Université de Toulouse

THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :
Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

Discipline ou spécialité :

Doc. U. Écologie et Évolution des Populations et Communautés / Écotoxicologie

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Présentée et soutenue par :
Cândida SHINN

le : jeudi 30 septembre 2010

Titre :

Impact of toxicants on stream fish biological traits

JURY
Prof. Tinka MURK
Prof. Helmut SEGNER
Prof. Amadeu SOARES
Prof. Michèle TACKX
Prof. Eric Pinelli

Ecole doctorale :
Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénieries (SEVAB)

Unité de recherche :
Laboratoire Evolution et Diversité Biologique

Directeur(s) de Thèse :
Prof. Sovan LEK
Dr. Gaël GRENOUILLET

Rapporteurs :
Prof. Tinka MURK
Prof. Helmut SEGNER

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Ph.D. thesis

Cândida Shinn

Supervised by:

Prof. Sovan Lek

Dr. Gaël Grenouillet

Laboratoire Evolution Diversité Biologique

Université Paul Sabatier, Toulouse, France

Dedicated to
my parents

Christine and Raymond Shinn

Table of contents

Dissertation structure

The present dissertation is divided into six chapters:

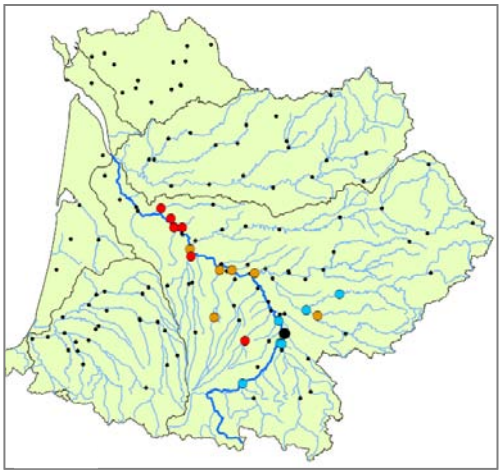
→ **Chapter 1** is a general introduction to the topic of water contamination and environmental assessment approaches, with reference to the main objectives of the research performed during the Ph. D. project.

→ **Chapters 2 to 5** are the development of each stated objective, in the structure of scientific articles, one already published, the remaining three as manuscripts to be submitted. Information needed to further detail the project objectives stated in chapter 1 is incorporated within the introductory sections of each article.

→ **Chapter 6** summarizes the main findings in a general conclusion.

1.

Introduction



1.1. A chemical Europe

In recent years, many scientific and political efforts have been made to assess the health of surface waters throughout Europe. The increasing number of new toxic substances appearing in the environment has caused concern for many decades. However, remedial and preventive action has been slow to take effect.

European legislative programs such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances) are the fundamental base of contaminant management in the European Union (EU). Although the EU regulation now gives greater responsibility to industry to manage the risk from chemicals and provide safety information on both old (existing) and new (emerging) substances, the implementation of the new controls on the authorisation or restriction of use of the estimated 100,106 commercialised chemicals will take many years to be implemented. In the meantime, many potentially hazardous chemicals are still being used or are sometimes used illegally, and thus continue to pose a threat to humans and the natural environment.

Of the 100,106 existing substances needing to be tested, 141 high-volume and/or most hazardous chemicals have been submitted for prioritisation by the European Commission and Member States, but only 39 have been selected as Substances of Very High Concern (SVHC) under REACH so far, although the restrictions applied to the existing 141 maintain. Regarding the hazard testing of chemicals on the market, minimum or very little data exists for 76% substances, no data for 21%, and sufficient data for only 3%.[1]

Historical pollution of persistent organic or inorganic substances is an additional load on the environment that must also be taken into account when considering the further release of contaminated effluents (such as industrial, urban and agricultural effluents) into already impacted environments.

1.2. European approach to water pollution

Clean water is undoubtedly vital for public health and ecosystems. In order to guarantee a basis for adequate water quality for humans and the natural environment, the EU has requested that all member states attain at least good ecological and chemical water quality status in all surface water bodies by 2015, stated in what is know as the Water

[1] Source of information on EU chemicals regulation: http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm (July 2010).

Framework Directive (WFD; Directive 2000/60/EC of the European Commission, 2000).[2] To support these goals the Directive foresees the elimination of priority hazardous substances within 20 years and sets limits on the concentration of specific pollutants identified by the EU as priority substances. Ongoing water quality monitoring programs have thus been adapted and extended to reach these objectives, in collaboration with national and international research programs (e.g. EU-funded projects such as Keybioeffects, ModelKey, NoMiracle, OSIRIS, and REBECCA).

Another Directive, published in 2008, establishes limits, known as Environmental Quality Standards (EQS), for the 33 priority substances that have been identified to date, and for an additional 8 substances regulated under previous legislation. An innovation of the current WFD in relation to preceding EU legislation is the inclusion of the ecological status, which addresses other perturbations such as dams built on rivers and water abstraction for industry or irrigation.[3]

The WFD is supported by other EU environmental legislation, in addition to the REACH Regulation controlling chemicals in products to reduce the contamination of water bodies. The Directives on Plant Protection Products (pesticides) and on Biocidal Products control pollution from agricultural chemicals and from pest-control and anti-microbial substances used in other sectors. The Nitrates Directive limits nitrogen pollution from fertilisers and manure, whilst the Directive on Industrial Pollution Prevention and Control regulates pollution from factories and other facilities.[3]

The WFD's 2015 target of good chemical and ecological status for all member state surface water bodies is a significant challenge. An assessment in early 2008 estimated that at least 40% of the EU's surface water bodies are at risk of not meeting this objective, and a further 30% are in need of additional data for assessment (Fig. 1).

[2] According to the WFD, a surface water body is a section of a river, a lake, transitional waters or coastal waters.
[3] Source of information on the EU strategy against chemical pollution of surface waters: http://ec.europa.eu/environment/water/water-dangersub/index.htm

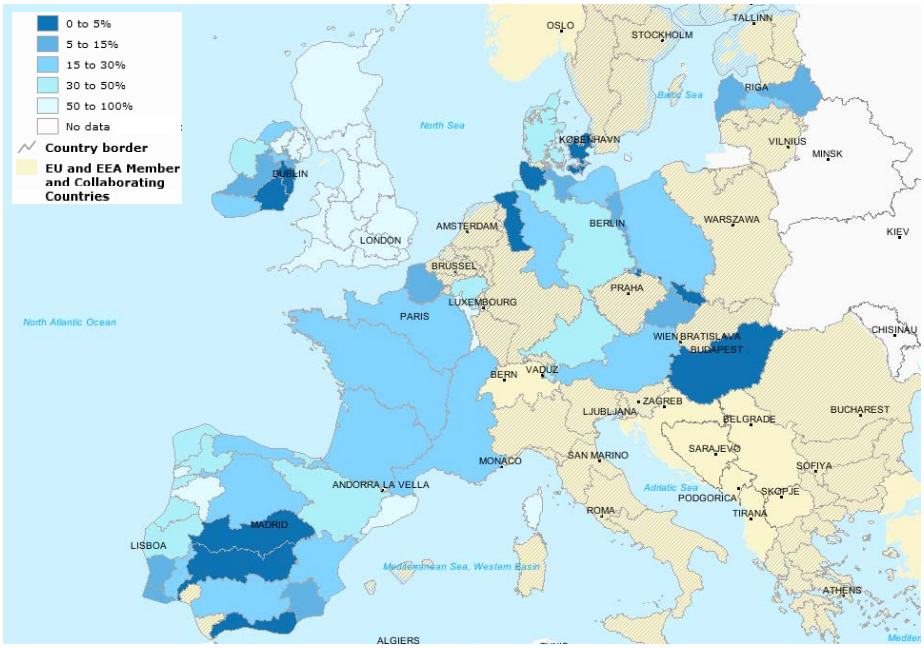


Figure 1 *- Percentage of Surface Water Bodies in each National River Basin District classified as not at risk, as of reporting of member states in 2009 (EEA, 2010).*

To simplify the EU reporting on the ecological status of water bodies, member states are required to classify each water body into a 5-scale classification key, upon integration of measured biological, chemical and hydromorphological parameters (Fig. 2). Each level is calibrated according to the deviation from reference conditions, specific to a type of water body. Ecological status assessment therefore facilitates detection of adverse ecological effects, while acting at the community level and integrating multiple stressors.

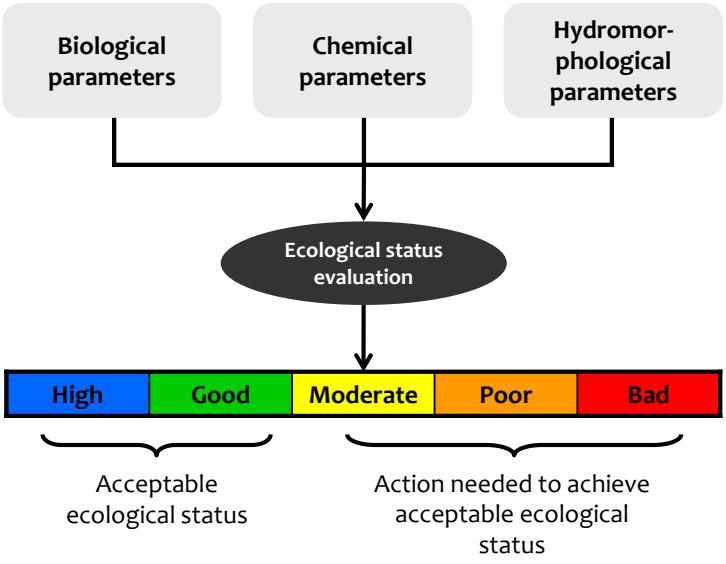


Figure 2 *- The ecological status evaluation process of surface water bodies.*

1.3. Status evaluation of rivers

With respect to rivers, their health changes along their course, generally being near-pristine at higher altitudes near the source and gradually becoming more polluted (due to agriculture, industry and urbanization) and transformed (by water extraction, dams, flow control and deviation) further downstream. A river's background geology and size can also vary geographically. It is therefore important that rivers be evaluated accordingly to their different longitudinal gradients and that multiple assessment points are considered along the various and differently impacted sections. In failing to do so, a river that may have an overall good status (average of high, good, and moderate status along the river), will not receive the necessary attention to the sections that are of lower status (Fig. 3).

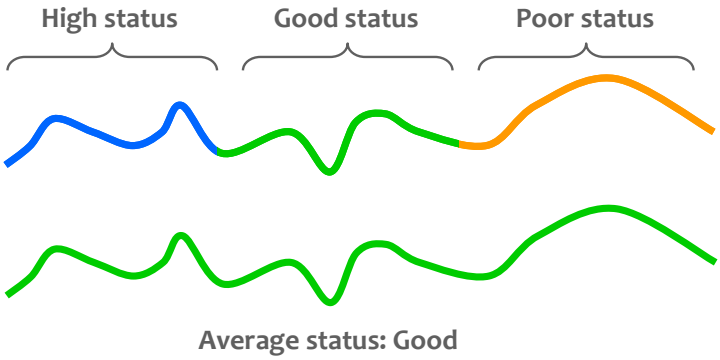


Figure 3 *- Illustration of a river classified by multiple segments (above) and by the overall status when averaging the quality of all segments (below). Adapted from EC Water Note 2 (2008).*

Ecological status concerns the quality of the structure and functioning of aquatic ecosystems. Therefore, sensitive biological measurement tools, also named bioindicators or biomarkers, are needed to adequately assess the integrity of rivers. Such tools will aid water managers in assessing, protecting and if necessary remediating such an important resource. The process through which biological and other tools are incorporated within the water body status evaluation process is based on three types of environmental monitoring, clearly distinguished by the WFD (Fig. 4): *surveillance monitoring* helps validate risk assessments and detect long-term trends, such as those resulting from historical contamination or climate change; *operational monitoring* allows for establishing the status of water bodies and whether they meet their environmental objectives, as well as monitor changes in their status; and *investigative monitoring*, on a case-by-case basis, determines the cause of failure or risk of failure to achieve good status (when this information cannot be obtained via

operational monitoring) and investigates the magnitude and impact of accidental pollution (EC, 2000; Dworak et al., 2005).

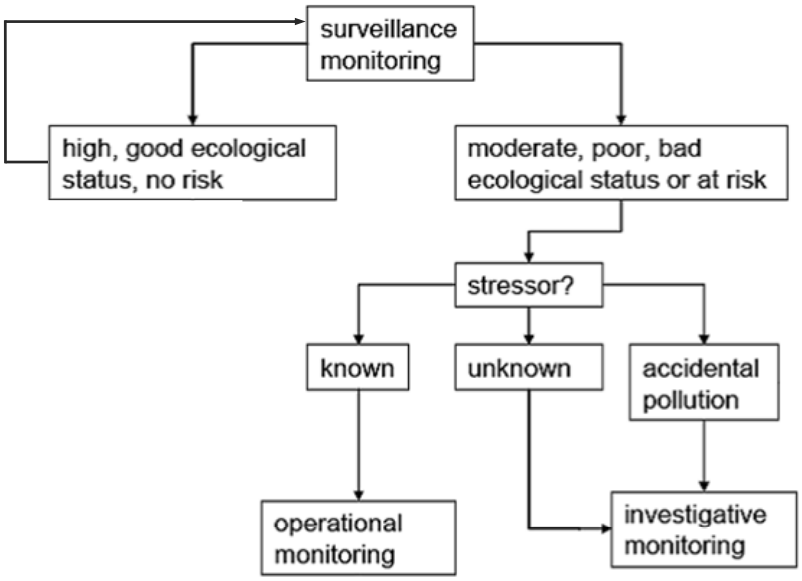


Figure 4 *- Decision tree regarding water body status and subsequent types of monitoring programs suggested by the WFD required. Adapted from Alterra MONSTAR (2010).*

1.4. Tools from ecotoxicology

The field of ecotoxicology contributes to the further understanding of the effects of toxic substances, natural or synthetic, on constituents of ecosystems (Truhaut, 1977; Chapman, 1995). Knowledge on the ecotoxicological effect of certain substances or mixtures of substances on organisms supports the development of methods that can be applied in environmental monitoring programs (Sanchez and Porcher, 2009). In this way, biomarkers, biosensors or whole-organism bioassays complement the information provided by more conventional approaches to environmental assessment and monitoring (Allan et al., 2006). Bioindicator species or, more specifically, individual-based biomarkers measured in exposed and non-exposed organisms, are highly integrative tools that link biological effects and the concentrations of environmental contaminants. Establishing this link is the basis to answer WFD targets related to the present and future assessment of ecosystem health.

Due to difficulties in attributing observed effects to environmental contamination, it is often difficult to find a clear relationship between the contamination and the response of wild populations (Schulz and Liess, 1999). Aquatic ecosystems are continuously being shaped by physical and chemical dynamics as well as ecological processes, which can have parallel

effects on communities and act as confounding factors in assessing the effects of pollutants (Nedeau et al., 2002). Such a multitude of interactions poses considerable challenges to ecotoxicological studies, examples of which are listed in table 1.

Challenges	**Examples**
Low concentrations of pollutants and long exposure times (chronic effects)	• Endocrine disruption • DNA damage/mutagenesis • Deficiencies in the immune system • Neurological effects
Multiple effects by single pollutants	• Multiple target sites and multiple modes of toxic action • Time- and tissue-dependent • effects
Complex mixtures of pollutants	• Wastewater treatment plant effluents • Field runoff • Pollutants and their degradation products • Complexes of chemical compounds
Multiple stressors	• UV and pollutants • Temperature and pollutants • Habitat alterations and pollutants • Pathogens and pollutants
Ecosystem complexity	• Variations in species sensitivities • Effect of propagation from organisms to populations and ecosystems • Identification of the stressor–effect relationship

Table 1 - *Examples of current challenges in ecotoxicology. Adapted from Eggen et al. (2004).*

It is therefore important to study a range of different biological variables in exposed and non-exposed (from reference conditions) organisms, as well as a suit of additional environmental parameters, so that cause-effect relationships between contamination and ecosystem response can be correctly established. Furthermore, while acute pollution generally has immediate and visible effects, and the cause is obvious and usually pinpointed, there is now evidence that chronic, sub-lethal pollution entails longer-lasting and less easily detected ecological consequences (Eggen et al., 2004). If chronic pollution and its effects are not assessed in a timely manner, consequences may go from disruption of a particular habitat or community, to long-term decline and eventually risk of extinction (Tanaka, 2003).

In addition to surveys carried out in the wild, classical ecotoxicological tests performed under controlled laboratory or semi-natural conditions contribute with fundamental knowledge of the direct effect of toxicants on organisms. The long-lasting debate on the extrapolation of laboratory-based conclusions to natural conditions may never be resolved (Kimball and Levin, 1985; Seitz and Ratte, 1991; Selck et al., 2002), but the importance of toxicant-orientated, single or multiple compound tests is irrefutable within environmental risk assessment (Chapman, 2002; Breitholtz et al., 2006). In order to verify whether a biological response does indeed occur when organisms are exposed (and not occur when slightly or not exposed, i.e., in reference conditions), bioassays must be developed and thoroughly tested. Ultimately, inter-calibration (between different testing institutions) and standardization (e.g. publishing of official test guidelines) of new bioassays can be performed in order to validate their integration in regular surveillance monitoring and/or situation-specific Environmental Risk Assessment (ERA).

1.5. Fish as bioindicators

Easy to capture and fairly easy to maintain and rear in captivity, freshwater fish are remarkable indicators of aquatic ecosystem health status. One only has to remember the visual impact that mass fish mortality in a number of acutely polluted rivers and lakes (Varshney, 1971; Chin Sue, 2002; Maheshwari, 2005; Chellappa et al., 2008) has on society and the media, and the political consequences they sometimes entail (Clark, 1995), to recognize their value as messengers of perturbed environments. The importance of fish communities for the balance within the aquatic ecosystem as well as their economic value has lead to their increasing use in routine monitoring of continental waters.

Either with economic or nature conservation intent, most developed countries have implemented national programs to survey fish populations on a yearly basis, collecting data on fish assemblages (presence/absence of species) and population size (abundance of fish; e.g., ONEMA in France). Such surveys are often performed in parallel to periodic surveillance monitoring of ecosystem characteristics such as water quality and hydromorphology, performed by local water agencies. Both types of monitoring are crucial in keeping a clear record of the evolution of fish populations and their surrounding habitat.

1.6. Current fish-based tools for environmental assessment, at the individual or population level

Fish are generally one of the most long-lived organisms in aquatic ecosystems. They thus integrate the history of the evolution of their surrounding habitat. There are an increasing number of studies using fish as ecological sentinel species, and specific responses of these organisms as integrators of past and existing environmental conditions, through multi-marker and multi-level of organisation approaches. Assessment can be performed at different levels of organisation, from whole fish communities (e.g. fish assemblages) down to the molecular level (e.g. gene expression), as is illustrated in figure 5. Different biological indicators have different levels of ecological relevance due to their varying capacity to translate the effects of, for example, physiological change on an individual, and consequently of individual responses on populations and communities (Jobling and Tyler, 2006). Ecologically-relevant endpoints are important in the ecological risk assessment process and also in environmental compliance and regulatory assessment (Adams and Greeley, 2000). Some biomarkers have broader response times, such as reproduction, as the effect of a contaminant can either have an effect on organisms' reproduction at a later stage of its life (long response time) or more immediately at a particular reproduction event (short response time). Cellular-level responses are generally more sensitive than organism or population-level responses, as the latter are more likely to be affected by interfering factors such as other environmental parameters, competition, predation, etc. Bioindicators not only track and reflect changes at higher levels of biological organization and function, suggesting causal relationships between these levels, but also function as sensitive early-warning indicators of improvement in the health of sentinel species.

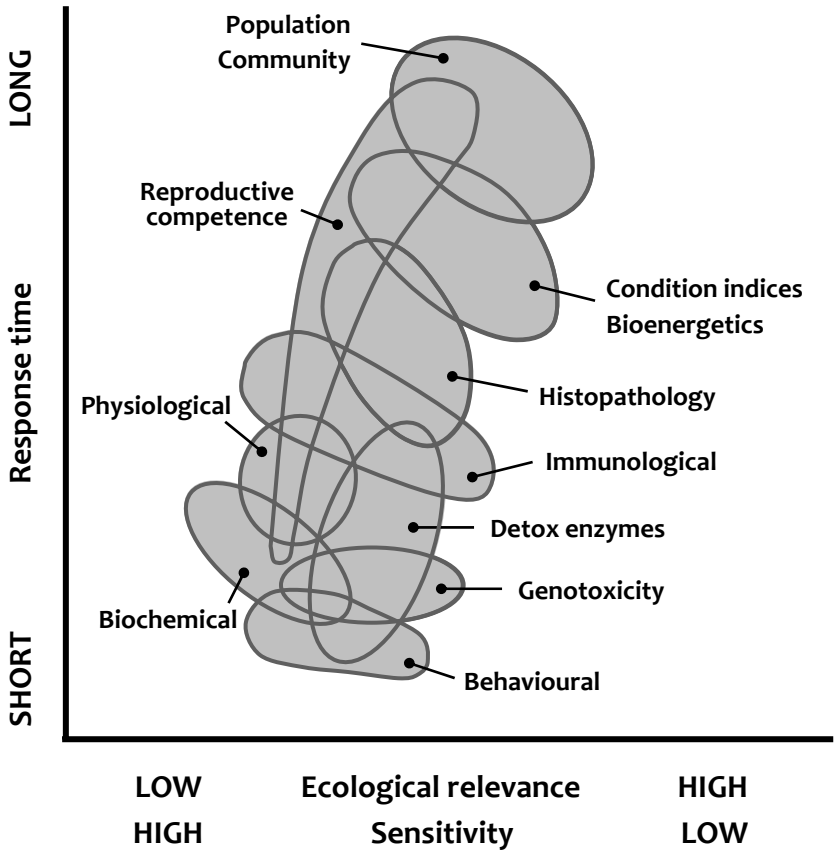


Figure 5 - *Position of biological indicators - like fish response to environmental stressors - according to their specificity, ecological relevance and response time. Adapted from ORNL (1995) and Sanchez and Porcher (2009).*

The ideal water body ERA campaign would include biomarkers from all levels of organisation (Table 2) and species representing different habitat compartments, as well as control for as many environmental and biological interaction factors as possible. Over that last decade, several large monitoring networks have been developed in an attempt to cover most aspects of integrated environmental studies, two examples being: Biomonitoring of Environmental Status and Trends (BEST; comprehensive guideline published by Bauch et al., 2005) and Programme for the Assessment and Control of Pollution in the Mediterranean Region (MEDPOL; marine environment).

Biochemical	Physiological	Histopath	Individual	Population	Community
MFO enzymes	Creatinine	Necrosis	Growth	Abundance	Richness
Bile metabolites	Transaminase enzymes	Macrophage aggregates	Total body lipid	Size & age distribution	Index biotic integrity
DNA integrity	Cortisol	Parasitic lesions	Organo-indices	Sex ratio	Intolerant species
Stress proteins	Triglycerides	Functional parenchyma	Condition factor	Bioenergetic parameters	Feeding types
Antioxidant enzymes	Steroid hormones	Carcinomas	Gross anomalies	Reproductive Integrity	

Table 2 - *Representative bioindicators measured at six major levels of biological organization (Adams and Greeley, 2000). The list does not include all possible biological effects that can be measured in bioassessment programs, but rather those that the authors have noticed that work best in a variety of aquatic systems (streams, rivers, lakes, estuaries) under a variety of environmental stress situations. MFO, mixed-function oxygenase detoxification enzymes.*

Although the above-stated "ideal water body ERA" is prohibitive from a practical and economical standpoint, there are examples of multi-marker approaches at smaller-scale sites, using not only fish (Stein et al., 1992; Adams et al., 1999; Adams and Greeley, 2000; Flammarion et al., 2002; Sanchez et al., 2008), but also biofilms (Sabater et al., 2007; Bonet et al., 2010), macroinvertebrates (Pinel-Alloul et al., 1996; Rogers et al., 2002), diatoms (Debenest et al., 2010), or several of those organism groups (Manny and Kenaga, 1991; Hering et al., 2006; Statzner and Bêche, 2010).

The capability to identify relationships between contaminants and fish responses increases when contaminant burden in biological tissue is measured, confirming the actual presence of the toxicants in the same organisms for which the biomarkers are studied. If contaminant levels in the environment are then strongly linked to different types of responses in the organism as well as to tissue concentrations, a relationship between environmental contamination and organism health can be evidenced.

1.7. Framework of the present study

1.7.1. Heavy metals over time

In the past, the impact of industrial contamination on aquatic environments has been of major focus in ecotoxicological studies, mainly as a result of the rapid development that

occurred since the industrial revolution. Persistent contaminants such as polychlorinated biphenyls, heavy metals, and dioxins, have been released into the environment from the dawn of the industrial revolution. Concerns regarding public, and eventually ecosystem health have lead to extensive environmental assessment of the impact of industrial contaminants, as well as the establishment of protective measures such as restrictive legislation. Although, currently, the number of reported studies in this field is enormous, it nevertheless remains important to continue monitoring the status of both pristine and impacted water bodies. In this way we are able to survey the degradation, maintenance, or improvement of ecosystem status and, if necessary, intervene in a timely manner.

In the context of this framework, I studied, via surveillance-type monitoring, the presence of heavy metal pollution in an impacted river over time (**Chapter 2**). The River Lot in southwest France has a history of heavy metal contamination due to mining activities in an upstream section of the watershed. Over two decades ago, a study was conducted to assess the extent of heavy metal accumulation in fish species and the environment. Here I report results from field monitoring performed 20 years apart, at the same sampling sites and with the same fish species (roach, *Rutilus rutilus*; bream, *Abramis brama*; perch, *Perca fluviatilis*).

1.7.2. Pesticide gradients and mixture toxicity

A fast growing world population has lead to the expansion of agriculture, and with it the development of a vast range of plant protection products. However, it is only over the past few decades that the impact of agriculture on the environment has become of greater concern. Furthermore, the development and usage of a vast range of plant protection products has resulted in a large diversity of substances that reach the aquatic environment. For most of these substances, no or very little information is known regarding their toxicity to aquatic organisms. And because pesticides have mostly not been designed to affect fish, few studies have focused on the side-effects they may indeed have on fish species.

Using investigative-type monitoring, I assessed the impact of agrochemical pollution on wild fish populations. More than half of the Adour-Garonne catchment area in southwest France is covered by agricultural land (Tisseuil et al., 2008). The Adour-Garonne water agency monitors pesticide concentrations throughout the watershed, information that is used here to evaluate the potential toxic pressure of the pesticide contamination on aquatic

organisms. The pesticide toxicity level is then related to different biological parameters studied in feral gudgeon (*Gobio gobio*) and chub (*Squalius cephalus*) captured at sampling sites with different pesticide levels. Gudgeon were evaluated for pesticide-related morphometric differences among sites, by accounting for a number of potentially confounding variables such as genetic differentiation between sampling groups, environmental parameters, and geographical distances between sites (**Chapter 3**). Chub were assessed for differences in biological indices (condition factor, hepato- and gonado-somatic indices), accumulation of pesticides in liver and muscle, and hepatic histological signs of adverse effects among the sampling sites (**Chapter 4**).

The Adour-Garonne pesticide concentration dataset was also used to select a mixture of three co-occurring pesticides – atrazine, linuron and metolachlor – to test in laboratory conditions. To assess if environmentally relevant levels of the mixture affected fish, the behaviour of juvenile rainbow trout (*Oncorhynchus mykiss*) exposed for 5 days was observed (**Chapter 5**). The quantitative behavioural endpoints observed were compared between control and exposed groups.

With these studies, different aspects of fish biology are used to evaluate the impact of contamination on freshwater fish species. The direct application of these studies within European Union's Water Framework Directive (WFD), although not immediate, is expected to contribute to the evaluation of the usefulness of fish species as sentinels of river water quality.

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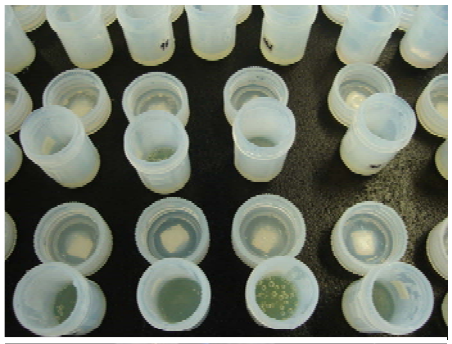
Temporal variation of heavy metal contamination in fish of the River Lot in southern France



Ecotoxicology and Environmental Safety 72 (2009) 1957–1965

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Temporal variation of heavy metal contamination in fish of the river lot in southern France

C. Shinn [a,*], F. Dauba [b], G. Grenouillet [a], G. Guenard [a], S. Lek [a]

[a] EDB (Laboratoire Evolution et Diversité Biologique), UMR 5174, CNRS-Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse cèdex 4, France
[b] ENSAT (École Nationale Supérieure Agronomique de Toulouse), Avenue de l'Agrobiopole, BP 32607 Auzeville-Tolosane, F 31326 Castanet-Tolosan Cedex, France

ARTICLE INFO

Article history:
Received 21 February 2009
Received in revised form
17 April 2009
Accepted 12 June 2009
Available online 14 July 2009

Keywords:
Heavy metals
Historical pollution
Freshwater fish
Muscle
Liver
Quality index

ABSTRACT

The present study aims at assessing the current situation of the heavy metal contaminated River Lot (SW France). Several fish species were captured in October 1987 and 2007 at three sampling sites. The concentration of copper, zinc, cadmium and lead were quantified in fish muscle and liver as well as in environmental samples (water, sediment, moss). The decrease in heavy metal concentrations in fish tissue between 1987 and 2007 reflects the decrease of heavy metal concentrations in the environment. Concentrations found in 2007 are comparable to those published by a study conducted in the 1990s. The situation of the River Lot has improved over the last 2 decades, although there is still margin for amelioration according to US EPA criterion to protect freshwater aquatic life. The average concentrations of cadmium in fish muscle in 2007 were above the maximum safe for human consumption defined by the European Commission.

1. Introduction

Used either as a means of transportation, as a source or water, or, worse still, as a sink for waste, the aquatic environment no doubt suffers the consequences of industrial activities. Albeit efforts – to a greater or lesser extent – to contain waste and reduce environmental impacts, a river system will naturally drain all areas surrounding it, washing into the aquatic environment many harmful substances. Many river systems in the industrialized world are thus the focus of regular monitoring of their contaminant load. In the European Union (EU), all member states must comply with water quality standards. When surpassing the maximum permissible levels, efforts must be undertaken to further control emissions. The present water framework directive in force in the EU works towards obtaining environmental qualities that will ensure high protection for aquatic ecosystems against toxic substances (Directive 2000/60/EC of the European Commission, 2000).

The River Lot, an affluent of the Garonne in southwest France, is known for its long history of intense heavy metal pollution and has been the focus of studies dating back to the 1970s (Audry et al., 2004; Blanc et al., 1999; Labat et al., 1977). In an effort to determine a recent Cd (cadmium) budget of the Lot-Garonne River system, Blanc et al. (1999) estimated that approximately 85% of the Cd in the River Lot is derived from anthropogenic origin. As such, the main sources of heavy metal pollution are the now closed mining sites situated along the Riou-Mort River, a tributary of the Lot in the Decazeville area. Apart from significant continuous input of Zn (zinc) and Cd between 1842 and 1987 due to the activity of a Zn ore manufacturing center (14.6 t/year of Cd until 1986, Dauta et al., 1999) there occurred an accidental spillage at this site in 1986. On that occasion, contaminated waste released from the area into the Riou-Mort River caused the mortality of several tonnes of fish inhabiting downstream from the accident site. In response, the Agence de l'Eau Adour-Garonne (AEAG; water agency for the Adour-Garonne River-basin) requested a follow-up on the concentrations of heavy metals in the tissues of native fish species (part of the present study). Almost two decades later, concern about the health of the local consuming population resulted in a survey and report on the risk of consuming the exposed fish (Ricoux and Gasztowtt, 2005). In addition to the historical heavy metal pollution affecting the River Lot, the natural flow of the river has been altered due to the construction of many locks and a few dams. The presence of barriers to the water flow leads to the accumulation of particulate matter to which trace elements are bound (Dauta et al., 1999). When the reservoirs release water, large amounts of heavy metals become water-borne and are transported downstream (Grousset et al., 1999; Lapaquellerie et al., 1995). Such dams are thus an additional source of heavy metal contamination to the watershed. As a result, heavy metals bioaccumulate to considerably higher concentrations in fish species of the Gironde estuary, where the

* Corresponding author. Fax: +33 561556728.
E-mail address: shinn@cict.fr (C. Shinn).

