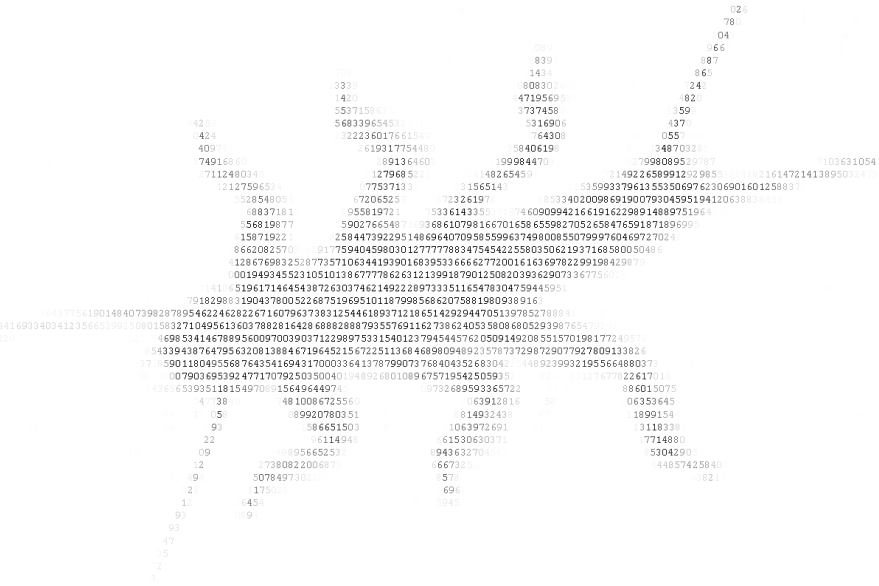
Plant protection product risk assessment for aquatic ecosystems

Evaluation of effects in natural communities



Alessio Ippolito

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The research presented in this thesis was carried out at the department of Environmental and Landscape Sciences (DISAT), Università di Milano-Bicocca, Milano, Italy.

Cover image: observed by far, the figure is the outline of a stonefly (*Agnetina capitata*), however, by a closer look, it is also a long string of values, representing the codification of the organism traits. The same happens with ecology and ecotoxicology: a holistic, comprehensive view is needed to understand what is the meaning and the overall relevance of any process, but a reductionist approach is often appropriate to get a mechanistic understanding. The challenge proposed by this thesis is to achieve a correct balance between holism and reductionism, finding the right distance to see both the stonefly and the string.

UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA
Facoltà di Scienza Matematiche, Fisiche e Naturali

Dottorato di ricerca in Scienze Ambientali
XXIV ciclo

Plant protection product risk assessment for aquatic ecosystems

Evaluation of effects in natural communities

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A Dino

CONTENTS

1 General introduction[1]

1.1 Ecological Risk Assessment for chemicals

Ecological risk assessment (ERA) is defined as a process that evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors (U.S. EPA, 1992). Particularly, chemical risk assessment requires a multidisciplinary approach, integrating environmental chemistry, classic toxicology and ecology. The high number of variables and many trade-offs make this kind of evaluation extremely complex. For this reason current regulations are based on simplified procedures. In the European Union (EU), these official procedures are reported in the Technical Guidance Document (TGD) in support of Commission Directive 93/67/EEC, Commission Regulation (EC) No. 1488/94 and Directive 98/8/EC of the European Parliament and of the Council (EC, 2003). Risk assessment guidelines for plant protection products are reported in the Annex VI of the Directive 91/414/EC, which contains the "Uniform Principles", the harmonized criteria for evaluating products at national level. All these risk assessment methods are based on a step-wise tiered procedures comprising the effect assessment, the exposure assessment and the risk characterization.

The effects assessment comprises the 1) hazard identification or identification of the effects of concern 2) dose (concentration)/response (effect) assessment. The evaluation of the effects, performed for each compartment of concern, largely relies on laboratory assays. Particularly, for the aquatic compartment, tests are carried out on standard species representative of three different levels of the trophic chain (producers, primary consumer and secondary consumer). The outcomes of these assays are expressed as ecotoxicological endpoints such as L(E)C50 (Lethal/Effect Concentration on 50% of the population) or NOEC (No Observed Effect Concentration). Using these evaluations a PNEC (Predicted No Effect Concentration) is determined dividing the obtained endpoints by an appropriate safety factor.

The exposure assessment evaluates the amount (concentration) of the chemical in each compartment of interest, trough direct measurements and model application. The result of both methodologies would be a Predicted Environmental Concentration (PEC). Predictive models are based on

[1] Part of this chapter has been published in: Ippolito A, Vighi M (2011) Introducing the vulnerability into pesticide ecotoxicology. Proceedings of the XIV Symposium in Pesticide Chemistry. 30th August-1st September 2011, Piacenza, Italy

standardized scenarios at different scales (local, regional and continental). All potential emission sources (point and diffused) are analyzed, and the fate of the substance is also considered.

The risk characterization consists in a comparison of the effect and the exposure assessments. Particularly, in the TGD, quantitative risk characterization is calculated by comparing the PEC with the PNEC (PEC/PNEC ratio). Within the Directive 91/414/EC framework, risk is quantified through the calculation of the TER (Toxicity/Exposure Ratio), i.e. the ratio between an exposure indicator (e.g. PEC) and an indicator of the effect (e.g. EC50).

1.2 Site-specific risk assessment: the new frontier of ERA

The introduction of the Water Framework Directive (EC 2000) posed intriguing challenges to ecotoxicology, and it has important implications about the way Ecological Risk Assessment is carried out. One of the main target posed by this regulation is the achievement of a 'good status' for all surface waters by 2015. 'Good status' means both 'good ecological status' and 'good chemical status'. Attempts to harmonize the WFD with the current regulation on pesticide registration are difficult and still in progress (see for example Brock et al. 2006).
Nevertheless, it is interesting to notice that the WFD moves the focus of ERA from the chemical (to be placed on the market) to the ecosystem (to be protected). This is certainly an important change of perspective, which should encourage ecotoxicologists to do more research about effects of chemicals in real environment. Indeed, it is well acknowledged that the level of alteration in response to the same level of exposure can vary greatly among different ecosystems. These differences are determined by some characteristics of the ecological systems. It means that the real effects provoked by chemicals are not only relying on their "absolute toxicity" and on their concentration, but also, to a large extent, on the degree of alterability of a certain system toward a specific stressor, or, in other words, on the vulnerability of the system. Thus, in site-specific ERA, the protection is shifted from a generic scenario to a real ecological system, with all its peculiarities that make it more or less vulnerable toward a certain stressor.
Vulnerability is a well-established concept in all the disciplines that deal with risk assessment, not only those which are concerned with environmental sciences. Geology as well as economics and social sciences contemplate the concept of vulnerability, despite facing very different types of risks. However, vulnerability is often overlooked in ecotoxicology, and it is never cited in current risk assessment procedures.

Nevertheless, in the 30-year-old definition of ecotoxicological risk and hazard, Cairns (1980) distinguished between the two terms. In his formulation the hazard is the combination of the exposure to a toxic chemical and the toxicological effects produced on a living organism. In this view, the hazard represent just the potential of the risk, not the risk itself. To determine how this potential could affect a certain system, the knowledge of its vulnerability is needed (Figure 1.1).

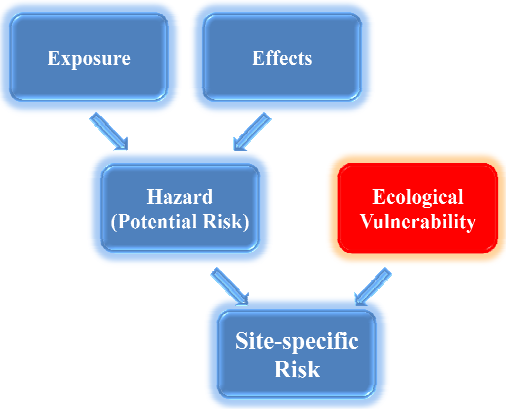

Figure 1.1 Theoretical framework for site-specific risk assessment procedure.

On a generic environmental level, the mathematical formula of risk as the product of hazard and vulnerability is also reported in the official glossary of the European Environmental Agency (EEA – definition available at http://glossary.eea.europa.eu).
Several definitions of vulnerability are present in literature, and different multidisciplinary groups of expert are still working to refine the concept (i.e. MOVE - Methods for the Improvement of Vulnerability Assessment in Europe). Turner et al. (2003) defines the concept as "the degree to which a system, subsystem, or system component is likely to experience harm due to exposure to a hazard, either a perturbation or stress/stressor", while the EEA proposes the following one: "the degree to which a system is susceptible to, and unable to cope with, injury, damage or harm". Both show interesting insights: the first one in particular highlights the very important aspect that vulnerability is not only concerned with systems, but even with single parts of them. The second definition instead implicitly suggests that vulnerability doesn't concern the resistance of the system only, since the capacity to cope with harm can be expressed even by a fast resilience.
However, none of this definitions highlights the specificity of the concept: vulnerability should always be considered as stress-specific. The vulnerability which a certain system (or part of it) shows toward a stressor

could be extremely different for another source of disturbance. No absolute determination is possible.
Although the discussion on the best definition of vulnerability is still in progress, many disciplines have already developed sound criteria and methodologies to assess this parameter. Ecotoxicology instead is still behindhand on this path: debates are still open about which aspects are determinant for the assessment of vulnerability. The same word "vulnerability" was often used in the past just like a synonymous of either sensitivity and susceptibility. Only recently some authors (e.g. De Lange et al. 2010) started to work in order to give "ecological vulnerability" a defined, wide acknowledged meaning within the field of ecotoxicology.
One of the key point of this view is the consideration that "ecological vulnerability" is a term which can regard several hierarchical level of organization (organism, population, community, ecosystem).
Despite each hierarchical level is characterized by its own prerogatives, is it possible to define a general framework for ecological vulnerability assessment, which can be always followed. Previous studies, especially assessing vulnerability to stressors linked to global change (Schröter et al. 2005, Adger 2006), consider vulnerability as a function of three variables: exposure to a stressor, effect (sensitivity) and recovery potential (resilience).
Similarly, Van Straalen (1993) identified three components in his population vulnerability conceptual model: external exposure, intrinsic sensitivity and population sustainability.
The study of the ecological vulnerability is a clear example of how risk assessment, as becoming site-specific, needs more ecological knowledge (Baird et al. 1996). In this path, the use of ecological and biological traits of organisms has proven to be a promising approach to evaluate the ecological vulnerability at different level of biological organization. A comprehensive framework for the use of traits in the vulnerability assessment has been recently provided by Rubach et al. (2011), where the new frontier of TERA (Trait-based Ecological Risk Assessment) announced by Baird et al. (2008) is starting to take shape.
One key point is the possibility to establish a mechanistic relation between the system (which might be an organism, a population, a community or even an entire ecosystem) and the response (in terms of sensitivity and vulnerability) of the same system to a certain stressor.
The potential power of biological traits resides mainly in their independence from the standard taxonomy, which has been used for a long time as the only measurement unit in ecotoxicological indexes. While standard taxonomy depends on phylogenetic evolution, the response of organisms after a potentially harmful exposure (both short and long-term response), depends on their current characteristics.
Biological and ecological traits are a representation of the *status quo* of the species, without historical perspective, which is nonetheless the basis of the

functional role played in the community ("Whereas taxonomy can be regarded as a higher-level expression of the genetic composition of organisms, traits can be seen as their functional consequence", Baird et al. 2008). The use of ecological and biological traits can be seen as the "missing link" between the structure and the function of the community.
An important change of view is also required for what concern the endpoints to be measured. First of all, at individual level, it is necessary to remember that each organism is strongly conditioned by a huge number of interactions with other components (biotic and abiotic) of the ecosystem. The usual endpoints of standard (lethal or sub-lethal) toxicity tests may be insufficient to assess the alterations induced by the exposure to a chemical on natural populations or on the structure and the functions of ecosystems. Thus, more ecologically relevant endpoints have to be considered, especially focusing on higher level of organisation (i.e. community, ecosystems), to achieve a better ecological realism. This requires the development of endpoints representative of the whole biological community (or even ecosystem), able to give safe indications of the chemical-induced alteration. A crucial point is that these endpoints should be able to detect a stressor-specific alteration, without being disturbed by any other natural or anthropogenic confounding factor.

