reach high concentrations during the first three days post-application. In addition, as we have not detected AMPA, we can presume we have taken only glyphosate in our samples because it would not have had enough time to decompose in the environment.

Many factors influence the characteristics of invertebrate assemblages, as channel form and materials, floodplain connectivity and riparian vegetation, water temperature and chemistry, availability of nutrients and energy resources, biotic interactions as well as the evolutionary history of species in the community and the legacy of past disturbance events and land use activities (Reeves et al., 1995; Harding et al., 1998; Allan, 2004). Nevertheless, streamflow may limit biological conditions at some sites where other factors would allow, for example, a higher abundance of organisms or a greater number of taxa (Konrad et al., 2008). However, biotic responses to streamflow may be conditional as they depend on the broader ecological state of a site (Konrad et al., 2008). Regarding the main biological quality indexes, the described environmental scenario is likely to be responsible for poor water quality. Habitat degradation, flow regulation, salinization and sewage discharge were probably the major factors explaining a less diverse community dominated

by tolerant species (Damásio et al., 2008). As a consequence, no differences were found at structural level in these organisms before and after herbicide treatment.

The toxicity of technical-grade glyphosate to aquatic invertebrates is known to be only minor (LC_{50} values of >55 mg/l glyphosate, WHO, 1994). However, an increased toxicity of glyphosate formulations has been reported and related to its surfactants or additives (Giesy et al., 2000; Tsui and Chu, 2003, 2004; Bringolf et al., 2007). For example for one of the most used glyphosate formulations (Roundup Ultra), toxicity thresholds of LC/IC_{50}<2 mg/l a.i. were determined for the amphipod *Hyalella azteca* and the copepod *Acartia tonsa* (Tsui and Chu, 2003, 2004). Recently Contardo-Jara et al. (2009) reported that both pure and formulated gyphosate were able to challenge the xenobiotic enzymatic defensive system of *Lumbricus variegates* (GST, SOD, CAT) at moderate doses (50–500 µg/l).

In the present study biomarker responses of the benthic macroinvertebrate species *H. exocellata* indicated oxidative stress, which was evidenced by a significant decrease in the ratio GSH/GSSG (Peña-Llopis et al., 2002) or increased levels of lipid peroxidation at three days post-application. Oxidative stress effects of glyphosate formulations have also been reported in lab exposed fish and tadpoles (Costa et al., 2008; Langiano and Martinez, 2008); thus it seems a common mechanism of action. Observed high activities of LDH in *H. exocellata* individuals sampled from site L2 three days after pesticide application indicated an increased rate of organism's anaerobic metabolism, suggesting a rapid need of additional energy to cope with increasing environmental stress levels (Moreira et al., 2006). Site differences in *H. exocellata* biomarker responses also agree with previous work conducted five years before in similar sampling locations (Barata et al., 2005). These included low levels of lipid peroxidation in site G and the effect of salinization on CAT and GST activities.

On the other hand, in the present study we reported that in a real field scenario environmentally measured glyphosate concentrations ranging between 20 and 137 µg/l in water affected *D. magna* feeding rates and the activity of biotransformation (GST, CbE), antioxidant (GPX), metabolic (LDH) and/or anticholinergic enzymes of the two studied invertebrate species.

Thus, these significant biological responses among the three studied sites before and following glyphosate application suggested interactive combined effects of naturally

occurring factors and the herbicide application. Indeed the studied sites were characterized by difference in organic pollution (L2 received the sewage of a nearby small industrial park) and salinization (G was highly impacted by an excess of Cl and SO_4; Barata et al., 2005; Damásio et al., 2008). It is worth noting also that the above mentioned studies showed that an excess of ammonium coming from WTP, salts and changes in oxygen levels affected the studied behavioral and biochemical responses. More specifically, the observed higher and lower proportional activities of GST and CAT in *D. magna* deployed at site G, respectively, were related with salinization and an excess of oxygen (Barata et al., 2007; Damásio et al., 2008).

There is little data on how to select appropriate application regime of a herbicide (Spencer et al., 2008). Farmers combine different commercial herbicides together with glyphosate to increase its levels of toxicity (Daurich et al., 2001). Further studies that evaluate glyphosate for managing giant reed indicate that a single late-season application of 3% or 5% onto the foliar mass was the most effective and least hazardous to biota (Spencer et al., 2008). In the present study we provide evidence that glyphosate had only marginal effects on the structure of communities, but specific detrimental effects on keystone species such as *H. exocellata* cannot be ruled out.

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Chapter 6

Biochemical responses of benthic macroinvertebrate species as a tool to diagnose the water quality of polluted rivers

6. Biochemical responses of benthic macroinvertebrate species as a tool to diagnose the water quality of polluted rivers

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ABSTRACT

Biological indexes based on benthic macroinvertebrate species are currently used world wide to measure river water quality. These indexes assign a global ecological status of the biotic community, but not necessarily may detect specific effects of water pollutants. Conversely biochemical markers measured in keystone macroinvertebrate benthic species may inform us about small changes in water quality. This is especially interesting in moderately polluted sites, where stressors are already affecting communities but not too strongly to be detected by biotic indexes. Up to ten different markers and 42 contaminants measured in ten populations of the caddisfly *Hydropsyche exocellata* were assessed across a polluted gradient in the industrialized Mediterranean river basin of Besós (NE, Spain). Ten populations were selected to include macroinvertebrate communities representing the five water quality degrees defined by the Spanish Environmental authorities for the biotic metric Iberian Bio-Monitoring Working Party (IBMWP). Results evidenced a clear deterioration of the ecological water quality parameters and benthic communities towards downstream reaches. At the same time, nine of the ten markers analyzed varied significantly across the studied populations and were able to differentiate populations within communities having a good and deteriorated ecological state. Principal Component Analyses revealed biochemical markers that co-varied with changes in macroinvertebrate assemblages, such as, the activities of B-esterases and DNA strand breaks. Others, such as, antioxidant enzymes, metabolizing enzymes and lipid peroxidation levels, responded differently and were closely related to high and presumably toxic levels of accumulated organic pollutants. Therefore our results indicate that the use of markers sensitive to water pollution may provide complementary information to monitor changes in water quality.

KEYWORDS: Biomarkers, Pollution, Water quality, Hydropsyche, River

INTRODUCTION

The past thirty years has seen enormous growth in the collection of environmental data, of all types, by European researchers. One of the most active areas of data-gathering has been the monitoring of surface waters, and consequently catchment-scale management has a good science basis in Europe. As a result of this monitoring effort, a large quantity of matched hydrochemical and biological information concerning the ecological status of these habitats exists today. In Europe, the appropriate use of such data is at the core of the European Union's Water Framework Directive (WFD, Directive 2000/60/EC), an ambitious piece of legislation that seeks to achieve good ecological status for European surface waters by 2015. The approach adopted by the WFD is revolutionary in changing the focus from chemical-based regulation to ecological effects-based regulation, and from site-specific controls to the consideration of impacts at a catchment or river basin scale (Hering et al., 2006). Such holistic approaches to integrated surface water management are essential to ensure the sustainable use of ecosystem goods and services (Barbour and Paul, 2010) although care has to be taken in the interpretation of the indicators used (Basset, 2010).

One of the particular and unique aspects of this integrated management is the use of ecologically-based instruments to assess and predict the ecological impacts of environmental pressures on water quality. In general, ecological status assessment involves sampling the aquatic community, and comparing against a reference prediction for that water body type. This approach is currently used in many countries (see reviews of Bonada et al., 2006; Dodelec and Statzner, 2010). Various biological metrics exist to quantify change in community composition (e.g. EPT taxa, Simpson-Index, Shannon-Wiener-Index, Margalef-Index) and these are often combined in multi-metric indices to improve the chances of detecting adverse changes. Among them, those focussing on assemblages of benthic macroinvertebrates are the most widely used (Rosenberg and Resh, 1993; Bonada et al., 2006). These metrics assign a global ecological status of the benthic macroinvertebrate community in different categories. This approach is currently used in the implementation of the WFD in Europe with the aim to improve the ecological status for European surface waters by 2015. However structural metrics although can detect the degradation of surface waters, do not detect specific effects of water pollutants neither

moderate changes in water quality (Barata et al., 2005; Damásio et al., 2008). Therefore, there is a need to complement the biological metrics actually used with other biological measures that may serve as descriptors of cause-effect or may inform about further degradation (or improvement) of the water.