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doi:10.1016/j.ecoenv.2009.06.007

Garonne River joins the Atlantic Ocean, than in several other heavily polluted estuaries throughout the world (Durrieu et al., 2005).

Organisms that inhabit an aquatic environment are useful bioindicators of the impact of the presence toxic heavy metals (Arain et al., 2008). However, the presence of such pollutants at high concentrations does not necessarily present a threat to the exposed organisms as they may not be uptaken by the organism at all or, if uptaken, its metabolism can deal with (tolerate and/or excrete) the load (Fernandes et al., 2008). Therefore, coupling the concentration of pollutants in tissues of exposed organisms to the concentrations found in the environment, while taking into consideration relevant abiotic factors (e.g. water hardness, temperature, particulate matter) as well as the tolerance capacity of the organisms, are key processes in environmental risk assessment.

Cu (copper) and Zn are essential elements for growth and development and their uptake from the environment is regulated according to nutritional demand through homeostatic control (Couture and Rajotte, 2003; Watanabe et al., 1997; Wiener and Giesy, 1979). However, Cu and Zn become toxic at concentrations above the limits of homeostatic control (McKim et al., 1978; Sinley et al., 1975). Cd and Pb are non-essential heavy metals that do not have dedicated regulatory mechanisms (Viarengo, 1989)and are therefore more toxic to organisms. As such, Cd and Pb (lead) are classified as priority substances by the European Commission (COM, 2006). The tissues selected for bioaccumulation analysis were of liver and muscle. The liver is the main site of accumulation, biotransformation and excretion of pollutants in fish (Moon et al., 1985; Triebskorn et al., 1997). The axial muscle is the section of fish that is consumed by human beings; therefore it is required to verify whether contaminants like heavy metals are within the recommended limits for human consumption.

Most studies on the impact of heavy metals on exposed organisms focus along a gradient of pollution in one moment in time (Andres et al., 1999; Fabris et al., 2006; Fernandes et al., 2008; Labat et al., 1977; Reynders et al., 2008) or compare two different seasons (Mzimela et al., 2003). Fewer studies have addressed the long-term temporal variation of such impacts (Bietz et al., 1997; Maes et al., 2008; Nakhle et al., 2007). This knowledge is nevertheless important to characterize the evolution of the environmental quality over long periods of time. The situation in the River Lot is continuously monitored for heavy metal loads in sediment and water. However, the contamination of biota is less documented. The objective of the present study was thus to assess how heavy metal levels in fish species from the River Lot have evolved over the last 20 years.

2. Material and methods

2.1. Study area and sampling

The present study focuses on the most downstream section of the River Lot (Fig. 1) in the southwest region of France. The Lot is an affluent of the Garonne, the largest river of the Northern side of the Pyrenees Mountains. Three sampling sites were selected: Cajarc, Luzech and Le Temple. All sites were downstream from the confluence with the heavily polluted Riou-Mort River (Fig. 1) and located immediately upstream from locks/dams, considered as possible reservoirs of contaminated sediment (Dauta et al., 1999).

In all studies, fish were handled in accordance with national and international guidelines for the protection of animal welfare (Directive 86/609/EEC of the European Commission). Fish surveys took place in October 1987 and October 2007. Fishing was performed by Electrofishing (Electro-Pulman, 300–400 V, 1.5 A max. current); progress was made via fishing boat. In the deeper and calmer areas fishing was performed with monofilament nylon gill-nets of different mesh sizes (18–33 mm). Of the fish species captured, only those that were present at all three sites in both surveys are considered here. The selected species are roach (*Rutilus rutilus*), bream (*Abramis brama*) and perch (*Perca fluviatilis*). Sampling of liver and muscle was carried out in the field in 1987 and in the laboratory in 2007. Fish were carefully conserved on ice during transportation and while awaiting dissection the following day. From each fish, liver and muscle samples, $1.1 \pm 0.36\%$ and $11.2 \pm 3.62\%$ of their total body weight, respectively, were taken and pooled into composite samples of 1–6 individuals. Fish in each composite sample did not differ in total length by more than 25%. Composite samples were necessary to reduce the final number of samples, and hence reduce the costs for heavy metal quantification. The composite samples were weighed and kept at −20 °C in clean 100 ml high-density polyethylene vials until analysis.

Data on environmental variables were provided by the AEAG for each sampling site: conductivity, pH, water temperature and concentration of Cu, Zn, Cd and Pb in water, sediment and aquatic moss (Table 2; Fig. 2). As the AEAG did not monitor water concentrations of heavy metals at those sites in 2007, the environmental data considered in the present publication are from 2006. This did not pose a problem as during the 10 preceding years (1996–2006), fluctuations in average yearly water temperature (16.79 ± 0.93 °C), pH (7.7 ± 0.12) and conductivity ($222.16 \pm 22.39\ \mu S\ cm^{-1}$) were minor, and, with the exception of Zn concentrations in the sediment, heavy metal concentrations in the water, sediment and aquatic moss have maintained relatively constant (Fig. 2). Over the period of years between 1982 and 2006, noticeable fluctuations occurred in heavy metal concentrations of Cu_{water} (until 1997), Cu_{moss} and $Zn_{sediment}$. $[Zn]_{water}$ and $[Zn]_{moss}$ decreased considerably until 1989 and then maintained relatively constant thereafter. Apart from these exceptions, there was a general decrease in heavy metal concentrations in all three compartments.

2.2. Heavy metal quantification

2.2.1. Sample preparation and analysis in 1987

Samples were immersed in a solution of NH_4NO_3 (10%), using a ratio of 2 ml/g of sample. They were then dried until no weight loss occurred (110 °C) to determine the water content and calcined (450–550 °C) for 2 h. The ashes were then mixed with 5 ml of water, 10 ml of HCl and 5 ml of HNO_3 (6.3%), boiled for 10 min and dried at 110 °C until no weight loss occurred. The residues were further treated with 20 ml of HNO_3 (6.3%), boiled and filtered with paper filter (0.45 μm). Chemical analysis was performed on the filtrates by the AEAG on an Atomic Absorption Spectrometer (Perkin Elmer 4100ZL).

2.2.2. Sample preparation and analysis in 2007

The frozen samples were freeze-dried for 48–72 h to determine the water content and then homogenized using a mortar and/or knife. Tissue mean water content was $80 \pm 4\%$. In total 100 mg of dry sample was weighed in pre-weighed polytetrafluoroethylene vials. Liver samples were treated with 0.1 ml H_2O_2 (30% ultrapure, Merck) for 15 min. prior to further digestion. All samples were ultrasonicated for 30 min with 0.5 ml bidistilled concentrated (biΔ conc.) HNO_3 (home-distilled in quartex system). Another 500 μl biΔ conc. HNO_3 was added and the closed vials were heated for 12 h at 90 °C. After cooling down, 0.5 ml H_2O_2 were added and the closed vials were heated for 12 h at 70 °C. Finally, the samples were dried at 60 °C for 3–4 days. The resulting dried matter was dissolved in 2 ml biΔ conc. HNO_3. Aliquots were further diluted in HNO_3 2% and, according to the dilution factor, a certain amount of In/Re (indium/rhenium) was added as internal standard. Blanks with no fish tissue were run with each batch of samples to monitor contamination of used reagents. A fish standard (Standard Reference Material® 1947, Lake Michigan Fish Tissue) for As (arsenic), Cu and Zn was prepared with the same protocol to check the validity of the analytical method. The heavy metal concentrations found in the fish standard analyzed with our protocol were thus compared with the certified values. Heavy metal measurements were performed on an Inductively Coupled Plasma Mass Spectrometry (ICP-MS 7500 ce, Agilent Technologies) at the Laboratoire des Mécanismes et Transferts en Géologie, Toulouse, France. Cu, Zn, Cd and Pb average concentrations in the blanks and the ICP-MS detection limits were, respectively, 0.213/0.031, 1.832/0.021, n.d./0.003 and n.d./0.013 μg/kg (n.d.—not detected).

2.3. Data analysis

The datasets did not fulfill the assumptions for parametric analysis even when transformed; therefore non-parametric analyses were performed. Differences between heavy metal concentrations in muscle and liver samples between years (species pooled), species (years pooled) and years per species were tested via analysis of variance (GLM; Poisson distribution with χ^2 test or quasipoisson distribution with Fisher's test). In order to assess differences between pairs of species and between-year differences in concentration of the heavy metals in water, sediment and moss, and between-year differences in water temperature, pH and conductivity Mann–Whitney *U*-rank tests were performed. Significance level of 0.05 was adopted in all analysis. To verify whether heavy metal concentrations found in the environment were more related to those found either in fish muscle or in the fish liver, co-inertia analysis was performed with data from both years. Co-inertia analysis performs simultaneous analysis of two datasets. This method maximizes the product of the obtained correlation between the two datasets (in

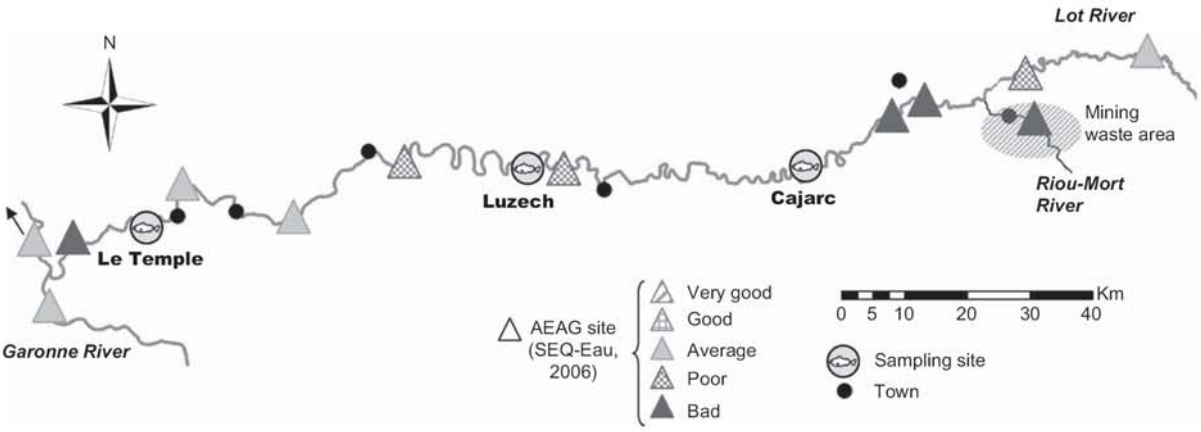

Fig. 1. Map depicting location of the fish sampling sites and the SEQ-Eau classification of the water agency's sampling sites according to heavy metal toxicity to organisms in 2006 (see discussion for further explanations).

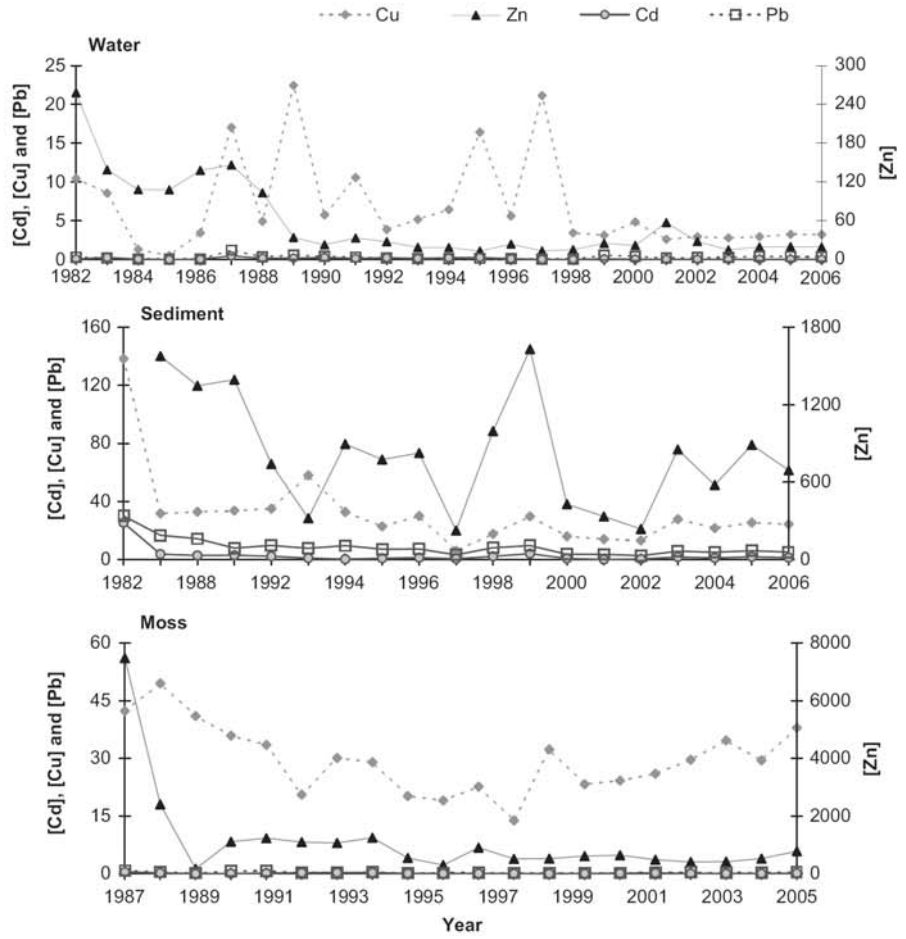

Fig. 2. Average concentrations of Cu, Zn, Cd and Pb in water ($\mu g\,L^{-1}$), sediment ($mg\,kg^{-1}$ wet weight) and moss ($mg\,kg^{-1}$ dry weight) of the River Lot between 1982 and 2006. Concentrations in sediment are missing for 1983–1986, 1990 and 1991, and in the case of Zn for 1982. Concentrations in moss were only available from 1987 onwards.

this case heavy metal concentrations in the environment and in fish), resulting in a coefficient of global similarity (RV coefficient). The closer to 1 the RV coefficients are, the more strongly linked the compared variables are. Monte-Carlo permutation tests indicate whether the co-structure between the two datasets is significant. All statistical analyses were performed using *R* (R Development Core Team, 2008) at a 0.05 significance level.

3. Results

Temperature and pH along the studied segment of the River Lot did not undergo significant changes between registered values in 1987 (17.93±0.92 °C; 7.81±0.04) and in 2006 (17.70±0.87 °C; 7.70±0.20) ($p = 0.2752$ and $p = 0.5127$, respectively). There was a slight increase in conductivity in 2006 (1987: 200.67±29.74 μS cm^{-1}; 2006: 312.33±53.29 μS cm^{-1}) ($p = 0.0495$), although values in both years are within the normal range of the conductivity found in the Adour-Garonne River-basin and favorable to aquatic life (DRI, 2007). Heavy metal concentrations in the water, sediment and moss significantly decreased between the two study periods (Table 2). Exceptions were $[Cd]_{water}$ (concentration of cadmium in the water; $p = 0.5127$), $[Cu]_{moss}$ ($p = 0.8273$) and $[Pb]_{moss}$ (0.5127) that presented no significant differences between the two sampling years.

Analyzed/certified (±uncertainty for certified value, with 95% confidence) As, Cu and Zn concentrations of the fish standard analyzed in 2007 were, respectively, 0.30/0.73 (±0.039), 0.48/0.41 (±0.029) and 3.88/2.66 (±0.08) mg kg^{-1} ww (wet weight). The method therefore provided concentrations that were within the certified ranges for As and Cu, but 46% above for Zn. Higher than standard Zn concentration is more likely a consequence of a contamination that may have occurred outside the clean-room upon ICP-MS analysis. All blanks presented concentrations of heavy metals close to or beneath the detection limit, except for Zn. However, the extent of Zn contamination via reagents and material is insignificant (<0.1%) in comparison to the concentrations found in all fish tissue samples.

Average total lengths of the sampled fish were similar in both the 1987 and the 2007 surveys, validating the comparison between years (Table 1). In general, Cu/Zn/Cd/Pb concentrations in fish liver and muscle decreased between the two study periods (Table 3; Fig. 3). However, $[Cu]_{liver}$ was found to be at similar levels in the liver of the fish captured in both years. Significant differences between species were found only for $[Cu]_{liver}$, bream presenting significantly higher concentrations than perch ($p = 0.0097$; Table 3; Fig. 3). $[Zn]_{liver}$ of bream maintained the same between the two periods (Table 3; interaction with $p<0.05$), whilst of perch and roach the concentrations decreased between 1987 and 2007 (Fig. 3). Heavy metal concentrations were systematically higher in the liver than in the muscle, for both study years and all species ($p<0.05$).

Co-inertia analysis indicated that environmental heavy metal concentrations were clearly related to those found in fish tissues, as co-structure between compared datasets (RV coefficient) was always significant (Table 4). Environmental concentrations were more strongly linked to concentrations in the muscle than in the liver, as the respective RV coefficients were higher.

Table 1
Number of fish sampled, number of fish per composite sample, and average fish length per species and year.

Species	Year	N fish captured	N composite samples	Average length (mm)
Bream	1987	20	3	290.42±76
	2007	17	8	245.19±53
Perch	1987	11	3	273.61±71
	2007	8	5	279.6±45
Roach	1987	52	4	214.54±26
	2007	56	15	206.87±44

Discussion

With the present study, we aimed at assessing the 20-year evolution of heavy metal concentrations in fish from the

Table 2
Average (±SD, for this study) concentrations of Cu, Zn, Cd and Pb in different compartments of the river system, measured along the study area in 1987 and 2006, in other rivers/lakes throughout the world, and the US EPA Criterion for Maximum and Continuous Concentrations. (For further explanations on the criterion refer to Section 4.)

	Water				Sediment			
	Cu	Zn	Cd	Pb	Cu	Zn	Cd	Pb
This study, 1987	17.03±3.59*	146.27±98.79*	5.54±5.76	14.47±2.66*	31.67±14.57*	1576.67±406.74*	41.67±20.50*	188±55.24
This study, 2006	3.22±2.03	19.38±1.07	0.5±0.01	4.33±0.58	24.35±7.65	692±313.00	14.75±8.65	57.5±19.50
Lake Boeuf, USA[a]	–	–	–	–	9.08	53.92	–	17.58
Boulder River, USA[b]	11.96	159.88	0.54	1.74	107.63	424.00	3.28	110.33
Asturian rivers, Spain[c]	3.45		0.13	3.65	5.02	–	0.14	9.81
Dipsiz stream, Turkey[d]	0.37	1.05	0.17	0.41	10.83	30.83	0.67	69.67
Orontes River, Turkey[e]	40.30	39.00	11.00	27.00	–	–	–	–
Ontario lakes, Canada[f]	15.67	8.27	0.30	–	–	–	–	–
Lake Beyşehir, Turkey[g]	100.00	160.00	–	–	5.97	–	33.18	–
CMC[h]	13	120	2	65				
CCC[h]	9	120	0.25	2.5				
	Moss							
	Cu	Zn	Cd	Pb				
This study, 1987	42.33±17.01	7477.33±7276.23*	60.47±35.44*	105.00±64.65				
This study, 2006	39.30±10.69	837.67±333.79	17.17±7.74	52.00±9.64				

Values for the present study are the annual average of the three sampling sites. Only data for non-reference sites have been considered when reporting other studies. Concentrations in water and sediment are in μg L^{-1} and mg kg^{-1} wet weight (converted from dry weight by dividing concentrations by 1.2), respectively. *Significant differences between present study sampling years ($p<0.05$; Mann–Whitney test). –, data were not available in publication or variable was not studied. CMC, Criterion Maximum Concentration; CCC, Criterion Continuous Concentration.

[a] Aucoin (1999).
[b] Farag et al. (2007).
[c] Linde et al. (1998).
[d] Demirak et al. (2006).
[e] Yilmaz and Dogan (2008).
[f] Rajotte and Couture (2002).
[g] Tekin-Ozan (2008).
[h] US EPA (2006).

Table 3
Result of analysis of variance taking into account the effect of year, species and their interaction.

			Effect					
			Year		Species		Year × species	
Tissue	Element	Test	Resid. dev	*p*-Value	Resid. dev	*p*-Value	Resid. dev	*p*-Value
Liver	Cu	F	92.35	0.759	67.62	**0.006**	66.79	0.598
	Zn	F	147.17	**0.033**	142.17	0.516	140.21	**0.018**
	Cd	F	32.91	**0.001**	31.23	0.497	30.10	0.568
	Pb	Chisq	4.36	**0.004**	1.87	0.288	1.57	0.864
Muscle	Cu	Chisq	7.41	**0.001**	7.31	0.950	7.18	0.939
	Zn	F	37.74	**0.000**	36.42	0.522	33.71	0.257
	Cd	Chisq	4.20	**0.000**	3.46	0.690	3.43	0.989
	Pb	Chisq	2.57	**0.004**	1.04	0.465	0.96	0.962

Given are residual deviances (Resid. dev) and *p*-values from respective tests (F, Fischer's test. Chisq, Chi-square test). *p*-Values <0.05 are in bold.

historically polluted River Lot. Data were obtained from fish surveys conducted in 1987 and 2007 at the same river sampling points, and compared. With the exception of $[Cu]_{liver}$, heavy metal concentrations in fish tissues decreased from 1987 to 2007, a trend that is reflected in the decrease of heavy metal concentrations in fish tissue.

$[Cu]_{liver}$ did not vary significantly between the two sampling years, possibly due to the fact that Cu is well regulated by fish below liver concentrations of ca. 10 mg kg^{-1} wet weight (Couture and Rajotte, 2003), which is the case in both surveys of our study. The colloidal fraction of Cu in the River Lot is considerable. In total 30–40% of Cu complexes are hydrophobic, thus with higher affinity to dissolved organic matter (Lemaire et al., 2006), as can be found in most studies reporting the affinity of Cu to organic phases (Mantoura et al., 1978; Benedetti et al., 1996; Lemaire et al., 2006, Santos-Echeandia et al., 2008). This could contribute to an increased bioavailability of Cu to aquatic organisms, including fish. Furthermore, despite a clear decrease between 1987 and 1997 and stabilization since 1998 in $[Cu]_{water}$, and a gradual, albeit small, decrease in $[Cu]_{sediment}$ over the years (Fig. 2), $CuSO_4$ is still heavily applied as a fungicide in vineyards located throughout the surrounding terrestrial basin, contributing to a constant input of Cu into the river basin (Kraepiel et al., 1997). In 2006 River Lot $[Cu]_{water}$ was in general lower than concentrations found in other rivers whilst $[Cu]_{sediment}$ was slightly higher (Table 5). An additional source of Cu could be the erosion of ore deposits in the valley of the River Lot (Schafer and Blanc, 2002). Higher concentrations in the liver than in the muscle in both study years are consistent with studies such as those performed by Andres et al. (2000) and Fernandes et al. (2008).

$[Zn]_{liver}$ in bream was as high in 1987 as it was in 2007, contrary to the decrease observed in perch and roach. Bream is generally a bottom-feeding species whilst perch is carnivorous and roach a water-column dweller. The fact that breams have closer contact to the sediment may explain the nondecrease of the concentration of Zn in their liver. Although $[Zn]_{sediment}$ in 1987 has decreased to less than half in 2006, it has fluctuated considerably. This fact provides an additional explanation to why bream captured in 2007, having been exposed to those fluctuations (given their size, thus age, upon capture), presents as much Zn in their liver as fish captured in 1987.

4.1. Comparison with other studies

A study performed by Andres et al. (2000) on the River Lot reports average concentrations of Cd and Zn in several fish species (including the three in our study) between 1995 and 1997. This study took place 10 years prior to our 2007 survey, and in the same area: from a reference site 41 km upstream of the confluence with the Riou-Mort River, down to Carjarc, the most upstream site in our study.

$[Cd]_{liver}$ and $[Zn]_{liver}$ in the Riou-Mort fish of 1995–1997 are higher than the 1987 averages in the downstream segment of the River Lot (our study). The higher concentrations in the liver are a direct response of higher concentrations of the trace elements in the water at Riou-Mort: ca. 29 and 980 $\mu g L^{-1}$ of Cd and Zn, respectively, as reported in Andres et al. (2000). With regard to bioaccumulation, the liver, as an organ of continuous detoxification, provides a more immediate assessment of the current environmental levels of pollutants (Bruslé et al., 1996). However, Riou-Mort $[Cd]_{muscle}$ and $[Zn]_{muscle}$ in 95–97 are in general lower than the downstream River Lot averages in our study. The muscle can be considered as an indicator of chronic exposure (Albaigés et al., 1987); the lower concentrations in 95–97 may indicate that the fish were coping better with the high levels of pollution (i.e. eliminating more and storing less) in the Riou-Mort than with the equally high levels of the downstream River Lot in 1987. Fish acclimation to chronic heavy metal contamination has been observed in other fish species, such as mosquitoefish (Klerks and Lentz, 1998), iberian-roach (Lopes et al., 2001) and rainbow trout (Chowdhury et al., 2005).

Overall, average 2007 concentrations of Cd and Zn in the muscle and liver of all three species are lower than those found in 1995–1997 at the Cajarc site, and 1987 concentrations are clearly higher. More specifically, [Cd] and [Zn] in breams and roaches captured at Cajarc in 2007 (data not discriminated in the present study) are in general lower than in fish captured at the same site in 1995–1997. Such differences may be due to the fact that the results presented by Andres et al. (2000) are an average of successive surveys covering different seasons (autumn and spring) whilst our surveys took place only in autumn. The average water temperature of the River Lot in spring and autumn differs substantially (average from 7.9 to 17.7 °C) and it is known that temperature influences the uptake and metabolism of heavy metal elements by fish (Cogun et al., 2006; Foster et al., 2000; Regoli and Orlando, 1994). Furthermore, $[Zn]_{water}$ at the reference site in 95–97 is higher, and $[Cd]_{water}$ lower, than the averages found further downstream in 2007.

Albeit higher concentrations found in fish tissue in 1995–1997 (Andres et al., 2000) than in 2007 these differences are not substantial with respect to the marked decrease observed from 1987. Remediation efforts after the 1986 accident have resulted in an important decrease of heavy metal concentrations in water, sediment and moss. However, since 1997, Cu, Zn, Cd and Pb concentrations in the sediment have stabilized, being still 1.2, 7.0, 37.2 and 1.7 times higher, respectively, than the pre-industrial background concentrations of the River Lot (Audry et al., 2004). This stabilization is reflected in the similar concentrations of heavy metal elements found in fish liver and muscle in 1995–1997 and 2007.

When assessing the contamination of biota from an impacted area it is often interesting to compare the extent of the contamination with studies performed in other polluted areas in the world (Tables 2 and 5). Heavy metal concentrations found in the three species in our study in 2007 are lower than those found in muscle of brook trout from the Boulder River watershed by Farag et al. (2007) and than in liver of brown trout from rivers in Asturia, Northern Spain (Linde et al., 1998). However, water concentrations in Boulder River watershed are in general much higher (except reference sites) than those found in the River Lot and the Northern Spain Rivers, which in turn have comparable concentrations between them. The fact that brown trout from the

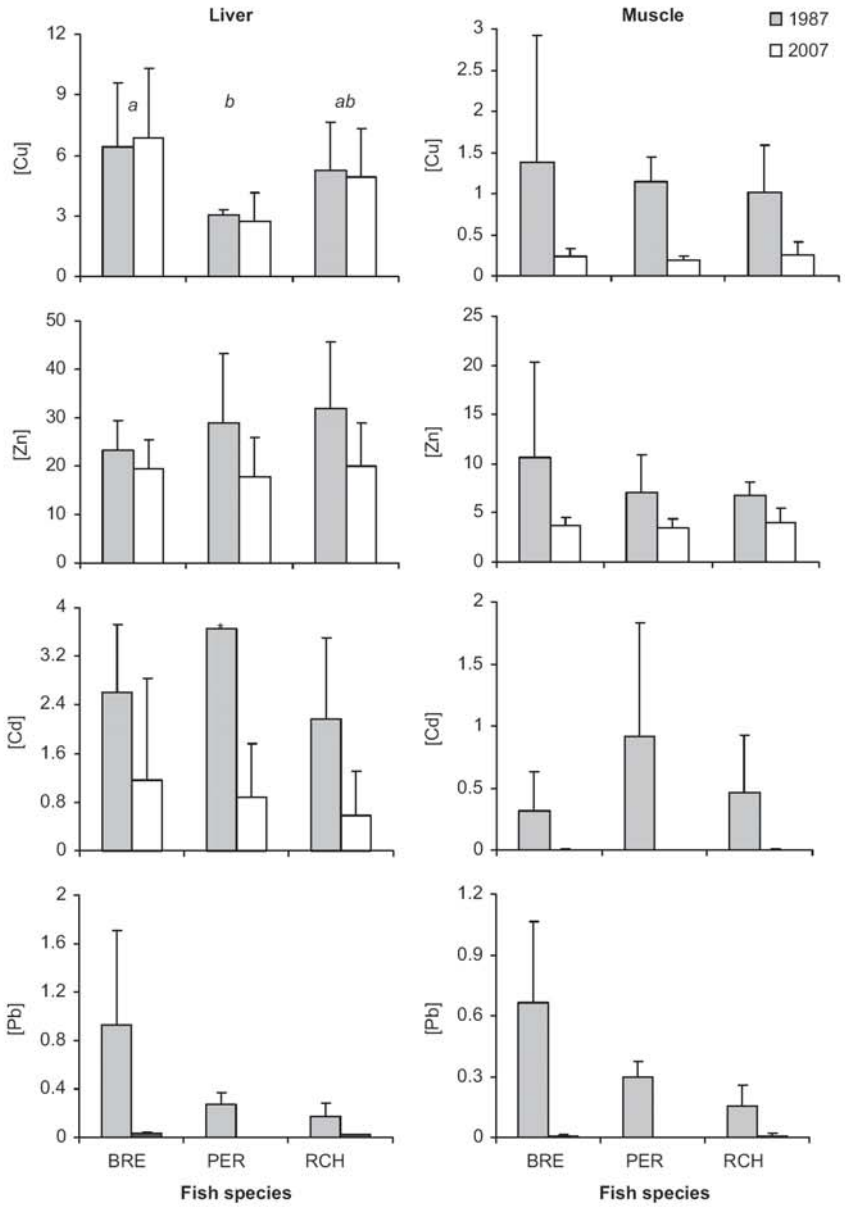

Fig. 3. Average concentrations (mg kg^{-1} ww; ±SD) of Cu, Zn, Cd and Pb in muscle (right) and liver (left) of the three fish species sampled along the River Lot in the 1987 and 2007 surveys. BRE: Bream; PER: Perch; RCH: Roach. * sample size = 1. A different letter indicates presence of significant differences between species (Mann–Whitney test).

Table 4
RV coefficients of co-inertia analysis comparing concentration of heavy metals in the environment (sediment, water and moss) and fish tissues.

	Sediment	Water	Moss
Liver	0.31***	0.4***	0.24*
Muscle	0.64***	0.63***	0.35***

* $p<0.05$.
*** $p<0.001$.

Asturian rivers presented higher heavy metal content than the species in our study, despite water concentrations being similar, maybe due to the different physiology and feeding habits of these species. Another factor could be physical–chemical processes taking place in the Asturian rivers, processes that increase the bioavailability of heavy metals to aquatic organisms. Indeed, the authors state that in these rivers there occur periodic floods in spring and summer, events that often lead to the resuspension of trace elements accumulated in the sediment and consequent increase of bioavailable levels. A study conducted by Rajotte and

Table 5
Other studies' heavy metal concentrations in the liver and muscle of fish and the maximum permissible levels in fish muscle in Europe.