1.3 Outline of the thesis

In this work the issue of the ecological vulnerability has been considered from several different perspectives, using multiples methodologies and working at completely different scales. The leading thread is to show how an ecologically based approach can enhance our understanding of environmental processes and thus improving risk assessment methodologies.

Particularly, in **chapter II**, a definition of the concept of ecosystem vulnerability is given, providing a theoretical framework for its evaluation. A description of a new versatile index for the Ecological Vulnerability Assessment is presented. In the same chapter the abovementioned index is applied to a case-study involving two Italian rivers, in order to illustrate the potential use of this tool.

The theoretical framework of the Ecological Vulnerability Assessment is based on three different elements: the susceptibility to exposure, the physiological sensitivity and the recovery. Existing indexes (e.g. SPEAR, Liess and Von der Ohe 2005) already used ecological traits to evaluate the first and the last term. However, the linkage between species traits and physiological sensitivity is much weaker in the literature. In **chapter III** this linkage has been explored for three classes of pesticides, using a huge bulk

of information about sensitivity data and biological traits of freshwater invertebrate taxa. To explore such a relationship an advanced chemometric approach was followed.

A stronger site-specific approach was adopted in **chapter IV** and **chapter V**. In those works two different studies were carried out, with the common scope of evaluating the actual pesticide risk for the benthic invertebrate community in a mountain stream sited in a region of Northern Italy. Particularly, an experiment with some artificial rivers was carried out to mimic a realistic exposure pattern for the area of interest, minimizing any other stressor. The response of the community structure was evaluated using different metrics.
The results of this experiment were used to interpret a 2 year monitoring campaign on the real stream, comparing the trends of the community structure (in terms of abundance of vulnerable taxa) with a more classical risk evaluation.

In **chapter VI** the concept of ecosystem vulnerability has been upscaled to the maximum possible extent. A spatially explicit model was used to evaluate the global distribution of the main abiotic variables influencing the vulnerability connected to events of pesticide runoff. A global vulnerability map was produced and compared with another map reporting pesticide hazard (thus derived from actual pesticide application patterns worldwide). Crossing the two maps a potential risk map was produced as a final outcome of the work. This chapter has been intended as an exercise to show how the integration of vulnerability and hazard to evaluate the risk is possible at any scale.

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2 Ecological vulnerability analysis: a river basin case study

Abstract
Assessing and quantifying ecosystem vulnerability is a key issue in site-specific ecotoxicological risk assessment. In this paper, the concept of vulnerability, particularly referred to aquatic ecosystems, is defined. Sensitivity to stressors, susceptibility for exposure and recovery capability are described as component of vulnerability of biological communities. The potential for habitat changes must also be considered in ecosystem vulnerability assessment. A procedure based on the application of an ecosystem vulnerability index is proposed. The method allows the assessment of vulnerability of riverine ecosystems to multiple stressors. The procedure is applied to two river systems in northern Italy: River Serio, subject to strong human pressure, and River Trebbia, in semi-natural conditions, as reference system. Macrozoobenthos is chosen as the indicator community. The actual quality of River Serio was evaluated as the result of the multiple stressor pressure on the reference system. Values and limitations of the approach are discussed.

Keywords: Ecological vulnerability assessment, river ecosystems, macrozoobenthos, river quality, multiple stressors.

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2.1 Introduction

Ecosystem vulnerability is an underdeveloped concept in ecotoxicological risk assessment.
The objective of most procedures required by European regulations on dangerous chemicals is assessing the risk for "general" European ecosystems. For example, for plant protection products, the objective of the Pesticide Directive (Directive 91/414; EC 1991) is "the placing of plant protection products on the market" and, in this frame, assessing a potential danger for European aquatic and terrestrial ecosystems. The FOCUS (FOrum for the Co-ordination of pesticide fate models and their USe) group for pesticides developed a number of standard scenarios, assumed as representative of different agronomic and environmental characteristics of different European regions (FOCUS 2002). This approach allows assessing risk considering different environmental scenarios, however, many characteristics of real ecosystems are not taken into account.
The recently approved REACH (**R**egistration, **E**valuation and **A**uthorisation of **Ch**emicals, Regulation 1907/2006; EC 2006), establishing common rules for "new" and "existing" chemicals, is based on a Chemical Safety Report (CSR) describing exposure scenarios that may vary from "generic" to "very specific". Exposure scenarios must include risk management measures that ensure that the risks from the use of the substance are adequately controlled. However, environmental risk assessment is based on the comparison between a PEC and a PNEC, and the latter is traditionally derived from laboratory toxicity tests. Extrapolation of laboratory standard tests to the field situation is hampered by lack of information for site-specific representative species, as well as for interactions related to structure and functioning of the ecosystem (indirect effects, homeostatic capability, recovery mechanisms, etc.). Therefore, in standard risk assessment procedures the community characteristics of actual ecosystems are poorly considered, if at all.
However, ecotoxicological risk assessment is not only required for general objectives, such as the continental-scale regulation of chemical substances. The scale of environmental management is usually smaller and requires assessment on relatively small geographic units (hydrographic basins, local administrative units, etc.), where site-specific approaches are required for protecting specific ecosystems. The responses of different ecosystems to a particular stressor may be very different. Therefore, information on the characteristics of potentially endangered ecosystems is essential in site-specific risk assessment.
Site-specific approaches are also required by the Water Framework Directive (WFD, Directive 2000/60; EC 2000) asking for tools capable to describe and assess the site-specific ecological status of European water bodies. The ecological status of aquatic ecosystems is the result of natural environmental

conditions and of the pressure of anthropogenic stressors. The deviation of ecosystem status from natural (reference) conditions is a function of the intensity of stressors and of ecosystem vulnerability. It follows that assessing sensitivity and vulnerability of ecological systems is a key issue in ecotoxicology. However, in spite of this relevance, few examples of vulnerability assessment have been presented in the literature. A state of the art overview is described by De Lange et al. (2010).
Ecological vulnerability must be assessed at different hierarchical levels (population, community, ecosystem, landscape). Some definitions are given by De Lange et al. (2010). The problem is not easy; particularly if one considers that the responses of different populations are generally different as a function of different stressors. Moreover, ecosystem vulnerability considers the response at the community level. The characteristics of a community are not merely the sum of the characteristics of individual populations; structure and function of the community are also regulated by emergent properties that are not easily described and predicted from lower hierarchical levels. According to van Straalen (2003), the community is the entity with the lower predictability, among the different ecological hierarchical levels. Assessing ecosystem vulnerability represents a challenge for modern ecotoxicology.
In this paper, the concept of ecosystem vulnerability is elaborated, with particular focus to aquatic ecosystems. A numeric “Vulnerability index” is developed, in order to evaluate the potential response of ecosystem features to multiple stressors.
The index has been applied to the case study of two river ecosystems subject to different levels of human pressure. The response of the aquatic communities is discussed as a function of their vulnerability to multiple stressors.

2.2 Vulnerability of ecosystems: definition and specific elements

Ecosystem vulnerability assessment is a complex process that needs a number of factors to be considered. In this paper, vulnerability is the set of properties of an ecosystem that determines its potential for being damaged by a specific stressor.
Each ecosystem consists of a community of species living in a specific biotope (characterised by its own physical, chemical, climatic, geographical and morphological features). Therefore ecosystem vulnerability assessment should comprise both community and habitat aspects.
Both evaluations are closely related: if a stressor can induce relevant habitat changes, then this could result in direct or indirect disturbance of the biological community, and vice versa.

2.2.1 Community vulnerability

Vulnerability assessment of a biological community must start from the analysis of three characteristics of the different populations:

- Susceptibility to exposure
- Sensitivity for a particular stressor
- Recovery potential at population and community levels

While species are the units that react first to the stressor on the basis of their specific traits, the impact to the community follows from population responses and changes in interspecific relations.
The final objective of environmental management is protecting structure and function of communities and ecosystems. Thus, the assessable characteristics of a biological community should not only comprise population characteristics, but preferably also include emerging properties and relationships and interaction among populations that determine community function and structure.

2.2.1.1 Susceptibility to exposure assessment

The Predicted Environmental Concentration (PEC) in a given compartment (water, air, soil) is the most frequently used exposure indicator in risk assessment. In some cases, the time variability of the PEC may be accounted for. Nevertheless species have intrinsic traits (behavioural, physiologic, metabolic, etc) that determine the probability for exposure.
Stressors characterised by discontinuous exposure may have a fully different effect on organisms continuously living in a given compartment in comparison with species with a polymorphic life cycle, changing living environment from one life stage to another (e.g. aquatic larvae of insects).
Other behavioural factors may also be relevant, such as mobility, seasonal behavioural changes, etc. All such factors together determine the probability to be exposed to a stressor. At present, precise criteria for assessing and quantifying the susceptibility to exposure have not been developed. A more detailed study on physiological and behavioural traits relevant in different exposure conditions would be necessary.

2.2.1.2 Sensitivity assessment

The sensitivity of different species in the community could be represented in probabilistic terms using the Species Sensitivity Distribution (SSD) method (van Straalen and Denneman 1989; Posthuma et al. 2002). Lack of experimental data on community species could be overcome trying to predict responses of different species to a specific stressor on the basis of some biological traits (Baird 2007). Another system to predict effects are QICAR (Quantitative Interspecific Chemical Activity Relationships, Tremolada et al. 2004; Dimitrov et al. 2000). However, in risk assessment procedures, the SSD approach is developed on the basis of data available in the literature on

organisms representative of a generic (aquatic or terrestrial) environment. For site-specific sensitivity assessment, SSD may be applied to assess the potentially affected fraction of the community, but cannot predict the actual species at risk.
Secondly, the sensitivity of a community is not a simple combination of the sensitivity of populations. An important point is evaluation of emergent properties and indirect effects, i.e., consequences on structure and on functioning of the community determined by alteration of relationships between populations (competition, predation, etc) after a disturbance. On this topic, considered one of the more complex aspects of modern ecotoxicology, only little methodology is presented in literature.

2.2.1.3 Recovery capability assessment
Sensitivity is an expression of resistance, but does not give any information about the response in the time after exposure. The assessment of resilience, i.e. the capability of a system to return in the pristine state of structural and functional organization after an alteration induced by a stressor, is particularly important if exposure is not continuous or not constant.
While functional recovery is due to feature of the whole system, structural recovery depends on the resilience of each population in the community. Population recovery depends on genotypic and phenotypic properties of individuals (age, sex and biomass distribution, fecundity, etc) and on collective species properties (meta-population structure, mobility, territoriality, seasonality, iteroparity, etc).
As for susceptibility to exposure, precise criteria for a quantitative assessment of recovery capability are not available. However, for a qualitative preliminary assessment, some relevant traits are the reproductive capability, the biotic potential, the length of the life cycle, the capability for genetic, physiologic and behavioural adaptation.

2.2.2 Habitat vulnerability assessment

Ecosystem vulnerability assessment is the result of the previous community vulnerability assessment and the habitat vulnerability. In this context, habitat vulnerability represents the intrinsic predisposition of a biotope to be altered by natural or anthropogenic stressors, considering both biotic and abiotic factors.
To assess habitat vulnerability, qualitative criteria should consider issues due to available space reduction, structural and morphological changes, alteration of physical and chemical conditions as well as modification of microclimate.

2.2.3 Vulnerability assessment and ecological quality

One of the requirements of the Water Framework Directive is the assessment of reference conditions in order to classify ecological quality of water bodies. According to the WFD (EC 2000) the ecological quality of a water body is defined as follows:

- High ecological status: The values of the biological quality elements for the surface water body reflect those normally associated with that type under undisturbed conditions and show no, or only very minor, evidence of distortion.
- Good ecological status: The values of the biological quality elements for the surface water body type show low levels of distortion resulting from human activity, but deviate only slightly from those normally associated with the surface water body type under undisturbed conditions.