There are several studies showing that the use of physiological responses of keystone macroinvertebrate species may provide additional information about changes in water quality (Barata et al., 2005, 2007; Damásio et al., 2008; Faria et al., 2010). Among the available methods, the integrated use of chemical analyses and biochemical and cellular responses to pollutants is a sound procedure for detecting impact of anthropogenic contaminants in freshwater systems. Moreover, since in real field situations aquatic organisms are currently being exposed to multiple chemical contaminants involving different toxicity mechanisms, each contributing to a final overall adverse effect, the use of a large set of biochemical responses may allow us to identifying potential hazardous contaminants in the field (van der Oost et al., 2003; Bocchetti et al., 2008). Recently, several biochemical markers were developed and used in field collected caddisflies of the tolerant species *Hydropsyche exocellata* and in transplanted *Daphnia magna* to monitor metallic pollution and ecological quality of water in Mediterranean Rivers. Contrary to macroinvertebrate assemblages, measured biochemical responses of *H. exocellata* and *D. magna* did not respond to changes in habitat quality, but were sensitive to small changes of chemical pollutants in water (Barata et al., 2005; Damásio et al., 2008).

The main objective of this study was to address if the use of multi-biomarker responses in keystone benthic macroinvertebrate species may complement existing biotic and multi-metric indices that are used to assess changes in ecological status of river biota. In particular, we assessed and compared biochemical responses of field collected caddisfly of the tolerant species *H. exocellata* with biological indices calculated from taxa assemblages of benthic macroinvertebrates in seven different sites in one basin with a total of ten different samples (in some places summer and spring samples were available when the river didn´t dries up in summer). Samples came both from pristine and different levels of polluted sites. Biomarkers included the phase II gluthatione-S-transferase activity (GST); glutathione reductase (GR), which aids maintenance of GSH levels recycling oxidized glutathione; antioxidant enzymes involved in detoxifying reactive oxygen species, such as, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX);

markers of oxidative tissue damage (lipid peroxidation and DNA strand breaks) and lactate dehydrogenase (LDH) as an indicative of the metabolic state of the animal (Puértolas et al., 2010). The activity of B-esterases was also measured to diagnose exposure to organophosphorous pesticides among other chemicals (Barata et al., 2004). As a secondary aim, the study also attempted to characterize metals and organic contaminants present in caddisfly larvae, to allow identifying major pollutants affecting the observed responses. This study was conducted in the Besós river basin (NE Spain), which is a good example of an intensively used Mediterranean stream system, receiving extensive urban, agricultural and industrial waste water discharges from the area of Barcelona (Prat and Munné, 2000).

MATERIAL AND METHODS

Study sites

The study was performed in seven locations within the Besós river basin, NE Spain (Fig. 6.1) and during two periods (13-14/4/2005 and 27-28/07/05, spring (sp) and summer (su), respectively), the sites belong to two different river types according to the classification used for the purposes of the WFD by the Water Catalan Agency (from now ACA) (Munné and Prat, 2005). Five of the study sites, B3 – B7, were located in tributaries belonging to the lowland Mediterranean river type with variable and low discharges and two of them, B1 and B2, were situated in small mountain streams. The ecology of the Besós river has been extensively studied (e.g.; Prat and Munné, 2000; Prat et al., 2002; Prat and Rieradevall, 2006; Damásio et al., 2008) and since 1994 a surveillance monitoring program is being carried out in this river supported by regional government (Diputació de Barcelona: http://ecobill.diba.cat/) and the ACA (http://mediambient.gencat.net/aca/). According to the previous studies, the selected sites had communities belonging to the five ecological quality types defined in the WFD: very good, good, fair, poor and very poor (Munné and Prat, 2009).

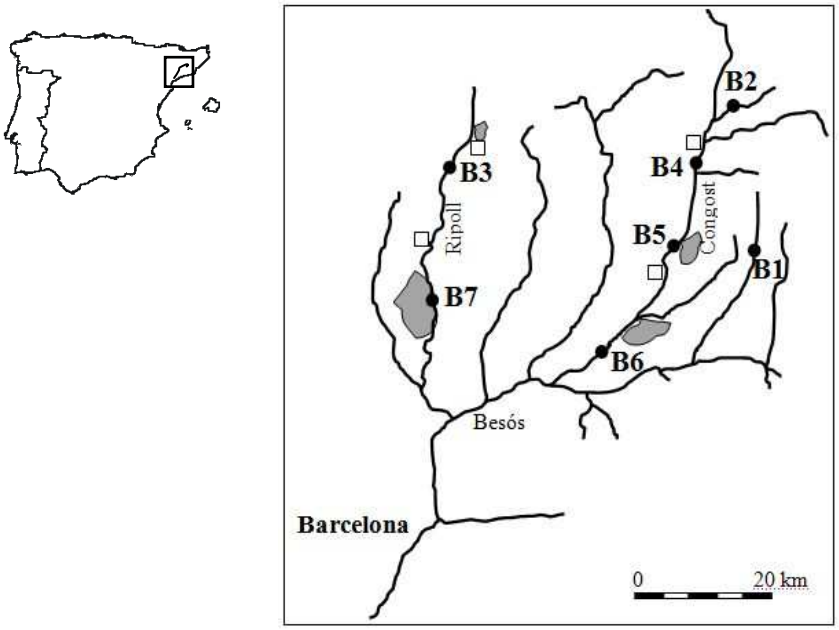

Fig. 6.1. Map of the sampling sites, which includes the main channel of Besós, the tributaries Congost and Ripoll rivers, the main urban nucleus (Sant Llorenç Savall, La Garriga, Granollers and Sabadell close to sites 3, 5, 6 and 7, respectively) and Waste Water Treatment Plants (WWTP), depicted as squares.

Physicochemical water measurements

A set of environmental variables were measured on each sampling occasion. Water physicochemical parameters including flow rate (l/s), temperature (T, ^{o}C), pH, conductivity (μS/cm) and dissolved oxygen (O_2, mg/l) were measured in situ with a Mini Air probe (Technika-Schiltknecht). Sulphates (SO_4, mg/l), chlorides (Cl, mg/l), N-ammonium (NH_4, mg/l), N-nitrites (NO_2, mg/l), N-nitrates (NO_3, mg/l) and P-phosphates (PO_4, mg/l) were obtained following well-established methods for water physicochemical parameters ASTM (1998) procedures.

Biological conditions and specimens collection

Benthic macroinvertebrates, riparian vegetation and habitat quality at the studied sites were assessed in order to establish the ecological status of the sites using the Guadalmed protocol (Jáimez-Cuéllar et al., 2002; Damásio et al., 2008). The quality of the riparian vegetation was measured using the Riparian habitat Ecological Quality Index (QBR) and the in-stream habitat quality was measured by the Fluvial Habitat Index (IHF). Regarding the quality of the macroinvertebrate communities, several metrics were assessed including the Iberian Bio-Monitoring Working Party Biological Index (IBMWP), the number of

taxons (S) and the Iberian Average Score Per Taxon (IASPT, the result to divide IBMWP by S).

Benthic macroinvertebrates were obtained quantitatively by sampling all available habitats with a kick net of 250 μm. Just after collection, 50-200 caddisfly larvae of the species *H. exocellata* were separated from other macroinvertebrate species and kept in the same river water for 1-2 hours to allow detritus to be removed from their body and the partial clearance of their gut content, rinsed several times with distilled water, frozen in liquid nitrogen and stored at –80^{o}C until further analysis. The rest of benthic macroinvertebrate species were preserved in formalin (5%) and identified to the family level to calculate the structural metrics. Not in all seasons it was possible to sample enough *H. exocellata* individuals to fulfill sample requirements, thus the number of communities studied were restricted to 10.