Species	Location	Liver				Muscle			
		Cu	Zn	Cd	Pb	Cu	Zn	Cd	Pb
Bluegill sunfish	Lake Boeuf, USA[a]	–	–	–	–	0.93	14.49	–	2.44
Bream	This study-1987	6.45	23.24	2.60	0.93	1.38	10.68	0.38	0.67
Bream	This study-2007	8.67	21.56	1.17	0.03	0.24	3.64	0.00	0.00
Brook trout	Boulder River, USA[b]	–	–	–	–	118.25	38.30	6.38	0.06
Brown trout	Asturian rivers, Spain[c]	153.90	–	2.23	2.46	–	–	–	–
Chub	Dipsiz stream, Turkey[d]	–	–	–	–	0.79	11.06	0.01	0.23
Crucian carp	Dniester River, Moldova[e]	–	–			3.66	8.43	0.03	1.24
Himri	Orontes River, Turkey[f]	45.72	89.59	2.80	1.55	4.55	9.89	0.12	0.12
Perch	This study-1987	2.82	18.82	3.66	0.20	1.14	7.00	0.90	0.30
Perch	This study-2007	2.72	17.88	0.87	0.00	0.19	3.44	0.00	0.00
Perch	Dniester River, Moldova[e]	–	–	–	–	2.66	9.85	0.03	2.16
Yellow perch	Ontario lakes, Canada[g]	7.64	27.33	1.98	–	1.06	5.91	1.20	–
Roach	This study, 1987	5.03	34.78	2.46	0.13	1.05	6.81	0.83	0.11
Roach	This study, 2007	4.91	20.06	0.58	0.02	0.25	4.04	0.00	0.00
Siberian roach	Dniester River, Moldova[e]	–	–	–	–	2.47	7.66	0.04	2.51
Tench	Lake Beyşehir, Turkey[h]	85.67	27.33	–	–	–	7.40	–	–
Maximum permissible levels for human consumption in Europe[i,j]		–	–	–	–	0.10	100<	0.05	0.30

Only data for non-reference sites have been considered when reporting other studies.
All tissue concentrations are in mg kg^{-1} wet weight. Tissue concentrations found in dry weight were converted to wet weight by multiplying by a factor of 0.2 (considering an average water content in fish tissues of 80%). –, data were not available in publication or variable was not studied.

[a] Aucoin (1999).
[b] Farag et al. (2007).
[c] Linde et al. (1998).
[d] Demirak et al. (2006).
[e] Sapozhnikova (2005).
[f] Yilmaz and Dogan (2008).
[g] Rajotte and Couture (2002).
[h] Tekin-Ozan (2008).
[i] Commission Regulation No. 1881/2006 (2006).
[j] Ricoux and Gasztowtt (2005).

Couture (2002) reveals heavy metal concentrations in muscle and liver of yellow perch from three polluted lakes in Ontario, Canada, that are in average slightly above those in perch analyzed in the present study in 2007. The heavy metal concentrations that were found in the contaminated lakes were comparable to those in the River Lot in 2006, with Cu slightly higher and Zn slightly lower.

4.2. Aquatic community effects

Concentrations of Cu, Zn and Cd in the river water in 1987 were higher than US EPA Criterion Maximum Concentration (CMC) reflecting the state of high pollution levels (US EPA, 2006; Table 2). The CMC is an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed briefly without resulting in an unacceptable effect (US EPA, 2006). This situation no longer prevailed in 2006 where the concentrations had unanimously dropped below their respective CMC. In general, concentrations in 1987 were lower than a previous study conducted in 1975 on the River Lot (Labat et al., 1977), indicating that the community had been exposed to concentrations well above the CMC already for several years. Concentrations of all four heavy metals in 1987 and of Cd and Pb in 2006, in the water of the studied area, exceeded the recommended Criterion Continuous Concentrations (CCC). The CCC is an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect (US EPA, 2006; Table 2). Therefore, it can be concluded that, according to the US EPA recommendation criteria, the studied segment of the River Lot in 2006 was still contaminated by Cd and Pb to an extent that is harming the aquatic community.

The water quality of the Adour-Garonne River-basin has been monitored by the AEAG for several decades. In order to assess the occurrence of contaminants in the aquatic environment, the AEAG has developed a five-level (ranging from 1: good, to 5: bad) index of heavy metal perturbation, the SEQ-Eau, which pools data on contaminant concentrations in water, sediment and water moss (MEDD, 2003). The SEQ-Eau for heavy metals incorporates a classification of the sites according to the water's appropriateness for drinking-water, aquatic sports and aptitude for aquatic life, with regard to 14 different trace elements (including the four elements studied here). This classification is performed for each environmental compartment (water, sediment and aquatic moss) and each heavy metal, and the worst classification is retained. For the most downstream section of River Lot in 2006 (this study) the SEQ-Eau is average to bad (3–5; data provided by the AEAG; Fig. 1). No sites are indexed as 1 (very good) or 2 (good). Downstream from the mining area of Decazeville, the Riou-Mort River has a bad SEQ-Eau. Up to ca. 42 km upstream from the confluence with the Riou-Mort, the Lot has a SEQ-Eau of average to poor (3–4). Some authors adopt points in this section of river as a reference to sites downstream from the Riou-Mort River (Andres et al., 1999; Audry et al., 2004). However, the adequacy of such practice should be questioned, as it possibly affects the conclusions of those studies. Despite all these uncertainties, local fishing authorities are still promoting fishing activities and the attribution of personal fishing permits along the River Lot (Fédération du Lot-et-Garonne pour la Pêche et la Protection du Milieu Aquatique, personal communication).

4.3. Sediment quality

In average, the section of the River Lot studied here was, in 2006, according to Müller's geoaccumulation index (Audry et al.,

2004; hereafter referred to as I_{geo}) unpolluted, except in the case of Zn for which it is strongly polluted. I_{geo} is a qualitative indicator to classify sediments according to their pollution intensity. In this classification, the authors consider the sediment heavy metal concentrations in the reservoir immediately upstream from the confluence with the Riou-Mort River as representative of the background concentrations of the River Lot (Audry et al., 2004). However, as proposed by Andres et al. (1999), there may also be inputs of heavy metals via diffuse spills from cities located in the upstream watershed of the River Lot. This is, in fact, reflected in the concentration of Cd in breams (Andres et al., 2000) from a reservoir upstream from the Riou-Mort confluence and only 41 km downstream from the reference site of Audry et al. (2004). However, sediment concentrations of heavy metals in that same section of the River Lot in 2006 (data not discriminated in the present study) were, according to the I_{geo}, unpolluted or unpolluted-to-moderately polluted. On the other hand, sediment concentrations of Zn and Cd in both study years were higher than the probable effect concentrations (PEC; MacDonald et al., 2000), above which adverse effects on the benthic community are expected to occur. Incidentally, these two trace elements were the ones most intensively explored until the closure of the metallurgical activities. Sediment-associated metals may constitute a long-term source of contamination, and thus risk, to higher tropic levels (Eimers et al., 2001; Farag et al., 2007).

Depending on the type of classification applied, and thus which factors are taken into account (toxicity to aquatic or benthic organisms, levels in sediment, water and/or moss, scaling of pollution intensity), the same river location can be classified differently. This indicates that not only one type of classification is sufficient to adequately assess the impact of a contamination on the environment. When possible, environmental risk assessment should include a combination of indexes that cover all environmental compartments concerned and the different biological groups involved. The scaling of such indexes would facilitate inter-index comparisons over time at the same location, and between locations.

4.4. Human health risk

In the 1987 campaign only the average concentration of Zn in the muscle did not exceed the established European Commission regulatory concentration for human consumption (Table 5). Average concentrations of Cu, Zn and Pb in fish muscle in 2007 were below the maximum established European regulatory concentrations. The average $[Cd]_{2007}$ was above the EC maximum, although the concentration in the most consumed species out of the three studied here (perch) was not.

In a humanitarian risk study conducted in 2004 by several official entities of the Adour-Garonne River-basin (of which the River Lot is part of), concentrations of Cd and Pb in muscle of carnivorous fish and eels were in average comparable to those found in the three species sampled in the 2007 campaign (Ricoux and Gasztowtt, 2005). The authors conclude that care must be taken upon consuming certain fish species captured in the Adour-Garonne River-basin, especially in the case of infants that have a diet of high consumption of river fish. The recommendation of the French agency for sanitary security of foodstuffs (Agence Franç aise de Sécurité Sanitaire des Aliments) is to diversify the origin of consumed fish, not preferring too often fish captured in the Adour-Garonne Rivers and avoiding consumption of fish that originate from heavily contaminated areas (Ricoux and Gasztowtt, 2005). These recommendations are based on prevailing concentrations of Cd, Pb and Hg (mercury) in the edible portion of carnivorous fish and eels from the Adour-Garonne River-basin.

5. Conclusion and outlook

As can be seen in the present study, the heavy metal contamination in the River Lot aquatic system has improved over the last 20 years. However, the situation is still not complying with established ecological and chemical requirements of the European Union. As such, the main source of heavy metals to both surface and ground waters – the upstream Riou-Mort River basin – is currently being assessed and measures to control the situation are being taken. The Adour-Garonne Water Agency and the society responsible for the disposal of industrial wastes (UMICORE) are aiming at reducing heavy metal loads, especially Cd, to reach the 2015 limits established by European Union's Water Framework Directive (Prefecture of Aveyron, 2008). Contaminated soils stored within the industrial area are being removed and properly disposed of and/or treated. The local population is being informed of the contamination and a health study has been performed in 2008 to evaluate current accumulation in and exposure of babies, children and adults to arsenic, Cd and Pb. Depending on the results of the study the local health authorities will inform the population on procedures to reduce human exposure. It will be interesting to see how the clean-up of the industrial area will affect the fish populations and the concentration of heavy metals in their tissues.

Acknowledgments

The present study was performed within the KEYBIOEFFECTS project (MRTN-CT-2006-035695) of the 6th Framework Programme funded by the European Union. The authors would like to thank the Agence de l'Eau Adour-Garonne for providing the environmental monitoring data, the laboratory technicians at the Laboratoire des Mécanismes et Transferts en Géologie in Toulouse where part of the chemical analysis was performed, and colleagues at Évolution et Diversité Biologique laboratory for their help in collecting and dissecting fish specimens.

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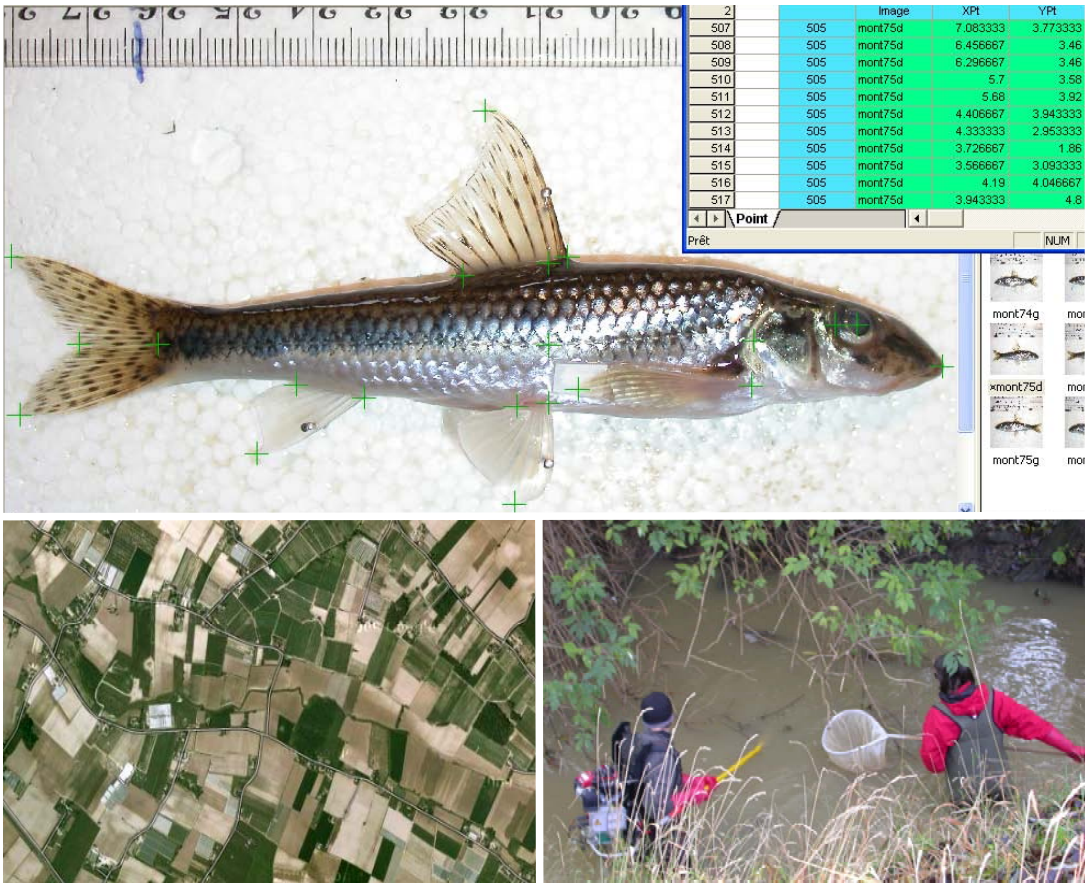
3.

Phenotypic variation as an indicator of pesticide stress in gudgeon (*Gobio gobio*): accounting for confounding factors in the wild



3.1. Introduction

Environmental stress, be it natural or anthropogenic, is broadly recognized as a driving force of population and individual level responses in natural environments (Bickham et al., 2000; Belfiore and Anderson, 2001; Van der Oost et al., 2003). Such responses are regularly used in the assessment of ecosystem health, as an indication of system functioning impairment or, more specifically, of the direct impact of stressors on species (Niemi and McDonald, 2004b; Posthuma and De Zwart, 2006; Doledec, 2009; Dobiesz et al., 2010). Concerning chemical stressors, a punctual, acute exposure to contaminants can engender immediate responses on wild organisms and many techniques and guidelines are now available to detect and quantify those effects (e.g. OECD and US-EPA guidelines for toxicological testing). However, long-term exposures to low levels of contaminants are widespread and in need of additional tools for adequate impact assessment. The currently enforced chemical substances directives require that toxicological testing be performed at the individual level but do not focus nearly as extensively on the importance of such studies at the population or community levels (Attrill and Depledge, 1997; Clements, 2000; Liess and Von der Ohe, 2005).

At the individual level, phenotype integrates the multiple effects of one or several stress factors upon the development of exposed organisms (Barlow, 1961; Langerhans, 2008). It has been shown that structural characteristics (phenotype) determine part of the ecological success (fitness) of organisms (Koehl, 1996). Therefore, via studying phenotypic variation between conspecifics exposed to different environments, we may gain insight from variation in fitness linked to environmental conditions. Phenotypic changes have thus been used as biomarkers of present or past chronic exposure of various organisms to pollutants (e.g. Nunes et al., 2001; Leusch et al., 2006) as well as when facing habitat degradation (e.g. Smakulska and Górniak, 2004).

The developmental stability – reproducible development of a genotype under given environmental conditions (Moller and Swaddle, 1997) – of the individuals of a population can be assessed by studying the degree of morphological variation (direct measurements between body landmarks; Pecinkova et al., 2007), or by calculating the level of fluctuating asymmetry (deviation from perfect bilateral symmetry; Van Valen, 1962). While some studies have shown that developmental instability increases with increase of environmental stress (Von Dongen, 2006; Almeida et al., 2008), others failed to establish clear relationships

(Bjorksten et al., 2000; Utayopas, 2001). The lack of evidence in the latter cases is subject to recent discussion of the adequacy of morphometric traits as a biomarker of environmental stress (Leung et al., 2000).

Because human pressure generally modifies more than one environmental factor at a time, and pressures from several sources often coincide, a multiple-stressor approach is the most adequate when conducting studies in the natural environment (Ormerod et al., 2010). Toxicological assessment of the presence of contaminants in natural environments provides on-site information of the processes and responses actually occurring within wild populations. However, it is often the case that a number of variables other than the toxic stressors are not taken into account, introducing error in the final assessment of the status of natural populations. Many studies refer to these so-called confounding factors as an explanation to weak relationships found between response and potentially causative variables (e.g., Ewers, 2006). For example, the genetic diversity of seven wood mouse populations in Belgium, was found to be unaffected by heavy metal contamination (Berckmoes et al., 2005). It was thus suggested that either the contamination was not strong enough, or not for a long enough time, to induce population genetics response. Alternatively, the authors also proposed that gene flow between populations could have masked the effects. Taking into account geographical distance between populations, for example, could help reduce the masking effect. Just as these factors can obscure detection of effects, they can also enhance them, similarly leading to erroneous conclusions. Confounding factors are also generally alluded to when seemingly contradictory results cannot be explained by methodological or environmental differences between studies alone.

In fact, few studies propose and apply methods dealing with the confounding effect of interfering variables. In environmental assessment, the determination of causality of toxic effects requires specificity of association, i.e., differentiation between stressor effects and environmental variability (Suter, 1993; Theodorakis, 2003). Experts in this field now stress the importance of considering the impact of confounding factors on the measured responses, in order that natural variability (e.g. biology and physiology of selected organisms) and pollution-induced stress may be distinguished (Sanchez and Porcher, 2009). Field studies are especially susceptible to this problem as their number of varying parameters is often larger in comparison to that of laboratory set-ups. Alternate hypothesis that may explain observed patterns can easily be overlooked within the field context if the

study design and data analysis do not take into account nor test for environmental and/or biological effects on the collected data (Belfiore and Anderson, 2001).

Despite their small number, examples can be found in the literature of research in which the authors designed their study in order to account for (some) confounding factors (Behrens and Segner, 2005; Langerhans, 2007; Jensen et al., 2008). For instance, Jenkins (2004) reported a study in which data was pooled from replicates of contaminated areas and from reference areas in order to lessen the effect of confounding factors. Confounding factors are particularly present in exposure scenarios with low or moderate contamination, or in the absence of clear, contrasting temporal and spatial gradients (Rogers et al., 2002).

As pointed out by Short et al. (2008), many species are able to actively avoid contaminated areas due to their high mobility, leading to presence of previously exposed individuals in less contaminated locations, or vice-versa. Moreover, spatially varying environmental factors may alter bioavailability of pollutants, weakening the strength in the interpretation of relationships between organism response and levels of contaminants. Migration of mobile organisms between contaminated and non-contaminated sites can also interfere with outlining cause-effect relationships. Although rarely considered in ecotoxicological studies, confounding factors such as mobility can adequately be accounted for by examining the population genetic structure (Theodorakis et al., 2001; Bourret et al., 2008). As it allows assigning individuals to biological populations rather than to a pre-determined sampling site, genetic data is well suited to deal with factors interfering at the populatione level.

An alternate but non-exclusive way of accounting for confounding factors is to include those factors within an appropriate statistical framework. A number of studies implement multivariate statistics in order to account for co-variables – especially when in large numbers – that are seemingly important in the structuring of response variables (e.g. Van den Brink and Ter Braak, 1999; Vila-Gispert et al., 2002; Koel and Peterka, 2003; Guasch et al., 2009). This kind of approach allows for the identification of factors contributing to the predictor-response relationship, for which partial correlations can then be tested.

Regarding the impact of contaminants on population, a first confounding factor concerns the possible relationships between contamination levels and populations' genetic structure. Recent studies have begun to show that environmental contamination can directly or indirectly affect genetic variability and allele frequencies of populations, resulting in

changes in gene flow, selective pressures, mutations or demographic history (review: Bickham et al., 2000; Staton et al., 2001; Theodorakis et al., 2006; Bourret et al., 2008). Most ecotoxicological studies including population genetic parameters aim at testing the relationship between contamination and genetic erosion (De Wolf et al., 2004; Bourret et al., 2008; Fratini et al., 2008; Gardeström et al., 2008; Ungherese et al., 2010). Although we recognize the importance of this kind of approach, namely for the preservation of genetic diversity that allows populations to adapt to environmental changes, we include a genetic component in our study in order to rule out the interfering effect of potential population structure that may differ greatly among geographical (according to site selection) and morphological clusters.

Besides being a key tool in ruling out noise due to genetic differentiation among populations, genetic data can be used to directly compare genetic differentiation with morphological differentiation. This approach provides insight on selective pressures that may be acting upon the populations exposed to different conditions. The among-population fixation index, F_{ST}, for neutral loci markers is a standardized measure of the degree of genetic differentiation among populations (Wright, 1951; Nei, 1987). Quantitative genetic differentiation for natural populations (P_{ST}), is based on phenotypic data derived from wild individuals (Leinonen et al., 2008; Raeymaekers et al., 2007). The P_{ST} index is the analog of the F_{ST} index that quantifies the among-population divergence between genes that code for quantitative traits (Merilä and Crnokrak, 2001; Leinonen et al., 2006), such as morphometric traits. If P_{ST} were estimated from allele frequencies at the loci determining the quantitative trait, P_{ST} would be expected to be equal to F_{ST} in the case where the trait carried an exclusively additive genetic basis (i.e., no gene interaction or epigenetic effects) and no linkage disequilibrium were to be present (Wright, 1951; Latta, 1998). The most common result is that $P_{ST} > F_{ST}$, meaning that directional/divergent natural selection has resulted in different phenotypes in different populations, as the level of quantitative trait differentiation exceeds that attained by genetic drift only (Merilä and Crnokrak, 2001; López-Fanjul et al., 2003; Raeymaekers et al., 2007). Morphometric traits are known to present considerable additive genetic variance (Crnokrak and Roff, 1995; Merilä and Crnokrak, 2001). When directly measured in the wild, P_{ST} cannot separate additive effects from environmental or nonadditive genetic effects on quantitative trait variation (Merilä and Crnokrak, 2001; Leinonen et al., 2008). A statistical design that accounts for the effect of external factors

(e.g. environmental conditions), can allow separating genetic *vs.* environmental effects. To our knowledge, no studies have yet attempted at achieving this with in wild populations.

In addition to intra-population genetic variability, it is also important to take into account other factors that the studied fish populations are subjected to and that vary between sampling sites. Water physical-chemical parameters are key factors in determining species' distribution in aquatic environments (Menge and Olson, 1990; Poff, 1997). Furthermore, sites differing in pollution levels are often distinct regarding other environmental factors such as dissolved oxygen, organic suspended matter, ions, etc (Ruse, 1996; Dyer et al., 1997; Rogers et al., 2002; Guasch et al., 2009). We therefore include a range of physical-chemical environmental parameters to correct for this component of variation. Given that the selected sampling sites are distributed over several rivers of the river basin studied here, geographical distances between sites are included, also helping to rule out genetic isolation-by-distance (Wright, 1943).

In this study, we tested whether wild populations of gudgeon (*Gobio gobio*) exposed to different levels of pesticide pollution presented significantly different phenotypes (assessed via intra-specific morphological variation) as a consequence of that exposure. The challenge here was to take into account multiple factors that were co-varying with contamination levels. We used an original method rooted on the partial Mantel test framework (Manly, 1991; Legendre, 2000) to test the effect of pesticide contamination on the morphometry of different gudgeon populations, while simultaneously taking into account the influence of a set of other potentially influencing factors: genetic differentiation between populations, various physical-chemical parameters for each sampling site geographical distances between sites, and site catchment area. General linear models were used to that respect. The consequence of not accounting for confounding variables is discussed, as well as the applicability of this approach for ecotoxicological assessment.

3.2. Material and methods

3.2.1. Site selection and characterization

Sampling sites were selected according to the pesticide water concentrations detected during field surveys performed in 2006 by the Adour-Garonne water agency (hereafter referred to as AEAG), throughout the Adour-Garonne river basin (South-western

France). Due to the intense agricultural activity and the extensive hydrographic network of the Garonne watershed (60 % of the total catchment area is used for agriculture, half of which for cereal crops (Tisseuil et al., 2008)), pesticide runoff and leaching into adjacent water bodies is a potential threat to aquatic organisms (Devault et al., 2009a; Morin et al., 2009a; Taghavi et al., 2010). In a diffuse, agricultural landscape such as the Garonne river basin, there is no clear pollution gradient along any particular river. Therefore, the 11 selected sampling sites were geographically dispersed throughout different rivers of the basin (Fig. 1), covering a range of varying pesticide levels. The sampled rivers varied in width, depth and turbidity. In general, better quality sampling sites were located on larger rivers (bigger width and depth) with low turbidity, whilst worse quality sites were located on smaller tributaries with turbid waters. The better quality sites were found on larger rivers most probably due to dilution of toxicants in a larger flow of water.

In order to characterize each sampling site, the AEAG pesticide concentration databases between 2006 and 2008 were used to calculate two toxicity indices: the msPAF (multi-substance Predicted Affected Fraction; Van Zelm et al., 2009) and TU (Toxic Units; Von der Ohe et al., 2008). The msPAF quantifies the toxic pressure put on an ecosystem due to the presence of a mixture of chemicals, indicating the fraction of all species that is predicted to be exposed above an effect-related benchmark, such as the EC50 or the NOEC. As pesticide concentrations varied within and among years, an average msPAF value for each sampling site was calculated according to Posthuma and De Zwart (2006) for 2006, 2007 and 2008, and the maximum value of the three years was retained in our analysis. The Toxic Units approach reveals whether the measured concentrations are higher than the known EC50 (median effect concentration), for three different species: the invertebrate *Daphnia magna*, the algae *Scenedesmus vacuolatus*, and the fish *Pimephales promelas*. TU calculation followed Von der Ohe et al. (2008) and the maximum TU value for each species at each sampling site was used. Then, a principal component analysis (PCA) was used to eliminate the colinearity between these two indices. The first axis of the PCA, accounting for 60.1% of the total variation, was kept as a synthetic index of toxicity. Pairwise differences between the toxicity of all pairs of sites were calculated (hereafter referred to as the “TOX” matrix). The chosen time period corresponded to the immediate years prior to field sampling of fish, for which the AEAG has performed an extensive survey across the river-basin.

For the same time period, data of 16 physical-chemical parameters (NH_4, calcium, Cl^-, conductivity, biological organic demand, chemical oxygen demand, hardness, Mg^{2+}, solid matter, NO_3^-, NO_2^-, HPO_4^{-2}, dissolved oxygen, pH, SO_4^{-2}, temperature) were normalized according to Pesce and Wunderlin (2000) and averaged for each sampling site. Two integrative environmental variables (Env1 and Env2) were derived from the first two axes of a PCA on the 16 environmental parameters (Appendix 1), accounting for 46.2 and 23.5% of the total variation, respectively. Pairwise differences were calculated between Env1 and Env2 values respectively, for all pairs of sites (matrices "ENV1" and "ENV2"). A high degree of colinearity among environmental variables in multivariate analysis may bias the results (Ter Braak et al., 1995). As ENV1 was found to be strongly correlated with TOX ($r_{ENV1*TOX}$ = 0.5430), it was removed from all subsequent analysis.

Sampling site catchment areas were obtained using the geographical information system in ESRI© ArcMap™ 9.2. Here we assumed that taking into account site catchment area adjusts for river size. Pairwise differences between catchment areas of all pairs of sites were calculated ("BAS" matrix). Geographical distances between all pairs of sampling sites ("GEO" matrix) were calculated using ArcMap. Pairwise differences between average water velocities measured at each sampling site for 2006 constructed the "FLOW" matrix.

3.2.2. Fish sampling and morphometric data

The gudgeon, *Gobio gobio* (L.) is a benthopelagic cyprinid fish common in both polluted and non-polluted areas in Western Europe (Flammarion and Garric, 1997; Knapen et al., 2009). We considered that the fish captured at a certain site have been exposed to the conditions measured at that site, because the gudgeon has a limited home range (±100 m; Stott, 1963). This has been confirmed by Bervoets and Blust (2003) and Van Campenhout et al. (2003) showing that metal concentrations in gudgeon tissue reflect levels measured in environmental samples.

Between August and November 2008, electrofishing was performed on foot or by boat. Up to 20 gudgeon individuals (Table 1) were captured, sacrificed on-site and transported on ice to the laboratory where they were kept, individually wrapped in aluminium foil, at -20°C until further processing.

To obtain morphometric traits of gudgeons, after unfreezing, both sides of each gudgeon - placed beside a metric ruler for scaling - were photographed. Both pelvic fins of

each fish were then removed and stored in 95% ethanol for DNA analysis. Photographs were analysed using Visilog 6.4 Demo® to obtain X-Y coordinates of the landmarks intended for morphometric measurements (see footnote of Fig. 2). 17 euclidean distances between 18 landmarks (Fig. 2) were calculated for both sides of each fish. All subsequent analysis (except measurement error estimation) was performed using Aitchinson (Aitchinson, 1986) log-ratio transformed measurements to account for individual size-effects (Peres-Neto and Magnan, 2004). The transformation follows the equation $Y_{ij} = \log x_{ij} - 1/p \cdot \sum_{i}^{p} \log x_{ij}$ (1), in which *p* is the number of morphological traits and x_{ij} the value for the i^{th} individual and the j^{th} trait. Based on the left-right differences of morphological traits X to XVII (not subject to asymmetry due to developmental instability), the dataset presented an average measurement error of 2.74 % (minimum 0.70 %, maximum 5.56 %). Gudgeon measured in average 8.48 cm (standard deviation: ±1.81). Quantitative trait differentiation - P_{ST} - values were estimated using the following equation $P_{ST} = \alpha_b^2 / (\alpha_b^2 + 2\alpha_w^2)$ (2), in which α_b^2 is the between-population variance and α_w^2 the within-population variance of the right-side measurement of each fish, per sampling site (*P_{ST}-I* to *P_{ST}-XVII*) obtained by analysis of variance on each trait. P_{ST} values were computed in the same way for left-right differences of morphological traits I to IX and averaged over the 9 traits (*P_{ST}-ASY*).

3.2.3. Microsatellite analysis

Genomic DNA was extracted from the pelvic fins following the salt-extraction method Aljanabi and Martinez, 1997. The markers selected by Blanchet et al. (2010) for gudgeon were used here: Ca01[a], Gob12[b], Gob15[b], Gob16[b], Gob22[b], Gob28[b], MFW1[c], Rhca20[d] (primer references: [a]Dimsoski et al., 2000; [b]Knapen et al., 2006; [c]Crooijmans et al., 1997; [d]Girard and Angers, 2006). Briefly, Blanchet et al. cross-amplified a set of markers and conserved only those that displayed highly readable and repeatable profiles. Loci that presented null-alleles were removed from the final list.

The selected loci were co-amplified using the QIAGEN® Multiplex PCR Kit (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) reactions were carried out in a 10 µL final volume containing 5–20 ng of genomic DNA, 5 µL of 2xQIAGEN Multiplex PCR Master Mix, and locus-specific optimized combination of primers (detailed recipes are available

upon request). PCR amplifications were performed in a Mastercycler PCR apparatus (Eppendorf®, Hauppauge, NY, USA) under the following conditions: 15 min at 95°C followed by 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C and finally followed by a 60 min final elongation step at 72°C. Amplified fragments were then separated on an ABI PRISM® 3130 automated capillary sequencer (Applied Biosystems). Allelic sizes were scored using GENEMAPPER™ v.4.0 (Applied Biosystems).

3.2.4. Discriminant analysis of morphometric data

We used linear discriminant analysis (LDA) to illustrate the main morphological differences among the 11 sampling sites, and to identify the traits that discriminate the sites. LDA was performed on all right-side morphometric traits (I to XVII, Fig. 2) using the R software (R Development Core Team, 2007, package ade4). The statistical significance of sites discrimination was assessed using a Monte-Carlo permutation test (1,000 permutations).

3.2.5. Genetic variation and population structure

For each sampling site, observed and expected heterozygosity (H_O and H_E) as well as inbreeding coefficient (F_{IS}) were estimated using GENETIX 4.05.2 (Belkhir et al., 2002). Number of alleles (A) and allelic richness (AR; based on minimum sample size) were calculated using the program FSTAT 2.9.3.2 (Goudet and Buchi, 1995). Departure from Hardy-Weinberg equilibrium and genotypic linkage disequilibrium between all pairs of loci for each population were checked using FSTAT, with significance levels adjusted for multiple comparisons (Bonferroni procedure; Rice, 1989). Differences of H_E and allelic richness between populations were tested using Kruskal-Wallis multiple comparisons test. F_{IS} averaged over populations was tested regarding difference to zero via a Student's t-test. Allelic frequencies were estimated and differences among populations calculated by Fisher's exact test, both using GENEPOP 4.0 (Rousset, 2008).