Therefore, high ecological status, representing the reference condition, is characteristic for a water body where human pressure is absent or negligible and the biological community is typical for pristine conditions.
Structure and functions of such a community depend on natural environmental factors and are different in different typologies of water bodies. It follows that reference conditions must be described for all the different typologies of a water body and for the different European ecoregions.
The set up of water body typologies and the definition of reference conditions is one of the objectives of the European Common Implementation Strategy for the Water Framework Directive. The definitions, methods, principles and criteria to be used for establishing reference conditions for the various typologies and for setting boundaries between high, good and moderate ecological status for inland surface waters are described in a specific Guidance Document (EC 2003).
The vulnerability of the communities typical for the different reference water bodies may be substantially different in relation to different stress factors. For example, the reference community of a cold mountain creek would be more vulnerable to oxygen depletion in comparison with those typical for a warm lowland river. Therefore, assessing the vulnerability of reference communities to potential stressors would be relevant to attain WFD standards.

2.3 Methodological approach

In this paper, a method to assess river ecosystem vulnerability is developed. As a first step, a general framework provides a qualitative description of the processes involved in the assessment. In a second step, a preliminary quantitative scoring system is proposed. The method is based on a stepwise

procedure and may be applied with different levels of detail, depending upon the information available and on the requested level of refinement.

Step 1. Characterizing different river typologies, from spring to mouth
The first step of the procedure may be obtained by applying the Huet model (Huet 1949), based on hydromorphological features (slope, river width), that divides rivers in 4 typology classes of water body (from high mountain to lowland river). The Huet model has been applied for mapping pesticide risk in Italian river basins (Sala and Vighi 2008).
A more detailed system of classification (RIVPACS) is provided by Wright et al. (2000). It is derived from four predictors (latitude, longitude, drainage area, and stream-channel slope).
The Annex II of the WFD proposes a system based on a list of main features:

- Distance from spring (indicator of the extent of water body)
- Morphology of riverbed
- Perennity and persistence of the flow
- Origin of water body
- Possible influence of watershed upstream

The WFD approach may lead to a large number of typologies; a rationalization may be needed here for practical purposes.
Among the three methods, the choice is related to the availability of hydromorphological data and to the required resolution.

Step 2. Reference water body
The actual community of a polluted river has been modified as a result of the impact determined by one or multiple stressors over time. However, management aims to establish and protect the potentially highest ecological quality. A suitable reference typology in natural or semi-natural conditions is required to perform vulnerability assessment. A proper selection of reference river should consider several characteristics like: length, extent of watershed, geographical position, average flow rate, average slope, etc.

Step 3. Characterizing biological communities
Each river segment (river typologies identified in Step 1, from here indicated with r.s.), is characterized by a potential biological community. The River Continuum Concept (Vannote et al. 1980) suggests a theoretical model where different species are distributed as consecutive Gaussian curves, the maxima coinciding with optimum habitat conditions of the species.
The knowledge of some characteristics of a specific ecosystem allows focusing on the populations that are more representative or that play a determinant role in the system (keystone species). This is a tentative, preliminary approach, because, up to date, more precise approaches capable to characterize the whole community are lacking.

Macrobenthos community is an excellent indicator of water quality. The EBI (Extended Biotic Index) is one of the most common water quality indicator (Woodiwiss 1964) routinely used since a long time in monitoring campaign. Different taxa cover several trophic levels and some of their characteristics (sensitivity to oxygen depletion as well as to some toxic chemicals, etc.) are quite well known.

Step 4. Characterizing stressors

Each event or process that can induce a change in the structure or functioning of a biological system must be considered a stressor. As vulnerability is not an absolute quality but is related to a particular stressor, vulnerability assessment has to be considered stressor-specific. Within this paper, vulnerability is assessed for each single stressor, even if one must be aware that combined stressors and interactions among stressors should be taken into account in a further development of the procedure.

Characterization of possible stressors acting on ecosystems should be developed with a qualitative-quantitative approach. That could be obtained following an adaptation of DPSIR, the causal framework for describing the interactions between society and the environment adopted by the European Environment Agency: Driving forces, Pressures, States, Impacts, Responses. (EEA 2009). It has been chosen because of its proven appeal to policy makers (Stanners et al. 2007) and applied at some extent in the WFD context (Borja et al. 2006).

Starting point are the driving forces (D): urban, agriculture, industry, hydromorphological factors, others (landfill, climate change, invasive species, etc). These produce pressure (P) generating stressors able to modify the state (S) of the water body. Impact (I) is related to potential alteration due to the combination of vulnerability and magnitude of stressors (as explained in par 2.4.6). Responses (R) have to be developed by further phases of risk management and mitigation.

Every stressor must be considered individually. Characterization of potential stressors should take into account:

- Variability in time (continuous, intermittent, pulse, etc)
- Variability in space (point source, diffuse source, fixed, mobile, etc)
- Typology (chemical, physical, biological, etc)

Step 5. Evaluation of vulnerability

Evaluation of ecosystem vulnerability to a specific stressor is performed according to the scheme of Figure 2.1. Each component of vulnerability (sensitivity, recovery capability, susceptibility to exposure and potential for habitat changes) is evaluated individually for each potential stressor.

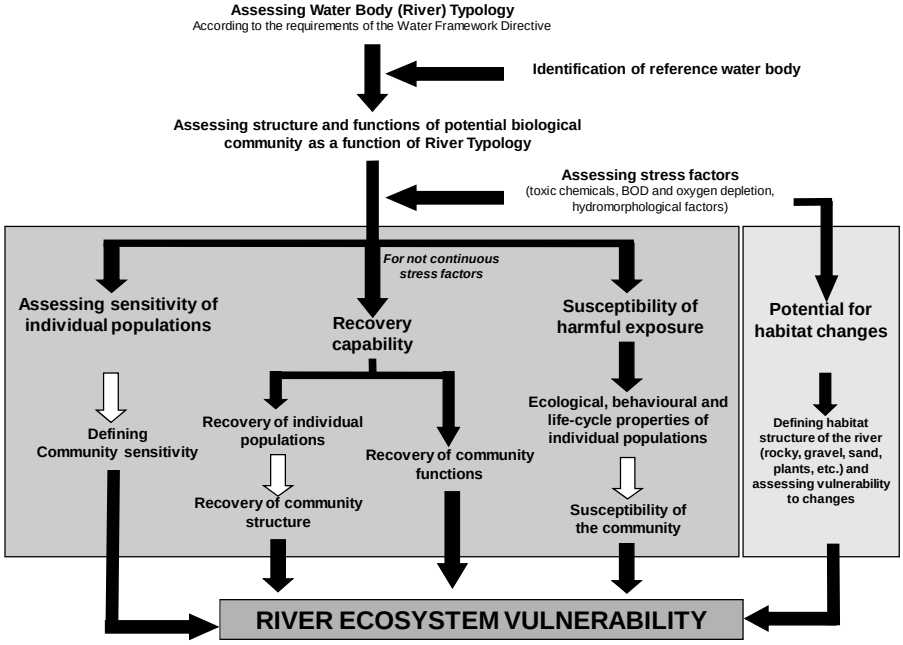

Figure 2.1 General scheme for river ecosystem vulnerability assessment. Left box is referring to community vulnerability assessment, while right box indicates habitat vulnerability assessment. Both evaluations are closely related, as one stressor acting on habitat could have indirect effects on the community and vice versa (Long term impacts, De Lange et al. 2010). White arrows indicate most critical issues arising at the change of scale from population to community level.

A simple scoring system from 0 to 3 has been developed to estimate the influence that a potential stressor can produce on a given component of the vulnerability (Table 2.1). Note that vulnerability is positively related to exposure susceptibility, sensitivity, and habitat alteration, while recovery capability contributes inversely.

Table 2.1 Scores attributed to the components of ecosystem vulnerability.

Scores	Influence on Se, Su, HA*	Influence on R*
0	No influence	High influence
1	Low influence	Medium influence
2	Medium influence	Low influence
3	High influence	No influence

*Se: Sensitivity; Su: Susceptibility of exposure; R: Recovery capability; HA: Habitat alteration.

A vulnerability assessment on a certain community has to cope with a lack of information at community level, so an expert judgment is required to provide a synthetic assessment on the community derived from exiting literature and data at population level. Some details on the procedure used for the scoring in the present case study are described in section 2.4.5.
Scores are used as inputs for the development of the following "Vulnerability index":

$$V_x = \frac{Se_x \times Su_x}{1 + R_x} + HA_x \quad (2.1)$$

Where:

V_x = Vulnerability of ecosystem to stressor X

Se_x = Score attributed to the influence of the stressor X on the Sensitivity of the community

Su_x = Score attributed to the influence of the stressor X on the Susceptibility to exposure

R_x = Score attributed to the influence of the stressor X to Recovery capability

HA_x= Score attributed to the influence of the stressor X on the Habitat Alteration

The index ranges from 0 (ecosystem not vulnerable to stressor) to 12 (ecosystem highly vulnerable to stressor). Community vulnerability is expressed by the first term of the index, while the second term expresses habitat vulnerability. Ecosystem vulnerability derives from the sum of these two components.
The equation assumes that community vulnerability varies linearly with sensitivity (Se) and with susceptibility to exposure (Su), because these are the elements that determine the immediate response of a community to a stressor. Resilience could mitigate alteration caused by the stressor, but only in a longer timescale. That is why R parameter could never bring to zero community vulnerability values if Se and Su are not null. When susceptibility to exposure or sensitivity is zero, community vulnerability is null and ecosystem vulnerability is only determined by habitat changes. When recovery capability is zero, community vulnerability is highest, as a function of Se and Su.
Most ecosystems are potentially affected by several simultaneous stressors, acting separately or in interaction. The index should be applied to all the stressors identified in the river, according to the list of Step 4. An example of multistress vulnerability scheme is shown in Table 2.2. The table is a simplification of the potential stressors corresponding to different pressures. For example urban sewage may contain a number of toxic chemicals

(pharmaceuticals, detergents, etc.). In this assessment, only the major stressors have been considered.
According to the DPSIR scheme, the impact (I) is the combination of ecosystem vulnerability and the magnitude of the hazard produced by the stressor. The probability of impact determines the risk.

Table 2.2 Example of application of vulnerability assessment to a real ecosystem. Only the main stressors have been considered. The empty boxes should be filled according to the scoring system of Table 2.1. The output of this scheme is a list of scores indicating ecosystem vulnerability referred to each stressor considered.

			Ecosystem Vulnerability			
			Community Vulnerability			**Habitat Vulnerability**
Driving force	**Pressure**	**State**	Susceptibility to exposure	Sensitivity	Recovery capability	Potential Habitat Alteration
Urban	Urban sewage	Oxygen depletion				
Industrial	Wastewater	Chemical PEC				
Agricultural (crop)	Pesticide	Chemical PEC				
Agricultural (animal farms)	Manure	Oxygen depletion				
Hydromorphological	Flow rate alteration	Reduction of flow				

2.4 Application to case study (River Serio - River Trebbia)

The method was applied to two river systems of northern Italy: River Serio, subject to high pressure from multiple stress factors, and River Trebbia, in semi-natural conditions, assumed as reference system. The vulnerability assessment procedure was applied to the macrobenthos community of River Trebbia, while macrobenthos of River Serio has been assumed as the resulting community as a consequence of the pressure of multiple stressors.

2.4.1 Characterizing different river typologies, from spring to mouth

According to the Annex II of WFD, five different river typologies have been identified from spring to mouth on the basis of some hydrological and morphological feature. So, the water bodies were divided into five river segments (r.s.) which have been considered comparable in the two rivers (Figure 2.2). Each r.s. corresponds to a sector of the watershed.