Biomarker analysis

Biomarkers were determined in larvae of *H. exocellata*, a tolerant species widely distributed within Besós river, whose biomarker responses has been previous applied (Barata et al., 2005). Samples were homogenized in ice-cold 100 mM phosphate buffer (PBS), pH 7.4, containing 100 mM KCl and 1 mM EDTA. *H. exocellata* heads were separated from the body, homogenized in the proportion 1 head: 200 μl PBS, centrifuged at 10 000 *g* for 30 min and supernatants used for Acetylcholinesterase (AChE) determination. All the others biomarkers were determined in the body. Bodies were homogenized in 1:8 proportion wet weight: PBS volume and centrifuged at 10 000 *g* for 30 min. Catalase (CAT) measurements were carried out using a spectrophotometer Cecil-CE 9200 (Cambridge, England), whereas all the rest of the biomarkers were determined using a Multi-Detection Micro- plate Reader, BioTek® (Vermont, USA). Assays were run at least in duplicate.

Catalase (CAT) activity was measured by the decrease in absorbance at 240 nm due to H_2O_2 consumption according to Aebi (1974). The reaction solution contained 80 mM PBS (pH 6.5) and 50 mM H_2O_2. Glutathione-S-transferase (GST) activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was measured as described by Habig et al. (1974). The reaction solution contained 100 mM PBS (pH 7.4), 1 mM CDNB and 1 mM of reduced glutathione (GSH). The formation of S-2, 4 dinitrophenyl-glutathione conjugate was

evaluated by monitoring the increase in absorbance at 340 nm. Superoxide dismutase (SOD) activity was determined according to Mc Cord and Fridovich (1969) based on the measurement of the degree of inhibition, caused by SOD, of cytocrome *c* reduction by free oxygen radicals (O^{-2}) released by the xanthine oxidase/hypoxanthine reaction. SOD units were determined using a standard curve of 0 - 1.5 SOD units/ml. The reaction contained 50 mM PBS (pH 7.8), 0.1 mM EDTA, 25 μM hypoxanthine, 1.82 mU/ml xanthine oxidase and 10 μM cytochrome *c*. Total Glutathione peroxidase (GPX) activity was determined according to Lawrence and Burk (1976), following the decrease in NADPH concentration (at 340 nm), which is consumed during the generation of GSH from oxidized glutathione, using cumene hydroperoxide as substrate. The reaction solution contained 100 mM PBS (pH 7.4), 2 mM GSH, 2 U/ml glutathione reductase and 3mM cumene hydroperoxide. Glutathione reductase (GR) activity was determined according to Carlberg and Mannervik (1985) by following the oxidation of NADPH at 340 nm. The reaction solution contained 100 mM PBS (pH 7.4), 0.07 mM NADPH and 0.7 mM GSSG.

Lactate dehydrogenase (LDH) activity was determined according to Diamantino et al. (2001) by monitoring the absorbance decrease at 340 nm due to NADH oxidation. The reaction contained 100 mM PBS (pH 7.4), 0.15 mM NaOH, 1.18 mM pyruvate and 0.18 mM NADH. Lipid peroxidation (LPO) was determined according to Esterbauer et al. (1991) using the Malondialdehyde (MDA) assay, which is based on the reaction of the chromogenic reagent 1-methyl- 2-phenylindole with MDA at 45˚C, giving rise to a chromophore with absorbance at 586 nm. Sample was incubated in (1:3) methanol: 5 mM 1-methyl-2-phenylindole dissolved in acetonitrile, 6% HCL and 0.012% BHT at 45˚C, for 40 min. Absorbance was then read at 560 nm vs. a standard solution of 1,1,3,3-tetramethoxypropane (TMP) treated similarly. DNA strand breaks were quantified according to Lafontaine et al. (2000) using the DNA alkaline precipitation assay. Tissue homogenates were incubated in a solution of 2% SDS, 50 mM NaOH, 10 mM TRIS, 10 mM EDTA and 0.12 M KCl at 60 ºC for 10 min. After centrifuging the sample, levels of stranded DNA were determined in the presence of 1μg/ml Hoechst dye at ex/em 360/450 nm, using a DNA standard curve.

Acetylcholinesterase (AChE) activity was determined by a modification of the Ellman method adapted to microplate (Barata et al., 2004). The reaction solution contained 100 mM PBS (pH 7.4), 0.33 mM acetylthiocholine and 0.17 mM 5,5-dithiobis-2-

dinitrobenzoic acid (DTNB), and the increase of absorbance was measured at 405 nm. According to the same method, carboxylesterase (CbE) activity was determined using 0.3 mM S-Phenyl Thioacetate as substrate. Proteins were measured by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Contaminant analyses

Metal concentration levels of As, Cu, Pb, Zn, Al, Cd, Cr and Ni were determined in single or in groups of two freeze dried larvae (mean ± SD; 19.13 ± 5.65 mg dry weight) following Barata et al. (2005) procedures. Dried larvae were digested with 0.75 ml concentrated nitric acid and 0.25 ml hydrogen peroxide (instra quality, Baker) using 60 ml Teflon bombs at 90 ^{o}C overnight. Within each digestion series, appropriate blanks with no insects were also subject to the same procedure to account for background contamination levels. Cooled digested samples were diluted to a standard volume with deionized water. Trace metal analysis were determined using a Perkin Elmer model Elan 6000 inductively coupled plasma mass spectrometer (ICP-MS). Calibration standards and a reagent blank were analyzed with every ten samples to monitor signal drift. In every instance, the signal typically changed by 3-5% throughout an analytical run. Additionally, rhenium was used as an internal standard to correct for any non-spectral interferences. Samples of similar weight of a certified reference material (lobster hepatopancreas, Tor 1, national Council of Canada, Ottawa) were digested during each analytical run; measured trace metal concentrations were within the certified range for the metal. Except for Cd, metals levels exceeded ten fold the analytical detection limit. For Cd, only those levels exceeding two fold the analytical detection limit (0.01 μg/g d.w.) were considered.

Up to 35 different organic compounds were analyzed in freeze dried larvae following Sánchez-Avila et al. (2010) procedures. Due to analytical constrains, pools of 10-20 individuals were used without replication. Extraction and clean up surrogate standards (4-n-NP-D_8, NP_1EO-D_2, 4.4-DDD $^{13}C_{12}$, DPP D_4, PCB 209) were added to a sample aliquot of 0.1 g, to get a final concentration of 1000 ng/g. Sample were homogenized and kept at 4 ^{o}C overnight and subsequently extracted by sonication (10 min) one time with hexane/dichloromethane (1:1, v/v) in a ratio of 100 ml per gram of dried mass. A twice extraction (same ratio) was performed with a mixture of hexane/acetone (1:1, v/v). After each extraction step, samples were centrifuged for 10 min and the extracts were combined

and concentrated to approximately 1 ml under a nitrogen current using a TurboVap at 25 °C. Extracts were subsequently cleaned up by solid phase extraction (SPE) cartridges with 5 g of Florisil, previously conditioned with 20 ml of hexane/dichloromethane (1:1,v/v) and 20 ml of hexane/acetone (1:1, v/v). The sample extract was eluted with 20 ml of hexane/dichloromethane (1:1, v/v) and 20 ml of hexene/acetone (1:1, v/v). The eluent was concentrated to a volume of less than 1 ml under a nitrogen current at room temperature and reconstituted with ethyl acetate to a final volume of 1 ml, with addition of 1 μg/ml of the internal standard Anthracene D_{10}. Final extracts were analyzed by gas chromatography – electronic impact – mass spectrometry in tandem (GC-EI-MS/MS). Analyzes were performed using an Agilent 7890A GC System (Agilent Technologies, Palo Alto, CA, USA) interfaced to a 7000A triple quadrupole mass spectrometer system (Agilent, USA). Separation and detection method parameters were similar to those reported by Sánchez-Avila et al. (2010). For increased sensitivity and specificity, peak detection and quantification was performed in Selected Reaction Monitoring (SRM) mode using two transitions per each compound. Internal standard quantification was performed using the surrogate standards method. A 9 points calibration curve was constructed at 100, 250, 500, 750, 1000, 2500, 5000, 7500 and 10000 ng/g with good linearity over this concentration range ($R^2 > 0.994$). The limits of detection (LOD) of the analytical method were calculated with the minimum concentration of analyte that produced a signal-to-noise ratio (S/N) of 3:1 and 6:1, respectively. Sample blanks were rigorously performed to eliminate any external source of contamination. Blank samples were below detection limits except for phthalates, where blank values were below quantification limits.