The degree of genetic differentiation among populations was assessed using the standardized F_{ST} approach. F_{ST} were calculated using FSTAT for each pair of sampling sites, based on the same principle as for P_{ST} calculation, i.e. comparing within and among-population variance (equation 1 applied to allelic data). Using the same software, the

statistical significance of F_{ST} values was tested by 55,000 permutations and the significant level was adjusted by the Bonferroni procedure (α = 0.0009). F_{ST} values were used to construct a F_{ST}-ratio distance matrix ("FST"). F_{ST} ratios were calculated as follows: $F_{ST}/(1-F_{ST})$ (3).

Population genetic structure was assessed via the Bayesian clustering method in STRUCTURE 2.3.3 (Pritchard et al., 2000a; Falush et al., 2003). Irrespective of sampling location, STRUCTURE allocates genotypes (individuals) to a number of genetic clusters (*K*), so as to minimize deviations from linkage and Hardy-Weinberg equilibrium within clusters. This method allows regrouping individuals according to their biological population, instead of predefined sampling sites. Ten replicates of each run from *K* = 1 to *K* = 11 were performed using the admixture model, *K* being the number of genetic clusters. Each replicate was run for 20,000 Markov chain Monte Carlo (MCMC) generations (initial burn-in of 20,000 generations). Posterior probabilities *L(K)* were estimated using the output of the runs, and *ΔK* calculated according to Evanno et al. (2005) as a complimentary method. When using *L(K)*, the *K* with the highest likelihood is considered as the optimal number of genetic clusters. Alternatively, *ΔK* is the measure of the second order rate of change in the likelihood of *K*, to select the most likely number of clusters *K*. As recommended by Evanno et al. (2005), the height of the modal value of the *ΔK* distribution was used here as the signal for the uppermost hierarchical level of genetic structure in our data set.

In addition, we considered the fractional membership (*q*) of each individual in each group (Pritchard et al., 2000a), also computed by STRUCTURE. Two categories of populations were differentiated according to their *q* values when considering *K* = 2 (clusters C1 and C2): those with a *q* higher than 70 % for either of the clusters were considered to belong to that cluster; and populations that do not present any *q* values above 70 % were considered as sharing membership between clusters.

3.2.6. Relating toxicity and morphometry

In order to study the relationship between two variables, many studies resort to the use of Mantel tests (Mantel, 1967), regression analysis which assess the strength of correlation between the distance or dissimilarity matrices of both variables (Legendre and Fortin, 1989; e.g. Vila-Gispert et al., 2002; Fratini et al., 2008). Partial Mantel tests (Manly,

1991; Legendre, 2000) are implemented to check if two variables are similarly correlated when controlling for a third variable (Gizaw et al., 2007; Raeymaekers et al., 2007; Willi et al., 2007; Bourret et al., 2008). However, this kind of test is limited in the number of variables that can be tested at the same time (maximum three). We thus extended the partial Mantel regression to more than three distance matrices by using general linear models (GLM).

The vectors of all distance matrices – FST, TOX, ENV2, GEO, BAS, FLOW, P_{ST} I to XVII, and P_{ST}-*ASY* (traits I to IX individually and average) - were extracted and the data scaled (transformed values are centered around zero and have a unit variance). For P_{ST} I to XVII and for all P_{ST}-*ASY*, GLMs was used to test, after 1,000 permutations, the relationship between TOX and morphometry, simultaneously taking into account FST, ENV2, GEO, BAS and FLOW. GLM output provided the significance of the correlation coefficients of simple (permuting one of FST, ENV2, GEO, BAS, and FLOW, excluding TOX) and composed (permuting TOX, including all others) models (see table 3). Significance levels were adjusted for multiple comparisons following the Bonferroni procedure. For all composed GLMs that were statistically significant, Pearson's correlation coefficient between trait measurements and TOX were calculated, thus obtaining the tendencies of those relationships.

3.3. Results

3.3.1. Morphological variation

LDA revealed 3 clusters of sampling sites apparently separated along the first axis: MUR, TRC and GUP to the left of the centre, AVN and RAB to the right, and the remaining 6 sites in the centre (Fig. 3). Correlation values of each morphometric trait with the first two axis of the LDA are shown in appendix 2.

3.3.2. Genetic variation and population structure

The microsatellite allele dataset did not reveal departure from Hardy-Weinberg equilibrium nor present genotypic linkage disequilibrium for any pairs of loci. From 3 to 17 alleles per locus were detected, at an average of 19.4 over all loci. Among the 11 sampling sites and for 8 loci, 155 microsatellite alleles were detected. The minimum total number of alleles over all loci was observed for DAD (65 alleles) and the maximum for SAV (82). Allelic

richness varied between 6.48 at DAD and 7.48 at MUR (Table 1). Differences between H_E and AR were non significant (p>0.05) for all population comparisons respectively. Average F_{IS} was significantly different to zero (p-value < 0.001).

F_{ST} ranged from 0.0006 to 0.1107, with an overall average of 0.0395 (Table 2). Significant differences were detected in 34/55 (63.6 %) of the comparisons.

The $F_{ST}/(1-F_{ST})$ ratio was not correlated with geographical distances between sites (r = 0.2009, p-value = 0.1414). Out of the 17 morphological traits, 15 presented larger average P_{ST} values than the average F_{ST}, 6 of which were significantly larger (95 % confidence intervals beyond the upper F_{ST} 95 % CI level; Fig. 4).

FST were strongly related to 6 of the 17 morphological PST (GLMs permuting the FST component and without additional components; Table 3). Complete GLMs on PST*FST, permuting FST and accounting for all other variables gave the same significant results as simple PST*FST models, except for PST-II (data not shown). Of the 6 significant FST-PST relationships, 4 also presented significantly larger PST than FST (traits III, IV, XVI, and XVII; Fig. 4).

Different K values assumed in the STRUCTURE software produced $L(K)$ values with a maximum likelihood at K = 4, although not much stronger than at K = 2 (Fig. 5). Amongst the steepest increases between successive K values, the lowest standard deviation was between K = 1 and K = 2. The method proposed by Evanno et al. (2005) also suggested a strong division at K = 2, corresponding to the modal value of ΔK (Fig. 5). The high values of cluster membership (q) when K = 2 also indicated that this division was the most probable. We thus considered the existence of two clusters (C1 and C2) the highest level of genetic structure. Regarding membership to each cluster (q), 5 populations were strongly assigned to one cluster: GAG, MUR and TRC to C1 (q > 70 % in 10/10 runs); AVN and DAD to C2 (q > 70 % in 10/10 and 5/10 runs, respectively). The remaining 6 populations (with q < 70 %) can be assigned to a cluster based on their highest q: GUP and SAV in C1; HER, MOIS, MONT and RAB in C2 (Fig. 3). All populations had a q in one cluster of at least 58 % over all the runs.

A general conformity is observed between the distribution of sampling sites according to morphology (LDA) and genetic data (STRUCTURE results) (Fig. 3). For example, MUR, TRC and GUP are closely positioned within the LDA plot, whilst most individuals in those populations were allocated to the same cluster regarding membership values.

3.3.3. Relationship between toxicity and morphometry

Complete GLM permuting TOX and including all other components, revealed significant slopes for comparisons of toxicity and 3 out of 17 morphological trait P_{ST} differences between sites (Table 3). No significant slopes were detected for any P_{ST}-*ASY* (only results for average P_{ST}-*ASY* are shown in table 3). With increasing toxicity differences, differences in fish eye radius (P_{ST}-II) and body height (P_{ST}-XVII) increased significantly, whilst differences in distance between snout and operculum (P_{ST}-III) and in dorsal fin base length (P_{ST}-XIII) decreased significantly.

When testing the correlation between toxicity (principal components of the 1st axis of the PCA performed with toxicity indices) and the measurements of each significantly related morphological trait, eye radius significantly decreased (r = 0.2710, p < 0.001) and body height significantly increased (r = -0.4263, p < 0.001) with increasing toxicity. No significant correlation was found between operculum position and toxicity (r = 0.0845, p = 0.2216) or between dorsal fin base length and toxicity (r = 0.0843; p = 0.2227).

3.4. Discussion

In the present study we aimed at assessing the relationship between morphometric traits of eleven wild gudgeon populations and the levels of pesticide toxicity they had been exposed to, whilst taking into account confounding factors such as genetic, geographical and physical-chemical differences between sites.

3.4.1. Morphometry and genetics

First we studied the morphometric and genetic differentiation between populations separately. A general conformity is observed between the distribution of sampling sites according to morphology (LDA) and genetic data (STRUCTURE). For example, MUR, TRC and GUP are closely positioned within the LDA plot, whilst most individuals in those populations were allocated to the same cluster regarding membership values. This type of pattern suggests that it is important to include genetic data in studies comparing populations. Without this information we would not have been able to conclude that the significant relationships found between morphometry and toxicity were the result of the toxicity *per se* or that of innate genetic differences between populations.

The gudgeon populations studied are in general weakly differentiated genetically: significantly different genotypes (i.e. high F_{ST}) corresponded to low (70.9 % of comparisons) or moderate (29.1 % of comparisons) levels of population differentiation (Wright, 1978; Hartl and Clark, 1997). This is not surprising in view of and the fact that gudgeon are present in a large range of riparian conditions typical of temperate rivers (Mastrorillo et al., 1996) thus presenting a broad ecological niche (Knapen et al., 2009), with high prevalence in rivers in South-West France (present in 79 % of all AEAG sampling sites; Gevrey et al., 2009). A tendency for isolation-by-distance (IBD; Hutchinson and Templeton, 1999) with a slight increase of genetic differentiation with distance was found for the ensemble of site comparisons, thus indicating regional equilibrium between gene flow and genetic drift. However, when testing for IBD within the clusters identified by Bayesian structuring analysis, the pattern showed no significant correlation between genetic differentiation and geographical distance, presenting high variance (results not shown here). According to Hutchinson and Templeton (1999), such a pattern indicates lack of regional equilibrium with genetic drift being much more influential than gene flow within the identified genetic clusters. In addition to various types of more-or-less insurmountable physical barriers along rivers of the Garonne basin (Eau France, 2010), gudgeon present a strong homing behaviour (Stott et al., 1963; Stott, 1967), which would explain reduced gene flow via migration.

As genetic drift is influencing genetic differences (F_{ST}) in our study, and F_{ST} and P_{ST} are correlated for some morphological traits, part of the variation observed in P_{ST} may also be driven by genetic drift. However, 6 morphological traits presented significantly greater differences than the corresponding F_{ST}, observed in neutral markers. This is a recurrent result in quantitative genetic studies and is generally understood as a result of directional natural selection in shaping patterns of quantitative trait differentiation (Merilä and Crnokrak, 2001). Although tests with laboratory-reared specimens would be necessary to tease apart the effects of selection and plasticity, we can nevertheless hypothesize that we are observing adaptation to the local environment, which in this case is characterized by having different degrees of pesticide contamination over sites.

The genetic-morphometric conformity found with the LDA (morphometric data) and STRUCTURE (microsatellite data) analysis is also apparent in the FST*PST GLM tests, for which 6 of the 17 morphological traits were significantly correlated to FST. Despite the fact that a third of F_{ST} values show moderate population differentiation, the modelling approach

applied still revealed a significant relationship between morphometry and toxicity when including all variables (composed GLMs). The strong correlations between FST and some PST found here suggest that the extent of genetic differentiation in neutral marker loci can be considered fairly predictive of the extent of differentiation in loci coding for quantitative traits (Merilä and Crnokrak, 2001).

It would thus be interesting to continue this study using quantitative trait loci (QTL) analysis on markers (genes) that affect certain phenotypic traits (Gelderman, 1975; Tanksley, 1993). For example, Raeymaekers et al. (2007) compared genetic divergence in three-spined stickleback populations based on neutral microsatellite markers, quantitative traits, and QTLs, along an upland-lowland (estuarine) gradient. It was found that some traits under divergent selection for P_{ST} were also so for QTL, and the extent to which those traits were developed was concordant with the position along the upland-lowland gradient. However, this was not the case for most P_{ST}-QTL comparisons. There is an interesting potential in this kind of approach when traits can be linked to one major gene `i.e. non polygenic; also supported by Macnair, 1991) or a specific breeding design is experimentally implemented (Merilä and Crnokrak, 2001; Raeymaekers et al., 2007), although not easily applied to wild populations. QTL could be used as a tool to identify phenotypes that are variably sensitive to exposure to environmental contaminants. If functional characteristics or morphological traits that confer individuals an advantage in more stressful environments can be linked to specific genes, ecological status of wild populations can then be screened using either a quantitative or genetic approach.

3.4.2. Morphometry as indicator of pesticide stress

In our study we tested a modelling approach that is capable of taking into account a range of co-varying factors. Composed GLMs to assess the relationship between morphological traits (P_{ST}) and toxicity, while including all other variables in the model were performed. Upon removal of confounding effects, 4 of the 17 morphological traits studied were significantly correlated with pesticide toxicity, suggesting a reaction of these traits in response to the agricultural stress. Although the simple TOX GLM would, for some variables, lead to the same conclusions as the complete GLM, this could well not be the case for other biological traits and/or when studying other species. Furthermore, significant genetic differentiation was observed between most sampling sites. Despite this, the model enabled

us to identify morphological traits that are related to sampling site toxicity levels after removal of genetic, environmental and geographical confounding effects.

Gudgeon eye-diameter and body height were the two morphological traits that presented a response to pesticide toxicity levels: increasing differences (between sampling sites) in those traits corresponded to increasing differences in toxicity. Eye-diameter significantly decreased with increasing toxicity levels. Previous studies have found that fish inhabiting more turbid areas than their conspecifics tend to present smaller eyes, compensated by the development of accessory sensory organs (Blaber, 1981; Bruton, 1985). However, correlations between gudgeon eye-diameter and toxicity with suspended matter levels were both non-significant in our study. On the other hand, the initial stages of eye development in fish have been previously shown to be disturbed by the presence of environmental contaminants, both in laboratory and natural settings (Weis and Weis, 1998; Nakayama et al., 2005; Kruitwagen et al., 2006). Thus our result corroborates previous findings although the mechanism behind the response cannot be determined here, nor which contaminants are responsible.

Gudgeon body height significantly increased with the level of toxicity. As previous studies have found negative relationships between water flow and fish body height (Pakkasmaa and Piironen, 2000; Imre et al., 2002; but see also Peres-Neto and Magnan, 2004), we checked the relationship between water flow and toxicity. Average water flows registered for year 2006 at the studied sampling sites did not exceed those suitable for gudgeon (according to preference curves reported by Lamouroux and Capra, 2002). We found that sampling sites with higher toxicity levels also presented lower average water flow. However, the GLM analysis we implemented here allowed eliminating water flow as a determining factor of fish shape, thus we can hypothesize that toxicity does in fact affect development of exposed gudgeon, in this case body height.

3.4.3. Confounding factors in river health assessment

It is impossible to account for all varying factors playing a role in field conditions. For this reason confounding factors are often referred to when an explanation is put forward regarding unexpected results in studies performed in natural conditions, or even in certain laboratory settings. Environmental variables, whether they are considered stressors or not, often interact with each other, synergistically or antagonistically, producing unexpected and

often unpredictable effects (Underwood, 1989; Folt et al., 1999). Not accounting for confounding factors can bias ecological interpretation of data and lead to erroneous conclusions. For example, in a review, Ewers and Didham (2006) concluded that confounding factors may greatly mask effects induced on species response to habitat fragmentation, leading to the underestimation of present and future consequences of habitat degradation and loss.

There are relatively few studies that in fact attempt to include confounding factors within the data analysis methodology. It is however clear that with the increase of the number of variables included in the data analysis, the robustness and ecologically relevance of the conclusions will equally augment (although not necessarily with increased statistical significance). This inadvertently leads to higher certainty regarding management and protective decisions based on environmental surveys. Downes (2010), as part of a special issue on multiple stressors in freshwater ecosystems (Ormerod et al., 2010), elaborated an interesting appeal to the use of "forgotten" tools and principles that can help distinguish between multiple stressors associated with human impacts. Part of the solution to correctly infer whether a set of stressors does in fact have an impact on wild organisms was suggestively based on the statistical approach for analyzing the dataset. Interesting, simple and apparently abandoned methods do indeed exist (e.g. quantile regression) that would greatly contribute to the better assessment of river health.

3.4.4. Conclusion and implications

Morphology is one of many phenotypic traits that can be studied in order to quantify (or simply detect) organism responses to environmental stressors. Our results underline the importance of taking into account the different sources of phenotypic variability between organisms when identifying the stress factors involved. The separation and quantification of the independent effect of such factors provides an interesting outlook regarding the use of these evaluation metrics in the natural environment. We do not however claim to have included the totality of interfering factors in one single study. Depending on the availability of the data, many other factors could be added (e.g. habitat quality, invasive species, inter-specific competition, seasonal variability). It would be interesting to apply a model such as our composed GLM to the response of two co-occurring species of the same group (i.e. two fish species, for example), presenting different sensitivities to a stressor, given that prior

knowledge on their sensitivity to that particular stressor is fairly reliable. In this way, the response of the sensitive species could be related to that particular stressor, so long as the non-sensitive species reveals indifferent to the stressor levels.

One important requirement - among others (for an overview see Statzner and Bêche, 2010) - regarding the applicability of a biomonitoring tool is the stability of the phenotypic trait(s) studied across large spatial scales. The inclusion of additional factors that quantify differences between geographically distanced sites can overcome this problem. This is especially important when the reference sites are not within the same eco-region as the impacted sites, as is often the case due to the widespread character of pollution sources. The use of transformed data (e.g. scores of a particular axis of a principle component analysis as a synthetic index of toxicity) for the implementation of a model such as the one we developed here, allows for the inclusion of a multitude of different sub-components within each factor index. For example, other types of contaminants could be included in the development of the toxicity index, or additional physical-chemical parameters in the general environmental variable. So not only is our approach expandable to different bio-geographical zones, it is also adaptable to a broad range of stressors.

Although here we attempted to offer insight in the causality relationship between pesticide stress and morphological changes in exposed gudgeon, the practical utility of the study mainly concerns the development of tools that can be easily implemented in environmental assessment programs. The fact that such a tool has incorporated in its design a range of generally interfering factors confers robustness to the final conclusions and demonstrates that adequate statistical approaches can greatly simplify integration of multiple factors. We hope that more studies in environmental assessment of river systems will adopt similar approaches, as environmental risk analysis and subsequent management and protective measures can but benefit from such improvements.

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3.6. Tables

Table 1 – Number of fish sampled, toxicity index (Tox = 1st axis of PCA; see text for details) and average genetic parameters for each sampling site according to eight microsatellite loci: H_E, unbiased expected heterozygosity; H_O, observed heterozygosity; F_{IS}, inbreeding coefficient (W&C); A, number of alleles per locus; AR, allelic richness. Standard errors in brackets.

Site	N fish	Tox	H_E	H_O	F_{IS}	A	AR
AVN	19	-0.46	0.759 (0.154)	0.631 (0.243)	0.198 (0.251)	8.50 (1.25)	6.73 (0.96)
DAD	20	0.921	0.807 (0.067)	0.720 (0.096)	0.112 (0.078)	8.13 (1.09)	6.48 (0.67)
GAG	20	1.009	0.782 (0.130)	0.747 (0.152)	0.045 (0.124)	9.38 (1.56)	7.20 (1.03)
GUP	19	-2.93	0.835 (0.092)	0.784 (0.139)	0.065 (0.114)	9.25 (1.19)	7.48 (0.83)
HER	20	0.479	0.784 (0.103)	0.649 (0.151)	0.184 (0.140)	9.00 (1.27)	7.04 (0.88)
MOIS	20	1.113	0.800 (0.095)	0.648 (0.154)	0.201 (0.153)	8.75 (1.31)	6.65 (0.76)
MONT	20	1.130	0.787 (0.097)	0.717 (0.121)	0.089 (0.121)	8.88 (1.25)	6.89 (0.78)
MUR	13	1.124	0.809 (0.121)	0.766 (0.188)	0.062 (0.159)	8.75 (1.19)	7.79 (0.95)
RAB	20	1.119	0.775 (0.127)	0.685 (0.194)	0.131 (0.202)	9.25 (1.37)	6.95 (0.81)
SAV	20	0.119	0.807 (0.100)	0.717 (0.124)	0.113 (0.128)	10.25 (1.46)	7.46 (0.87)
TRC	20	-3.62	0.754 (0.148)	0.737 (0.197)	0.031 (0.147)	8.88 (1.26)	6.83 (0.87)

Table 2 - Pairwise genetic (F_{ST}) and geographical distances between sampling sites. F_{ST} values are below the diagonal and geographical distances in kilometres are above. F_{ST} significance levels are indicated by asterisks: blank, non-significant; * $p< 9x10^{-4}$; ** $p<1x10^{-4}$; *** $p<3x10^{-5}$.

	AVN	DAD	GAG	GUP	HER	MOIS	MONT	MUR	RAB	SAV	TRC
AVN		227.52	191.53	12.35	186.00	130.83	156.00	218.86	208.36	177.40	20.74
DAD	0.0398**		168.55	230.19	163.03	96.69	71.52	195.88	19.16	154.42	222.69
GAG	0.0954**	0.0495**		194.20	16.76	71.86	97.03	27.33	149.40	18.20	186.70
GUP	0.0291**	0.0419***	0.0475***		188.67	133.50	158.66	221.52	211.03	180.07	23.41
HER	0.0199	0.0231	0.0352**	0.0384***		66.34	91.50	44.09	143.87	12.67	181.17
MOIS	0.0055	0.0185*	0.0570	0.0293*	0.0083		25.17	99.19	77.54	57.73	126.00
MONT	0.0108	0.0310***	0.0816***	0.0277*	0.0214	0.0017		124.36	52.37	82.90	151.17
MUR	0.0990**	0.0467*	0.0006	0.0439**	0.0527*	0.0574	0.0812**		176.73	45.53	214.03
RAB	0.0239*	0.0230*	0.0542***	0.0371**	0.0140	0.0162	0.0185	0.0688***		135.27	203.54
SAV	0.0388	0.0367**	0.0223	0.0240***	0.0069	0.0147	0.0320*	0.0320	0.0135		172.57
TRC	0.1107***	0.0744***	0.0156*	0.0528***	0.0559***	0.0792***	0.0971***	0.0145	0.0759***	0.0236***	

Table 3 - Slopes of general linear models performed with all P_{ST} (I to XVII and ASY) permuting different components of the model. § indicates the complete model: permuting TOX and including all other variables. Significant tests after 1,000 permutations are in bold and marked with an asterisk (significance level adjusted according to the Bonferroni procedure; p = 0.0029). Refer to legend of Fig. 2 for landmarks used for each P_{ST} and to Material and methods for explanation of variables.

P_{ST}	TOX §	TOX	FST	ENV2	GEO	BAS	FLOW
I	0.1334	0.2075	**0.7392** *	0.3440	0.0705	-0.0736	0.0159
II	**0.4048** *	**0.3944** *	**0.4009** *	**0.6841** *	0.0534	-0.0405	0.1148
III	**-0.3729** *	-0.1190	**0.4568** *	0.1161	0.1237	-0.1636	0.0042
IV	-0.1648	0.0478	**0.5812** *	0.3041	0.0992	-0.1448	0.0616
V	-0.3055	-0.0662	0.2962	-0.0014	0.1265	-0.1218	0.0918
VI	-0.1895	-0.1826	-0.0251	-0.0335	0.0279	-0.0085	-0.1521
VII	0.3063	-0.2339	-0.0831	0.1908	-0.0337	-0.1451	-0.1409
VIII	0.0192	-0.0597	-0.0662	0.2205	-0.1110	0.0920	-0.0903
IX	0.0044	0.0799	0.2618	0.3934	0.0184	-0.0285	-0.0274
X	0.0342	0.0481	0.1615	0.2511	0.1342	-0.0669	-0.0925
XI	-0.0182	-0.0470	0.0615	-0.0063	0.0445	-0.1425	-0.1498
XII	-0.0232	-0.1537	-0.0438	0.2093	-0.1104	**0.3961** *	0.1063
XIII	**-0.4062** *	-0.2364	-0.1353	-0.0403	0.1385	0.1215	0.0107
XIV	-0.0708	0.0442	**0.7322** *	0.0946	0.0903	-0.1083	-0.0576
XV	-0.1834	-0.1636	-0.046	0.0838	-0.0542	0.0021	-0.0918
XVI	-0.2520	-0.1649	0.0956	0.0992	0.1092	-0.1391	-0.1566
XVII	**0.3840** *	**0.4374** *	**0.4896** *	**0.6202** *	0.1476	-0.2467	0.1231
ASY	-0.1829	-0.0102	-0.0998	-0.1536	0.1120	-0.1386	-0.0749

Appendix 1 - Correlation of each environmental variable with the first two axis of the principle component analysis. Absolute values greater than 0.5 are in bold.

	1st axis	2nd axis
Ammonium	-0.491	-0.045
Calcium	**-0.822**	0.360
Chloride	**-0.863**	0.434
Conductivity	**-0.780**	0.483
Biological O_2 demand	**-0.667**	-0.112
Chemical O_2 demand	-0.311	0.064
Hardness	**-0.849**	0.457
Magnesium	**-0.741**	0.172
Suspended matter	**0.571**	**0.769**
Nitrate	**-0.865**	0.271
Nitrite	**-0.850**	-0.255
Orthophosphate	**-0.689**	-0.023
Dissolved O_2	**0.622**	**0.729**
pH	**-0.563**	**-0.522**
Sulphate	-0.136	**0.919**
Temperature	**-0.567**	**-0.754**

Appendix 1 - Correlation of each morphometric trait with the first two axis of the linear discriminant analysis. Absolute values greater than 0.5 are in bold.

Trait	1st axis	2nd axis
I	-0.159	0.377
II	0.154	0.271
III	-0.007	**-0.830**
IV	0.341	**0.543**
V	-0.196	-0.024
VI	-0.274	-0.071
VII	0.134	0.234
VIII	0.041	-0.419
IX	0.137	0.197
X	-0.199	**0.718**
XI	0.321	-0.127
XII	0.063	-0.027
XIII	0.023	0.213
XIV	-0.364	0.069
XV	0.107	0.035
XVI	0.297	**-0.767**
XVII	**-0.558**	-0.194

3.7. Figures

Figure 1 - Selected sampling sites throughout the Garonne river basin (grey area in insert map).

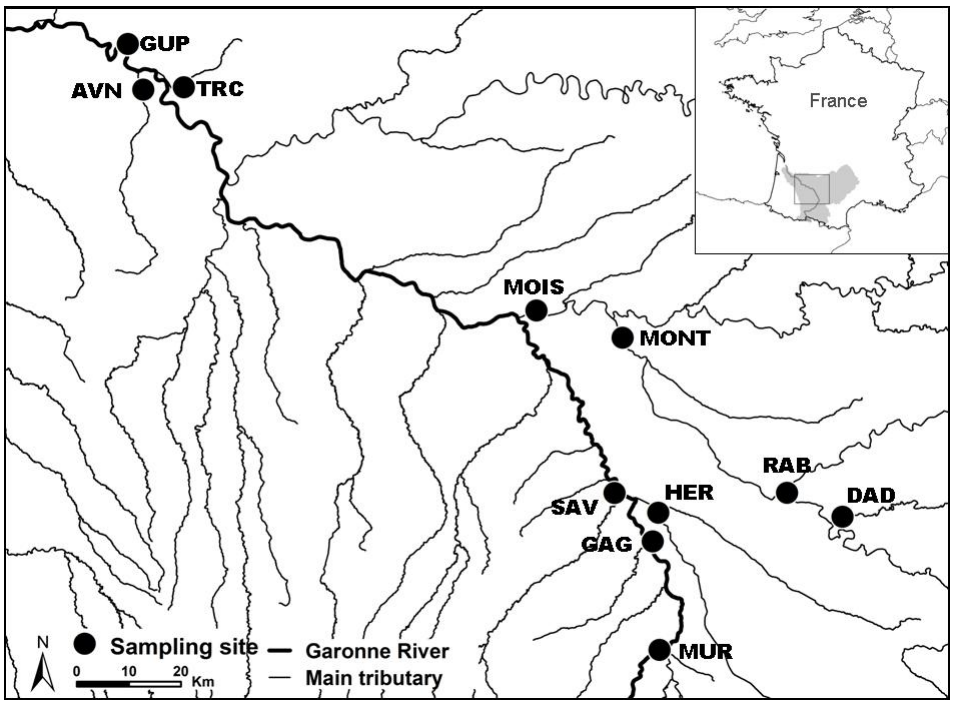
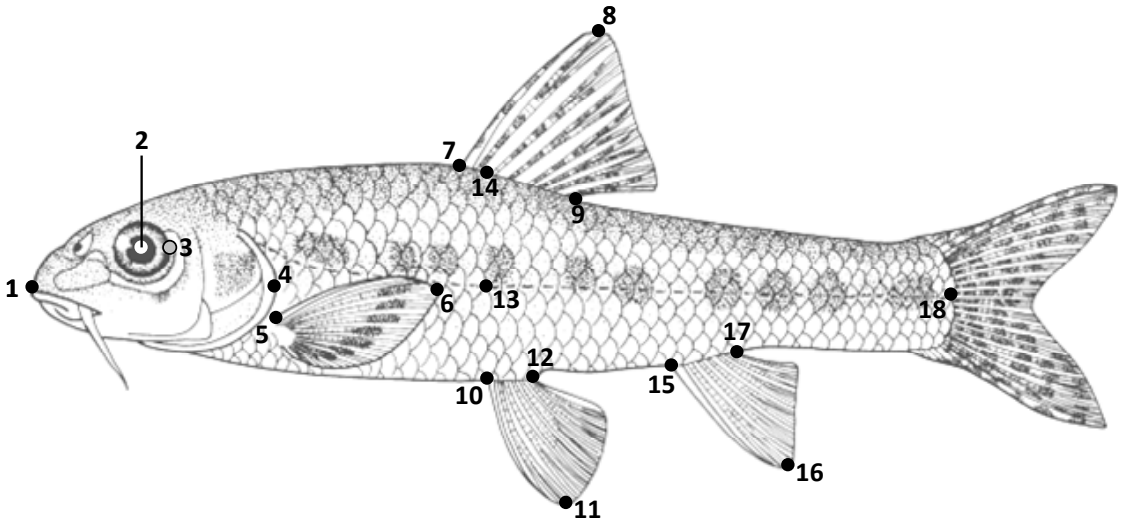


Figure 2 - Placement of landmarks on a gudgeon specimen sketch (Illustration by Susan Laurie Bourque, reproduced with permission from the Canadian Museum of Nature, Ottawa, Canada).



Footnote: The following morphometric traits were measured: I (1-2), II (2-3), III (1-4), IV (4-5), V (5-6), VI (1-10), VII (10-11), VIII (10-12), IX (13-14), X (1-18), XI (1-7), XII (7-8), XIII (7-9), XIV (15-16), XV (15-17), XVI (1-15), XVII (10-14).

Figure 3 - Distribution of the fish specimen according to the first two linear discriminant functions based on the 17 right-side morphometric measurements of gudgeon captured at all sampling sites. Point shading indicates the inferred genetic cluster to which individuals belong when q > 70 % (identified using STRUCTURE; K=2). Ellipses group individuals from each sampling site (Monte-Carlo test after 1,000 permutations: p = 0.001).