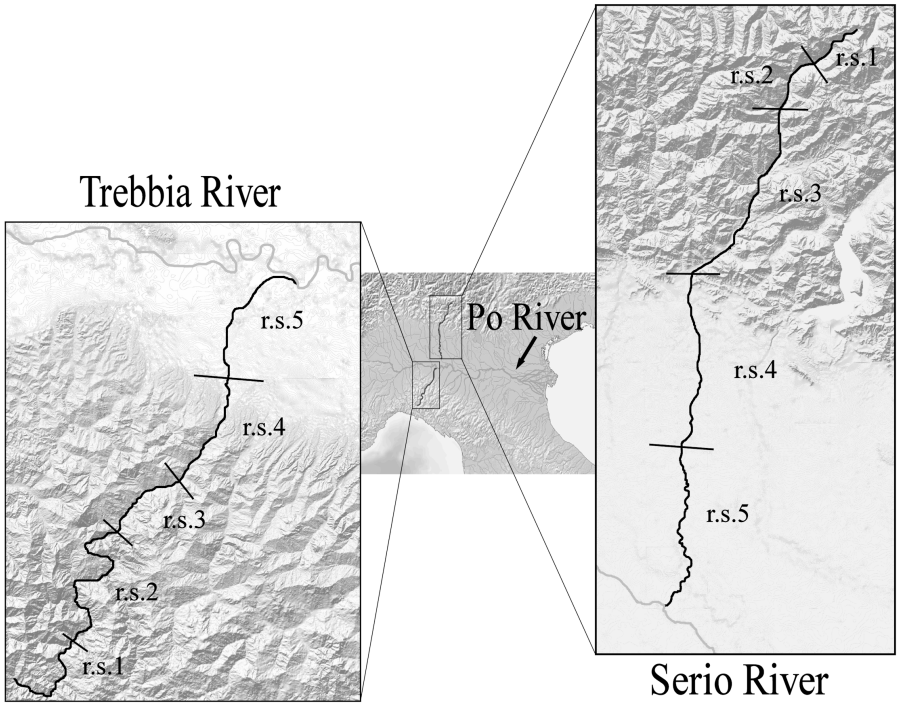

Figure 2.2 Identification of 5 river segments related to water body typologies on River Serio and River Trebbia from spring to mouth.

2.4.2 Finding reference water body

The two rivers are in the same climatic area, are comparable for morphological and hydrological characteristics (Table 1 in *Appendix 1*) and a comparable sequence of ecosystem typologies can be identified: both originate in mountains, flow through hills and then, for a large extent, in the Po Valley. Both rivers belong to the Po basin, and that makes them comparable even on a geographical point of view (Figure 2.2).
On the contrary, human pressure is extremely different. River Serio is subject to relevant urban, industrial and agricultural emissions. Moreover, water use for electric power production and agriculture irrigation, poses serious flow rate problems. River Trebbia presents a low human pressure and it is still in semi-natural conditions. The watershed is considered of high natural value as confirmed by the presence of a SCI (Site of Community Interest) from Perino to Bobbio, in r.s. 3. Therefore, River Trebbia has been chosen as a reference system.
The ecological status of River Serio is changed since a long time: the communities present now in the river are derived from pristine natural communities that should be similar to those of River Trebbia.
The aim of this case study is to assess vulnerability of the macrobenthos communities of River Trebbia, assumed as the potential pristine communities of River Serio, toward effective stressors acting (or that have been acting) on the riverine system. The comparison with the present community of River Serio would provide an example of the changes likely to occur as a function of vulnerability and of intensity of stressors.

2.4.3 Community characterization

Monitoring data on macrobenthos community (EBI – Extended Biotic Index - values, presence of different taxa, number of systematic groups for each taxon) have been obtained from ARPA (Agenzia Regionale per la Protezione dell'Ambiente - Regional Environmental Protection Agency) Emilia Romagna and Provincia di Genova for river Trebbia, from ARPA Lombardia and Provincia di Bergamo for River Serio (ARPA 2009; Provincia di Bergamo 2001; Provincia di Genova 2003). Data refer to 11 sampling stations on River Trebbia and 7 sampling stations on River Serio, covering all identified r.s. (Figure 2.3). In both rivers monitoring data were available for 9 sampling years (from 2000 to 2008). No time trends were observed, so the composition of macrobenthos reported in Table 2.3, represents simple arithmetical averages of abundance of systematic groups for each taxon in the 9 sampling years.

Table 2.3 Composition of macrobenthos communities of each river segment (r.s.) of River Trebbia and River Serio. Number of systematic units (S.U.) for each taxon are reported. For River Serio records of 3 stations belonging to segment 3 have been kept separated because a clear gradient is present. Sums of systematic units consider even minor groups not reported in the table.

r.s.	Plecoptera	Ephemeroptera	Tricoptera	Coleoptera	Odonata	Diptera	Crustacea*	Gasteropoda	Oligochaeta	Tricladae	Heteroptera	S.U.	EBI
RIVER TREBBIA													
1	4.3	8	5.2	2.2	0	7.3	0	1	1.7	0.3	0	30	10.5
2	3	4.7	4.1	2.5	0.5	5.6	0.3 (0.0; 0.3)	0.3	2	1	0.1	24	10.6
3	2.3	5	2.8	1.8	1.1	4.3	0.1 (0.0; 0.1)	0.1	1.4	0.6	0.3	19.7	9.1
4	2.2	5.5	3.1	2	0.9	4.4	0.4 (0.0; 0.4)	0	1.2	0.5	0	20.4	9.5
5	1.5	4.4	1.3	0.7	0.3	2.8	0.5 (0.0; 0.5)	0.1	1.2	0	0	13	7.9
RIVER SERIO													
1	3	2	2	3	0	3	0	1	2	1	0	17	10
2	2	3	2	1	0	5	0	1	0	0	0	14	8
3	1.2	2.1	1.6	0.4	0	3.1	0	0.6	1	0	0	10	6.9
	0	1.1	0	0	0	2.2	0	0.8	1.1	0	1	6.2	4.7
	0	1.3	0	0	0	2	0	1.1	1.1	0	0.9	6.4	4.9
4	0	1	0	0	0	1.8	0.1 (0.1;0.0)	0.3	1.3	0.1	0.7	5.3	4.2
5	0	1	0.7	0.2	0.2	1.4	1.4 (0.8;0.6)	1.5	1	0.3	1.4	9	5.8

* numbers in brackets are the systematic units of Asellidae and Gammaridae respectively.

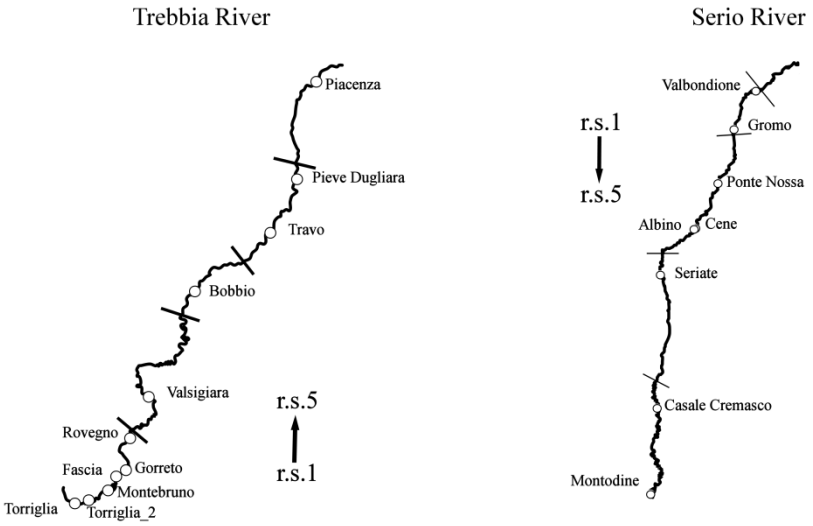

Figure 2.3 Position of the different macrozoobenthos sampling stations on both rivers.

2.4.4 Stressor characterization

Five drivers have been considered: Urban, Industrial, Agricultural (crop), Agricultural (animal farms), Hydromorphological. For Urban and Agricultural (animal farms), the combined action of organic sewage and related solid suspended matter has been considered. For the Industrial driver, only generic toxicity of chemical substances has been considered. For Agricultural (crop), only toxicity of plant protection products (especially insecticides) have been considered. The Hydromorphological driver is referring to flow rate alteration (reduction of natural flow, no consideration have been made about increase of natural flow). Urban, Industrial and Agricultural (animal farms) produce continuous stressors, while Hydromorphological and Agricultural (crop) produce discontinuous stressors. All driving forces listed in Table 2.2 are present, with different intensity, in River Serio.

2.4.5 Evaluating vulnerability

For each r.s., the vulnerability of the reference community to the five stressor clusters, listed in Table 2.2, has been assessed according to the vulnerability index of equation 2.1 (Table 2.4).

1st segment. Two third of the reference community (Table 2.3) is represented by taxa particularly sensitive to the oxygen concentration, so the community is highly sensitive to “urban” and “animal farms”. Susceptibility to exposure

is highest because urban and animal farms wastewaters are always present during the year and could affect each part of the river ecosystem. Influence on recovery is high as these stressors are continuous, so recovery cannot occur. Habitat could be significantly altered by solid particulate matter in a river typology where sediment is usually poor.
As for "industrial" stressor, sensitivity to toxic input should be relevant but not very high, as a good number of non-arthropods, relatively resistant to toxic chemicals, is present. Furthermore no sensitive crustaceans are present. No habitat alteration should be produced.
Agricultural stressors are discontinuous, so susceptibility to exposure is lower than others stressors and recovery is possible. All species considered are characterized by r-strategy with high reproductive potential, so recovery capability is high. Solid deposition producing habitat alteration is possible.
Hydromorphological issues are generally related to flow rate alteration producing a reduction of habitat. Habitat reduction should have species-specific effects that could be related to life stage of individuals. Those effects may affect the population density and may lead to further complication. However, at this stage we considered a generic effect on all macrobenthos taxa.
Moreover, reduced dilution increases the concentration of toxic substances. Richness of species in this segment requires a large number of different habitats, so influence of habitat reduction is very high. Susceptibility to exposure has been evaluated as intermediate because it depends on the relation between the distribution of critical flow over the year and the growing rate of singles populations. This kind of stressor is discontinuous, so recovery is possible.

2nd segment. Community and habitat are substantially the same as the first segment, so vulnerability is always the same.

3rd segment. The number of total systematic units in this segment has been reduced by one third (from 30 to less than 20), so habitat requirements should be reduced. Furthermore the river became wider and deeper so alteration in flow rate should have less influence on the community.

4th segment. The presence of crustaceans (particularly Gammaridae) increases sensitivity to agricultural stressor (particularly to insecticides) from 2 to 3. Habitat alteration produced by urban or agricultural activities reduces its influence since natural quantity of sediments increases a lot.

5th segment. Sensitivity to urban and animal farms drivers diminishes as the number of systematic units particularly sensitive to oxygen depletion decreases (Plecoptera, Tricoptera, Ephemeroptera).

Since the total number of species decreases, less variability of habitat is required while river in this segment has the biggest average flow rate. So influence of water flow alteration on the habitat vulnerability decreases.

2.4.6 Validation of vulnerability assessment through macrobenthos data

The effect of actual stressors present in each segment of River Serio related to the vulnerability of the reference communities determined on River Trebbia (Table 2.4) can be checked by examining the present macrobenthos community of Serio. A quantification of the magnitude of the actual stressor is needed in this phase.

Agriculture (crop) - The intensity of the stressor has been estimated calculating the agricultural surface. Land use is reported in Table 2.5 and is divided in five classes: agriculture (all permanent and non permanent crops); industry (industrial, commercial, and handicraft activities); urban; other - possible stressors (hospital, mining, road and infrastructure, airport); other – no stressors (forest, uncultivated, grassland, meadow).
In Serio watershed, crops are mainly corn and other cereals. So, the main stressors are: pesticide use (mainly herbicides) and manure/fertilizer that may affect the oxygen demand. In *Appendix 1* the maps of land use and the number of agricultural enterprises for each river segment are presented.

Agriculture (animal farms) - Animal farms are present in each river segment, particularly in segments three and four. Figure 2.4 shows the animal farm density, expressed as number of farms per km^2, in each river segment of River Serio. The main stressor could be the release of organic matter, in the form of manure, and the related increase in oxygen demand.

Urban - The magnitude of the stressor is related to the total population present in the watershed (411,217 inhabitants). In Figure 2.4 the distribution of population density for each segment of the river is shown. Even if wastewater treatments plants are present, a relevant residual organic load has to be taken into account. A precise quantification is difficult.

Industrial - Industrial wastewater quality is a function of industrial typology. In the Serio watershed, 24,3 % of the enterprises are in construction sector, 23,1 % are retailers (shops and wholesale), 16,2 % are manufacturing, mainly textile, activities. In *Appendix 1*, the distribution of industrial activities is reported. Therefore, in the watershed the main industrial stressor may derive from manufacturing activities. The density of manufacturing activities in each watershed portion is shown in Figure 2.4, the distribution of all activities is provided in Figure 2 of *Appendix 1*.

Table 2.4 Vulnerability assessment for each river segment and each kind of driver.