Data analysis

Biochemical responses and metal levels across sites and seasons were compared using one way ANOVA followed by post-hoc multiple comparison tests (Zar, 1996). Data was log transformed prior to analysis to improve normality and variance homocedasticity. Due to sample size constrains measurements of organic contaminant levels in *H. exocellata* samples were not replicated and hence could not be compared statistically. Principal Component Analyses (PCA) on biochemical responses and on the whole data set of biological and phsyco-chemical measurements were also performed. The former was used -considering all replicates within sites to classify the studied populations according to their

biochemical response patterns. The latter was performed considering only the mean responses and aimed to identify relationships. Due to the large number of pollutants measured and the existence of empty (non detected) values, only metal levels that were detected in all samples were considered (i.e. As, Cu, Pb, Zn, Al). Organic contaminants were grouped in seven categories: alkylphenols (AP), that included octylphenol, nonylphenol technical and two nonylphenol ethoxylates (mono- and di-); polychlorinated biphenyls (PCBs), with seven congeners, 28, 52, 101, 118, 138, 153, 180; polycyclic aromatic hydrocarbons (PAHs), that included 14 compounds (naphthalene, acenaphthene, fluorene, fluoranthene, anthracene, phenanthrene, pyrene, chrysene, benzo (a)pyrene, benzo (a) anthracene, benzo (b)fluoranthene, benzo (g,h,i)pyrene, indeno (1,2,3-cd)pyrene, dibenzo (a,h)anthracene); dicloro difenil trichloroethanes (DDTs) with five compounds that included parental and degradation metabolites (2,4 DDD; 2,4 DDT; 4,4 DDD; 4,4 DDE; 4,4 DDT); endosulphan (ENDO) with two isomers (alpha and beta); hexachlorocyclohexane (HCH) with the four isomers (alpha, beta, gamma, delta). In both analyses, since variables were very different (physico-chemical, quality indices and biochemical responses) and/or they were not measured using the same scale units, the data was auto-scaled prior to analysis (each element was subtracted by its column mean and divided by the standard deviation of its column).

RESULTS

Physico-chemical water parameters

In general, water flow decreased dramatically in summer and nutrient load and conductivity increased substantially from upper to downstream reaches due to the discharge of effluents coming from waste water treatment plants (Table 6.1). The observed quite large variation of temperature, water flow and oxygen levels across sites were related to the inclusion of two river ecotypes, small mountain streams and lowland Mediterranean rivers with variable and low discharges and data from two different sampling periods. The sites are a good representation of the different levels of pollution present in the area, from pristine reaches (B1, B2) to very polluted (B7).

Table 6.1. Ecological and physicochemical water quality parameters (Mean) at the studied seven sites across seasons.

	Sudied sites									
	B1 sp	B1 su	B2 sp	B3 sp	B4 su	B5 sp	B5 su	B6 sp	B6 su	B7 su
IBMWP	205	180	135	101	45	35	39	39	66	24
S	33	33	23	21	12	10	10	11	17	7
IASPT	6.2	5.5	5.8	4.8	3.7	3.5	3.9	3.5	3.8	3.4
QBR	100	100	72	90	40	25	20	10	10	15
IHF	92	88	79	73	60	67	75	68	53	48
Flow	12	3	2	3	26	56	5	69	2	66
T	16.1	17.5	8.8	10.1	26.5	9.4	23.6	21.2	29.7	26.7
pH	8.31	7.68	8.11	7.83	8.11	8.41	7.72	9.12	8.81	8.09
Cond	219	215	674	835	2200	1533	1510	887	2000	2340
O_2	10.58	8.94	10.25	10.10	9.01	9.24	6.35	15.55	12.50	11.90
NH_4	0.33	0.41	0.14	3.30	1.15	0.25	0.49	0.49	0.41	0.58
NO_2	0.01	0.03	0.01	0.40	0.29	0.03	0.03	0.02	0.01	0.08
NO_3	0.01	0.01	1.23	1.31	0.86	2.21	1.24	1.47	0.01	1.54
PO_4	0.01	0.03	0.01	2.70	1.88	0.62	0.67	0.49	0.51	1.71
SO_4	17.2	17.3	102.3	76.8	157	119	118	165	195	282
Cl	9	12	16	141	524	269	294	343	430	458

sp: spring; su: summer. Abbreviations are explained in the text.

Contaminant levels in organisms

From the eight metals analyzed in biological samples only five were detected in all samples (Table 6.2). ANOVA analyses restricted to these five metals denoted significant (P<0.01) differences across sites and seasons (Table 6.3). Organisms of site B6 followed by B5 had the highest levels of metals being from two fold (As, Cu, Zn) to almost two orders of magnitude (Pb) higher than those measured in other locations.

PCBs, PAHs, DDTs and ENDO showed higher levels for middle and downstream sites, than upstream ones (Table 6.2). PCB levels measured at site B6 were over two orders of magnitude greater than those from site B1; those of DDTs were undetected in upstream reaches, reaching levels of 14.0 – 82.0 ng/g d.w. in downstream locations. PAHs and ENDO showed only a moderate increase from upstream (405.5 – 507.9 ng/g d.w. and 138.1 – 241.3 ng/g d.w., respectively) to downstream sites (526.8 – 689.2 ng/g d.w. and 254.3 – 420.6 ng/g d.w., respectively). AP and HCH varied little across sites, with no a clear trend towards downstream locations.

Table 6.2. Levels of metals (Mean ± SE; µg/g d.w.) and organic pollutants (Mean; ng/g d.w.) measured in *H. exocellata* larvae collected at the studied sites across seasons. LOD: limit of detection. Different letters indicate significant differences following ANOVA and Tukey's posthoc tests. Abbreviations are explained in the text.

	Metals (µg/g d.w.)																					
	As			Cu			Pb			Zn			Al			Cd		Cr		Ni	
Sites	Mean	SE		Mean	SE		Mean	SE		Mean	SE		Mean	SE		Mean	SE	Mean	SE	Mean	SE
1 sp	4.4	0.6	a	17.1	0.3	a	1.8	0.3	a	130.5	1.8	a	333.5	28.7	a						
1 su	3.1	0.8	a	17.3	0.7	a	1.6	0.1	a	135.4	6.1	a	314.6	48.4	a						
2 sp	4.4	0.5	a	19.4	0.2	a	1.2	0.1	a	144.2	3.4	a	470.2	43.6	a						
3 sp	4.3	0.8	a	14.8	0.4	a	2.4	0.4	a	116.8	5.6	a	196.8	17.8	a						
4 su	3.5	0.5	a	17.4	0.7	a	1.8	0.0	a	136.5	13.9	a	278.0	35.4	a						
5 sp	5.1	0.3	a	19.1	1.7	a	4.7	0.8	a	130.2	3.9	a	931.3	154.6	b						
5 su	5.6	0.2	b	19.8	1.2	a	5.9	0.9	a	127.4	1.9	a	1025.0	79.1	b						
6 sp	7.7	0.7	b	35.8	1.9	b	97.1	15.1	b	222.4	18.5	b	875.4	132.9	b	0.4	0.1	8.0	1.0	5.0	0.8
6 su	8.2	1.0	b	29.8	5.1	b	102.8	11.4	b	232.4	40.9	b	991.7	71.1	b	0.2	0.1	5.8	0.8	6.2	1.1
7 su	3.6	0.3	a	20.4	0.3	a	1.7	0.1	a	155.4	6.2	ab	317.5	24.7	b			4.7	0.2	2.9	0.1
LOD	2.7			0.6			0.3			4.1			158.3			0.1		1.1		1.1	