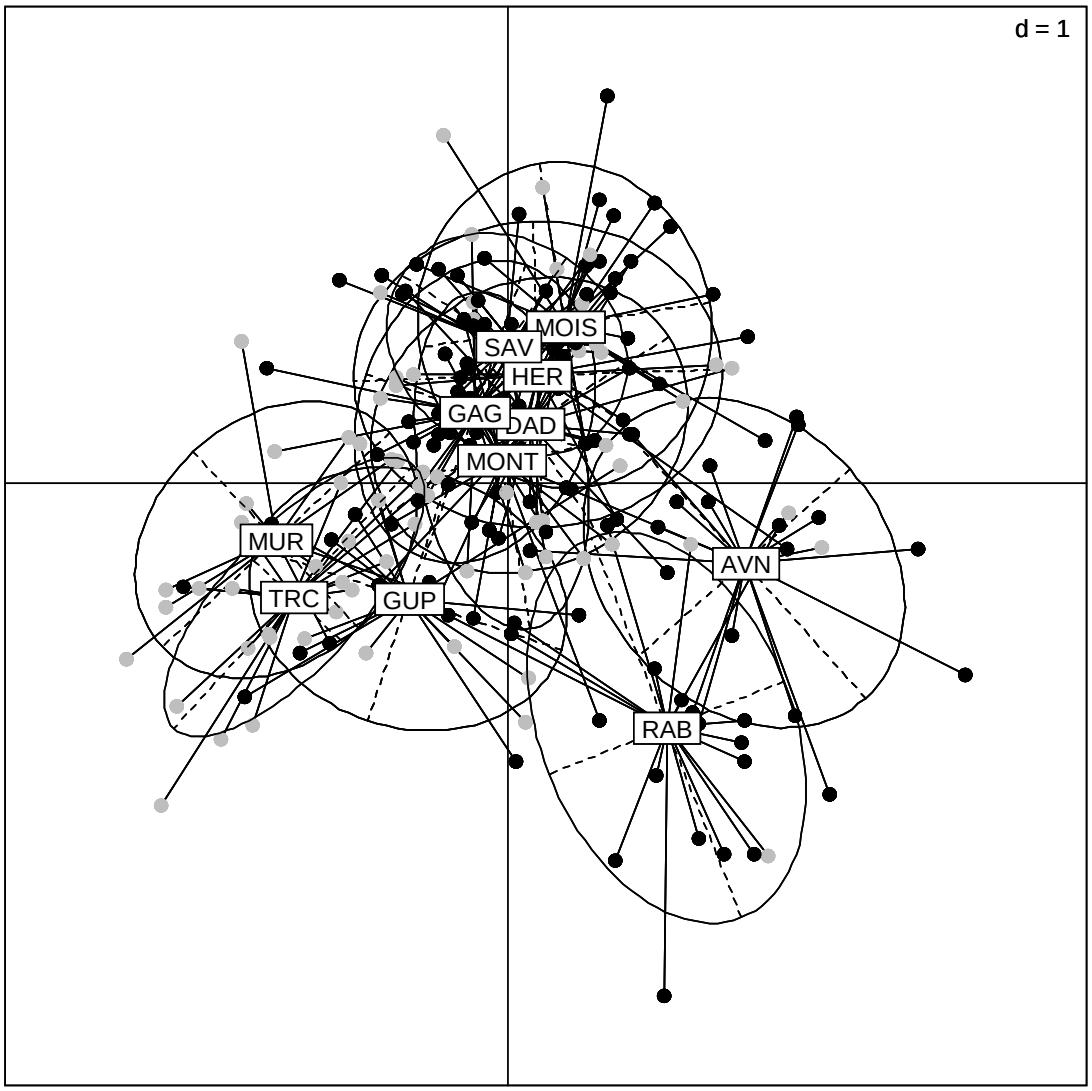


Figure 4 - Average P_{ST} (morphometric differentiation; black dots) for each morphological trait and respective 95 % confidence intervals (horizontal bars) for all populations pooled together. Full vertical line indicates average F_{ST} (genetic differentiation) of all populations and loci pooled together and dashed vertical lines the corresponding 95 % confidence interval.

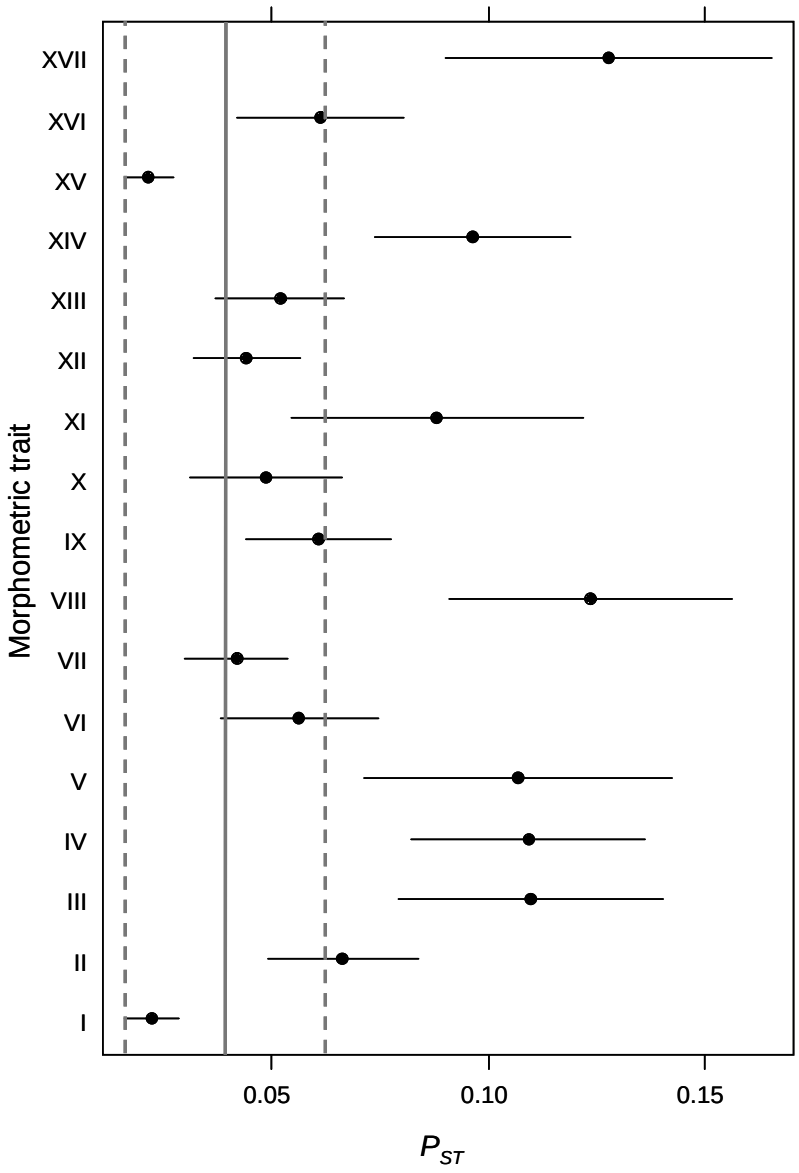
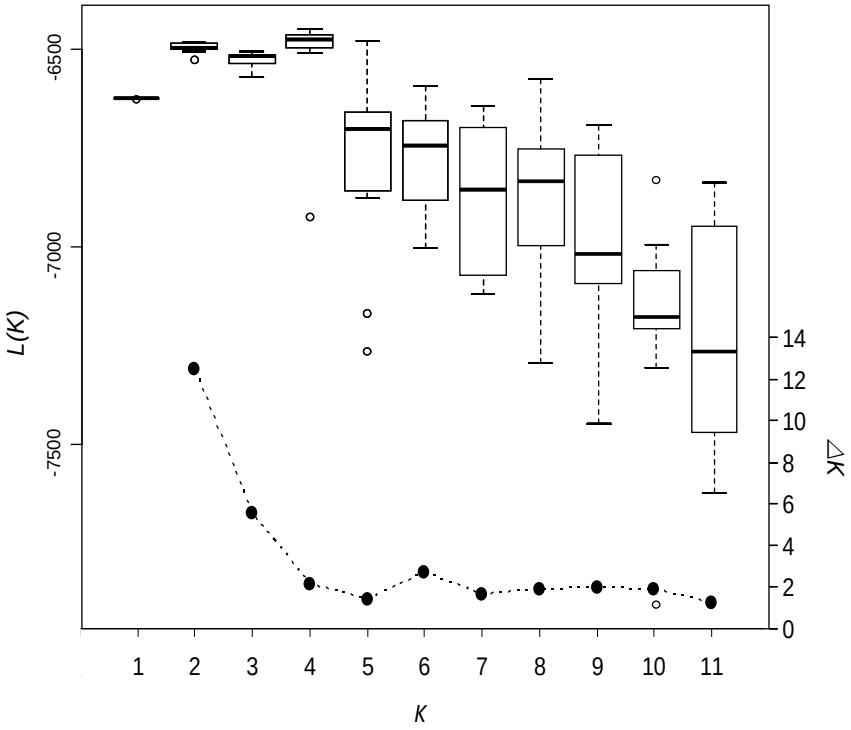


Figure 5 - Identification of population genetic structure based on Bayesian clustering. Given are posterior probabilities *L(K)* (box plots) and second order rate of change in the likelihood of *K* (*ΔK*, full dots) according to the number of genetic clusters (*K*). See Material and Methods for details.



4.

Biological traits of feral European chub (*Squalius cephalus*) along a pesticide gradient in southwest France

4.1. Introduction

A strong lobby on water quality problems in the European Union has resulted in the establishment of the Water Framework Directive (Directive 2000/60/EC of the European Commission, 2000), requesting all member states to attain at least good ecological and chemical status of all surface water bodies by 2015. Water agencies and partner ecosystem assessment institutions are therefore in need of chemical and biological tools to adequately assess the current status of the water bodies for which they are responsible.

In Europe the consumption of pesticides is decreasing, principally because more efficient substances are being applied in smaller quantities (EEA, 2010). Nevertheless, pesticides still have a significant impact on natural environments. This impact varies by the land-use of surrounding areas and by the type of pesticides used (Belden et al., 2007; Hrodey et al., 2009). However, it is not necessarily the amount of toxic substance used but rather the toxicity of an individual pesticide that determines its potential for environmental damage. Because pesticides are inherently ecotoxic - they have been designed to bestow a toxic effect on pest organisms - it is essential that their presence and concentration in the environment are continuously monitored, as well as the subsequent risk to non-target organisms. Furthermore, pesticides do not occur alone in the environment, but as part of a cocktail of substances and their metabolites that are of both natural and anthropogenic origin (from agriculture, industry, urbanization; Eggen et al., 2004; Chèvre et al., 2006). It has been demonstrated that the toxicity of a mixture of substances may still be considerable even if individual compounds occur at low concentrations (Walter et al., 2002; Backhaus et al., 2003; Lydy et al., 2004; Junghans et al., 2006; Belden et al., 2007a).

Aquatic organisms inhabiting contaminated environments are important sentinels of water quality and general ecosystem health (Adams and Greeley, 2000). By studying the response of biological parameters of exposed wild organisms as well as measuring environmental concentrations of toxicants of potential concern, a causal relationship can be established. Fish are commonly used for aquatic biomonitoring purposes, but because pesticides have mostly not been designed to affect fish, fewer studies have focused on the side-effects they may indeed occur in

fish species. In field surveys it is particularly important to study fish species that allow for measurement of biological parameters (biomarkers) from different levels of organisation (i.e. cellular, individual, population). The chosen species should be common so as to avoid time-consuming sampling and preferably wide-spread to cover a broad range of environments. European chub (*Squalius cephalus*) fulfil these requirements and it has previously been shown to be an adequate candidate for routine biomonitoring of pollutant-impacted rivers (Flammarion et al., 2002a, b; Krča et al., 2007; Frenzilli et al., 2008; Randak et al., 2009; Hinfray et al., 2010; Wenger et al., 2010).

Agricultural land in the Adour-Garonne river basin (southwest France) covers 60 % of the total catchment area, half of which cultivates cereal crops (Tisseuil et al., 2008). Due to the intense agricultural activity and the extensive hydrographic network of the Adour-Garonne watershed, pesticide spray-drift, runoff, and leaching into adjacent water bodies are a potential threat to aquatic organisms {Devault, 2009a; Morin, 2009a; Taghavi, 2010}. This system is thus an adequate case-study for the assessment of the impact of agrochemicals on biological parameters measured in exposed organisms. Ibarra et al. (2005) reported that species richness of fish assemblages was inversely proportional to non-point source pollution (a set of 20 physical-chemical water properties) in the Garonne basin. Studying the impact of pesticide pulses due to runoff events, Polard et al. (2010) found that caged Crucian carp presented signs of erythrocyte genotoxicity when exposed to river water from spring floods. To our knowledge, no other studies have focused on using biological parameters of fish species as a tool to evaluate river agrochemical contamination in the Adour-Garonne river basin. Therefore, organo-somatic indices, condition factor, and hepatic histopathological changes were assessed in feral European chub sampled from rivers of the Adour-Garonne catchment, presenting a range of pesticide levels quantified by two toxic pressure indices.

4.2. Material and methods

4.2.1. Sampling site selection and characterization

Sampling sites were selected via the analysis of the pesticide water concentration database compiled from a 2006 field survey performed throughout the Adour-Garonne river basin by the Agence de l'Eau Adour-Garonne (AEAG; Adour-Garonne water agency). The AEAG assayed water samples for 120 pesticides (mainly herbicides, although also some insecticides and fungicides), 5 times over the course of the year, at 131 different sampling sites using gas and liquid chromatography with mass spectrometric detection (GC-MS and LC-MS/MS, respectively). The potential effect on water quality of pesticides was quantified by the SEQ-Eau index (Table 1), an environmental quality index developed by the AEAG. It ranges from 1 (good quality) to 5 (bad quality) for a given pesticide [see MEDD & Agences de l'eau (2003) for more details on the calculation of the SEQ-Eau]. In order to summarize the effect of many pesticides, we conducted a Principal Component Analysis on the SEQ-Eau value by sample site matrix. We limited the pesticides included in the PCA to those found at > 5 % of sample sites (N = 26 compounds), and we used the maximum SEQ-Eau value for these pesticides throughout the year. PCA scores on the first axis were extracted for each site and used to cluster sampling sites into one of 3 quality groups - good, average and bad (Table 1; Fig. 1). This quality index is hereafter referred to as QUAL'06.

Fish sampling was performed along a gradient of pesticide-related water quality with 3 bad, 2 average, and 2 good QUAL'06 sites. In a diffuse landscape such as the Garonne river basin, there is not a clear gradient of pollution along any particular river. The selected sampling sites were therefore geographically dispersed throughout different rivers of the basin (Fig. 1) covering a range of different pesticide levels. In order to assess pesticide exposure and potential adverse effects due to toxic pressure, the msPAF (multi-substance Potentially Affected Fraction of species) and Toxic Units (TU) indices were calculated (Table 2) using pesticide field concentrations reported by the AEAG from 2006 to 2008. The msPAF and TU allow for quantification of pesticide toxic impact on organisms over a continuous scale. A continuous toxic pressure classification allows for an assessment of a cause-effect

relationship over many different pesticide levels, thus resulting in a more realistic interpretation of the field situation. For all toxicity index calculations, reported concentrations below the limit of quantification were excluded to avoid overestimation of risks by including compounds that were likely to be absent.

The TU approach uses a risk quotient to reveal whether the measured bioavailable concentrations are higher than the known L(E)C50 (Lethal/Effect) for a certain species. Environmental quality is thus considered inadequate if the resulting TU is higher than 1. Fish-TU calculation followed von der Ohe et al. (2008) in which the measured pesticide concentration of a particular compound (C_i) was normalized by dividing by the corresponding 96 h-LC50 of the standard fish test species *Pimephales promelas*:

$$TU = C_i / LC\ 50_i \qquad (1)$$

As pesticide environmental concentrations varied within and between years, and toxic risk estimation should be designed to be protective, sampling site overall fish-TU corresponded to the maximum of the fish-TU for all compounds over the 3-year period. A logTU of -4 has been set as a minimum toxicity level where no effects on the fish community are expected, assumed to be protective of the community as it corresponds to TU below $1x10^{-4}$ of the acute LC50 (von der Ohe et al., 2008; Wenger et al., 2010).

In order to obtain an estimate of the potential ecological impact at the community level, thus taking the TU approach a level of complexity further, the msPAF is used. This index quantifies the toxic pressure on an ecosystem due to the presence of a mixture of chemicals, indicating the fraction of all species that is predicted to be exposed above an effect-related benchmark {Van Zelm, 2009}. Calculations of msPAF were performed according to Posthuma and De Zwart (2006). The calculation of PAF levels is based on chemical-specific species sensitivity distributions (SSDs) that describe the variation in sensitivities for a set of species under acute or chronic exposure to a certain compound. The single substance PAF (ssPAF) can be used as an approximation of the ecological risk of a single substance to the ecosystem at measured or predicted ambient concentration and is calculated by

$$ssPAF = \left(1 + e^{-(\log(C) - \alpha / \beta)}\right)^{-1} \quad (2)$$

where *C* is the environmental concentration of the compound under consideration and α and β characterize the (normal) distribution of the SSD. The β, or slope, of the species sensitivity distributions is assumed to be equal for compounds with the same toxic mode of action (TMoA; De Zwart, 2005). To aggregate ssPAF values to a single overall msPAF two toxicological models are applied: concentration addition (CA) and response addition (RA). CA is applied for compounds that have the same Toxic Mode of Action (TMoA). The cumulative PAF for mixtures of chemicals with the same TMoA (PAF_{TMoA}) is read by hazard unit (HU = $C/10^{\log(L(E)C50)}$) addition for a single TMoA and is calculated by

$$PAF_{TMoA} = \left(1 + e^{-\left(\log\left(\sum HU_{TMoA}\right) / \beta_{TMoA}\right)}\right)^{-1} \quad (3)$$

where $\sum HU_{TMoA}$ is the sum of the HU for all chemicals with the same MoA and β_{TMoA} is the MoA specific β. The pesticide environmental concentration dataset used here contained compounds belonging to 23 different TMoA groups. The MoA specific PAF or PAF_{TMoA} values are then aggregated to an overall msPAF by RA, assuming that the susceptibility of species for the (groups of) chemicals is statistically independent. msPAF values were calculated using the maximum values of PAF_{TMoA} obtained throughout the 2006 to 2008 period, again with the intent of evaluating the worst-case, thus most protective, scenario:

$$msPAF = 1 - \prod_{TMoA} \left(1 - PAF_{TMoA}\right) \quad (4)$$

An msPAF of 3 % is expected to place primary producers, invertebrates and fish at risk (Faggiano et al., 2010).

Based on Pesce and Wunderlin (2000) an integrated physical-chemical Water Quality Index (WQI) was calculated for sites using data for 16 physical-chemical parameters (NH_4, calcium, Cl^-, conductivity, biological organic demand, chemical oxygen demand, hardness, Mg^{2+}, solid matter, NO_3^-, NO_2^-, HPO_4^{-2}, dissolved oxygen, pH, SO_4^{-2}, temperature) from the AEAG's database for 2006 to 2008.

4.2.2. Fish sampling and processing

The chub (*Squalius cephalus*) is an abundant non-migratory cyprinid species inhabiting streams and lakes throughout most of Europe (Billard, 1997). No major threats are known for this species (Freyhof and Kottelat, 2008). Chub are omnivorous, feeding on plant detritus, vegetation, seeds and invertebrates when young, and preying on small fish as they mature (Billard, 1997). Chub specimens were collected between September and November 2008 using electrofishing (Electro-Pulman, 400V-DC, 1.5A max. current) performed on foot or from a boat. A maximum of 21 of the larger individuals from each sampling site were transported alive to the laboratory in large, opaque buckets containing cooled, aerated river-water.

Within 24 hours of returning to the laboratory, the removed chubs were individually euthanized and the following measurements were taken: length to fork (mm), whole fish-, intestine-, liver- and gonads weights (g), and sex. For age determination, scales from the upper, anterior body of the fish were removed and dry-stored. Scale samples were later cleaned in water and mounted, and the number of winter annuli was counted under a Canon EF 100mm magnifier to determine fish age. Muscle fillets of 2.5 g and liver samples of 0.5 mg were taken from male individuals, placed in polyethylene vessels, shock-frozen in liquid nitrogen and stored at -20°C in order to prevent degradation and alteration of pesticide levels. Only male specimens were considered for pesticide tissue bioaccumulation as higher variability is expected between females due to lipid mobilization in vitellogenin production {Henderson, 1984}. The remaining liver from each fish was stored in 4 % formaldehyde.

4.2.3. Statistical analysis

In order to assess whether chubs from more contaminated sites were more adversely impacted by pesticides than those from sites with lower contamination, three biological indices (BI) were calculated: condition factor, $CF = (W_B \times 100) / L^3$, hepato-somatic index, $HSI = (W_L / W_B) \times 100$, and gonado-somatic index, $GSI = (W_G / W_B) \times 100$ (W_B, somatic body weight, i.e. body weight after removal of gonad, gut

and liver; L, fork length; W_G, gonad weight; W_L, liver weight). CF as indicated here is frequently named somatic index in other studies. It has previously been found, both for chub and other temperate-living fish species, that juvenile and adult fish do not present the same seasonal patterns regarding body energy levels (e.g. Encina and Granado-Lorencio, 1997a, b). For this reason, small, immature, apparently young-of-the-year fish, for which the sex was not possible to identify, were excluded from all subsequent analysis.

A significant ($p < 0.05$) effect of sex was found on GSI, and of age on all three BIs. Analysis of GSI thus included only male fish as female fish were much fewer in number and likely to present more variation in gonad sizes than males. To account for the age effect, correlations between residuals of BI*age regression models (resulting in age-corrected BIs) and toxicity indices were performed and tested. Age-correction regression models were performed with Box-Cox power-transformed BIs to obtain normal residuals (Box and Cox, 1964). As both age-corrected GSI and CF were significantly correlated to the toxicity indices (see results section), the relationship between them was tested via analysis of variance. For this, GSI were re-calculated to account for fish length instead of somatic body weight, already included in CF calculation (i.e., $CF_{gonads} = (W_G \times 100) / L^3$). All analysis was performed in R 2.11.1 (R Development Core Team, 2010).

4.2.4. Pesticide quantification in fish tissues

Pesticide concentrations were measured in individual muscle and liver samples of four fish sampled at one non-polluted site (MUR) and four fish sampled at each of two polluted sites (AVN and TRC), using an original method developped at Ecolab campus ENSAT, F-31326 Castanet Tolosan (Castaing, 2006). Isolation of target analytes was carried out on an Accelerated Solvent Extractor (Dionex ASE 200). Still frozen fish tissue was homogenised with anhydrous diatomaceous earth (Hydromatrix ®, Varian) at a ratio of 1:3 (w/w) and extracted with a mixture of acetonitrile (ACN) and dichloromethane (DCM) at a ratio of 3:1 (v/v). Extraction was performed in two successive steps: the first at 50°C for 5 min, the second at 100°C for 5 min. Extracts from each phase were pooled into glass vials and placed at -20°C

overnight. Each sample was then filtered using a paper filter. Hexane was added to the filtrate at a ratio of 3:4(v/v), the sample mixed, and the mixture was placed at -20ºC overnight. After bubbling under nitrogen during 30 min, extracts were placed in a decanting vial and the ACN and hexane phases were separately recovered. Hexane was added to the ACN phase and ACN was added to the hexane phase. After manual mixing and ultrasonication, the two phases were again separately recovered via decantation. Water residues are removed from each phase via filtration with anhydrous sodium sulphate Pestipur (Carlo Erba SDS F -13124 Peypin).

The samples were then reduced to 2 ml by evaporation and purified. The hexanic phase was purified using Florisil cartridges (Sep-Pak) and 2.5 ml syringes; elution was performed successively with 20 ml hexane/diethyl ether (94:3, solvent A), 10 ml hexane/acetone (90:10, solvent B) – solvents A and B recovered in the same recipient -, and finally 20 ml hexane/acetone (1:1, solvent C). The ACN phase was purified using HLB cartridges (Plus Oasis), preconditioned with 2 ml hexane, 4 ml methanol, and 2 ml ACN. The sample was eluted with 5 ml ACN. Each of the 3 separate eluents (solvents AB and C from the hexanic phase and eluent from the ACN phase) was reduced to 2 ml via evaporation.

Final quantification of the tested pesticides was performed via GC/MS, Gas Chromatography (Thermo Scientific Trace GC; Phenomenex Column 5MS 30 m, 0.25 mm, 25µm) coupled to a mass spectrometer (Thermo Scientific, DSQII) operated in selected ion monitoring (SIM) mode. An aliquot of 1 µl of each sample extract was injected (splitless mode) at 280°C. The carrier gas was helium Air Liquid (Alphagaz2). Chromatographic conditions in the splitless mode (injector temperature: 280°C) were set up at an initial temperature of 45°C. The first step had a temperature increase rate of 35°C/min up to 180°C, then a second step at 8°C/min up to 280 °C and a final 10-minute plateau at 280°C. The detection conditions were: temperature, 300 °C; E.M.V., 2600 V.

Quantification was performed using external standard calibration mixtures: “Mix 44” from Dr. Ehrenstorffer provided by Clouzeau Info Labo F-33220 Ste Foy la Grande (16 molecules: s-triazines ,substituted ureas and chloracetanilides); and lab-made mixtures with individual standards composed of fungicides selected based on their usage in the studied area - “Mix Herbicides” and “Mix Azole”. Chromatograms

and spectrum data were analysed and quantified using Thermo Scientific Xcalibur 1.3 version. The pesticide level was estimated by the area report vs a calibration curve and taking into account the final volume of the extract and the fresh weight of the sample introduced in the cell of the ASE extractor.

The detection limit established was 0.001 µg/g. Recovery after sample preparation, extraction and purification obtained for each pesticide varied from 82.4 to 104.6 %, leading to a mean recovery of 95.4 ± 6.5%, with an acceptable repeatability of < 14 %. The efficiency of this method is confirmed by the test on organo-chlorine derivatives which gave a mean recovery yield of 98.5% in accordance to methods used in other studies.

4.2.5. Histological analyses

Formalin-conserved chub liver samples were fixed in Bouin fixative for 24 h, processed for histology, and stained with hematoxylin and eosin. Histological cuts were screened for cellular modifications at the Centre for Fish and Wildlife Health (Bern University, Switzerland). The screening protocol was qualitative and conducted in a subjective manner. Nevertheless, by considering the ensemble of histological images from good and bad quality sites, some conclusions can be drawn regarding the effect of water quality on liver morphology and functioning. It is known that the number and/or size of certain cellular alterations (such as macrophage aggregates) increase with age (Blazer et al., 1987). We therefore took care to compare fish of similar age and size within sites of each level of toxic pressure.

4.3. Results

4.3.1. Site water quality

Fish-TU and msPAF followed concordant patterns over all sampling sites (r^2 = 0.810, p = 0.0273). Both indices reflected the successive environmental degradation of sampling sites in the QUAL'06 groups (Table 1). All bad quality sites and one average quality site presented log fish-TU above the minimum toxicity level established for fish (-4). At bad quality sites 2.25 to 9.42 % of the community was

potentially affected, whilst only a maximum of 0.1 % was at risk at average quality sites and even less at good quality sites (Table 1).

4.3.2. Biological indices

A few chubs from bad quality sites presented external body lesions and both internal and external parasites (not quantified), whilst individuals from good quality sites were in apparent good health. The biological indices assessed were not significantly correlated with the physical chemical parameters presented in Table 1, or with the overall water quality index ($p > 0.05$). Fish presented a significant increase of GSI and decrease of CF with increasing toxic pressure (fish-TU and msPAF), whilst HSI did not show any significant tendencies (Fig. 2). The relationship between CF and CF_{gonads} was non significant ($p > 0.05$).

4.3.3. Pesticide bioaccumulation

Pesticide concentrations and the number of molecules detected were found to be higher in fish liver than in muscle (Fig. 3). The most abundant molecules in both tissues were isoproturon and linuron, whilst chloroacetanilides (metazachlor, alachlor, metolachlor), and tebuconazol were more present in the liver than the muscle. Only traces of s-triazine residues were found in both tissues and at all sites. Regarding the different sampling points, AVN (bad quality site) presented higher pesticide concentrations in the muscle whilst MUR and TRC (good and bad quality sites, respectively) presented higher concentrations in the liver. These tendencies were observed also regarding the log liver/muscle partition ratio that was negative (muscle > liver) mostly only for the two bad quality sites (Fig. 4).

4.3.4. Hepatic histopathology

Liver of fish from good quality sites were characterized by a homogeneous and compact cell structure. Hepatocytes presented basophilic appearance with nuclei of uniform shape and size and slightly granulated cytoplasm. A moderate amount of fat vacuoles was present and evident pathologies were absent (Fig. 5A).

In bad quality sites, fish presented a more irregular cell arrangement and less compact hepatic cell structure (Fig. 5B, C) than fish from good quality sites. Hepatocytes presented undefined cell borders, fewer fat vacuoles and moderate to severely granulated, cloudy cytoplasm. Multifocally the hepatocytes are detached (decreased contact between cells; Fig. 5B, C). Many hepatocytes presented cytoplasmic vacuoles with non-fatty, eosinophilic amorphous material (Fig. 5B, C), occasionally with brownish pigment, probably consisting of ceroid and lipofuscin (Fig. 3D). Presence of different stages of single hepatocyte degeneration, characterized by karyorrhexis, karyopycnosis or cell debris, was evident (Fig. 5B-D). In singular cases areas of focal necrosis were seen with leucocyte - lymphocytes, macrophages, and melanomacrophages - infiltration (Fig. 5D-F). Infiltration of lymphocytes, macrophages and melanomacrophages were also observed perivascular, pericholangiar and scattered in the parenchyma, (Fig. 5E, F). Occasionally, granulomas with fibrous cells surrounding cellular debris were detected (Fig. 5G). The cause of these granulomas remained obscure but a parasitic origin is possible.

4.4. Discussion

The objective of this study was to relate changes observed in various biological variables, measured in a fish species, with varying levels of environmental pesticide pollution, quantified by two toxic pressure indices (TU and msPAF).

4.4.1. Biological indices

Overall, chub general condition and gonado-somatic index, but not hepato-somatic index, seem to have been affected by the presence of an environmental mixture of pesticides, as shown by the altered condition factors (lower CF in more polluted sites) and gonad-body ratios (larger GSI in more polluted sites). This was shown for both fish-TU and msPAF.

Condition factor, HSI and GSI were comparable to values reported for European chub in previous studies, when considering indices of fish sampled at the same period of the yearly cycle (Encina and Granado-Lorencio, 1997a; Flammarion and Garric, 1999; Flammarion et al, 2002a). Decreases in CF due to toxicant exposure

have been reported in other studies of fish exposed to various types of contaminants (e.g. organic contaminants and metals: Sorensen and Bauer, 1984; Hontela et al., 1995; Couture and Kumar, 2003; Rowe, 2003; agrochemicals: Jenkins, 2004; Miller et al., 2009). Although it is generally expected that chronic contaminant exposure will decrease GSI (e.g. Grady et al., 1992; Friedmann et al., 1996; Flammarion et al., 2002a; Randak et al., 2009; Hinfray et al., 2010), higher GSI in males from bad quality sites in our study may be interpreted as a response to a more stressful environment. Over time, a more polluted site may have induced a selective pressure on individuals that are capable of preparing for an early start in the reproductive season. Chubs from more polluted sites may have gonads that mature earlier on, resulting in higher GSI values. Such individuals would have increased reproductive success as the young-of-the-year, having hatched earlier, would have more time to forage and find appropriate niches. Other studies have pointed out the fish are capable of adjusting certain life-history traits in response to environmental changes when inhabiting variable ecosystems (Ribbink, 1990; Smith, 1991; Van Winkle et al., 1993), either through innate phenotypic plasticity or via genetic selection over time (Knapen et al., 2004; Raeymaekers et al., 2007; Sæther et al., 2007).

On the other hand, greater investment in gonads may contribute to lower CF as a result of fish investing more energy in reproduction and thus leading to depletion of body reserves due to higher metabolic demand (Encina and Granado-Lorencio, 1997a, b). Although GSI and CF were correlated to toxic pressure indices in inverse manner, we did not observe a causal relationship between them. It is nevertheless important to compare fish captured during the same season, as reproduction and other seasonal factors such as environmental fluctuations (e.g. seasonal variations in food availability) have the potential to alter GSI, CF, and other biological parameters in different ways at different times of the year (Dygert, 1990; Encina and Granado-Lorencio, 1997, a, b; Santos, 2008, 2010).