	River segment 1					River segment 2					River segment 3					River segment 4					River segment 5				
Stressor Drivers	**Su**	**Se**	**R**	**HA**	**V**	**Su**	**Se**	**R**	**HA**	**V**	**Su**	**Se**	**R**	**HA**	**V**	**Su**	**Se**	**R**	**HA**	**V**	**Su**	**Se**	**R**	**HA**	**V**
Urban	3	3	0	2	**11**	3	3	0	2	**11**	3	3	0	2	**11**	3	3	0	1	**10**	3	2	0	1	**7**
Industrial	3	2	0	0	**6**	3	2	0	0	**6**	3	2	0	0	**6**	3	2	0	0	**6**	3	2	0	0	**6**
Agricultural (crop)	2	2	3	2	**3**	2	2	3	2	**3**	2	2	3	2	**3**	2	3	3	1	**3**	2	3	3	1	**3**
Agricultural (farm)	3	3	0	2	**11**	3	3	0	2	**11**	3	3	0	2	**11**	3	3	0	1	**10**	3	2	0	1	**7**
Hydromorphological	2	0	3	3	**3**	2	0	3	3	**3**	2	0	3	2	**2**	2	0	3	2	**2**	2	0	3	1	**1**

Table 2.5 Land use of the watershed of different segments of River Serio. “Other - possible stressors” are land uses that could hypothetically cause impacts but are not considered in this paper.

	Rs 1		Rs 2		Rs 3		Rs 4		Rs 55	
	Area (ha)	%	Area (ha)	%	Area (ha)	%	Area (ha)	%	Area (ha)	%
Agriculture	0.3	0.01	5	0.03	435	1.25	18399	64.66	10385	53.18
Industry	0	0	6	0.04	705	2.03	1905	6.69	555	2.84
Urban	32	0.54	176	1.12	2308	6.64	3547	12.47	1588	8.13
Other - Poss. Stres.	1	0.01	3	0.02	169	0.49	818	2.88	193	0.99
Other - No Stressor	5966	99.45	15534	98.8	31151	89.6	3785	13.3	6808	34.86

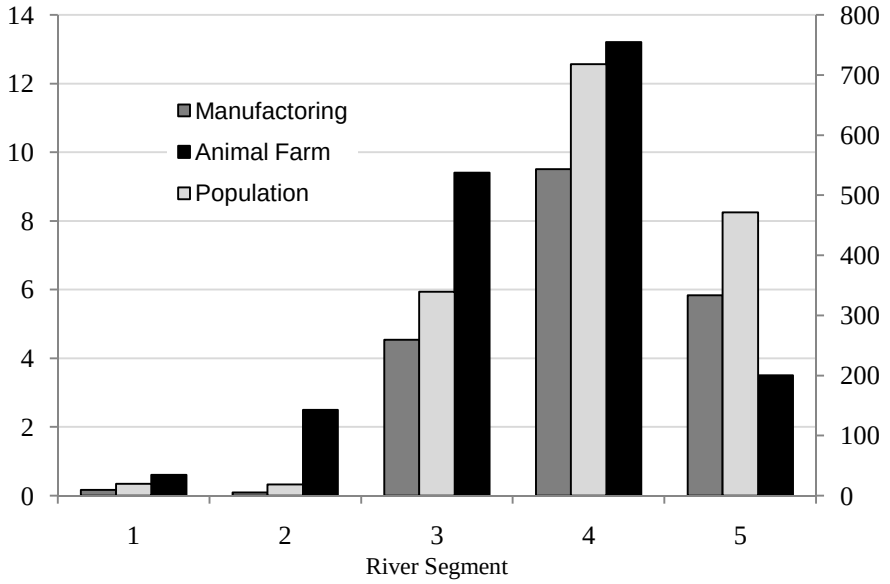

Figure 2.4 Density (number/km^2) of manufacturing activities, animal farms (left scale) and population (right scale) referred to each River Serio segment.

Hydromorphological - Water flow reduction is a known problem affecting all length of River Serio. Nevertheless, only a qualitative description of this driver has been provided, since no sound data about natural flow rate have been found. Water flow alteration is due to water withdrawals: 3 withdrawals are recorded in first r.s., 2 in the second, 19 in the third, 2 in the fourth and 4 in the fifth. Upstream (until r.s. 3) withdrawals are due to hydropower production, so water is returned downstream. From the second part of r.s. 3 withdrawals are due to irrigation, so water is not returned to the river. Major water flow alteration regards some parts of r.s. 4, that are often dry for several days a year. R.s. 5 shows less alteration because of the presence of some tributaries and springs.

To confirm the suitability of River Trebbia as a reference river, in Table 2.6, the number and typology of discharge points of the river are reported. Inhabitants in the watershed are 24500. 90% of the households are connected to wastewater treatment plants. Considering a total amount of 44 industrial discharge points, they are mainly in the last segment of the river. Agriculture is present especially in the plain area (related to river segment 5). It must be noted that the 5th r.s. probably suffers for some flow alteration due to water use in agriculture. So its use as reference system could be questionable.

Table 2.6 Number of discharge points, per typology, in the river segments of Trebbia (modified from ARPA 2008).

	Total number of discharge points	Number of urban discharge points	Number of industrial discharge points
Rs 1 and 2	174	174	0
Rs 3	41	40	1
Rs 4	62	44	18
Rs 5	51	26	25

To get a semi-quantitative description of the intensity of stressors, a scoring system, functional to this particular case study is shown in Table 2.7. The scoring system (with the exception of hydromorphological stressor) was built on the basis of qualitative observations of minimum and maximum values on a European scale for each parameter.

Table 2.7 Categorization of stressor indicators. Edge values are comprised in the major class. Classes 4 and 5 of hydromorphological stressor consider minimum flow requirements (MFR) as the last trigger.

	0	1	2	3	4	5
Percentage of agricultural land use	0 - 5	5-15	15-25	25-50	50-75	> 75
Animal farms density (farm/Km^2)	0-1	1-2	2-3	3-4	4-5	>5
Population density (inhabitants/Km^2)	0-100	100-500	500-1000	1000-2500	2500-5000	>5000
Manufacturing activities / Km^2	0-3	3-7	7-10	10-20	20-60	>60
Flow rate variation (%)	0-10	10-20	20-40	40-60	60-MFR	>MFR

Table 2.8 shows the application of the scoring system to the different river segments of River Serio. Scoring of flow rate changes is based on qualitative information, as no quantitative data have been found.

Table 2.8 Magnitude of stressors in River Serio for each river segment.

	1	2	3	4	5
Urban – population density (inhabitants/Km^2)	0	0	1	3	1
Industrial - (manufacturing activities / Km^2)	0	0	1	2	1
Agriculture- percentage of agricultural land use	0	0	0	4	4
Agriculture- Animal farms density (farm/Km^2)	0	1	3	4	2
Flow rate variation (%)	4	3	3	5	2

The combination of vulnerability related to each potential stressor and the magnitude of the stressors (considering the actual watershed conditions) lead to an evaluation of the potential alteration of a natural community in the river. This procedure was proposed to perform a preliminary validation of the presented methodology. Therefore, vulnerability scores (Table 2.4) may be multiplied times the stressor magnitude scores (Table 2.8), giving the results reported in Table 2.9. This table gives a semi-quantitative indication of the level of possible alteration produced by actual stressors on pristine macrobenthos community. A sum of all potential alteration for each river segment is also reported, in order to give an indication of the total pressure on the river segment. However, the total value must be taken with care, because effects of different stressors may not be additive.
By comparing community data in River Serio with those of River Trebbia (Table 2.3) and with potential alteration assessment (Table 2.9), the following comments can be made.

Table 2.9 Potential alteration in each River Serio segment.

	1	2	3	4	5
Urban	0	0	11	30	7
Industrial	0	0	6	12	6
Agricultural (crop)	0	0	0	10	10
Agricultural (farms)	0	11	33	40	14
Hydromorphological	12	9	6	10	2
Total	12	20	56	102	39

River segment 1. The community appeared in good quality, as indicated by the high value of EBI and the presence of sensitive species. However a decrease of systematic units, in comparison with reference community may be related to a non-specific stressor, like flow rate alteration.

River segment 2. Further reduction of systematic units and decrease of EBI value could be related to oxygen depletion due to the presence of animal farms.

River segment 3. This segment was represented by three sampling stations. However, the first one should be assumed as representative of conditions close to r.s. 2. In the other stations the quality of macrobenthos community substantially changed. All taxa particularly sensitive to oxygen concentration disappeared or reduced significantly while systematic units of other taxa remained more or less constant. This result fits well with the assessed influence of animal farms and urban stressor.

River segment 4. The community showed the biggest alteration with respect to the reference. The number of total systematic units decreased, reaching the lowest level of the entire river. EBI score was also the lowest. All the stressors reached the biggest score in this segment and each could have important effects on the community. Crustaceans appeared in this section, represented only by Asellidae, while in the reference community Gammaridae were also present. It is worth noting that Asellidae are very resistant to toxicity of some chemicals (e.g. insecticides) while Gammaridae are extremely sensitive (Bonzini et al. 2008; Sala et al. 2012).

River segment 5. The quality of the community in this segment is improved, as indicated by the slight increase of EBI and of the number of systematic units, as well as by the presence of some sensitive groups (Tricoptera, Gammaridae). Indeed, potential alteration substantially decreases for almost all stressors and is even lower than in segment 3. However, it should be considered that the river represents a continuum, so the quality of this segment may be influenced by the very bad quality of the previous one.

2.5 Discussion and conclusions

As highlighted by De Lange et al. (2010) there is a lack of methods for assessing and quantifying ecosystem vulnerability to human stressors, suitable to be used in site-specific ecotoxicological risk assessment. This paper describes a first vulnerability indexing method for the aquatic environment, suitable to assess different stressors likely to occur in riverine ecosystems. In spite of some simplifications, the application of the method to a case study seems to provide satisfying results. The composition of the community of River Serio fits quite well with the predictions possible on the basis of the vulnerability of the reference community and of the intensity of stressors. However, it must be underlined that the described procedure represents a preliminary proposal that need to be refined and improved and needs additional information for a better quantitative estimation of the data to be used in the assessment. An overview of the major needs for more knowledge and information and of some conceptual drawbacks is given below.

1. All indicators based on scoring systems and synthetic algorithms suffer for a large margin of subjectivity and arbitrariness. Moreover, some of the assumptions and comments have been based on “expert judgements” of the authors and not on more or less codified evaluation approaches. The effectiveness and reliability of the vulnerability index of equation 2.1, as well as of the whole procedure, must be validated and calibrated

through the application on many other aquatic ecosystems with different characteristics and typologies. Moreover, some more scientifically-based procedures should be developed for the comparison of the structure of biological communities, in order to evaluate the significance of some observed changes.

2. The sensitivity of taxa to different stressors is a key point for estimating the sensitivity of the community. For the macrobenthos community, this is well known for oxygen content and some reliable information is available for some toxic chemicals, such as some insecticide classes (Bonzini et al. 2008; Sala et al. 2012). In this paper, the sensitivity to insecticides has been applied in general to toxic chemicals. It has been an arbitrary choice, due to the need to apply the procedure without sound scientific information available. More information is needed for a sound science-based knowledge on species sensitivity distribution to different stressors in aquatic communities.
3. Community vulnerability assessment has been mainly based on considerations on individual populations, in particular on these populations that are significant for a given stressor. However, most critical issues arise moving from population to community level. For none of the three considered components, community properties could be inferred from the sum of single population features. Emergent properties and indirect effects, due to complex relationships between different species in ecosystems, prevent the use of a reductionist approach in this phase. It is recognized that, up to date, our descriptive and predictive capability of ecological hierarchical levels is the lowest at the community level (van Straalen 2003). The implementation of tools capable to provide integrated responses at the community levels is a challenge for ecotoxicology in the next future.
4. Interaction must be taken into account not only at the population-community level, but also at the stressor level. Even if some pragmatic approaches can be proposed for mixtures of toxicants (Verro et al. 2009), the interaction of multiple stressors (physical, chemical, etc.) is far to be additive. More knowledge is needed for assessing cumulative effect of multiple stressors.
5. All simplified approaches like those described above, presume that the response of bio-ecological entities (individuals, populations communities) to a stressor follows a continuous trend (linear, logistic, exponential, etc.). However, discontinuous responses are possible. For example, the Rivet Hypothesis (Ehrlich and Ehrlich 1981) presumes that each loss of a species affects ecosystem integrity to a small extent; if too many rivets are lost, the system collapses. This confirms our lack of knowledge on community functioning.
6. Other approximations are referred to the specific case study. The description of driving forces, pressures and stressors has been strongly

simplified. For example, for industry only chemical toxicity has been considered, whilst industrial pressure may be extremely variable as a function of industrial typology. This kind of detail requires a more careful assessment of land use and of activities in the watershed that was not the objective of this paper.