	Organic contaminants (ng/g d.w.)				
	AP	PCBs	PAHs	DDTs	ENDO	HCH
1 sp	740.1	18.5	405.2	1.4	138.1	4.4
1 su	883.4	25.0	420.5	1.4	140.5	2.3
2 sp	790.3	101.3	507.9	2.7	150.4	1.2
3 sp	854.0	70.4	455.4	1.4	241.3	6.6
4 su	1031.8	260.7	633.0	66.6	470.8	11.1
5 sp	650.5	324.9	526.8	14.0	259.6	7.3
5 su	791.2	246.1	679.1	28.1	340.6	4.1
6 sp	831.1	2993.7	619.7	56.4	254.3	4.8
6 su	831.1	1500.0	689.2	82.0	420.6	5.6
7 su	1359.4	399.1	618.4	19.4	355.7	12.7
LOD	1.5-108.2	0.4-1.7	0.4-4.3	0.2-0.6	9.8-14.1	0.4-3.6

Table 6.3. One way ANOVA results comparing log transformed metal levels and biochemical responses measured in *H. exocellata* larvae collected at the studied sites and seasons. Only the degrees of freedom (*df*), Fisher's coefficient (*F*) and significant levels corrected according to Bonferroni for simultaneous tests are depicted. *$P < 0.01$; **$0.005 < P$.

	df	*F*
Metal levels		
As	9,30	5.6**
Cu	9,30	11.9**
Pb	9,30	46.8**
Zn	9,30	7**
Al	9,30	19**
Biochemical responses		
CAT	9,42	22.3**
SOD	9,42	4.5**
GST	9,42	15.4**
GPX	9,42	3**
CBE	9,42	46.8**
CHE	9,42	21.6**
LDH	9,42	11.1**
GR	9,42	2 ns
DNA	9,42	15.3**
LPO	9,42	12.2**

Ecological status

The quality of the riparian habitat (FHI, QBR) decreased substantially from upper to downstream sites (Table 6.1) as the physicochemical parameters did. Closely linked with the deterioration of water chemistry and habitat conditions, macroinvertebrate communities were dominated by more diverse (S>20) and sensitive taxa (IBMWP >100, IASPT >5.4) in upper reaches and less diverse (S<10) and tolerant taxa to pollution (IBMWP <50, IASPT <4) in downstream sites. The exception being site B6 in summer that showed a relative quite high IBMWP score (66) but with an IASPT lower than 4 (which implies that most of the taxa are pollution tolerant). Consequently, the ten samples studied showed different degrees of deterioration and could be classified as having a very good (B1, B2), good (B3), fair (B4, B6 summer), poor (B5, B6 spring) and very poor (B7) ecological quality.

Biochemical responses of H. exocellata

Univariate ANOVA of the biochemical responses of *H. exocellata* showed that nine of the ten responses measured varied significantly across the studied populations (Table 6.3) and also denoted clear responses patterns of *H. exocellata* to pollution (Fig. 6.2). Activities of ChE and CbE decreased from upper to downstream reaches and the opposite trend was observed for the antioxidant, biotransformation and metabolic enzymes CAT, GST and LDH, respectively, and levels of lipid peroxidation and of DNA strand breaks (Fig. 6.2).

The first two components of a PCA analysis performed on the full data set of biochemical responses explained 56.6% of data variance and confirmed the findings of univariate ANOVAs. PC1 and PC2 defined a clear stress gradient separating upstream populations having high activities of SOD, ChE and CbE from those of middle and downstream reaches with high activities of CAT, LDH, GST and elevated levels of LPO and DNA strand breaks (Fig. 6.3). This means that caddisfly larvae collected from communities with a poor ecological status had lower activities of B-esterases, higher activities of antioxidant and metabolizing enzymes, and greater levels of tissue damage. By plotting the mean site scores and their 95% CI it was possible to discriminate six groups with no overlapped 95% CI: two for reference populations having a very good ecological status B1 and B2, one for population B3, that had a good ecological status, two for populations having a fair and poor ecological status (B5 and B4-B6) and one for population B7, that had a very poor ecological status. Interestingly, PCA scores of individuals collected from the same site but in different seasons were quite similar indicating that pollution sources responsible for the observed biochemical changes are acting continuously.

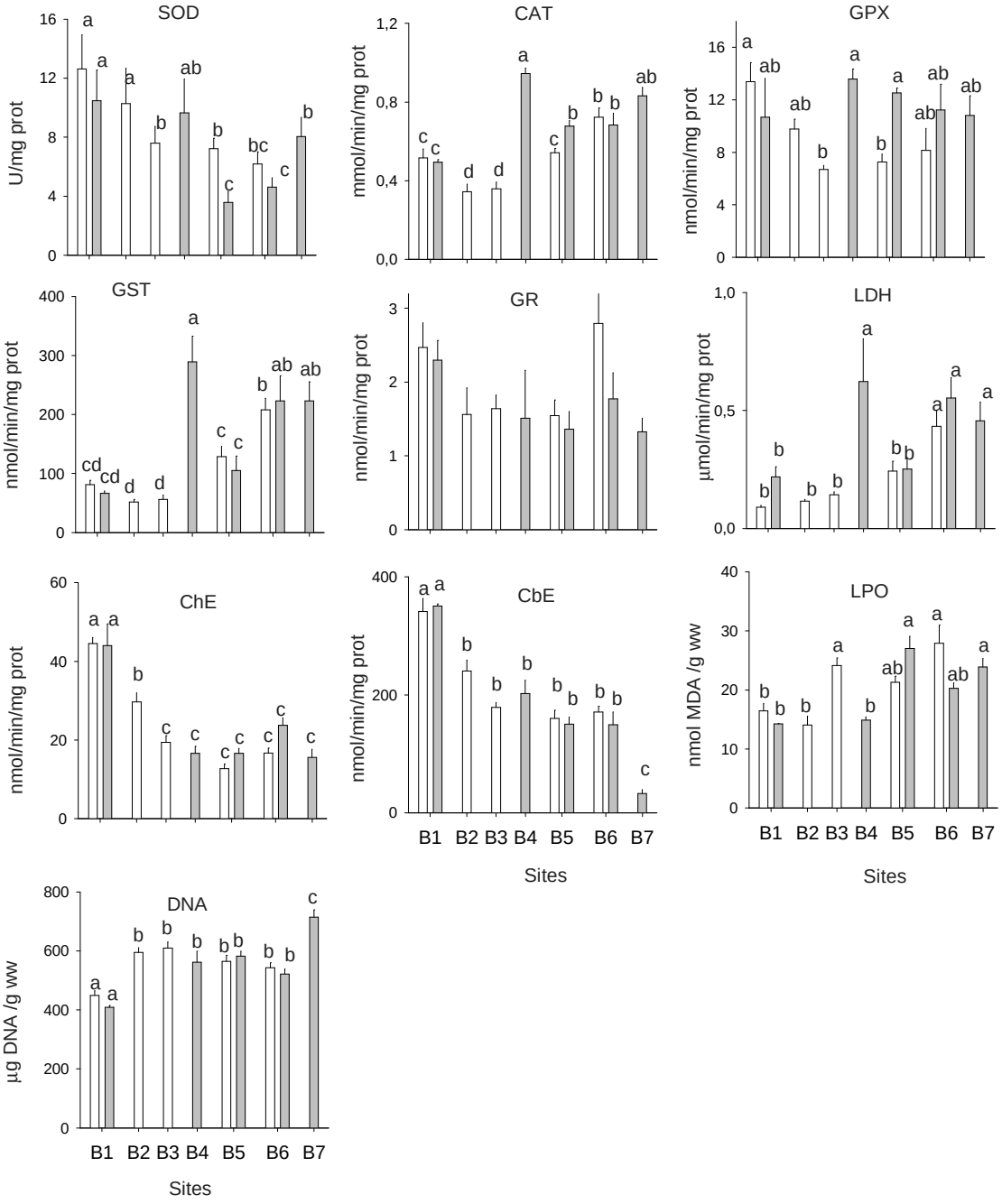

Fig. 6.2. Biochemical responses (Mean, SE) measured in *H. exocellata* individuals at the studied communities. Abbreviations are depicted in the text. Different letters indicate significant differences following ANOVA and Tukey's posthoc tests. GR graph has no letters since there were no significant differences across sites. White and grey bars indicate spring and summer samples, respectively.