4.4.2. Pesticide accumulation and liver histopathology

A larger number of molecules detected and higher pesticide concentrations and in fish liver, in comparison to muscle, is concordant with the accumulative and metabolic function of the liver towards xenobiotics (Preez and van Vuren, 1992).

Higher concentrations of isoproturon and linuron were likely due to the type of crops and treatment period, whilst low concentrations of s-triazines indicate a past but persistent contamination of the aquatic environment. Liver/muscle concentration ratios also indicated higher pesticide levels in the liver than in the muscle, especially for fish from good quality sites. An explanation for this could be a stronger metabolic capacity of individuals from good quality sites in comparison to fish from bad quality sites.

Macrophages are believed to act in the centralization of foreign material and cellular debris for destruction, detoxification or reuse, the storage of exogenous and endogenous waste products, the immune response, and iron storage and recycling (Wolke, 1992). Fish of larger size, with nutritional deficiencies, or in poor health tend to have more or larger macrophage aggregates (Agius, 1979; Agius and Roberts, 1981; Wolke et al. 1985b). Immune cells, as well as granuloma, were observed more frequently in chub from bad quality sites, which is concordant with the decrease of CF in these individuals. Similar trends have been reported in a number of studies on liver, spleen or kidney of wild fish exposed to a variety of contaminants (Poels et al., 1980; Khan and Kiceniuk, 1984; Wolke et al., 1985b; Khan, 2000; Blazer, 2001; Meinelt et al., 2007). Granuloma are fibrous structures that encapsulate metabolites resulting from the degradation of material of parasitic origin. The presence of such structures suggests that fish from more contaminated sites have decreased defences against the entry of parasites into the organism and are under increased immunological stress.

Sufficient energy storage is essential for organisms to remain healthy and resistant to disease (Bonga, 1997; Schreck et al., 2001), especially when faced with a stressful environment (e.g. over-crowded, aquaculture conditions; Binuramesh et al., 2005; Welker et al., 2007; Caipang et al., 2009). Stressful conditions can lead to allostasis (the ability to achieve stability through change; McEwen and Wingfields, 2003) in which the animal is adapting to a challenging situation, but at the expense of placing a strenuous load on the body, thus possibly becoming maladaptive regarding basic life functions and reproductive fitness (Smolders et al., 2009; Schreck, 2010). A disruption of the energy balance is apparent in the chubs studied here, with fish from more polluted sites presenting lower CF, more parasites,

decreased metabolic activity (possibly due to reduced functionality of liver cells), an increased number of hepatic immune-response cells, and a decrease in hepatic lipid vacuoles. An inverse relationship between plasma glucose levels - the energy source used to maintain homeostasis (Mommsen et al., 1999) - and environmental pesticide contamination has been previously reported (e.g. Bleau et al., 1996; Quinn et al., 2010), as fish metabolize glycogen to meet the increased energy demands of the chemical stressor.

4.4.3. Site toxic pressure levels

Faggiano et al. (2010), using the msPAF approach, reported that within the Adour-Garonne river basin sites with the highest toxic (pesticide) risk could reveal an impact on the entire ecosystem (primary producers, invertebrates and fish), mainly due to the strong influence of four different TMoA. In the current study, out of a selection of twelve TMoA assessed individually regarding the presence of a correlation with the BIs (data not presented here), one (seed growth inhibitor) was not significantly correlated to CF, and three (seed growth inhibitor, germination inhibitor, and uncoupler of oxidative phosphorylation) were not significantly correlated to GSI. None of the 12 TMoA assessed presented significant correlation with HSI. Of the four TMoA highlighted in the study of Faggiano et al. (2010), three were in common with those correlating significantly with CF and GSI, confirming their suggestion of an impact on the ecosystem, including fish. Regarding TU, Wenger et al. (2010) set a minimum fish toxicity (i.e. no effects on the fish community) at a log fish-TU of -4, which is slightly lower than some log fish-TU found for the sampling sites in our study.

Chub CF, as well as GSI, was correlated to both toxic pressure indices with comparable strength. The TU and msPAF evaluation of the ecological hazard of pesticide contamination revealed similar trends throughout the river sites studied here. It therefore seems that the pesticides at highest concentrations (maximum of dominant chemical used for TU estimation) also present the strongest TMoA, thus determining the strength of msPAF values. Indeed, of the 120 pesticides measured by the AEAG over the 3-year period considered, only half were above the detection limit and only a quarter were detected in more than 5 % of all samples. Furthermore,

of the 23 TMoA considered here, only 15 were above zero in 3 of the 7 sampled sites, and only 9 were above zero in 4 sites. Pesticide mixtures found in water bodies adjacent to agricultural areas with mainly cereal crops (as in our sampling area) are often dominated by a small number of compounds, namely herbicides and insecticides such as isoproturon, diuron, atrazine, metolachlor, and carbofuran (Gilliom, 2007; Schuler and Rand, 2008; Faggiano et al., 2010). Their high concentrations result from the large amounts applied to agricultural fields and in urban areas and occasionally also due to their environmental persistence (Gilliom, 2007; Debenst et al., 2010).

Wenger et al. (2010) did not find any significant effects of a gradient of general organic contamination (including Polyaromatic hydrocarbons and agricultural and other industrial/urban-originated compounds) on chub biological indices or histological parameters. However, different levels of dissolved oxygen were presented as the major determining factor in their study. It is thus of course of fundamental importance to check the relation of other environmental parameters with the stressors and biological responses studied, in order to avoid masking of effects that may otherwise be relevant bioindicators of stress. The absence of correlation between basic water parameters (temperature, dissolved oxygen, and conductivity) or a general water parameter quality index, and the biological indices studied here, indicates that responses are indeed determined by the presence of contamination.

4.4.4. Improvements to the current study

For further statistical robustness (i.e. clearer relationships between potential causes and biological responses), a larger number of sampling sites would be required, as well as an increase in the number of individuals sampled per site. The site selection process applied in the present study reveals that the initial collection of sites to be sampled must take into account the fact that a number of those sites may not be included in later assessment due to lack of representativity, as a result of the reduced number or absence of individuals from certain age-class, etc. The reference (or less polluted) sites selected for our study were not located on pristine, mountain

streams, as such sites would most probably not host the more tolerant species found at downstream polluted sites. Furthermore, if pristine reference sites were included in the survey, other environmental parameters would be even more divergent between good and bad quality sites, than between an all-downstream selection of sites.

Studying the impact of toxic pressure on a more tolerant species (such as the European chub in the present study) may reveal difficult to tease out possible effects, due to their capacity to deal with the stressors involved. On the other hand, the fact that a relatively tolerant species does show here a response to the environmental contamination considered, indicates that other more sensitive co-occurring species may be suffering to a larger extent. Multi-species assessments would therefore contribute with important information with regard to this issue.

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4.6. Tables

Table 1 - Sampling sites grouped by quality according to pesticide levels in 2006 (QUAL'06) and ordered by multi-substance Potentially Affected Fraction of species (msPAF). Indicated are maximum invertebrate and fish Toxic Units (TU) and msPAF values (see Methods for calculation explanations), average temperature, dissolved oxygen, conductivity, and Water Quality Index for the period 2006 to 2008. The number of pesticides used to calculate fish-TU and msPAF were, respectively, 59, 58 and 60. Percentage of raw msPAF values are given to facilitate interpretation.

Site	QUAL'06	log fish-TU	msPAF (%)	Temp. (ºC)	Oxygen ($mg.L^{-1}$)	Cond. ($\mu S.cm^{-1}$)	WQI	Catchment area (km^2)
MUR	good	-5.22	0.0001	15.20	10.84	265.00	83.40	5841
MONT		-5.23	0.0014	16.10	9.61	280.23	86.85	9802
MOIS	average	-4.94	0.0041	16.33	9.77	310.27	86.60	15729
HER		-3.83	0.0797	16.12	9.25	627.50	75.46	985
GUP	bad	-2.80	2.2451	14.23	9.56	826.68	79.35	132
AVN		-2.82	3.9207	14.43	9.90	445.27	85.63	406
TRC		-2.27	9.4212	15.18	10.12	776.23	76.90	174

Table 2 – Number (male/females), average age (years), size (mm), weight (g) and biological indices (CF, condition factor; HSI, hepato-somatic index; GSI, gonado-somatic index) of fish sampled from each studied site. Standard deviations are in brackets. Sites are grouped according to QUAL'06, as in table 1.

Site	N fish	Age	Length	Weight	CF	HSI	GSI
MUR	4/0	3.8 (0.96)	265.4 (30.51)	247.0 (73.35)	1.299 (0.04)	1.010 (0.20)	0.530 (0.09)
MONT	6/0	2.0 (0.89)	198.7 (58.38)	119.8 (100.01)	1.179 (0.09)	1.124 (0.18)	0.665 (0.24)
HER	5/3	3.0 (2.20)	265.2 (121.63)	362.6 (343.73)	1.195 (0.08)	1.343 (0.49)	1.295 (0.85)
MOIS	6/0	2.8 (1.72)	208.5 (64.32)	144.8 (100.24)	1.261 (0.08)	1.303 (0.43)	0.892 (0.15)
AVN	16/2	3.0 (0.91)	202.2 (29.52)	100.8 (51.57)	1.107 (0.07)	1.185 (0.26)	1.316 (0.77)
GUP	8/3	4.1 (1.87)	241.7 (48.99)	188.1 (127.46)	1.126 (0.08)	1.473 (0.40)	1.757 (0.66)
TRC	15/6	2.9 (1.09)	188.1 (46.01)	84.8 (86.50)	1.021 (0.05)	1.192 (0.17)	1.563 (0.77)

Footnote: Fish used to calculate GSI (males only) were, on average, 0.24 years younger, 8.60 mm shorter, and 19.87 g lighter than those used to calculate CF and HSI (males and females).

4.7. Figures

Figure 1 - Selected sampling sites throughout the Garonne river basin (grey area in insert map). Sampling site quality is shown according to the QUAL'06 classification (see text for explanation). Land coverage of the region (urban, agriculture and forest areas) was obtained with the CORINE database for year 2006 (Corine Landcover map; Institut Français de l'Environnement, IFEN, 2006).

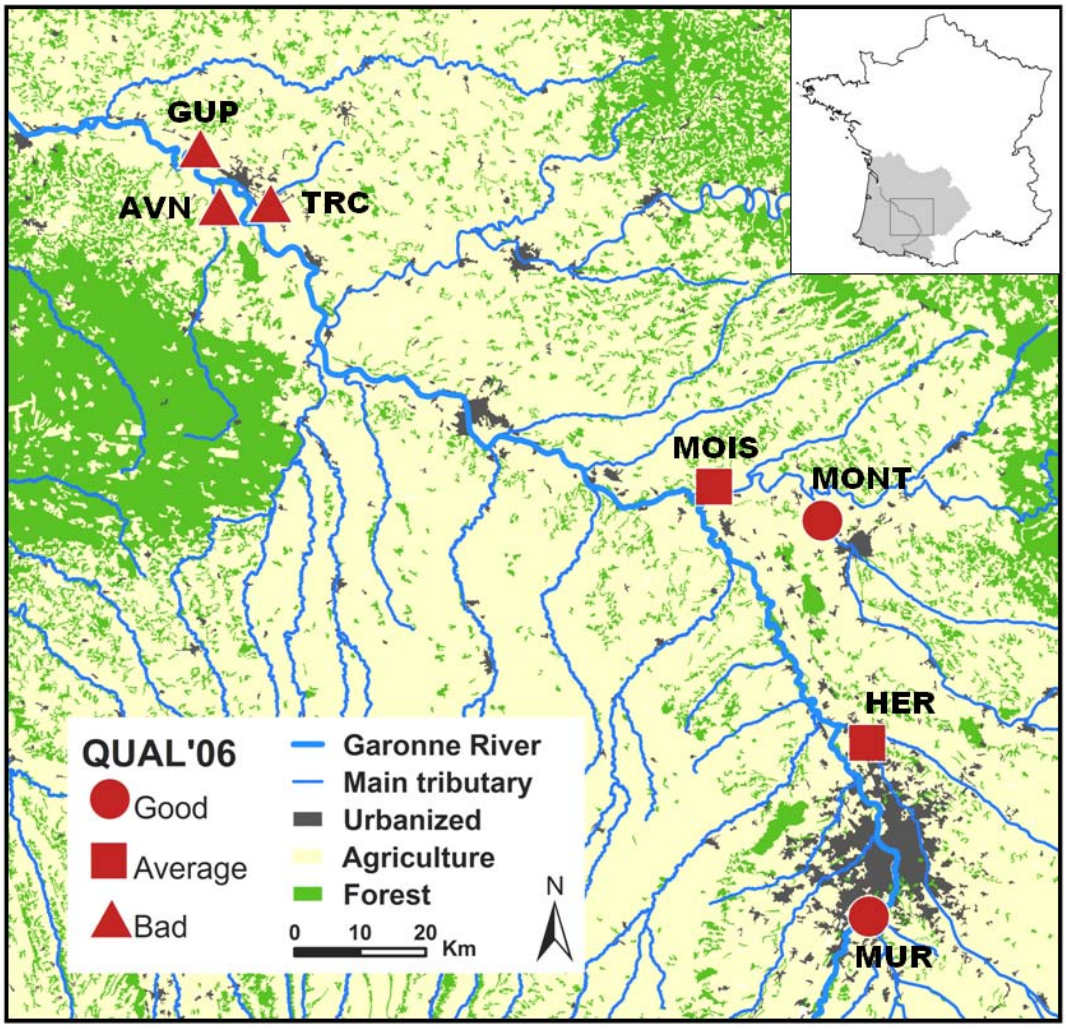


Figure 2 - Effects of fish-TU and msPAF on age-corrected condition factor (CF), hepato-somatic index (HSI), and gonado-somatic index (GSI). Average values for each site are given and error bars represent standard deviations. Higher TU and msPAF values (to the right of the x-axis) indicate higher toxic pressure. Logged values of raw (non-percentage) msPAF are used, to facilitate graphic visualisation. Vertical dotted lines correspond to established fish-TU minimum toxicity limit and 3 % of msPAF (log 0.03 = -1.522; see text for further details). At the top-left of each graph are the respective Pearson correlation coefficients (r^2) and p-values (p).

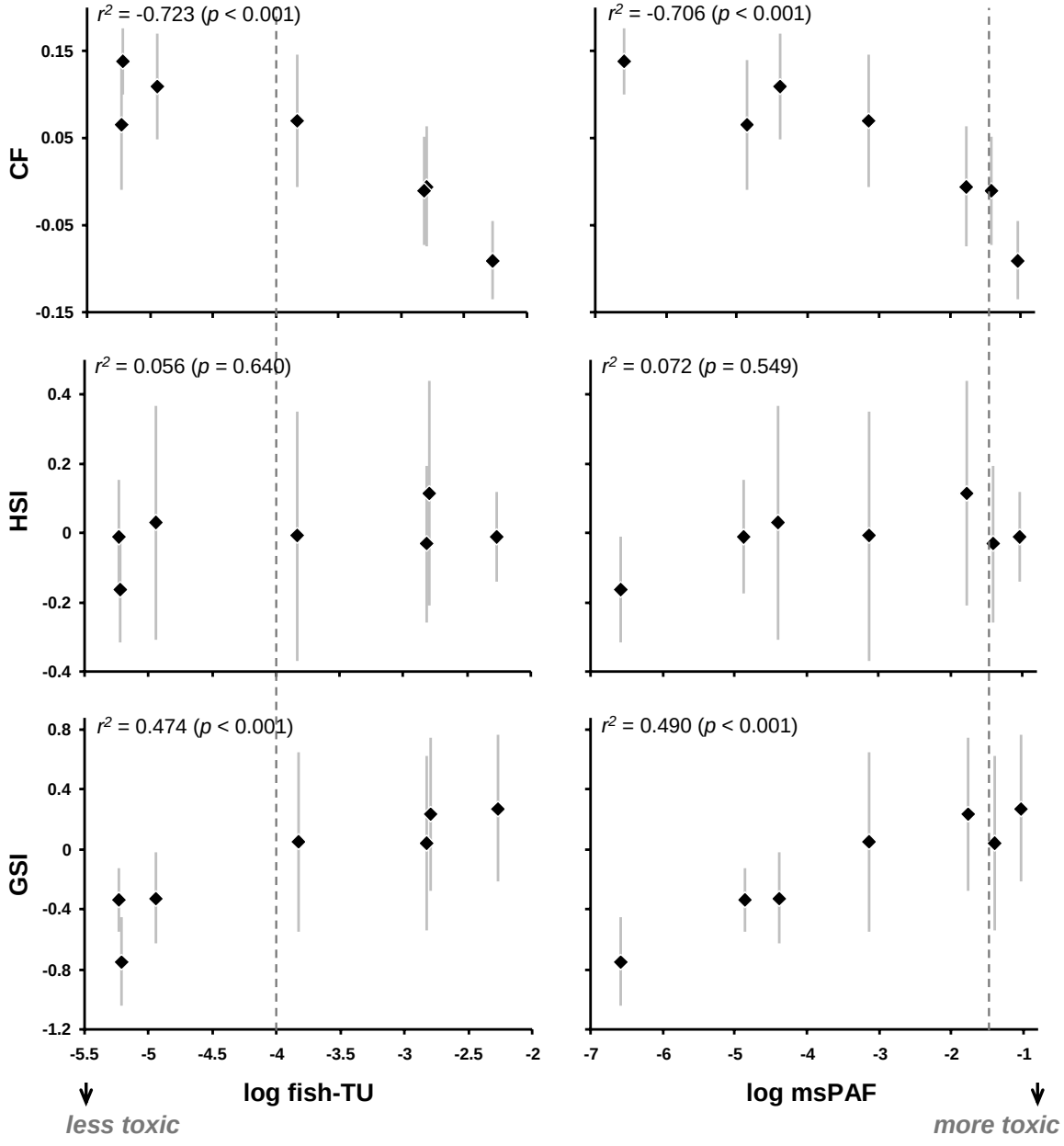


Figure 3 - Measured pesticide concentrations in muscle and liver of fish from one good quality site (MUR) and two bad quality sites (AVN and TRC). Vertical bars represent standard deviations (N = 4). DEA, desethylatrazine.

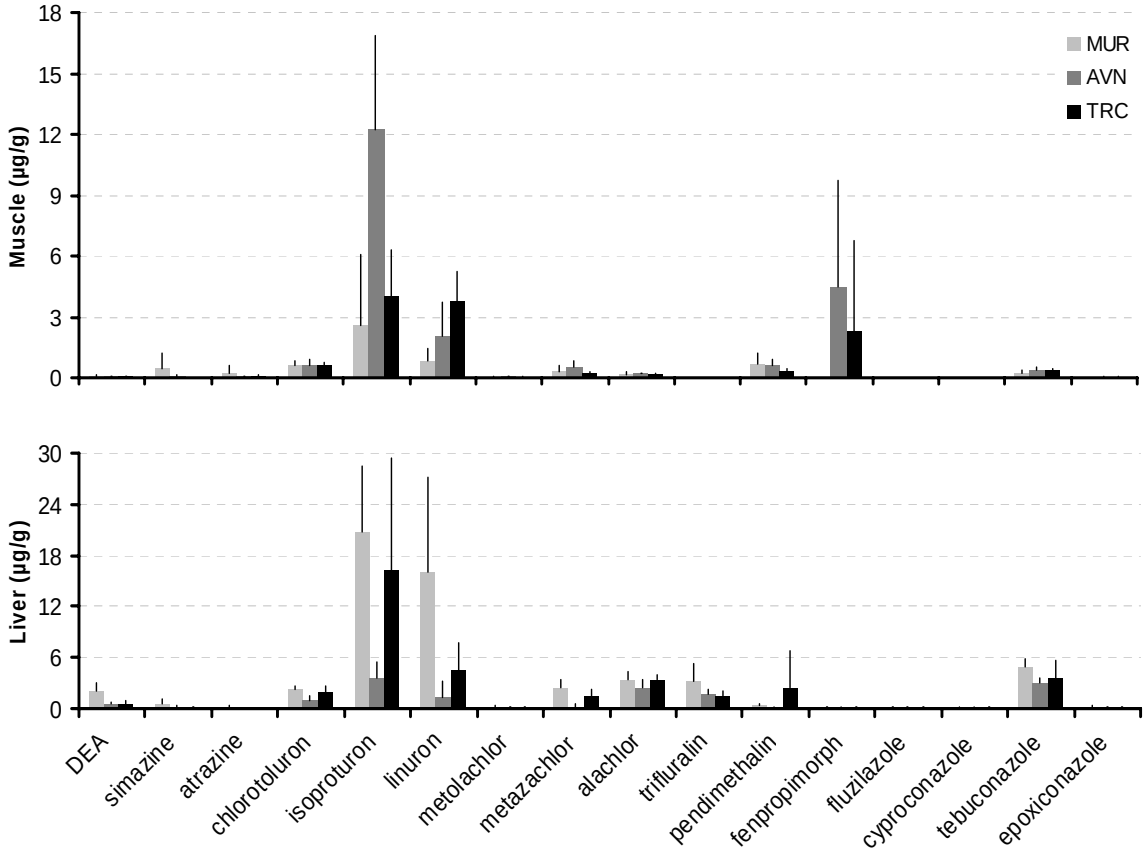


Figure 4 - Logarithm of the liver/muscle concentration ratios for each pesticide and sampling site. MUR, good quality site; AVN and TRC, bad quality sites. Full black horizontal line corresponds to log ratio=0. DEA, desethylatrazine.

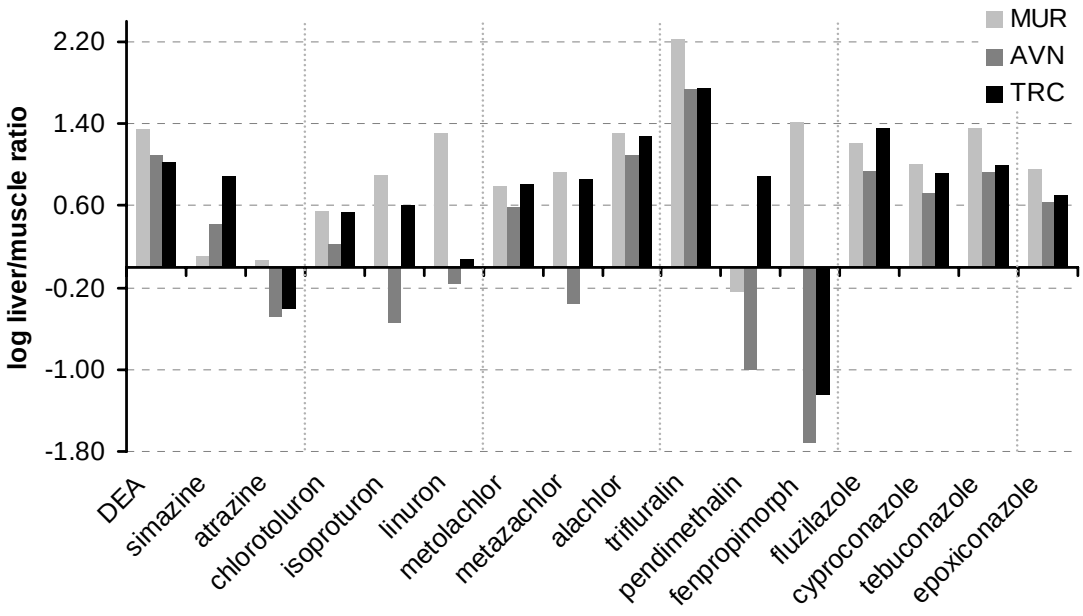


Figure 5 - Photographs of hepatic histological cuts from fish from good (A) and bad (B-G) quality sites. The dotted box in E is the area presented in F. h, hepatocyte (cytoplasm); hn, hepatocyte nucleus; hm, hepatocyte cell membrane; dh, degenerating hepatocyte; kr, hepatocyte undergoing karyorhexis; kp, hepatocyte undergoing karyopycnosis; fv, fat vacuole; am, amorphous material (in vacuoles); d, cell detachment; bam, brown pigmented amorphous material; em, eosinophylic matter; m, macrophage; mel, melanomarophage; ma, melanomacrophage aggregate; l, lymphocyte; cd, cell debris; * rim of leucocytes, partly degenerating; f, fibroblasts; bv, blood vessel; bd, bile duct; Amplification: F, 200x; E, G, 500x; A-D, 1000x.

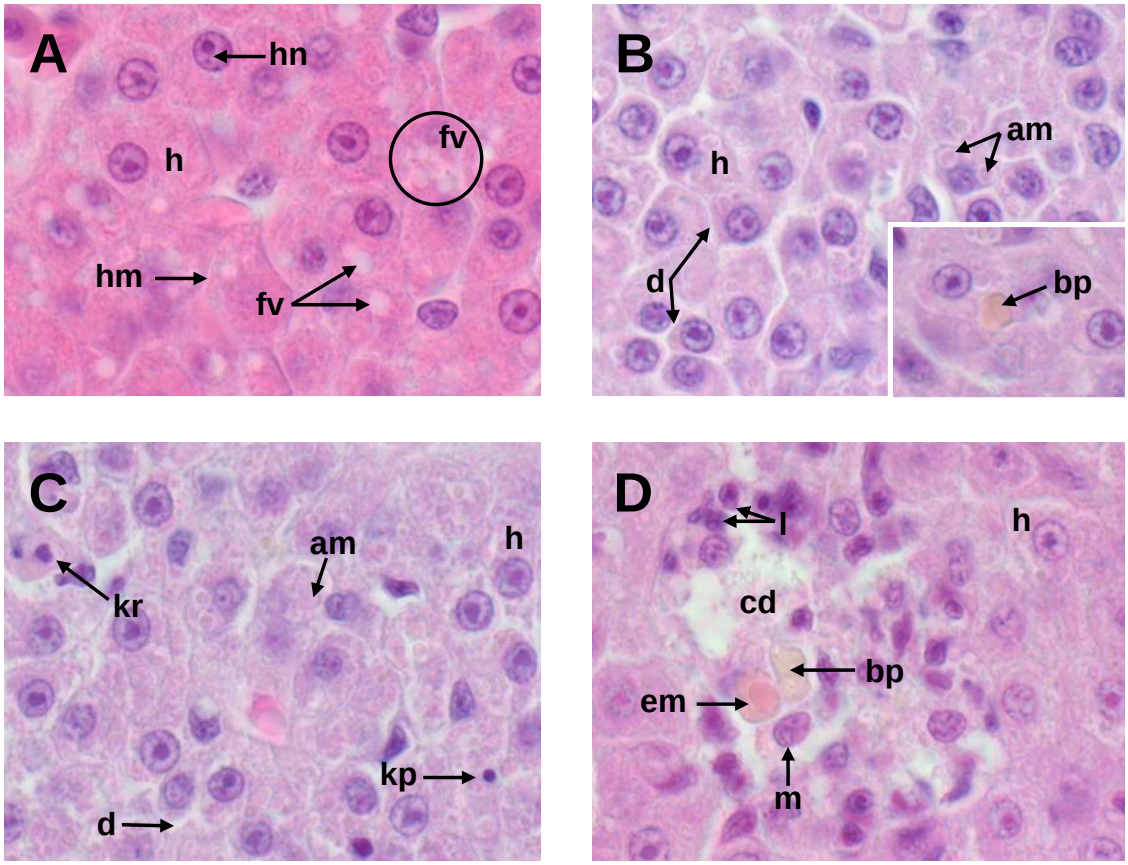
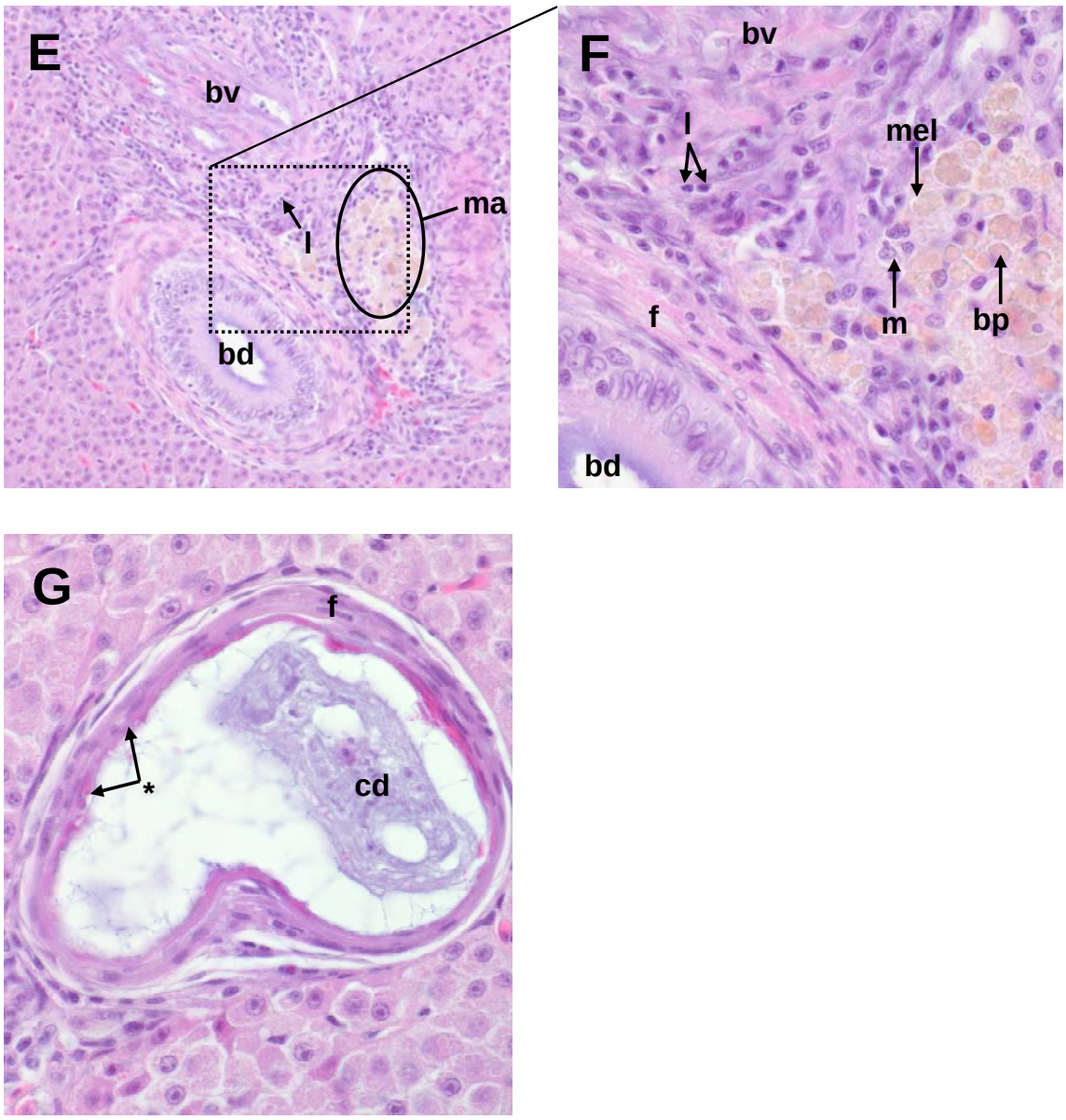
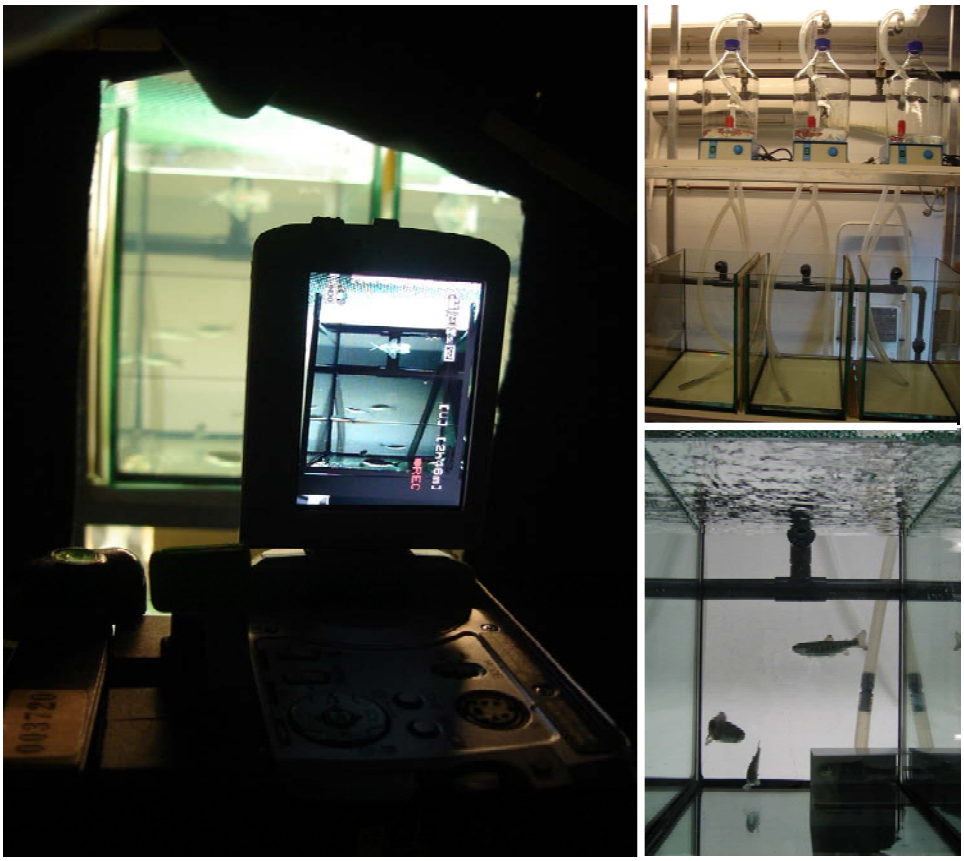


Figure 5 – (continuation)



5.