In spite of all these drawbacks, the method represents an attempt to estimate and to express in quantitative (numeric) terms a property of ecosystems extremely important for environmental protection and management, frequently overlooked risk assessment procedures.
Vulnerability assessment may also allow a better definition of Environmental Quality Standards of chemicals in surface water, as required by the WFD.
Comprehensive vulnerability assessment has to take into account all the potential stressors that may reach the water body as WFD addresses the potential ecological risk of all toxic chemicals. The proposed method allows considering the relative importance of a single stressor and the potential magnitude of the complex system of different stressors considering spatial differentiation.
Indeed, environmental risk assessment in the context of WFD is based on two assumptions: ecosystem sensitivity depends on the most sensitive species and protecting ecosystem structure protects community function (EC 2003).
Three main categories of effect could be taken into account for freshwater ecosystems: effect on ecosystem structure, ecosystem function, aesthetic and economic values. Therefore, ecological protection goals have to be fixed in order to guarantee also ecosystem services provided by the aquatic ecosystems.
Furthermore, sustainability of freshwater ecosystems involve not only their ecological properties but also their economic and social function and imply also the need of assigning to ecological vulnerability an assessment of economic and social issues, related to ecosystem capability of providing ecosystem services and their related economic and social values (Brock et al. 2006).
The ecosystem services and values assessment represent a relevant tool for addressing risk management. According to Brock et al. (2006), the following ecological impacts may be considered important from a scientific and stakeholder point of view: decrease in biodiversity; impact on ecosystem functioning; decrease in perceived aesthetic value and functionality to humans. The same authors also underline the relevance of spatio-temporal differentiation in ecological protection goals and propose the harmonisation of the different scientific approaches for ecotoxicological risk assessment adopted in guidance documents. A differentiation in the protection level of aquatic habitats, related to the level of vulnerability, may contribute to a

more focused risk assessment that takes into account perceived difference in functionality and intrinsic value of surface water.

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3 Sensitivity assessment of freshwater macroinvertebrates to pesticides using biological traits

Abstract
Assessing the sensitivity of different species to chemicals is one of the key points in predicting the effects of toxic compounds in the environment. Trait-based predicting methods have proved to be extremely efficient for assessing the sensitivity of macroinvertebrates toward compounds with non specific toxicity (narcotics). Nevertheless, predicting the sensitivity of organisms toward compounds with specific toxicity is much more complex, since it depends on the mode of action of the chemical.
The aim of this work was to predict the sensitivity of several freshwater macroinvertebrates toward three classes of plant protection products: organophosphates, carbamates and pyrethroids. Two databases were built: one with sensitivity data (retrieved, evaluated and selected from the U.S. Environmental Protection Agency ECOTOX database) and the other with biological traits. Aside from the "traditional" traits usually considered in ecological analysis (i.e. body size, respiration technique, feeding habits, etc.), multivariate analysis was used to relate the sensitivity of organisms to some other characteristics which may be involved in the process of intoxication. Results confirmed that, besides traditional biological traits, related to uptake capability (e.g. body size and body shape) some traits more related to particular metabolic characteristics or patterns have a good predictive capacity on the sensitivity to these kinds of toxic substances. For example, behavioral complexity, assumed as an indicator of nervous system complexity, proved to be an important predictor of sensitivity towards these compounds. These results confirm the need for more complex traits to predict effects of highly specific substances. One key point for achieving a complete mechanistic understanding of the process is the choice of traits, whose role in the discrimination of sensitivity should be clearly interpretable, and not only statistically significant.

Keywords: sensitivity prediction, pesticides, traits, freshwater macroinvertebrates, multivariate analysis, chemometrics

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3.1 Introduction

Current procedures of Ecotoxicological Risk Assessment (ERA) for chemicals rely on both the evaluation of generic exposure and effects. Exposure is often based on a predicted environmental concentration (PEC), while effects are usually extrapolated from measurements of toxicity on single species lab-tests. Nevertheless, actual responses of biological/ecological systems strongly depend on some complex characteristics they have. These characteristics determine the vulnerability of the system, from the low hierarchical level of organisation such as population, to the highest levels like community, ecosystem and landscape.
Vulnerability is usually considered as a function of three different elements: susceptibility to exposure, direct sensitivity and recovery capability (resilience) (Ippolito et al. 2010; De Lange et al. 2010; Turner et al. 2003). This general framework is essentially confirmed by the population vulnerability conceptual model proposed by Van Straalen (1993), which identifies three categories: external exposure, intrinsic sensitivity and population sustainability.
Direct sensitivity is certainly one of the most studied issues in ecotoxicology. Empiric-experimental methodology has led to a great number of laboratory tests, in which the toxicity of a huge number of chemicals has been "measured" over a relatively small number of species. This approach has been of great importance in the past, in order to determine more or less general guidelines for regulatory purposes. Nevertheless, it appears unsuitable to apply in the future, since ERA is moving towards more site-specific studies and evaluations. Site-specific risk assessment procedures are becoming common even on a regulatory level (the Water Framework Directive, Directive 2000/60; EC, 2000 is probably the most cited example in this sense). The main focus of this "new generation" of ERA procedures is on specific ecosystems, whose function and structure need to be preserved. It is widely acknowledged that the extrapolation of laboratory single-species tests to assess the vulnerability of ecosystems is a major factor of uncertainty in the risk assessment procedures. One of the main reasons for this, though not the only, is determined by our lack of knowledge about the intrinsic sensitivity of many species towards a certain exposure. Experimentally measuring the sensitivity of all the species present in a certain ecosystem towards exposure from each potential chemical (every possible species/compound combination) appears unsustainable. Even omitting ethic implications, the choice of performing such a huge amount of tests would cost too much in terms of time and money. Furthermore, ecotoxicological tests are potentially subject to errors, biases, unrepeatability, etc.
A proposal for a "rapid test" on complex multi-species communities has been made by Kefford et al. (2005). However, precise protocols for these

procedures have still not been developed and the comparability of the results is questionable.
To deal with this problem, several good alternative methods, often inferential, have already been developed in the past. Nevertheless, their use sometimes is unsuitable or anyway insufficient, mostly for site-specific risk assessment.
One good example is provided by Species Sensitivity Distribution (SSD, Posthuma et al. 2001), which allows to calculate the percentage of species at risk for a certain exposure (Hazardous Concentrations, HCs). Although this tool has shown to have a good predictive power on general evaluation of communities, it cannot accurately assess which population would be really at risk as a consequence of a certain exposure. Similar levels of uncertainty are unacceptable in some cases, especially when we deal with high conservation value communities (Baird and Van den Brink 2007) or in presence of keystone species. Another good tool for the prediction of sensitivity are the Quantitative Inter-specific Chemical Activity Relationships (QICAR: see Tremolada et al. 2004 and Dimitrov et al. 2000 for an earlier formulation of the concept), which, nonetheless, have only been tested on an extremely small number of species.
In order to perform accurate predictions, we probably need to go towards a mechanistic understanding of the processes which determine the sensitivity of organisms to different chemicals. It seems reasonable to think that the degree of intoxication of a certain compound in an organism is determined by some physico-chemical characteristics of the compound (that is the approach followed by QSAR - Quantitative Structure-Activity Relationship, see the examples of Van Leeuwen et al. 1992 and Todeschini et al. 1996), as well as by some morphological, physiological and metabolic features of the organisms.
This kind of approach has been recently proposed by Baird and Van den Brink (2007), which successfully related biological macroscopic traits with the sensitivity of a limited number of species toward chemicals without highly specific modes of action (narcotics, polar narcotics, metals). Since this path seems promising, it was adopted in this work on a broader scale, in order to relate some biological traits to the sensitivity shown by a greater number of species towards some insecticides, which usually have a specific toxicity. A similar attempt, with a slightly different methodology from what is proposed here, has been already made by Rubach et al. (2010).
The use of biological and ecological traits is one of the new frontiers of ecological risk assessment. Their first systematic use in ecology dates back to the early nineties, when traits distribution in rivers was related to habitat heterogeneity (Upper Rhone River case study, see for example Usseglio-Polatera 1994) following the habitat templet-environmental filter concept (Southwood 1977; Townsend and Hildrew 1994; Poff 1997). Only afterwards was traits distribution in rivers used to discriminate different

anthropogenic stressors with biomonitoring (Dolédec et al. 1999; Statzner et al. 2001). More recently, the biomonitoring of biological and ecological traits has been used to develop an index based on the vulnerability of macrobenthonic fauna. This index (SPEAR – SPEcies At Risk, Liess and Von der Ohe 2005), initially developed to assess pesticide-induced impacts, showed to have good predictive power even for other chemical stressors (Beketov and Liess 2008).
The line followed in this paper, the use of traits to predict sensitivity of organisms, is only the last approach of what has been already called TERA (Trait-based Ecological Risk Assessment, Baird et al. 2008).
The potential power of biological traits resides mainly in their independence from the standard taxonomy, which has been used for a long time as the only measurement unit in ecotoxicological indexes. While standard taxonomy depends on phylogenetic evolution, the response of organisms after a potentially harmful exposure (both short and long-term response), depends on their current characteristics. That would explain why phylogenetically closely related organisms can show huge differences in sensitivity to the same chemical, while phenomena of evolutionary convergence could explain similar responses of taxa which are far apart in phylogeny.
Biological traits are then a representation of the *status quo* of the species, without historical perspective, which is nonetheless the basis of the functional role played in the community ("Whereas taxonomy can be regarded as a higher-level expression of the genetic composition of organisms, traits can be seen as their functional consequence", Baird et al. 2008).
The use of ecological and biological traits can be seen as the "missing link" between the structure and the function of the community.

3.2 Materials and methods

The largest available number of freshwater macroinvertebrates (planktonic and benthic) has been considered in order to evaluate the actual power of prediction exerted by biological traits on the sensitivity of organisms. In this case we considered the sensitivity of macroinvertebrates toward three of the most common classes of insecticides: organophosphates, carbamates and pyrethroids.
Data have been collected for the implementation of two different databases: the first one gathers information on the sensitivity of species tested against the selected substances; the second one gathers biological information which is numerically coded for the same species considered in the first database. The second database has been used to analyze the biological proximity of the taxa, using the selected biological traits. The results have then been

compared with the standard taxonomy, in order to enhance the differences between the two methods of aggregation.
The two databases have been crossed with particular attention to variable selection, operated by genetic algorithms, in order to model the sensitivity of organisms on the basis of their biological traits (Figure 3.1).
Finally, the influence of the most significant variables has been evaluated, considering the differences between the mode of action of the chemicals.

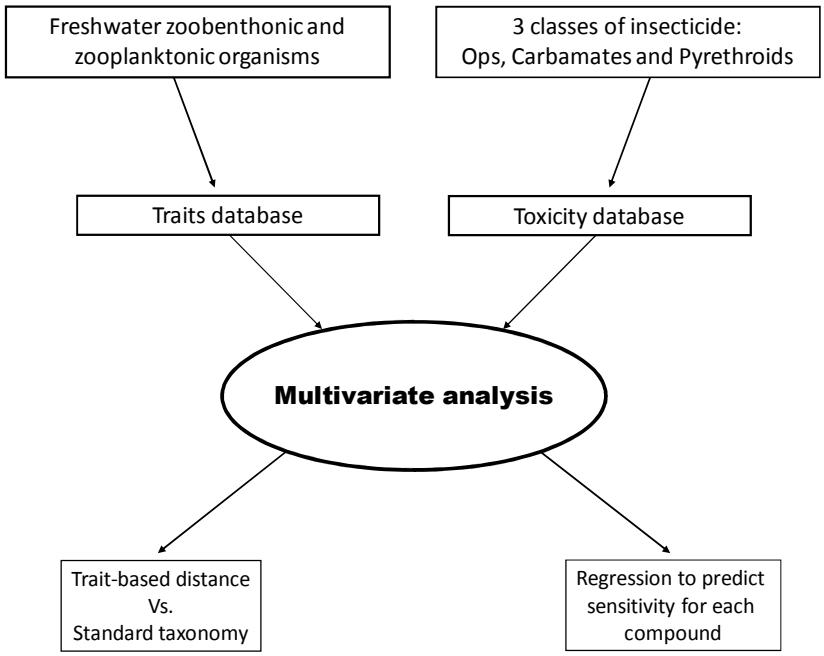

Figure 3.1 Work scheme followed: one database of toxicity data and another one of biological traits were implemented. The two databases were crossed with techniques of multivariate analysis to produce: 1) a trait-based dendrogram to evaluate the relative distance between taxa and 2) regression models to predict sensitivity of organisms to each compound.