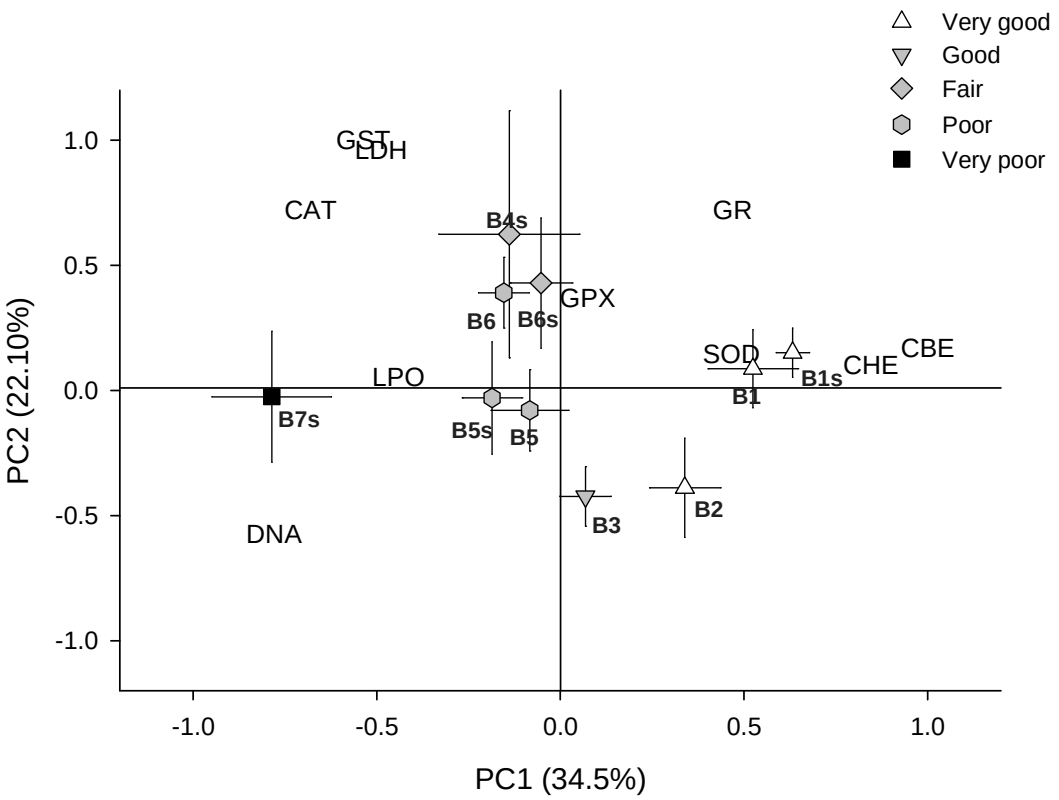

Fig. 6.3. Biplots of the first two components of a PCA performed on measured biochemical responses of *H. exocellata* collected at the studied sites and seasons. Loading abbreviations are depicted in the text. Population scores are depicted as mean values with their 95% CI. Symbols indicate the ecological quality groups of the studied macroinvertebrate communities according to WFD classification. Summer samples are identified by a "s" after the population code.

Relationships between biological and environmental factors

A PCA performed on up to 38 biological and physicochemical parameters identified the following relationships (Fig. 6.4). The ecological quality of macroinvertebrate assemblages was positively related with the quality of the riparian vegetation and habitat and inversely with nutrients and conductivity related parameters. The first two components, which accounted for 67.6 % of data variance, discriminated upstream from middle and downstream sites and evidenced that most oxidative stress and metabolic markers (CAT, GST, LPO, LDH) were positively related with the body burdens of most contaminants such as PCBs, DDT, PAHs and ENDO, as well as with conductivity parameters. DNA strand breaks were positively related with levels of HCH. The activities of B-esterases (ChE, CbE) followed a different pattern, being inversely related with contaminant body burdens and positively with good ecological quality parameters. Most populations were distributed along a diagonal line delimited by sites B1 and B7, the

exceptions being populations from site B3, which were affected by NH_4 and NO_2, and those of site B6, which despite of having the greater levels of metals, had apparently moderate biological effects.

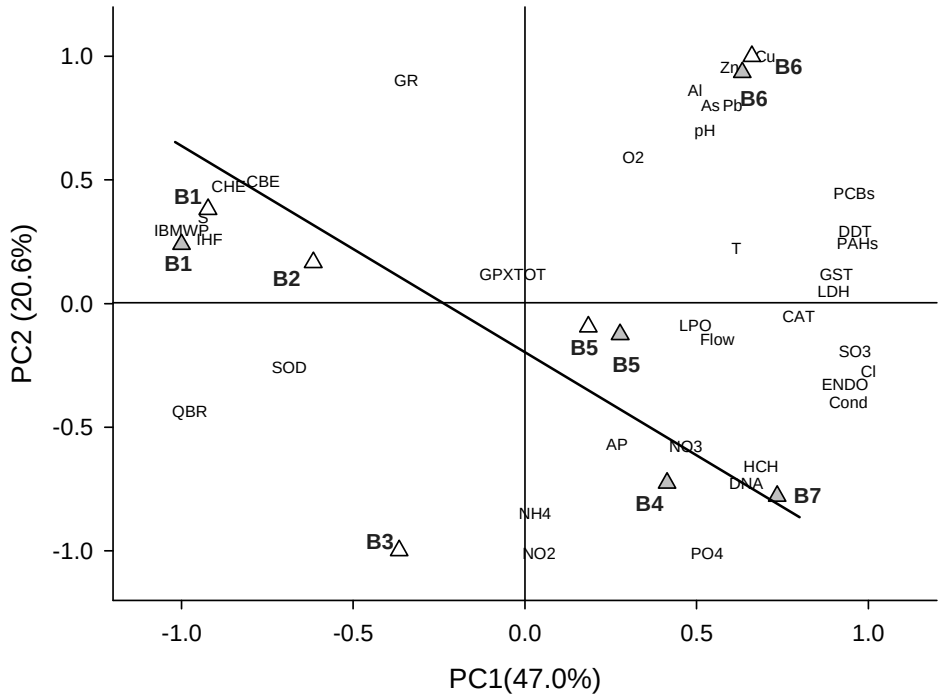

Fig. 6.4. Biplots of the first two components of a PCA performed on measured mean ecological, biochemical, water physico-chemical and contaminant body burdens measured at the studied sites and seasons. Loading abbreviations are depicted in the text. White and grey triangles indicate spring and summer samples. The regression line defined by site scores, excluding out those of B6, is also depicted.

DISCUSSION

Physicochemical water parameters and pollutant concentration levels determined in caddisfly larvae indicated a clear increase of organic and metal pollution from upper to downstream reaches in the Besós river system. These results are characteristic of Mediterranean regions, where intensive water resource use is frequently linked to the lack of water flow due to climatic constrains, and rivers can receive effluents from cities, industries and agriculture with null or scarce dilution in summer. In this situation, water quality is poor and measures that are effective for wet countries, such as the building of wastewater treatment plants, fail to recover river water biological quality (Prat and Munné, 2000).