Behavioural response of juvenile rainbow trout during a short, low-dose exposure to the herbicide mixture atrazine, linuron and metolachlor



5.1. Introduction

Pesticides are frequently detected in surface-waters in proximity of agricultural fields due to run-off, spray-drift or leaching. A mixed-crop farmland will typically be treated with a broad range of substances with different chemical activities (or modes of action). Thus, adjacent water bodies carry a mixture of chemicals that were applied aiming at specific organisms (pests) but may present risks to non-target species inhabiting those ecosystems. Herbicides, designed to inhibit germination, development and persistence of weeds, may indirectly affect aquatic fauna through disturbance of communities at the lower end of the trophic-chain, such as phytoplankton, macrophytes, etc (Butler et al., 1975; De Noyelles et al., 1982; Dewey, 1986; Schäfer et al., 1994; Solomon et al., 1996; Belden et al., 2007a; Daam et al., 2009). However, scarce information exists on their direct effect on invertebrates, fish, and other aquatic vertebrates, especially concerning environmentally-relevant concentrations.

One way to test whether a chemical at low concentrations has an adverse effect on fish and other organisms is to observe behavioural changes, a concept initially suggested by Warner et al. (1966). Behaviour is the result of the interactions of an organism with its external environment, integrating physiological, biochemical and metabolic processes with the environmental factors that stimulate behavioural responses (Grue et al., 2002). The ecological relevance of behaviour is considerable as it can be regarded as the functional interface between the individual and the population (Little, 1990). Indeed, inadequate behavioural responses to physiological and environmental stimuli owing to adverse effects of aquatic toxicants can have serious implications for survival (Weber and Spieler, 1994). And although behavioural responses are not as contaminant-specific as other biomarkers of lower complexity (Peakall, 1994), their attractiveness remains in a higher sensitivity in terms of dosage and response time (Adams, 1999), thus there potential as early-warning signals of effect (Hellou, 2010). Indeed, fish are capable of detecting, and sometimes responding by avoiding, water-borne chemicals at concentrations below the lowest observed effect concentrations, that in turn are several orders of magnitude lower than reported acute median lethal concentrations (Atchinson et al.,

1986; Diamond et al., 1990; Little and Finger, 1990). Furthermore, behavioural changes can occur up to 75 % earlier than the onset of mortality (Little and Finger, 1990).

A practical application of behavioural responses due to their sensitivity is the use of sentinel organisms in real-time water quality monitoring devices. An example is the commercialized Truitosem (Bougeois and Leger, 1998), a device designed to warn of the presence of peaks of contaminants in water intended for production of drinking water. The swimming activity of juvenile trout, exposed to a constant flow of the monitored water, is continuously registered and when significant changes are registered an alarm is signalled. Another application, although still far from being routinely used, is the inclusion of behavioural endpoints in regulatory frameworks. However, the lack of standardized and (field) validated test methodologies, difficulties in performing studies that are capable of showing effects on reproduction or survival, and thus impairment at the population or community level (determining ecological relevance of the measured behaviour), have hindered their inclusion in most hazard assessment programs (Grue et al., 2002).

Fish are convenient models for behavioural ecotoxicology studies as many of their behaviours that are easily observed and quantified under controlled conditions are *per se* ecologically relevant (Scott and Sloman, 2004). For example, social interactions such as schooling, courtship, and dominance hierarchies are directly linked to success in predator avoidance, reproduction, and food resources, respectively. Disruption of those behaviours will most certainly jeopardize the chances of succeeding in fundamental processes that are crucial to individual, and eventually population, continuity, and at considerably lower toxicant concentrations than those known to induce immediate mortality.

Dominance hierarchies are a key factor in ensuring enough resources (food and shelter) for individual fish optimal growth, and are established via intraspecific competition (Chapman, 1966). Fish establish and defend their territory through agonistic acts towards conspecifics, such as threats, nips and chases (Scott and Sloman, 2004). Alterations in agonistic acts due to the presence of toxicants may lead to either failure to maintain a territory (in the case of a decrease or absence of agonistic acts), or metabolic fatigue, if an extreme increase in the frequency of

agonistic acts occurs. The latter situation may also create higher levels of stress for subordinate fish, leading to fatigue through higher swimming demand and reduced capacity to feed, as well as higher probability of injuries inflicted by the attacker and thus increased risk of pathologies. The action of toxic substances can also directly interfere with feeding and predator recognition-and-escape behaviours through physiological interaction with sensory organs/cells, as has been demonstrated in a number of studies (e.g. Saglio and Trijasse, 1998; Tierney et al., 2007b). Fish swimming activity is also altered in presence of contaminants, either incited, repressed (Zhou and Weis, 1999; Steinberg et al., 1995), or altogether inhibited (Little et al., 1990), with consequences on other basic activities such as foraging.

The three herbicides studied here - atrazine, linuron and metolachlor - are representatives of three chemical groups and two modes of action, and all inhibit primary producers. They have been reported to co-occur in environmental water samples from a number of different watersheds (e.g. Frank et al., 1990; Hall et al., 1999; Steen et al., 1999; Gilliom, 2007; Schuler and Rand, 2008; Faggiano et al., 2010) due to their application to the same crops (e.g., corn, sorghum, soybeans; Peterson et al., 2001).

Atrazine ((2-chloro)-4-(ethylamino)-6-isopropylamino)-*S*-triazine), one of the most intensely used pesticides in the world, is part of the s-triazine chemical group. This herbicide inhibits photosynthesis by blockage of electron transport in the photosystem II (van Rensen, 1989). It is included in the EU priority substance list used for water chemical status definition due to its high mobility and persistence in the environment, causing adverse effects at low concentrations (Directive 2008/105/EC of the European Commission, 2008). The EU has established a Maximum Allowable Concentration of 2 µg/L of atrazine for inland surface waters. A lowest observed effect concentration (LOEC) of ≤ 5 µg/L has been reported for swimming behaviour of zebrafish (Steinberg et al., 1995). Saglio and Trijasse (1998) reported increased surfacing activity, decreased grouping behaviour, and decreased sheltering in response to an alarm signal in goldfish exposed to 5 µg/L for 24 hours. A 30-min exposure to 1 µg/L atrazine eliminated preference behaviour for a natural odorant in rainbow trout (Tierney et al., 2007a). In the same study, 10 µg/L atrazine significantly reduced l-histidine-evoked EOGs (electro-olfactograms).

Linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) is a phenylurea that acts upon photosynthesis in a similar way as atrazine (van Rensen, 1989). This herbicide in considered slightly, to moderately toxic to fish (US-EPA, 1995). The Canadian WQG for the protection of freshwater life against linuron has been set at 7 µg/L (Caux et al., 1998). Linuron is known to have anti-androgenic activity in rats (Lambright et al., 2000; Wilson et al., 2009) and is suspected to alter olfactory-mediated behaviours in fish. The structurally similar herbicide diuron was reported to alter olfactory-based behaviours in goldfish, such as the decrease of grouping behaviour in the presence of an alarm signal, after a 24-h exposure to 5 µg/L diuron (Saglio and Trijasse, 1998). Tierney et al. (2007b) detected reduction of l-serine-evoked EOGs in rainbow trout exposed to linuron at 10 µg/L for 15 minutes, suggesting that exposure to linuron has the potential to disturb predator avoidance and food location in salmonid fish.

Metolachlor (2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide) is a chloroacetanilide that promotes the inhibition of cell division in seedling shoots and roots (Takacs et al., 2002). It acts by inhibiting formation of very-long-chain fatty acids (VLCFAs, chains with more than 18 Cs) by the microsomal elongase system of the endoplasmic reticulum (Chesters et al., 1989). Metolachlor is listed by the U.S. RED as moderately toxic to freshwater fish. A 4-week exposure of fathead minnows revealed a NOEC for reproduction at 780 µg/L (Dionne, 1978), and due to lack of further chronic, sub-lethal studies, the CCME has established a Water Quality Guideline of 7.8 µg/L for the protection of aquatic life (1999a). This herbicide has been reported to affect the perception of chemical stimuli by the crayfish *Orconectes rusticus*, leading to inappropriate decisions in the exposed organisms regarding detection of food and response to an alarm signal (Wolf and Moore, 2002), as well as interfere with the ability of crayfish to respond to social signals involved in agonistic behaviours (Cook and Moore, 2008)

In the present study we investigated the effect of a low-dose mixture of three herbicides on the behaviour of juvenile trout (*Oncorhynchus mykiss*). Occupation of the water column, number of movements, and number of agonistic acts, observed at regular intervals throughout the experiment, as well as average growth rates, were compared between exposed and control organisms.

5.2. Material and methods

5.2.1. Pesticide mixture selection

Three herbicides, each belonging to 3 different chemical groups - s-triazines, acetanilides and phenylureas -, were selected to be assessed in the mixture toxicity tests. The selection was based on the pesticide concentrations measured by the AEAG throughout the Adour-Garonne river basin during the year 2007. Hierarchical clustering analysis was performed in R using complete linkage and Euclidean distances between the maximum concentrations of contaminants that were detected in more than 5% of the yearly samples. The contaminants were thus grouped according to their co-occurrence and concentration (Fig. 1). The selected herbicides were atrazine, linuron and metolachlor (CAS numbers 1912-24-9, 330-55-2, and 51218-45-2, respectively). Test concentrations of each compound were established according to the highest concentration found in year 2007, multiplied by 50 for atrazine and linuron and by 5 for metolachlor. The difference in multiplication factor was in order to avoid metolachlor dominating the mixture, and thus possibly determining fish response regardless of the presence of linuron and atrazine, both at otherwise one degree lower then metolachor. The selection of the final (nominal) concentrations of each chemical in the mixture was thus 10, 15 and 45 µg/L for atrazine, linuron and metolachlor, respectively.

5.2.2. Exposure setup

A flow-through system (Fig. 2) was constructed in a closed room with air at a constant temperature of 16ºC. Eight 40L (30x40x40 cm) glass aquariums received a continuous, controlled flow of aerated and refrigerated (LAUDA WK 4600) tap water. Aeration of water before distribution to test aquaria was installed to promote release of chlorine and to fully oxygenate, as no aeration was provided in the individual aquariums to avoid loss of contaminants. A multichannel peristaltic pump (Watson Marlow 205U), equipped with silicone tubes (63 µm internal diameter) delivered the mixture of pesticides to each mixing vessel at the desired rate. Water inflow, regulated by flow meters (Cole Parmer), was established according to the required test concentration and renewal rate (see details below). Mixing vessels -

containing a magnet and placed above a magnetic stirrer – received water and contaminants (during the exposure period), providing test water for two aquaria each. Passive outflow from aquaria was attained by constant overflow through an opening in the upper part of the rear of the aquarium, connected to a drain. When test substances were applied, the drain was connected to a self-assembled carbon filter device in order to remove contaminants prior to discharge into the sewerage system. Aquaria received a light:dark regime of 16:8 hours.

5.2.3. Pesticide mixture administration

Stock solutions of each pure compound (obtained from Sigma-Aldrich) were individually prepared in a carrier solvent (acetone; Carlo Erba). From each stock solution aliquots were taken to prepare concentrated aqueous mixture solutions containing all three pesticides. The aqueous mixture of pesticides was delivered to each mixing vessel at a rate of 0.15 ml/min. Mixing vessels received fresh water via the flow meters at a rate of 276 ml/min. Each mixing vessel provided water for two replicate aquaria at a rate of 7.80 L/hr, thus resulting in one complete water change of each aquarium every 5.13 hr. Control treatments equally received water from mixing vessels but without the addition of pesticides via the peristaltic pump. The amount of solvent in the final test water of spiked treatments did not exceed 0.1 ml/L (as recommended in OECD toxicity testing guidelines; e.g. OECD 215).

5.2.4. Fish acclimation and exposure

Juvenile rainbow trout (*Oncorhynchus mykiss*) aged 5 months old were obtained from a commercial fish farm in the Eastern Pyrenees in July 2009. The fish were acclimated to laboratory conditions in 160 L holding tanks for 7 days. They were then weighed and measured (Table 1) and randomly allocated to the 8 testing aquaria (8 fish per aquarium), already running on flow-through mode with non-spiked water. All weight measurements during this experiment were performed after a 12-hour fasting period. Care was taken to only include fish that did not differ more than 25 % in size between and within treatments. Fish were fed fish pellets (Neo start, Le Gouessant, France) twice daily with a total daily food-body weight ratio of

3%. This ratio resulted in optimal growth and best water parameters during a previous trial without contaminants and similar sized fish (unpublished data).

After 10 days all pre-selected fish were again weighed and measured, and the feeding rate recalculated to maintain the daily food intake at a food-body weight ratio of 3%. The fish were returned to their original aquarium to maintain groups identical to those of 10-day pre-exposure period. A resettling period of 24 hours was allowed before exposure was initiated. At the applied flow rates of water and spiking solutions, mixture concentrations in theory reached 70 % of nominal levels 24 hours after spiking was commenced (83 % after 48 hours). Exposure duration was of 5 days, at the end of which fish were measured and weighed.

Deposited faeces were removed daily by siphoning and aquaria walls were brushed on a regular basis. Any dead fish were removed as soon as they were detected. Dissolved oxygen, temperature, pH, conductivity, and nitrite were checked daily.

5.2.5. Analysis of pesticide concentrations

Using glass bottles, one sample of 1L test water was collected from each of two treated aquaria at 24 and 72 hours after start of pesticide exposure. The water samples were treated with analytical grade dichloromethane (10ml to 1L of sample; Carlo Erba) and stored at 4°C until chemical analysis.

Atrazine, linuron and metolachlor concentrations were measured in the stored water samples following a protocol developed at Ecolab campus ENSAT (F-31326 Castanet Tolosan, France; Devault et al., 2007). Liquid-liquid extraction was performed with 850 ml of non-filtered samples in a 3-step procedure: 70 ml dichloromethane (DCM; Pestipur, SDS-Carlo-Erba) in the 1st extraction step, and 60 ml DCM in both the 2nd and 3rd steps. After shaking, settling times were 30 min, 1hr and 1 hr, respectively, with decantation of the DCM phase after each settling time, collecting all 3 extracts in the same recipient. After extraction, water residues were removed from each sample using filters made of fibre glass and anhydrous sodium sulphate (SDS-Carlo-Erba). The samples were evaporated at 40°C, resuspended with hexane, placed in dark vials, and reduced to a known volume under a nitrogen

stream. Before analysis, an internal standard (Fenitrithion-D6, Ehrenstorffer provided by Clouzeau Info Labo F-33220 Ste Foy la Grande) was added to each sample extract at a ratio of 1:50 (internal standard:sample).

Final quantification of the tested pesticides was performed via GC/MS, Gas Chromatography (Thermo Scientific Trace GC; Phenomenex Column 5MS 30 m, 0.25 mm, 25µm) coupled to a mass spectrometer (Thermo Scientific, DSQII) operated in selected ion monitoring (SIM) mode. An aliquot of 1 µl of each sample extract was injected (splitless mode) at 280°C. The carrier gas was helium Air Liquid (Alphagaz2). Chromatographic conditions in the splitless mode (injector temperature: 280°C) were set up at an initial temperature of 45°C. The first step had a temperature increase rate of 35°C/min up to 180°C, then a second step at 8°C/min up to 280 °C and a final 10-minute plateau at 280°C. The detection conditions were: temperature, 300 °C; E.M.V., 2600 V.

The detection limit established was 0.001 µg/g. Recovery after sample preparation, extraction and purification obtained for each pesticide varied from 82.4 to 104.6 %, leading to a mean recovery of 95.4 ± 6.5%, with an acceptable repeatability of < 14 %. The efficiency of this method is confirmed by the test on organo-chlorine derivatives which gave a mean recovery yield of 98.5% in accordance to methods used in other studies.

5.2.6. Growth analysis

Differences in length, weight, and growth rates between exposed and non-exposed control fish before and after spiking started, were assessed via analysis of variance (ANOVA). For the same time-points, the effect of treatment on the difference between the heaviest fish of each aquarium (supposed as being the most dominant fish; Metcalfe, 1986; Grant et al., 1989) and all other fish in the aquarium, and between the heaviest and the lightest (supposed as being the most subordinate), was tested via ANOVA. The control-exposed divergence percentage of heaviest-lightest difference at the end of the experiment was also calculated.

5.2.7. Behaviour data collection and statistical analysis

Two horizontal black lines were drawn on the visible side of each aquarium such that the water column was divided into three equal parts. Five-minute video recordings of each aquarium were performed twice-daily - one between 10 and 12 a.m. and one between 3 and 5 p.m. - throughout the test period (i.e. from the start of test water spiking). The camera was positioned behind a dark, opaque curtain with openings for aquarium observation. Care was taken not to disturb the fish at least 2 hours prior to video recordings.

Videos were observed on a PC using standard media viewing software. Starting at 0 seconds, the number of fish in each section of the aquarium (top, middle, and bottom; presence data) was recorded at 30-sec intervals, throughout the 5-min video recording. The total number of agonistic behaviours (fish A approaching fish B, resulting in the escape of fish B; aggression data) were recorded during the whole video recording. All observations were performed by the same, singular person without knowledge of the treatment of each aquarium.

Using the presence data, the total numbers of movements between top and middle, and middle and bottom compartments of the aquaria were calculated for each aquarium/day/observation event and divided by the total number of fish present. This statistic was named M_F (movements per fish). The same data was used to estimate the proportion of fish per aquarium compartment per day/aquarium/observation event. A weighted water column height per number of fish was also calculated using presence data per aquaria section, referred to as *Hgt* (cm). Water depth here is the inverse of water column height, which was measured from the bottom up. The total number of aggressions per aquarium/day/observation was divided by the number of fish present and hereafter referred to as *Agr* (aggressions per fish). Pearsons' correlation tests were performed between pairs of variables M_F, *Hgt*, and *Agr*, for controls and exposed treatments separately.

To test the effects of the pesticide mixture on behavioural parameters, Generalized Linear Mixed Models (GLMMs) were used. Treatment (exposed and control) and observation hour (time since start of observations on day 1 of exposure; continuous) were set as fixed factors. Observation hour was scaled (transformed

values are centered around zero and have a unit variance) and aquarium was a random factor. GLMM with gaussian error distributions (model fit by residual maximum likelihood approximation) were used to evaluate all behavioural parameters. Statistical significance of model outputs were given by calculating the 95% confidence intervals (1000 runs of Markov chain Monte Carlo generations) and checking for overlap of the interval with zero that indicates a non-significant effect.

All statistical analyses were performed in R 2.11.1 (R Development Core Team, 2010), using the lme4 package for GLMM runs (Bates and Maechler, 2010), and level of significance set at $p<0.05$.

5.3. Results

The average test water temperature did not deviate more than 0.7 °C in each individual aquarium throughout the whole experiment, ranging from 11.9 to 13.4 °C over all aquaria. Dissolved oxygen (84-95 %), pH (7.70-8.05), conductivity (252-275 $\mu S/cm^2$) and nitrite concentrations (< 0.01 mg NO_2^-/L) maintained within recommended limits. Average measured concentrations of atrazine, linuron and metolachlor were, respectively, 1.9, 1.9 and 3.3 µg/L at the first sampling time (24 hours from start of exposure), and 4.0, 4.8 and 13.1 µg/L at the second sampling time (72 hours from start of exposure). The overall average nominal/maximum measured ratio was of 36 %.

Out of the 64 fish tested (8 per aquarium), 1 fish died in 3 of the aquaria during the acclimation period, and none died during the exposure period. Mortality was thus negligible and not related to a specific treatment. Prior to exposure, fish from all test aquaria grew in average 30% of their initial body weight, at a rate of ca. 4.3 % per day (Table 1). Feeding behaviour maintained apparently constant and after both the 10-day acclimation and 5-day exposure no significant differences of length, weight, and growth rates between exposed and control fish were detected. No inter-treatment significant differences were detected between the heaviest fish and the weight of all other fish, nor between the heaviest and the lightest fish. The heaviest-lightest weight difference resulted in an average 10.4 % divergence between exposed (45.5 % difference) and control (55.9 % difference) fish.

During the exposure period, no behavioural perturbations visible to the naked-eye were observed in the treated aquaria. In general, a hierarchy was established within each group per aquarium, with one visibly dominant fish. The difference between the weight of the heaviest fish in each aquarium (supposed the most dominant fish) and that of the lightest (supposed the most subordinate fish) did not reveal significant differences between treatments

Pearson's correlation tests detected significant relationships between *Agr* and M_F in controls, and *Agr* and *Hgt* in exposed fish (Table 2). An increase in aggressiveness co-occurred with an increase in the mobility of control fish (Fig. 3). In exposed fish, an increase in aggressiveness co-occurred with a decrease in the average water column height that the fish explored (Fig. 3). A positive correlation between M_F and *Hgt* was found to be almost statistically significant (p-value = 0.0634) among control fish.

Results of GLMMs revealed a significant effect of exposure to the pesticide mixture on M_F and *Hgt*, but not on *Agr* (Table 3). The average number of movements per exposed fish was significantly smaller than that of control fish (Fig. 4a). Exposed fish explored preferably the bottom compartment of the aquaria, whilst control fish were generally in the upper sections of the water column (Fig. 4b). The average number of agonistic acts was, although lower in exposed aquaria, not significantly different from control conditions (Fig. 4c).

5.4. Discussion

In this study we sought to identify whether herbicides affect the behaviour of fish by exposing juvenile rainbow trout to a low-dose mixture of three co-occurring substances during a 5-day test. Our hypothesis was that fish swimming activity, the use of the water column, and interactions between individuals would be modified due to exposure to the mixture. We studied these behaviours by observing fish twice-daily throughout the exposure period, registering the vertical distribution of fish in the water column and the number of agonistic acts between all individuals. This allowed for quantification of the number of movements per aquarium, the average height at which fish swam, and the number of aggressions per total number

of fish, and thus comparison of these parameters between exposed and non-exposed (control) organisms. Fish growth was also monitored in order to check for physiological changes at a broad scale, which could be the result of behavioural disruptions.

5.4.1. Pesticide exposure concentrations

The low measured pesticide concentrations, in comparison to those intended, may have been due to: biological degradation; adsorption of compounds on parts of the flow-through system (silicone tubing, glass mixing vessel and aquarium walls, etc) depite care to use material with lower adsorptive tendency; slow increase of concentrations in the aquaria (intended levels would be reached at a later point). However, the measured concentrations in samples taken towards the end of the exposure time were close to maximum levels allowed by European or Canadian regulation in surface waters (regulated limit/maximum measured for atrazine, linuron and metolachlor, respectively: 2/4.0, 7/4.8 and 7.8/13.1 µg/L. The concentrations that the fish were ultimately exposed to were thus within ecological relevant levels.

5.4.2. Fish mobility and space occupation

Fish exposed to the mixture spent more time in the lower parts of the aquaria and moved less in general, whilst control fish tended to explore at higher levels (lower depth) in the aquaria with increased mobility (number of movements per fish), reflecting better swimming activity. Eissa et al. (2006b) reported changes in spatial distribution of juvenile carps exposed to Cd^{2+}, namely reflected in alteration in the preferred swimming depth. In our study, it could be hypothesised that a greater number of fish movements was the result of a greater number of agonistic interactions (causing increased fleeing of attacked fish). However, if fish are performing more movements the probability of encountering another fish increases, and with it the probability of there occurring an agonistic act.

Inhibition of fish behaviour in exposed groups was detected via the decrease of average aquarium height explored with increase of agonistic acts, whilst in control

groups the number of agonistic acts did not correlate strongly with swimming height. Although more agonistic acts were occurring at lower levels of the aquarium in exposed groups, the number of movements per fish was not significantly correlated to swimming height in those groups. This ambiguousness in exposed fish could be the result of an effect of the pesticide mixture on the perception and integration of external stimuli such as the approach and aggressiveness of a conspecific. The disruption of external stimuli perception has previously been reported in previous studies exposing fish to contaminants (e.g., Fuiman and Magurran, 1994; Tierney et al., 2007a). Nevertheless, as a greater number of exposed fish tended to occupy lower levels of the water column (i.e. greater concentration of individuals), there was also an increased probability of encounters and thus increased frequency of agonistic acts. Registering in which compartment – and at what average height - occurred each agonistic act, thus knowing how many fish were in the respective compartment for a certain aggressivity rate, could help clarify the aggression-height linkage.

Hypoactivity may increase vulnerability to predation indirectly through reduced feeding and thus reduction of energy levels available for escape, or directly via reduction of active escape response (Laurence, 1972; Steele, 1983; Kramer, 1987). A certain degree of hypoactivity was detected in exposed fish, as those aquaria presented a significantly smaller number of movements per fish. Hypoactivity of contaminant exposed fish has been reported in other studies, such as for goldfish exposed to parathion (Rand, 1977c), rainbow trout exposed to carbaryl (Little et al., 1990), and mummichog exposed to lead (Weis and Weis, 1998). In an effort to classify chemicals according to general mode of action, Drummond and Russom (1990) used behavioural and morphological signs of stress in fathead minnow subjected to acute exposure, to then group chemicals into classes of toxic effects. The triazine group - for which they tested the triazine derivative 3-amino-5,6-dimethyl-1,2,4-triazine during 96hr - was classified within the hypoactivity syndrome class. This response syndrome was characterized by more extreme behaviours than those observed and quantified in our study, probably due to the much higher concentrations used in their toxicity tests (concentrations not mentioned in their study). Although we tested a mixture of pesticides, in contrast to

Drummond and Russom's single-substance tests, there is evidence of a similarity in the behavioural trends in both studies.

5.4.3. Fish growth and aggression

Fish species that form dominance hierarchies do so in order to fend off intruders that would otherwise compete for essentials such as food, shelter, and potential reproductive partners (Chapman, 1966). A socially dominant individual thus insures the necessary conditions for optimal growth (Metcalfe, 1986; Grant et al., 1989) and progeny. Previous trials in our laboratory, using the same experimental setup but without the addition of contaminants to the test water (unpublished data), showed that after 2 to 3 weeks in enclosures with 6 juvenile rainbow trout, dominant individuals were clearly bigger than subordinate fish and less prone to die from disease, most probably stress and starve-induced effects in subordinate fish. DiBattista et al. (2006) reported that the negative effect of social subordination on growth in rainbow trout was due to lack of feeding. Experiments with Nile tilapia revealed that the introduction of a dominant fish completely inhibited, via aggressive attacks, the feeding behaviour of subordinates, leading to a partial inhibition after a certain period of time (Vera Cruz and Brown, 2007). These authors thus also attributed alteration in growth rate between dominants and subordinates to changes in their feeding behaviour.

Within the test time of our experiment (acclimation + exposure), no significant differences in growth between exposed and control fish were detected, when considering all fish from each treatment, and also when comparing the weight of the heaviest fish (supposedly dominant) per aquarium and all other/the lightest fish (supposedly the most subordinate). At the end of the experiment, the weight difference between the heaviest fish in each aquarium and the lightest resulted in a 10.4 % average divergence between exposed and non-exposed fish, with exposed fish presenting smaller differences. However, this percentage is not considerable in face of the biological variability inherent to fish experiments (Woltering, 1984; Kolok et al., 1998). The presence of the pesticide mixture did not induce significant differences in the number of agonistic acts per fish in comparison to controls, which

could explain the absence of an important growth divergence between heaviest and lightest fish. However, the 10.4 % divergence could become greater if exposure were to have been for a longer period of time. A significant reduction of the dominant-subordinate size difference in exposed fish could in that case be interpreted as a disruption in the processes underlying the establishment of hierarchies, as dominants and subordinates may be growing at more similar rates due to hypoactivity of dominant fish, as a result of a weaker suppression of subordinates and allowing them to invest more in feeding. An indication that this could have occurred if allowing for a longer exposure period was the reduced number of movements per fish in treated aquaria in comparison to non-treated (only significantly correlated to aggressiveness in control individuals), suggesting a tendency to hypoactivity and eventually reduced feeding activity.

Apart from short exposure time, another reason for the non detection of significant changes in growth, and in dominant-subordinate size differences, could be individual variation in standard metabolic rate (SMR). Cutts et al. (1998) reported that individual differences in SMR of juvenile Atlantic salmon contributed to differences in aggression between fish. When grouping individuals of similar SMR, the authors found that aggression levels were highest within groups of high SMR, and no significant differences in mean growth were found between groups. Most importantly, the low SMR group presented less intra-group growth variability, suggested as a consequence of an even access to food among all individuals. In our study, such individual variability may have masked effects on growth that could otherwise be related to contaminant exposure.

Although the juvenile trout used in our experiment were from the same strain (breeding batch), there is evidence from other studies of within-strain variation regarding certain behaviours (Magurran, 1993; Pitcher and Parrish, 1993). Fish can present behavioural syndromes – suites of correlated behaviours across situations – that vary from individual to individual within the same population/strain (Huntignford, 1976; Sih et al., 2004). Behavioural syndromes have been suggested to explain non-adaptive behaviours in certain contexts as well as the maintenance of individual variation in behavioural types (Sih et al., 2004). Moretz et al. (2007) found that boldness and aggression in zebrafish were related but strongly depended on fish

strain. Studying each fish individually as well as comparing behaviour before and after exposure could help reduce variability via accounting for individual and between-group/treatment variance throughout the experiment.

5.4.4. Concluding remarks

Studies on the effect of pesticide mixtures are currently lacking, especially regarding the impact of environmentally realistic low doses on ecologically important and highly sensitive biological endpoints. Much work is still to be done to further support the inclusion of behavioural endpoints in the evaluation process of sublethal contaminant toxicity in organisms. Laboratory studies as the one presented here can contribute to the definition of the suitability of different behaviours for use in routine risk and hazard assessment. The behavioural endpoints chosen here were easily observed, and within a short timeframe, simple to quantify, and of biological significance and ecological relevance, overcoming some of the behavioural toxicology challenges outlined by Little et al. (1993a). High within-group variability remains a problem difficult to address without a considerable expansion of test facilities - in order to increase replicate numbers and include additional test conditions - and thus the imminent logistic and financial consequences.