3.2.1 Toxicity database

The main source of information for the gathered toxicity data was the U.S. Environmental Protection Agency (U.S. EPA) ECOTOX database, (http://cfpub.epa.gov/ecotox/, free access, data retrieved on 17-11-2009).
From the database it is possible to download the results of queries in .xls format. For this analysis the same array of input parameters (with the exception of the chemical entry) was always used. In order to have an exact identification of the chemicals the CAS (Chemical Abstracts Service) number was used as entry for the queries. CAS numbers were retrieved from the Pesticide Manual (Tomlin 2003).

Apart from the identification of the tested species, the CAS number of the substance, the indication of the measured endpoint and the results, the downloaded reports of the queries include a huge additional bulk of information for every single record; the indication of the exposure duration, the test medium, the life stage of the organisms and the original reference from which the data were retrieved showed to be of great importance among the others.
Besides the ECOTOX database, further data were found on a previous paper-database edited by the U.S. Fish and Wildlife Service (Mayer and Ellersieck 1986). A few data were retrieved from the Pesticide Manual, but to a much lesser extent.
The first selection of the gathered data was performed on the basis of the tested organisms. Only freshwater macroinvertebrates (planktonic and benthic) were considered. This choice is due to the abundance of information about these kinds of organisms, both from a toxicological and a biological point of view. Furthermore, it should be noted that many of these organisms are commonly used in several ecological quality indexes. Data gathered refer to insects (larvae and adult), crustaceans, molluscs, annelids and flatworms.
Data retrieved from the ECOTOX database presented a huge heterogeneity. No specific quality criteria are applied for the inclusion of a publication data in the database. The only criteria refer to the validity of the tested species (scientific names), validity of the chemical (ECOTOX staff must be able to locate the CAS# of the tested compound) and the indication of exposure duration
(http://cfpub.epa.gov/ecotox/help.cfm?help_id=GENERALFAQ&help_type=define&help_back=1#notdatatype).
In order to use only comparable information, with verified quality, a 5-step selection was performed on the retrieved data (Figure 3.2).

1. **Availability of the test procedure (binding criterion)**: as a first step the availability of the original paper from which the datum was derived was verified. If it was impossible to find it, correspondent datum was deleted. If the entire procedure of the test was reported, datum was kept; otherwise it was deleted. An exception was for the data produced with certified/standard procedures.
2. **Test typology evaluation (binding criteria)**: only acute toxicity data were considered, referred to an exposure duration from 24 to 96 h. Data were kept only if they referred to aquatic toxicity, not of other kinds of exposure (oral, topic, etc). Data were deleted if the measured endpoint was different from LC_{50} or similar (i.e. EC_{50} immobility). Since most of the data available are obtained trough static tests with almost constant (nominal or measured) concentration, no specific criteria were adopted for the selection.

3. **Experimental test design evaluation (non binding criteria)**: several parameters were considered in order to rate the quality and the reliability of each procedure. Non-compliance of one of these criteria did not lead to the automatic deletion of the datum. Each test was evaluated case by case in order to determine its reliability.
4. **Evaluation of the data analysis (non binding criteria)**: statistic methodologies used in each test to produce the data were evaluated. Again each test was evaluated case by case without rigid exclusive criteria.
5. **Selection of data within the same work (binding criteria)**: regardless of the quality of work, some data are referred to particularly resistant strains or to organisms selected in order to show strong resistance toward the tested chemical. In all these cases, data were not considered.

Since data were still referred to different exposure times (24, 48, 72, and 96 hours), in order to get a more homogeneous database, data were normalized after the selection. Most of the selected original data referred to 24-hours tests, so the entire database was normalized to this exposure time. Normalization was performed through the use of a conversion table (Table 3.1). The aim of this conversion was not to produce very accurate extrapolations, but the use of averages for each taxon helped to compare data which were otherwise incomparable.
The table provides a multiplicative factor for LC_{50}, for every taxon/exposure time combination derived from statistically significant correlations among toxicity data obtained on the same species at different exposure times (Sala et al. 2012). The obtained value is a reliable assessment of the 24 hours LC_{50}.

Table 3.1 Conversion table for the time normalization of toxicity data. The combination of test duration/ type of organism gives the multiplicative coefficient to be applied for the data. The coefficients refer to organophosphorus insecticides and are obtained from statistically significant correlations ($p<0.01$) (modified after Sala et al. 2012).

	24h	48h	72h	96h
Zooplankton	1.00	2.50	3.13	5.00
Benthic crustaceans	1.00	2.50	3.50	5.00
Insect larvae	1.00	2.22	2.78	4.89
Insect adults	1.00	2.50	3.50	5.00
Molluscs	1.00	1.50	2.17	3.33

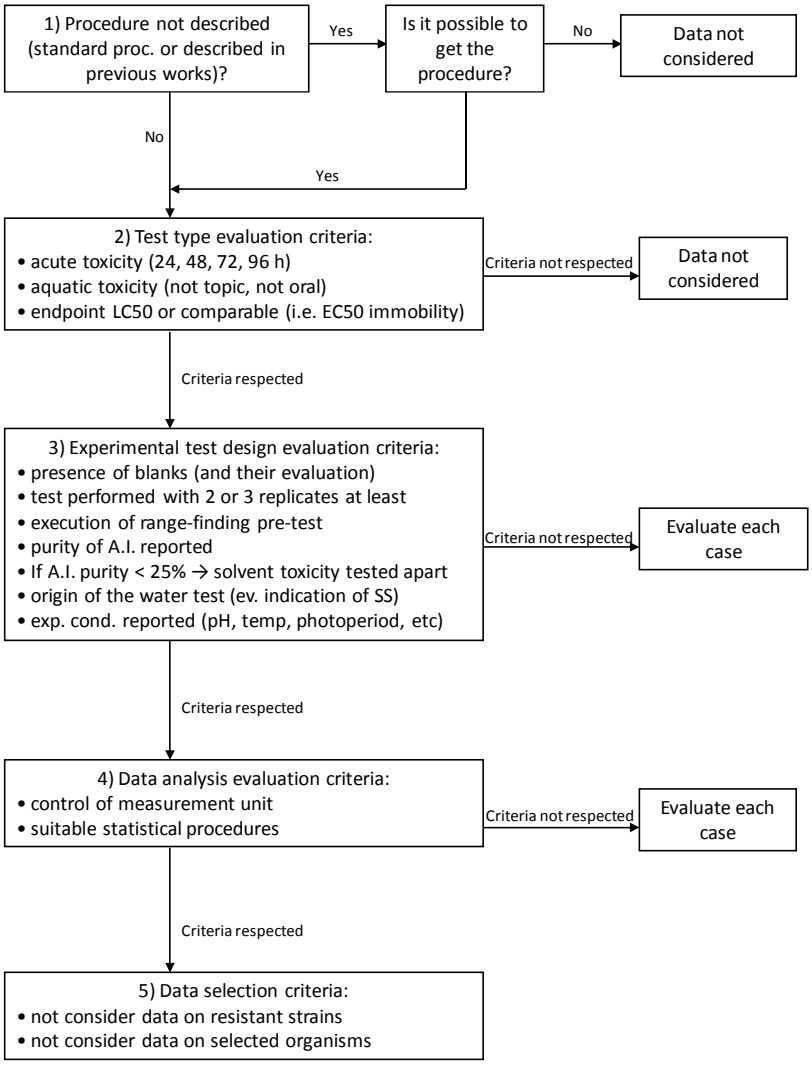

Figure 3.2 Flow chart of the methodology for the selection of toxicity data. Selection was performed on the basis of the quality of the original paper the data were retrieved from.

The reason for selecting short term acute toxicity data is not only their higher availability. Pesticide concentrations in surface water, particularly in rivers, are characterised by peaks corresponding to application dates and runoff events, so the aquatic community is generally exposed to pulses of high concentrations (sometimes at acute toxicity levels) instead of continuous chronic levels (Verro et al. 2009). Therefore, using the terminology adopted for the environmental quality standards under the Water Framework Directive (WFD EQSs), it is reasonable to refer to the risk as a maximum acceptable concentration (MAC), in order to protect against possible effects

from short term concentration peaks, instead of an annual average concentration (AA) to protect against the occurrence of prolonged exposure (EC 2011).
Normalized data were then aggregated at the species level. If more than one single value was present for each species/compound combination, then the available data were averaged through a geometric mean. In case data originally referred to a 24 hours test were present, they were preferred to other data originally referring to other exposure times (which were excluded from the averages). Different life stages of the same species were treated separately (as if they were different species), especially when precise information was available for each stage (i.e. average dimensions, etc.).
Average values, expressed in µg/L, were transformed in µmol/L. Molecular weights of the respective substances were retrieved from the Pesticide Manual (Tomlin 2003).
Due to the extreme variability in the sensitivity shown by the considered organisms (up to 5 order of magnitude for the same chemical), each datum was expressed as the inverse of the logarithm (base 10). Using the inverse of LC_{50} we obtained a value (variable Tox., Eq.3.1) proportional to the toxicity of the compound.

$$Tox = LOG\left(\frac{1}{LC_{50}[\mu mol/L]}\right) \qquad (3.1)$$

A big number of the most common substances for each class of insecticides was initially considered.
The substances were then selected on the basis of the quantity of available data (after the first 5-step selection). At first, substances with less than 50 observations were not considered for the statistical analysis; this was done to avoid possible biases due to small datasets. Nevertheless, no pyrethroids out of the 17 initially considered met this criterion after the data selection, so the trigger was dropped to 30 observations for this class. Obviously, this choice leads to a weaker analysis in statistical terms, but we refused to exclude such an important class of pesticides.
Two pyrethroids (cypermethrin, deltamethrin), four OPs (organophosphates) (malathion, chlorpyrifos, parathion, fenitrothion), and only one carbamate (carbaryl) were finally investigated

3.2.2 Trait database

The need for a shared, unifying encoding system for biological traits of organisms has been widely acknowledged. Nevertheless, current available trait-databases show inconsistency in trait definitions (Baird et al. 2008). Furthermore, these databases usually provide information referred to the high taxonomic level (usually family level or superior, very rarely at genus

or species level). For our aims, however, the best available resolution level is needed, due to the significant differences in sensitivity between species within the same taxon (or even life stages of the same species).
Considering these issues, different sources were investigated. Some very important references were other trait databases which try to harmonize information available in the literature: two already published databases (Tachet et al. 2002; Vieira et al. 2006), one free on-line database (Henegan et al. 1999) and one restricted-access on-line database (Schmidt-Kloiber and Hering 2010). To improve our own database and to get information at the lowest available taxonomic level, a huge number of different sources were also considered: peer-reviewed scientific papers, grey literature and even non-scientific material (especially websites). The same papers evaluated for the toxicity database, sometimes provided useful information even for this database. A complete list of all references used for the implementation of the traits database is available in the *Appendix 2*. Despite the efforts, it was not possible to find all the information at the species level. When information was not available for a species, the information related to the closest species, or to the immediately higher taxonomic level (e.g. genus, family) was used.
10 traits were considered, mostly derived by Tachet et al. (2002) and Vieira et al. (2006), which in our opinion could have some possible influence in determining the response of organisms toward toxic chemicals. More biological traits were considered, overlooking most of the ecological characteristics. Some of these characteristics (salinity preferendum, temperature preferendum, pH preferendum, etc.) could have a certain influence in determining the sensitivity measured in standard conditions. For example, a static test at 20°C could determine a situation of stress (though not detectable in the control) in a species which prefers fast current velocity and lower temperature. The stress can artificially enhance the sensitivity of the organism. Nevertheless, our analysis aims to find a traits-sensitivity relationship which can be used in the environment, where species are likely to be found in their ecological optimum. Other ecological traits, such as "dispersal ability" are simply not conceptually related to sensitivity, i.e. even if a correlation between this kind of traits and sensitivity is found, no deterministic hypotheses can be made about how the former can influence the latter. The focus then was on biological traits which can have an understandable influence on the sensitivity of organism. Seven traits were taken from Tachet et al. (2002) and Vieira et al. (2006):