Measured physicochemical water parameters including conductivity and nutrient load along the studied sites (Table 6.1) were high compared to levels usually found in undisturbed rivers within Europe (Chapman and Kimstach, 1996). In particular, conductivity and phosphate levels observed in downstream reaches exceeded 1000 µS/cm and 0.4 mg/l, respectively, thus denoting high organic and saline pollution compared with reference sites in the same basin (B1, B2). It is worth noting that the relatively high (>1000 µS/cm) conductivity levels found in most downstream locations are likely to be related to waste water effluents coming from treatment plants and industrial activities (Prat and Munné, 2000). Physicochemical water characteristics thus indicate sub-optimal conditions in many of the studied sites for biological communities. Indeed the IBMWP and IASPT scores obtained for the benthic macroinvertebrate community inhabiting the studied sites denoted a good ecological status for upper reaches and a poor ecological status (and very tolerant taxa) for middle and downstream reaches (Table 6.1). In this situation, caddisfly assemblages of the river are dominated by the stress tolerant species *H. exocellata* (Bonada et al., 2004).

Except Pb at site B6, measured trace metal concentrations in whole *Hydropsyche* larvae collected from the Besós River basin were in the same range of those reported for reference sites (Cain and Luoma, 1998; Cain et al., 2004; Solà and Prat, 2006). Indeed Solà and Prat (2006) reported metal body burdens of As, Cu, Zn and Cd in *Hydropsyche* larvae, collected from a mine river, one to two orders of magnitude higher than those observed in this study. The quite high levels of Pb measured in site B6 were comparable to those reported by Solà and Prat (2006) in *Hydropshyche* larvae collected from a mine river (i.e. 100 µg/g d.w.). Therefore, the previous reported information indicates that the metal levels measured in Besós river, except Pb, were far below those challenging their survival. On the other hand, organic contaminant levels of PCBs and DDTs, measured in whole *Hydropsyche* individuals collected in downstream sites, were quite high compared with reported information in related aquatic insect species. Bartrons et al. (2007) reported levels of PCBs, DDTs and HCH in trichoptera larvae collected from alpine lakes of 20, 5, 2 ng/g d.w., respectively. Kovats and Ciborowski (1993) reported levels of PCBs ranging from 100 to 300 ng/g d.w. in hydropsychidae larvae collected along St Clair, Detroit and Niagara North American Rivers. Bizzotto et al. (2009), in macroinvertebrate taxa with a similar foraging behaviour (i.e. collectors) as *Hydropsyche*, found PCB, DDT and HCH

levels of 16-190, 8-210, 1-12 ng/g d.w, respectively, in Alpine river streams from Italy. PAHs levels were of similar magnitude of those reported in larvae of aquatic insects collected from oil contaminated wetlands (Wayland et al., 2008). Therefore, it is likely to state that the organic contaminant levels measured in Besós river were close to those challenging their survival.

Biomarkers and chemical contaminants measured in feral organisms have been widely used worldwide to biomonitor detrimental effects of pollutants in the field. In few occasions, however, biomarkers have been used to determining the ecological water quality of surface waters according to WFD (Hagger et al., 2006, 2008; Jemec et al., 2009; Sanchez and Porcher, 2009; Solimini et al., 2009; Vighi et al., 2006). The biochemical based classification obtained in this study (Fig. 6.3) match quite welly with the five ecological quality types estimated according to the ACA (Munné and Prat, 2009). Our biochemical based classification was able to differentiate two reference populations having very good (B1, B2), one having a good (B3) quality scores, two more groups of populations with moderate and poor ecological qualities (B4-B6, B5) and other with population B7 that had a very poor ecological quality.

A further analysis combining biochemical and ecological traits and a broad range of environmental factors and contaminants identified different associations and contributing factors (Fig. 6.4). The principal one, defined by 7 out of the 10 communities studied, was delimited by macroinvertebrate assemblages and *H. exocellata* individuals having a good ecological status and high activities of B-esterases versus those having a poor ecological quality and high levels of DNA strand breaks. Interestingly, nutrients such as PO_4 and NO_3 and conductivity were highly correlated with DNA strand breaks and with a poor ecological status. The second and third source of variability was associated with high levels of NH_4 and NO_2 at site B3 and of metals in *H. exocellata* individuals in site B6. Markers such as high activities of CAT, GST, LDH and lipid peroxidation levels were highly related with accumulated levels of PCBs, DDTs, PAHs, ENDO and to a lesser extent with a poor ecological status and water micro-contaminants such as NH_4, NO_2, NO_3, PO_4. Therefore, markers such as CAT, GST, LDH and LPO were affected differently than macroinvertebrate assemblages to environmental stress and hence they could provide additional information to assess ecological quality of riparian communities. It is noticeable that as expected and according to the low levels of metals measured in *Hydropsyche*

larvae, most measured biolochemical responses, thus the PCA based biochemical-contaminant associations provided here agrees with measured toxic levels of contaminants in *Hydropsyche* samples. The antioxidant and phase II metabolizing enzymes CAT and GST act detoxifying reactive oxygen species and secondary metabolites (Livingstone, 2001) that may also include peroxidated products (Ketterer et al., 1983), thus they are physiologically linked with lipid peroxidation levels, which is a marker of oxidative stress mediated tissue damage (Halliwell and Gutteridge, 1999). Accordingly the relationship of these three markers with pro-oxidant factors such as organic pollutants and conductivity related parameters is expected to occur (Di Giulio et al., 1995; Halliwell and Gutteridge, 1999). High activities of lactate dehydrogenase have been associated to increased metabolism under stressful conditions (Menezes et al., 2006; Moreira et al., 2006), thus its association with the previous enzymes and pollution is also reasonable.

One of the greatest efforts of environmental state agencies for the implementation of the WFD have been to develop robust and harmonized ecological monitoring tools to assess the water quality of surface waters across EU countries (Munné and Prat, 2009), which implies a considerable effort, time and money. Biomarkers, although not incorporated in the WFD, are among the emerging biological monitoring tools considered for implementation of the WFD (Allan et al., 2006; Mills et al., 2007). By 2020, EU member states will have to improve the quality of their surface waters and report those changes to the WFD. In this sense, the use of markers sensitive to water pollution may provide useful information on small changes in water quality specially in the border of moderate to poor quality. Here, the studied biochemical responses provided a more fine classification than the currently used biological indexes, differentiating populations within communities having a good and deteriorated ecological state. Interestingly, our approach was specially robust non differentiating populations from the same site, collected in different months, indicating that pollution sources responsible for the biochemical responses are acting continuously.

The determination of biochemical markers in sites that are in good or moderate state will give us valuable information of the health of their populations. Sites in good ecological status with populations that have some of their biomarkers afected will imply that the site may change to a lower status in the future and therefore measures can be taken to improve the water quality. This study, however, was limited to a river system impacted

basically by industrial and domestic pollutants coming from waste water treatment plants. Further studies including more river types and environmental stresses conditions are needed to generalize our findings and to establish thresholds of biomarkers that should not be trespassed, this thresholds may act as sentinels of further degradation of the ecological condition of the streams.

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Chapter 7

Concluding Remarks

7. CONCLUDING REMARKS

Biomarkers can be used to assess the health status of organisms and to obtain early-warning signals of environmental risks. They are a way of integrating the concentration of toxicants in environment with the bioavailability and effects to the target species, allowing us to approach to a dynamic picture of the exposure scenario (McCarthy and Shugart, 1990; van der Oost et al., 2005; Shugart et al., 1992). Until now, however, the use of biomarkers to diagnose ecological impairment in rivers is restricted to only few fish, but even fewer invertebrate species that, in most cases, do not represent local species. For this reason, there is an urgent need to develop biomarkers in key macroinvertebrate species within the river ecosystems that are the object of study. Furthermore, biomarker responses in these species can provide valuable information about the specific toxic effects caused by contaminant substances in the local macroinvertebrate communities and also the probable repercussions at higher community levels.