Despite such constraints, the analysis of behavioural responses to contaminant exposure can provide insight into various levels of biological organization, allowing for quantification of neural and mechanical disruption as a result of biochemical and physiological changes (Brewer et al., 2001; Scott and Sloman, 2004). Being several times more sensitive than high-dosed toxicity tests that provide information on lethality thresholds and other acute effect derived indices, behavioural endpoints can help determine adequate no and lowest observed effect levels, important for water quality standard definition.

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5.6. Tables

Table 1 - Weight and fork length of fish at start and end of acclimation period and at end of test period, and fish growth in percentage for each period.

	Weight (g)		Fork length (mm)	
	control	***exposed***	***control***	***exposed***
Start of acclimation	3.234 (0.554)	3.214 (0.628)	59.600 (3.688)	59.445 (4.407)
End of acclimation	4.188 (0.838)	4.116 (0.937)	66.335 (4.969)	66.061 (5.022)
End of test	4.939 (1.177)	4.860 (1.147)	70.299 (6.360)	69.882 (5.773)
Growth (% / day)				
acclimation	2.28	2.19	1.02	1.00
test	3.04	3.06	1.13	1.09

Table 2 - Pearson's correlation coefficients between M_F (movements per fish), *Hgt* (weighted average water column height), and *Agr* (aggressions per fish), for exposed and control fish (above and below the diagonal, respectively). Significant correlations (p-value < 0.05) are marked with an asterisk.

	M_F	*Hgt*	*Agr*
M_F	-	0.1962	-0.188
Hgt	0.3432	-	-0.392 *
Agr	0.4219 *	-0.063	-

Table 3 - Results of Generalized Linear Mixed Models performed on behaviour parameters measured during treatment period. In bold are significant effects (p-value < 0.05). See text for details on model construction. M_F, movements per fish; Hgt, weighted average water column height; Agr, aggressions per fish. CI, confidence interval.

		95% CI	
	t-value	lower	upper
M_F			
Treatment	-2.2700	**-1.3066**	**-0.1910**
Observation hour	1.3250	-0.0597	0.3229
Hgt			
Treatment	-1.2170	**-7.9552**	**-1.6319**
Observation hour	-1.8140	-0.9477	0.2973
Agr			
Treatment	-0.7180	-2.4859	1.1695
Observation hour	2.2760	-0.0009	0.8581

5.7. Figures

Figure 1 – Dendogram of hierarchical clustering analysis (performed using complete linkage and Euclidean distances) of pesticide concentrations occurring in the Adour-Garonne river basin in 2007. Grey boxes indicate pesticides selected for the mixture toxicity test. AMPA, aminomethyl phosphonic acid; 2,4-D, 2,4-Dichlorophenoxyacetic acid; 2,4-MCPA, 2-methyl-4-chlorophenoxyacetic acid; γ-HCH, γ-Hexachlorocyclohexane .

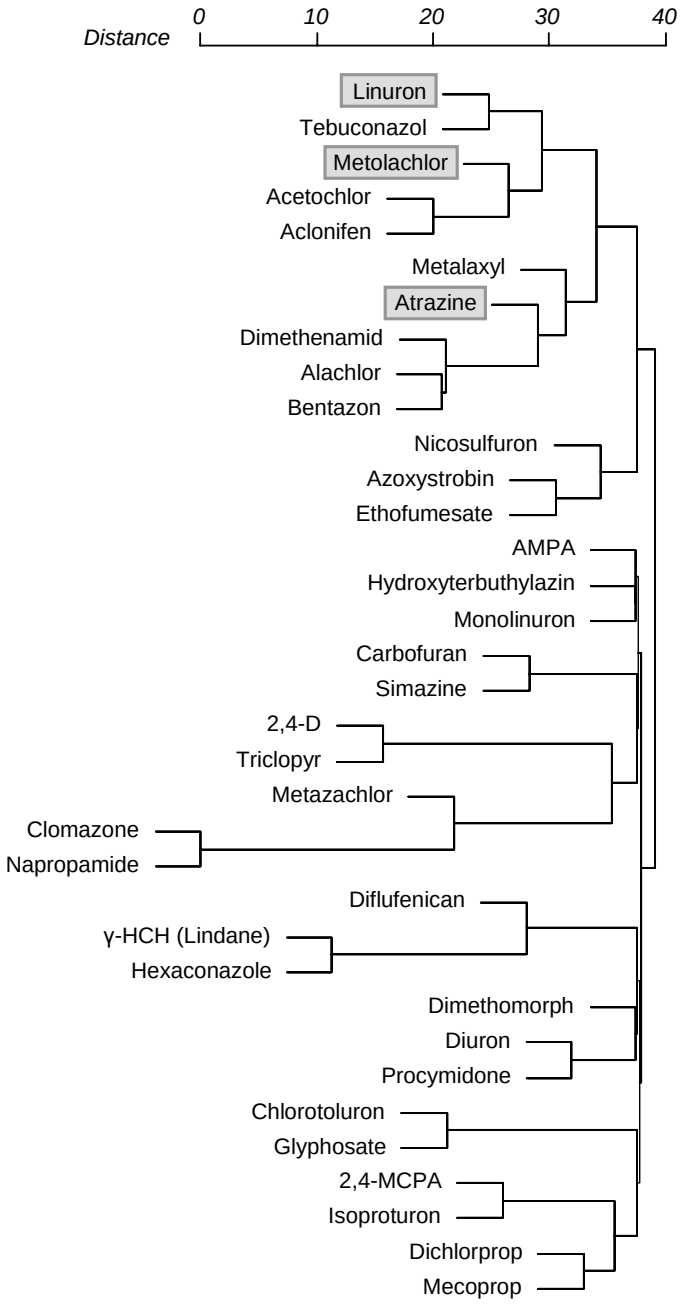


Figure 2 - Schematic diagram of flow-through system built for exposure testing with continuous renewal of test water.

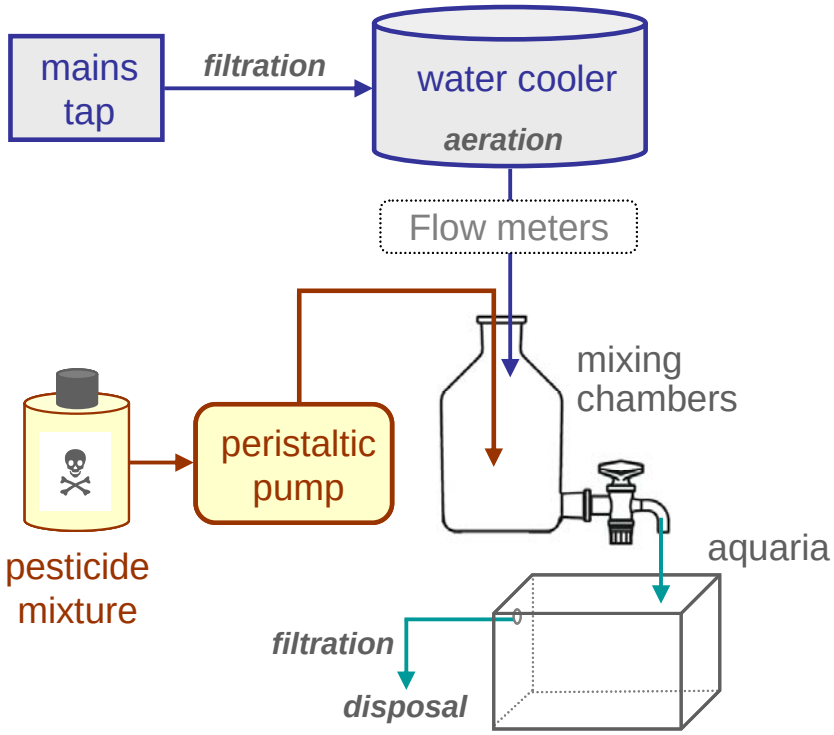


Figure 3 - Linear regression plot between average water column height (*Hgt*) and aggressivity (*Agr*), left, and movements per fish (T_M) and aggressivity, right. Grey lines and dots correspond to controls, and black lines and squares correspond to exposed individuals.

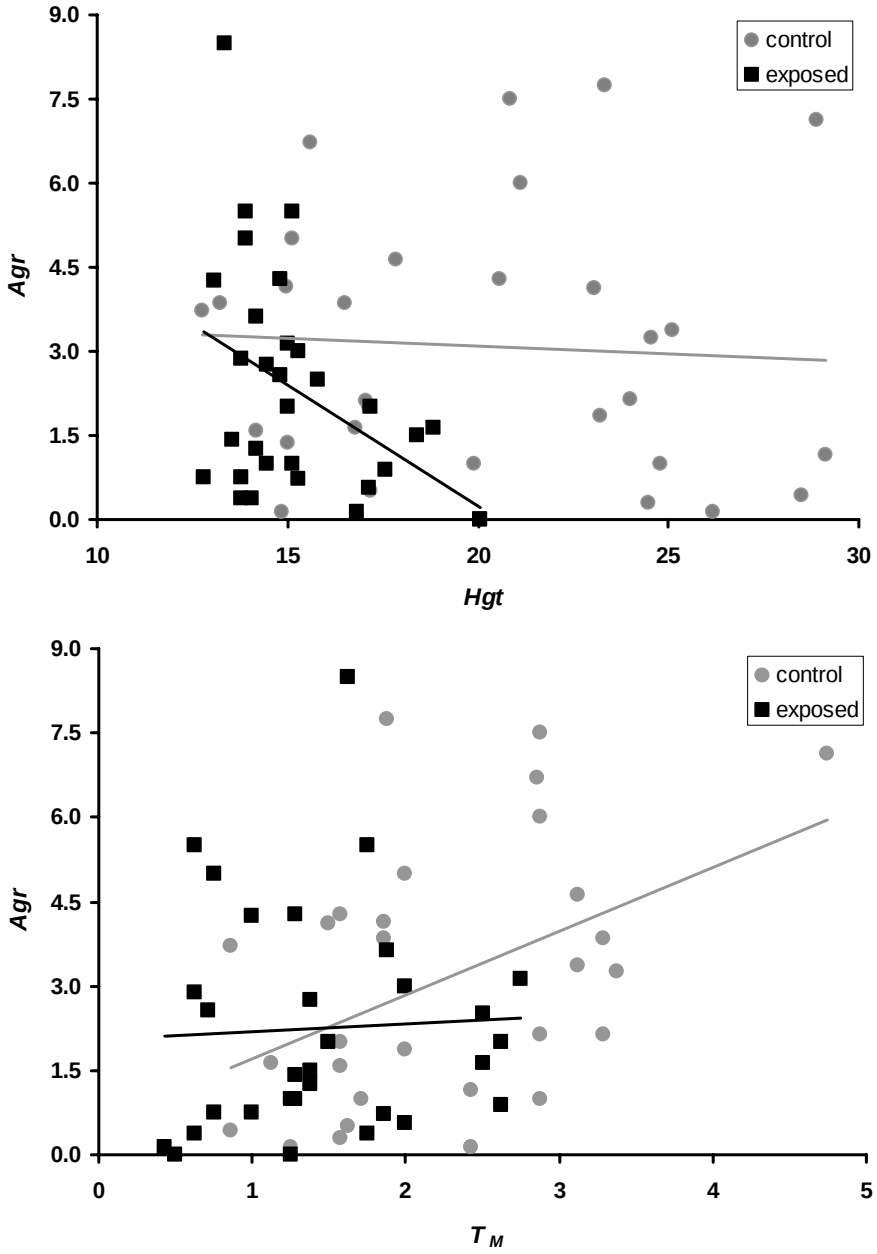
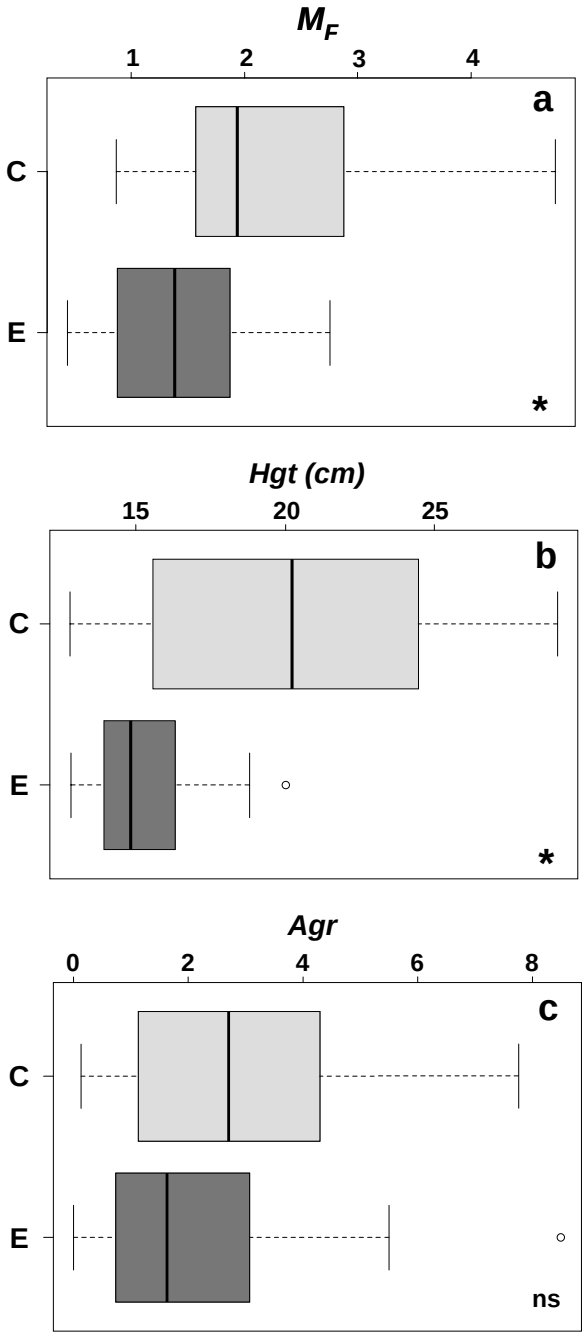


Figure 4 - Box-plots of quantitative behavioural observations of fish not exposed (C, control) and exposed (E) to the pesticide mixture: a, movements per fish (M_F); b, weighted average water column height (*Hgt*); c, aggressions per fish (*Agr*). Treatment significance as in Table 3 is indicated in the bottom-right corner of each plot (*, $p < 0.05$; ns, non significant).



6.

Conclusion

Freshwater rivers and streams are among the most imperilled ecosystems in Europe and across the world. Diminished water quality can have profound consequences for species in these ecosystems, both directly and through their linkages with other species and ecosystem components. Fish, being near the top of the food chain, are linked with many other species. This suggests both that they may be particularly sensitive to diminished water quality and that adverse effects on them may have consequences for many other species and the ecosystem as a whole. The main objective of this dissertation was to relate changes observed in freshwater fish to water contamination. A short summary of the results from each study is reported in the following sections.

6.1. Fish and environment of the River Lot are less burdened with heavy metals

In the first chapter of this dissertation, I show that accumulation of heavy metals (copper, zinc, cadmium and lead) in liver and muscle of three fish species decreased between 1987 and 2007, reflecting a general decrease that occurred in the environment. The results demonstrate that remediation efforts in the River Lot river basin have been at least partially successful. However, according to US-EPA criterion to protect freshwater wildlife, the River Lot aquatic community is still under threat. Sediment levels of zinc and cadmium were higher than the concentrations at which adverse effects on the benthic community are expected to occur. This highlights that heavy metal contamination in the River Lot may be affecting the aquatic ecosystem at different trophic levels. Further remediation programmes are currently taking place, aiming at reducing heavy metal loads, especially cadmium, to the limits established by the EU WFD for the year 2015.

6.2. Morphometric differences in wild gudgeon correlate with pesticide levels

The second chapter of this dissertation demonstrates that fish morphology can be significantly affected by diminished water quality, here being an increase in the concentration of toxic pesticides. While controlling for potential confounding

factors (genetic differentiation between populations, riparian distances between sites, site catchment area, various physical-chemical parameters, and water velocity), I show that 4 of the 17 morphological traits measured in captured gudgeons were significantly correlated with the level of pesticide toxicity, suggesting a response of these traits to environmental stress. For example, fish eye-diameter decreased with increasing toxicity levels, while body height increased. These results underline the importance of controlling for different sources of phenotypic variability among organisms when studying the impact of stress factors on morphology. Indeed, not accounting for confounding factors can bias ecological interpretation of data and lead to erroneous conclusions. While I attempted to directly verify a causal relationship between pesticide stress and morphological changes, the principal utility of this study concerns the development of tools that can be easily implemented in environmental assessment programs. The approach proposed here confers robustness to the final conclusions by accounting for a range of potentially interfering factors and demonstrates that adequate statistical approaches can greatly simplify the integration of multiple factors.

6.3. Environmental pesticides affect body condition, gonad weight and liver cells of feral chub

Next, I demonstrate that increasing toxic pressure of agrochemicals in the studied river sites is related to a decrease in chub condition factor (CF), an increase in gonado-somatic index (GSI), signs of increased immunological stress in hepatic cells, and higher concentrations of pesticides in fish muscle. No significant causal relationship was observed between GSI and CF. Signs of hepatic cell stress in fish from more polluted sites may be related to their lower CF. Although I show correlative links between environmental stressors and organism responses, direct causal relationships are still difficult to establish. Other unmeasured factors could always be playing a role. Nevertheless, the fact that a relatively tolerant species such as the European chub shows a strong response to the environmental contamination considered suggests that other more sensitive co-occurring species may be suffering to a larger extent

6.4. Juvenile rainbow trout undergo behavioural changes when exposed to a pesticide mixture

Lastly, I show that fish exposed to a mixture of pesticides were hypoactive and spent more time in the lower parts of the aquaria in comparison to non-exposed controls, reflecting inhibited swimming activity. Levels of aggression were comparable between the two treatments. Average swimming height of exposed fish decreased with the number of agonistic acts, whilst in control groups there was no significant relationship between the two behaviours. Growth did not differ significantly between exposed and control fish within the test time. In natural conditions, hypoactivity could result in increased predation risk, as well as susceptibility to malnutrition through reduced foraging activity.

6.5. Implications of ecotoxicological findings

This dissertation shows that fish are appropriate sentinels of water quality in aquatic systems, integrating toxic pressures that occur over different time scales and over a range of contaminant levels. Historical pollution of river systems, as in the case of the heavy metal contaminated River Lot, can be regularly monitored by screening environmental samples. However, it is only by assessing accumulation in biota that the extent of exposure can be evaluated, and the link to environmental contamination established. Surveillance monitoring based on both environmental and biological samples is thus essential for the complete and integrated assessment of ecosystem status.

Investigative monitoring – such as was performed here regarding wild gudgeon and chub populations exposed to agrochemicals – is an important step in evaluating the suitability of fish biological parameters as biomarkers of water quality. Significant differences detected in this study between biological parameters of fish exposed to distinct levels of pesticides demonstrate their suitability in the assessment of the health and toxicological status of fish. Such relationships provide valuable information for bridging the gap between chemical and biological monitoring. However, the interpretation of the signals reflected in the observed fish fauna is often a complex issue and a one-to-one relationship between a pressure or

impact and a fish metric will seldom exist. Furthermore, natural environments are characterized by many co-varying factors, which can potentially confound assessments of causal relationships. Thus the separate contributions of anthropogenic and natural variation, as well as other co-varying factors, are not readily disentangled and may remain unclear. An appropriate selection of sampling sites, particularly with regard to reference conditions, and knowledge of potential confounding factors in the study system can both help to improve analyses of causation.

Indirect community or ecosystem-level impact assessment can be estimated via calculation of toxicity indices of the pollutants found in the environment and via hypothesis drawn from observations on general fish condition. A valuable addition to such assessments would be direct community-level observations regarding model organisms of several different trophic levels and different organism groups, as well as ecosystem-level information concerning interactions between different species and between species and physical habitat quality. Data of this sort, although indeed challenging to obtain given the requirements of extensive sampling, would nevertheless increase the ecological relevancy (as presented in figure 5 of Chapter 1) of the ecological assessment. On the other hand, information on effects at the cellular/molecular level and biomarkers of exposure increase sensitivity and contribute to the early-warning aspect of ecological surveys. Indeed, in order to prevent extensive damage of contamination to aquatic ecosystems, the development of early-warning tools is essential. Arrays screening protein-level physiological responses of organisms and observation of behavioural changes in (artificially) exposed organisms have considerable potential in this area. The challenge – as indeed with most biomarkers – is to then relate changes observed at the individual level to consequences at the population level.

6.6. Link to the protection of European waters

Much research of the sort that is presented here is needed to improve interpretation of bioindicator/biomarker-based data. The combination of chemical and biological endpoints of various sorts provides weight of evidence that greatly

improves site-specific environmental risk assessment. The current WFD uses on one hand data on water chemistry and contaminant loads, and on the other general fish fauna parameters, a two dimensional approach to a multivariate problem. However, there is a wide range of biological parameters at different levels that have the potential to improve assessment by increasing the multivariate nature of the information used. The cause-effect linkages shown in the present thesis using for the most part individual-orientated variables (as well as a range of other environmental parameters), indicate that there is indeed a considerable amount of biological information that is being overlooked. Although a fish population may be found to be at a sustainable size with individuals of all age-groups to insure perseverance, a chronic exposure to a range of stressors (including chemical) may well impact individual fish at finer levels (health, behaviour, genetic variability), changes that in the long term could engender larger-scale effects.

Selecting appropriate biological markers to be used as tools within the scope of the WFD depends on the type of information required and the costs entailed. The number of variables assessed must thus be chosen in accordance with the complexity of the ecosystem being evaluated, within realistic and sustainable financial and time budgets - the early-warning signal can originate not only from the markers used *per se*, but also from the effectiveness of the evaluation process as a whole.

In order to include fish biological traits at the individual and population level (such as those studied here) within ecological status assessments required by the current WFD, an across-river catchment and across-country comparison and standardization is necessary. Such procedures have been performed in the past decade, for example in the scope of the FAME project (Fish-based Assessment Method for the Ecological Status of European Rivers). Instead of focussing on the more classical Index of Biotic Integrity (IBI) for fish populations using occurrence and abundance data (e.g. Oberdoff et al., 2002), scientists within the FAME project used 14 to 35 metrics (depending on the country), classified within the 3 major categories of the WFD: species composition (including metrics related to trophic composition, reproduction and condition), fish abundance and age-length structure (Kestemont & Gouffaux 2002). The chosen metrics were included in the assessment due to

ecological and statistical (ability to categorise and score the range of values observed against a valid reference condition) reasons, as well as based on understanding of the limitations of the sampling procedure used to assess the metric. Some metrics initially proposed were not included in FAME final assessments, namely those describing the health and condition status of fish, such as proportion of fish with disease, tumours, fin damages and other anomalies, and proportion of hybrid individuals.

However, as was shown here, there are indeed individual-based traits that present a linkage/response to environmental degradation due to the presence of contaminants. Further steps would be required in order to include such data within the WFD, remaining valuable in contributing with indications of the potential of fairly classical bioindicators. Studying all the different biological traits presented here – and more – for the same fish species and comparing between different species would add robustness to the assessment. Although while moving up the organizational ladder (from cellular to individual, etc) and including many other co-varying factors (hydromorphology, climate, habitat, etc), the specificity of a response towards a certain stressor decreases, the responses become more integrative.

To conclude, the combination of parameters at different scales allows for improved assessment of the ecological status and the driving factors involved. Holistic, multi-metric approaches to ecological risk assessment are no doubt preferable for more meaningful characterization of water body health (Hagger et al., 2008). Increasing certainty of risk assessments via including more complete and significant biomarkers/ bioindicators supports confident risk management

6.7. References

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Acknowledgements

The present study was performed within the KEYBIOEFFECTS project (MRTN-CT-2006-035695) of the 6th Framework Program funded by the European Union. The author would like to thank:

- The Agence de l'Eau Adour-Garonne (AEAG) for providing the environmental monitoring data and the Office National de l'eau et des Milieux Aquatiques (ONEMA) for assistance at certain occasions of the field sampling campaigns;
- Colleagues and friends at Évolution et Diversité Biologique laboratory for their support and help throughout the 3 years of research;
- The fantastic EDB accountants Sandra Méric and Peggy Leroy, and the PhD school secretaries Dominique Galy and Dominique Pantalacci;
- The EDB fish team Läetitia Buisson, Géraldine Loot, Simon Blanchet, Sebastien Brosse, and especially Clément Tisseuil, Loïc Tudesque, and Roselyne Etienne;
- My supervisors Gaël Grenouillet and Sovan Lek;
- Other researchers and professionals that were a great help and inspiration throughout the project: Eric Pinelli, Georges Merlina, Francis Dauba, Caroline Casademont-Gomiero, Antoine d'Oliveira, Emilie Wronka, Mirco Weil, Richard Gallampois, Dominique Guery, people at the "Atelier Générale de Mécanique" (UPS), Jean-Baptiste Ferdy, Helmut Segner, Michael Wenger, Heike Schmidt, John Sumpter and his team, Frédérique Candaudap, and Carole Boucayrand;
- The undergraduate students that provided hands-on assistance in different parts of the project: Mathieu Gondolo, Marine El Adouzi, Simon Scodavolpe, Marina Pi Sánchez, Carole Gleitz, Théo Robert, and Sarah Fourste;
- All the Keybioeffects team, especially Muriel Gevrey, Guillaume Guénard and Leslie Faggiano, and of course the project director and Helena Guasch and manager Anita Geiszinger;
- Friends in the beautiful city of Toulouse and beyond: ADLM scuba-diving group, Florent Bled, Olivier Navaud, Miroslav, Rui Narciso, Jean Luis, Martine and

swimming colleagues, Charles Boury, the Aachen bunch, Elyes Lamine and co., and the Veyssière family;

- And finally those special people whose presence, from near and afar, makes life all the better: Wafa Bouzid, Charlotte Veyssière, Luba Kovalova, Ana Margarida, Raquel Antunes, Iris Vilares, my family, and Kyle Dexter.

Figures on chapter front pages

Chapter 1 – Left, the Trec stream flowing amongst corn fields. The water is very turbid and the banks of the stream have been cut back in recent years. Nevertheless, fish were still abundant; top right, juvenile rainbow trout in a quarantine tank before being selected for the pesticide mixture exposure; bottom right, map of the Adour-Garonne river basin with main tributaries in blue, water agency survey sampling points as black, small dots, and pre-selected sites for field sampling of fish as coloured circles (red are bad quality sites, orange medium, and blue good quality sites).

Chapter 2 – Left, setting gill nets in the River Lot to capture fish for heavy metal analysis; Bottom right, dissection of captured fish and sampling of tissues; Top right, drying phase of acid digestion of tissue extracts.

Chapter 3 – Bottom left, Google maps satellite view of area around Trec stream as an example of the dense patchwork of agricultural fields; bottom right, electrofishing in the Gupie stream; top, treating gudgeon photographs in Visilog® by placing landmarks on chosen points and thus obtaining x-y coordinates for morphometric measurements.

Chapter 4 – Top, ONEMA field team electrofishing in the River Garonne at Muret (MUR sampling station); bottom right, chub specimens from a good quality site (above) and a bad quality site (below), the latter presenting external lesions (neat the tail and anal fin in this case), not observed in fish from good quality sites; bottom middle, amplified chub scale for age identification by counting the winter annuli, generally detected by a change in the shading and shape of the groups of lines; bottom left, parafinized liver samples of feral chub, ready to be embedded prior to cutting and staining for histopathological observation.

Chapter 5 – Top right, empty test aquaria with mixing vessels placed above, ready to be used for a test; bottom right, juvenile rainbow trout during acclimation period, with the over-flow collection system visible at the rear (dark tubing); right, filming of fish behaviour behind an opaque black curtain.

Chapter 5 – The River Garonne at Pont Neuf, viewed from the left bank, Toulouse.

Impact of toxicants on stream fish biological traits

Abstract

In order to guarantee a basis for adequate water quality for humans and the natural environment, the European Union has requested that all member states attain at least good ecological and chemical water quality in all surface waters by 2015, in what is know as the Water Framework Directive. Biologically-based tools are thus needed to adequately assess the ecological status of water bodies. Such tools will aid water managers in assessing, reporting, and eventually protecting such an important resource. The main objective of this thesis work was to evaluate the status of native fish populations in toxicant-impacted rivers in South-West France using a number of fish biological traits.

The historically polluted environment of the River Lot has been strongly impacted by heavy metals during and after mining activities took place in certain parts of the river basin. The concentration of copper, zinc, cadmium and lead were quantified in muscle and liver of 3 fish species, as well as in environmental samples (water, sediment, moss) in 1987 and 2007. The situation of the River Lot has improved over the last 2 decades although there is still margin for amelioration according to criterion for the protection of freshwater aquatic life. The average concentrations of cadmium in fish muscle in 2007 were above the maximum safe for human consumption defined by the European Commission.

More than half of the Adour-Garonne river catchment area is covered by agricultural land. In this context, the impact of agrochemical pollution on wild gudgeon and chub populations was assessed. A set of biological traits were measured for each species and the link to pesticide toxicity levels tested. Upon removal of confounding effects (genetic variation, geographical distance, water parameters), 4 of the 17 morphological traits studied on the gudgeon were significantly linked to pesticide toxicity. Such differences in body shape are susceptible to take place during early developmental stages, thus indicating that fish were indeed exposed to change-inducing stressors such as toxicants throughout their development. Chub general condition decreased while gonad size increased with increasing toxicity. Histopathological observations of liver tissue indicate that chubs from more polluted sites have reduced lipid storage and increased immunological stress.

In order to verify whether pesticides found in the Adour-Garonne river basin cause sub-lethal effects on fish, farm-raised juvenile rainbow trout were exposed in laboratory conditions to low levels of a mixture of herbicides (atrazine, linuron and metolachlor). During the 5-day exposure, trout behaviour was monitored. The increase in number of aggressions of control fish was reflected in an increase of movements per fish, whilst this relationship was not seen in exposed fish. In average, exposed fish tended to occupy lower compartments than control fish. Juvenile trout exposed to a mixture of pesticides thus presented a general hypoactivity, possibly a handicap to survival in the long term.

The overall results of these investigations indicate that fish are relevant as environmental bioindicators and as model test organisms to evaluate the effect of sub-lethal levels of contaminant mixtures, in the field and in laboratory conditions. When testing whether there is a significant relationship between contamination and a biological response, it is important to take into account multiple confounding factors, both environmental as well as innate to the organism. In this way, the link between presence/magnitude of contamination and effect on the ecosystem can be clearly established, and used to promote preventive and protective measures for species conservation and the protection of a basic resource - water.

Cândida SHINN

Impact of toxicants on stream fish biological traits

Directeurs de thèse :
Sovan LEK & Gaël GRENOUILLET

Thèse soutenue à l'Université Paul Sabatier, Toulouse III
le 30 septembre 2010

Résumé

Pour garantir une qualité d'eau appropriée à la population humaine et aux milieux naturels, l'Union Européenne a sollicité tous les états membres afin d'obtenir d'ici 2015 une qualité écologique minimale satisfaisante pour toutes les eaux de surface, définie par la nouvelle Directive Cadre sur l'Eau. L'objectif principal de cette thèse a été l'évaluation du statut de populations de poissons natives de quelques rivières du Sud-ouest de la France, impactés par les polluants (métaux lourds et pesticides), en utilisant des traits biologiques des poissons : morphologie, santé, condition et taux d'accumulation des contaminants. Globalement, les résultats de recherches ici menées démontrent que les poissons sont appropriés en tant que bioindicateurs environnementaux. Le lien entre la présence/magnitude de la contamination et l'effet sur l'écosystème peut donc être établi, et utilisé pour promouvoir des actions préventives et protectrices à des fins de conservation des espèces et de protection d'une ressource essentielle comme l'eau.

Écotoxicologie; Directive Cadre sur l'Eau; qualité de l'eau; métaux lourds; pesticides; poissons de rivière; bioindicateur

Discipline : Doc. U. Ecologie et Evolution des Populations et Communautés / Écotoxicologie

Laboratoire Evolution et Diversité Biologique