Chemical contaminants have multiple toxicological modes of action and hence affect many biochemical responses within the organisms. These responses include: changes on phase I and II biotransformation enzymes, that convert the parental toxicant into more soluble metabolites that are easy to be eliminated; oxidative stress, mediated by biochemical processes of enzymes as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR), associated to the presence of reactive oxygen species (ROS); consequences of oxidative stress, as lipid peroxidation, DNA damage and perturbed redox status; neurotoxic responses of B-esterases enzymes, acetylcholinesterase (AChE) and carboxylesterase (CbE); alterations on the metabolic state of the animal (van der Oost et al., 2003, 2005; Livingstone, 2003; Halliwell and Gutteridge, 1999). Therefore, monitoring programmes based on biomarker procedures should consider a broad range of biochemical responses.

On behalf of the above stated, this thesis had two main objectives, that were tested along Chapters 2 to 6, and that generated the following concluding remarks:

(1) The use of biomarkers to find out the physiological mechanisms of genetic adaptation to the organophosphate pesticide fenitrothion in *Daphnia magna* (Chapter 2). Genetic adaptation to pollution has been an important research line of the group leaded by

Amadeu M. V. M. Soares (CESAM & Biology Department, Aveiro University, Portugal) and of Carlos Barata (Consejo Superior de Investigaciones Científicas, Barcelona, Spain) for over two decades. Firstly, this line was focused on Risk Assessment and aimed to reduce the inherent genetic variability in tolerance present among different *Daphnia magna* clones (Baird et al., 1991, 1998; Barata et al., 1998, 1999; Soares et al., 1992). Secondly, it expanded to evolutionary genetics, and aimed to study the genetic and ecological consequences of adaptation to pollution of field populations of cladocerans (Barata et al., 2000, 2002a, 2002b, 2002c). In this thesis, this line was proceeded with the study of the physiological mechanisms of tolerance to pollution. In particular, it focused on the study of *Daphnia magna* tolerance to organophosphorous pesticides, to continue a previous work (Barata et al., 2001) started a decade ago with Lúcia Guilhermino (ICBAS & CIIMAR, Porto University, Portugal) and Amadeu M. V. M. Soares.

This study was performed in *D. magna* populations selected from Ebro Delta (NE Spain), an area devoted to intensive agricultural activities, principally rice culture, that poses important environmental threats derived from the extensive use of pesticides such as the insecticide fenitrothion (Escartín and Porte, 1996). From the rice fields of the Ebro Delta were collected sensitive and resistant clones to fenitrothion, whose tolerances differed up to six fold. Toxicity results were similar to those reported by Barata et al. (2001) and indicated moderate levels of tolerance compared with that found in *D. magna* for cadmium (over two orders of magnitude; Baird et al., 1991) or in insects and mite species for organophosphorous pesticides (over 100 fold; Roush and Mckenzie, 1987; Dunley et al., 1991; Morton, 1993). Biomarker results point towards that the differences on the bio-activation of the phosphorotionate OP pesticide to its active oxon metabolite contributed mostly in explaining the observed levels of resistance. This process is physiologically regulated by specific cytochome P450 mixed function oxidases (MFO). Therefore, the responses to fenitrothion of these *D. magna* clones can be a model system to study the biochemical processes involved in the development of population resistance of non-target aquatic invertebrates to pesticides, since it is crucial to understand the capability of wild populations to adapt and hence to avoid extinction under long term exposure to OPs.

(2) The use of biomarkers as diagnostic tools of pollution in riparian habitats (Chapters 3, 4, 5, 6). Likewise the previous objective, Amadeu M. V. M. Soares and Carlos

Barata had also been actively involved in developing complementary Environmental Risk Assessment monitoring tools to implement the existing ones in freshwater ecosystems. Most of their activity has been focused in in situ bioassays to allow determining subtle and functional effects on key ecological processes (Dos Santos et al., 2002; Faria et al., 2006, 2007a, 2007b; Lopes et al., 2007; Moreira-Santos et al., 2005; Pereira et al., 1999, 2000; Pestana et al., 2009; Rendón-Von Osten et al., 2006). This thesis aimed to complement this line of research with biomarkers.

In Chapter 3, *D. magna* in situ post exposure feeding responses combined with the study of biomarkers, water pollutants and biological indices were used in two rivers (Llobregat and Besós, NE Spain) to discriminate and identify sources of anthropogenic impairment and pollution. These two rivers provide water resources to the city of Barcelona and they suffer from an overexploitation of their water resources, destruction of their riparian communities and diffuse pollution coming from waste water treatment plant effluents. They have been exhaustively studied by the local environmental water authorities. Therefore, they offer an excellent system to test the purposed diagnostic tools. The results obtained allowed to discriminate different sources of anthropogenic impairment. In situ bioassays and those of macroinvertebrate assemblages were affected by distinct environmental factors. From up to twenty environmental variables considered, seven of them including habitat degradation, suspended solids, nitrogenous and conductivity related parameters affected macroinvertebrate assemblages. On the other hand, levels of organophosphorous compounds and polycyclic aromatic hydrocarbons were high enough to trigger the responses of *D. magna* in situ bioassays. These results emphasize the importance of combining biological indices with biomarkers and, more generalized and ecologically relevant (grazing), in situ responses to identify ecological effects of effluent discharges, from sewage treatment plants, in surface waters.

In Chapter 4, a large set of biomarkers and contaminants were analysed in Asiatic clams (*Corbicula fluminea*) transplanted along an area impacted by pesticides related to rice culture (Ebro Delta). Environmental hazards were assessed by measuring up to 46 contaminant levels and 9 biomarker responses during the main growing season of rice (from May to August). Measured biological responses showed marked differences across sites and months. Antioxidant and esterase enzyme responses were in most cases inhibited. Lipid peroxidation levels increased steadily from May to June in drainage channels.

Endosulfan, propanil and phenylureas were the chemical contaminants causing the most adverse effects in the studied species.

The aim of Chapter 5 was to evaluate the effect of the application of the herbicide Herbolex to control giant reed (*Arundo donax*), which has glyphosate as active ingredient, on the structure and function of a nearby river ecosystem. Specifically, the glyphosate environmental fate in the surrounding water was assessed, as well as its effects on transplanted *Daphnia magna,* in field collected caddisfly *(Hydropsyche exocellata)* and on benthic macroinvertebrate structure assemblages. Investigations were conducted in the industrialized and urbanized Llobregat river, before and after a terrestrial spray of the herbicide. Measured glyphosate levels in river or pore water lasted up to 12 days following herbicide application. Effects were only detected in transplanted *D. magna* and in field collected *H. exocellata* biomarkers. Effects included *D. magna* feeding inhibition and oxidative stress related responses, such as, increased antioxidant enzyme activities, related with the metabolism of glutathione, and increased levels of lipid peroxidation. These results emphasize the importance of combining chemical, ecological and specific biological responses to identify ecological effects of pesticides in the field.

Finally, in Chapter 6, a multibiomarker approach was applied on a macroinvertebrate keystone species (*Hydropsyche exocellata*) to try to diagnose subtle and specific effects of contaminants on macroinvertebrate communities having a good and poor ecological water quality. The study aimed to compare the ecological water quality classification provided by the Water Framework Directive (based on biological indexes) with that obtained with biomarkers. Results evidenced a clear deterioration of the ecological water quality parameters and benthic communities towards downstream reaches. Nine of the ten markers analyzed varied significantly across the studied populations and were able to differentiate more finely populations within communities having a good and deteriorated ecological stage. Some biochemical markers, such as, the activities of B-esterases and DNA strand breaks, co-varied with changes in macroinvertebrate assemblages. Others, such as, antioxidant enzymes, metabolizing enzymes and lipid peroxidation levels, responded differently and were closely related to high and presumably toxic levels of accumulated organic pollutants. Therefore, these results indicate that the use of markers sensitive to water pollution may provide complementary information to monitor changes in water quality.

In resume, the experimental approach and the results presented in this thesis indicate that the combined use of biomarkers with different metrics, such as biological indices and in situ tests, improve substantially our ability to diagnose detrimental effects of pollutants in riparian communities. Future work thus should be focused to develop biomarkers in more keystone species.

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