- Body length (as a proxy of general body size)
- Life cycle duration
- Number of reproductive cycles per year (potential)
- Respiration technique
- Feeding habits (feeding typology)
- Armor (degree of sclerification)
- Body shape

It is worth noting that "maximal potential size" (as it is in Tachet et al. 2002), was substituted by (actual) body length. This discrimination allows to explain differences in the sensitivity of two life stages of the same species. When no precise information was available about the length or the life stages of the tested organisms, average values retrieved from the literature were used.
Three new traits were introduced, which, to our knowledge, were never used before in this kind of analysis. These are:

- Degree of ramification (related to the surface/volume ratio)
- Internal mechanisms of O_2 transport (related to the capability of internal transport of chemicals)
- Behaviour complexity (proxy of nervous system complexity)

Since both acetylcholinesterase (AChE) inhibitors (OP and carbamates) and axonic toxicants (pyrethroids) exert their action on the nervous system of organisms, we decided to investigate whether structural nervous complexity can be related to sensitivity towards these chemicals. The idea is that complex, very specialized systems can be more vulnerable than simpler ones.
Nevertheless, assessing structural complexity is not an easy task: how can we quantify nervous system complexity? Deamer and Evans (2006), proposed an easy equation which quantifies nervous structural complexity on the basis of the number of neurons and interconnections between them. Though the equation is very simple, very scarce data are available in the literature. Some authors, in addition, criticized even the possibility of a reliable estimation of the total neurons of organisms (Laverack 1988).
Structural complexity of the nervous system is usually reflected in a behavioural complexity (Koch and Laurent 1999), so we tried to roughly quantify the behavioural complexity of the investigated organisms. To do so, we selected three very important behavioural patterns: main movement, feeding attitude and predation avoidance attitude.
The way an organism moves not only reflects on the degree of control which it has on the body, but it's also an indicator of the precision of the perception of the environment. A strong swimmer, for example, needs to quickly detect obstacles in a 3-d space, while crawlers usually move slower in a 2-d space; sessile animals do not even move, so they do not have the same need. Similarly, an organism could be a quasi-passive consumer (filter feeder for example) or have a more active feeding behaviour. In this view, the most complex behaviour is shown by predators, which feed on "active material". For the same reason, the predation avoidance attitude was investigated to assess behavioural complexity.
The surface/volume ratio was initially included in the analysis as another variable, since its importance in toxicokinetics is well known. Nevertheless, very poor information was found on such parameter. An attempt to assess

the ratio was made, approximating each organism to a solid 3-dimensional figure (ellipsoids, cylinders, spheres, etc.). Nevertheless, we had little chance to validate our assessment. Furthermore, the assessed values showed a strong collinearity with the "body length" variable. Due to these problems the surface/volume ratio was not included in the analysis.
In a first attempt each trait was quantified and coded using the "Fuzzy coding approach" (Chevenet et al. 1994), which uses positive scores (from 0 to 1) to describe the affinity of a species for different modalities (categories) of the same variable (trait). The sum of the affinity values of the same variable is always 1. This approach was shown to be effective for pure categorical (nominal) variables, while it was not for ordinal variables (both continuous numerical or categorical). For continuous variables the fuzzy coding approach divides the entire range of variation into a certain number of intervals, which behave like independent categories. Fuzzy coding hides the rank of organisms for a certain variable (trait) and really complicates the interpretation of the results. Then we used a mixed approach: all nominal variables were fuzzy-coded, while all ordinal variables were not divided into intervals, but they were assigned a single value from 0 to 1. This value was chosen in one case on a continuous scale (body length) while all the other variables were coded using discrete values (always in a range between 0 and 1). Coding criteria for all the variables are represented in Table 3.2.
For behavioural complexity we assign a score from 0 to 1 for each of the three behavioural patterns, and then we made an average of the three scores. For each pattern several categories are defined. The complete algorithm used to calculate the final value of the variable "behaviour complexity" (BE) is reported in eq.3.2.

$$BE = \frac{\sum_{k=1}^{3}\sum_{i=1}^{n} aff_{i,k} \times coef_{i,k}}{3} \qquad (3.2)$$

Where:
aff = values attributed to the affinity of a species for the *i*-ith category (from 0 to 1, the greater the value, the greater the affinity).

coef = coefficient typical of the *i*-ith category (from 0 to 1, the greater the coefficient, the greater the behavioural complexity determined by the category).

n = number of categories of *k*-ith pattern

Note that, for each behavioural pattern:

$$\sum_{i=1}^{n} aff_i = 1 \qquad (3.3)$$

Categories and relative coefficients for each behavioural field are reported in Table 3.3.

Biological information was collected on 253 species (or life stages of species), belonging to 144 genera, 79 families, 32 orders and 10 classes (see *Appendix 2*).

Table 3.2 Traits codification and quantification. For pure categorical (nominal) variables a value (from 0 to 1) is attributed to the different categories as a function of the affinity. The sum of the affinity values of a variable is 1.

Variables	**Categories (where present)**	**Values**	**Code**
1) Body length		Length (mm)/100	BL
2) Life cycle duration	< 1 year	0	LD
	= 1 year	0.5	
	> 1 year	1	
3) Reproductive cycle / year (voltinism)	< 1/year	0	VL
	= 1/year	0.5	
	> 1/year	1	
4) Respiration technique	Tegument		RT_te
	Gill	Affinity	RT_gi
	Plastron	(form 0 to 1)	RT_pl
	Aerial		RT_ae
5) Feeding habits	Deposit feeder		FH_df
	Shredder		FH_sh
	Scraper	Affinity	FH_sc
	Filter feeder	(form 0 to 1)	FH_ff
	Predator		FH_pr
	Parasite		FH_pa
6) O_2 transport	Tracheal closed		OT_tc
	Tracheal open	Affinity	OT_to
	Blood hemocyanin	(form 0 to 1)	OT_hcy
	Blood hemoglobin		OT_hgl
	Blood no pigments		OT_nopg
7) Degree of sclerification (armor)	None	0	AR
	Head sclerotized	0.25	
	Body partly sclerotized	0.5	
	Full sclerotized	1	
8) Behaviour complexity		*	BE
9) Body shape	Linear		BS_li
	Dorsoventrally flattened	Affinity	BS_dvf
	Laterally flattened	(form 0 to 1)	BS_lf
	Solid ellipsoidal		BS_se
10) Ramification	Almost none	0	RA
	Poor	0.3	
	Medium	0.6	
	High	1	

* Detailed explanation of Behaviour complexity values is reported in Table 3.3.

Table 3.3 Scoring system used for the assessment of behavioral complexity. Three behavioral patterns were evaluated. For each pattern several categories are defined. For each species the affinity value (from 0 to 1) for the different categories is attributed and is multiplied by the respective coefficient. Obtained values are summed within the same behavioral pattern. The final score is the average of the scores calculated for each pattern.

Behavioral pattern	Category	Coeff.
Main movement	Sessile	0
	Crawler	0.3
	Poor swimmer	0.6
	Skilled swimmer	1.0
Main feeding attitude	Passive consumer	0
	Active consumer on passive material	0.5
	Active consumer on active material	1.0
Predation avoidance attitude	Passive	0
	Active	1.0

3.2.3 Statistical analysis

A first, preliminary analysis has been performed on the trait database only. Euclidean distances (complete linkage) between organisms in the 25-dimensional space (representative of the 25 traits and traits categories, here treated like independent variables) were calculated and represented by a dendrogram. We assigned the same weight to all the traits, since we still ignored which traits had major influence for what concerns our purpose.
The obtained dendrogram was then used to compare Euclidean trait-based distances with taxonomic affinity. The aim of this analysis was to verify whether "actual" distances between taxa differ from phylogenetic ones, in order to justify on a quantitative level the suitability of a non taxonomic-based analysis.
The second analysis researched a relationship between biological traits and sensitivity for the selected substances. Variable "Tox" was our dependent variable, while traits were our predictors.
The aim then was to verify to what extent is true that:

$$Tox = f\left(trait_1; trait_2; ...; trait_p\right) \qquad (3.4)$$

The regression models were produced using the genetic algorithm technique searching for the best variable subset (Holland 1992, Leardi et al. 1992). In this optimization technique, each variable is represented as a gene. More genes form a chromosome. In this approach a chromosome is the representation of a point in the *p*-dimensional space of the independent considered variables.
In the genetic algorithm the variable selection is composed of three different steps:

- First step (initial phase): the dimension (N) of the population is fixed. The initial population is generated randomly extracting a certain number of chromosomes. The response of each chromosome is calculated, then the N best chromosomes enter into the initial population, ranked by the quality of the response.
- Second step (evolutive phase): a) chromosomes are coupled in order to breed a new generation of chromosomes; b) chromosome can mutate randomly. For both processes the correspondent response is calculated. If the response is better than the one of some chromosomes present in the initial population, the new chromosome (2^{nd} generation chromosome or mutated chromosome) enters the population, substituting the worst one present in the initial population. The processes of chromosomal cross-over and mutation continue iteratively .
- Third step (final phase): reaching of a stop criterion (fixed number of iterations, for example).

In our regression analysis, performed with MobyDigs software (Todeschini 2003), we used the following set-up parameters:

- Population size: 50
- Number of retained best model for each model size: 3
- Stop Criterion: 50000 iterations

Regression models with a dimension up to 5 variables were selected. Q^2 was used as the quality criterion for the selection, obtained by the leave-one-out validation technique. This parameter gives a quantification of the predictive power of models (unlike R^2, which is only a measure of fitting) through a cross-validation technique: each data is left out in turn and a model is derived using the other data. The model obtained is used to calculate the response of the data left out. The response is then compared with the true observed value (Leach 2001).
We kept the three best models for each dimension (from 1 to 5 variables), comparing values of Q^2. A further severer validation of the quality of the models was performed calculating Q^2 by the bootstrap technique.
The influence of each variable was evaluated considering the frequency (%) of appearance in the final best regression models found.
Each analysis was conducted on single substances with the largest available number of data. The analysis were performed at the species level and at the genus level. Data (both predictors and response variables) at the genus level were calculated just averaging data at the species level.

3.3 Results

3.3.1 Trait-based vs. phylogenetic taxonomy

The Euclidean distances between organisms on the basis of their traits quantification was calculated. The results of this calculation is reported in a dendrogram (Figure 3.3). The complete matrix (organisms vs. traits) used for this analysis, as well as the references used to compile it, are available in the *Appendix 2*.

Trait-based differentiation clearly indicates that standard taxonomy is not accurate enough to group animals on the basis of their morphological and functional features.

Even though general homogeneity is preserved, we found important differences in the same taxon already at the order level. For some groups of animals this can be easily expected, like for Diptera, which is well known to be composed of very different organisms; in the dendrogram three well-separated groups are present: one composed of Chironomidae, Ceratopogonidae and Chaoboridae, another one which included Culicidae and Simulidae, while the last one is formed by Athericidae. Other groups of animals, generally considered quite homogeneous, like Ephemeroptera and Plecoptera, also showed important differences. Moreover, these taxa are mixed in the dendrogram, with some families of Plecoptera, for example, more similar to some Ephemeroptera than to other Plecoptera. Within insects, Odonata was shown to be extremely heterogeneous. Crustaceans appears to be a bit more separated into orders, but with some remarkable exceptions: decapods, for example, are separated into two well divided groups, one of which is more similar to amphipods, and the other more similar to isopods. On the contrary, molluscs and anellids are much more grouped at order level. To a great extent this reflects what generally happens for sensitivity: while mollusc and anellids taxa are always the most resistant, arthropod orders generally present wide overlaps in sensitivity values

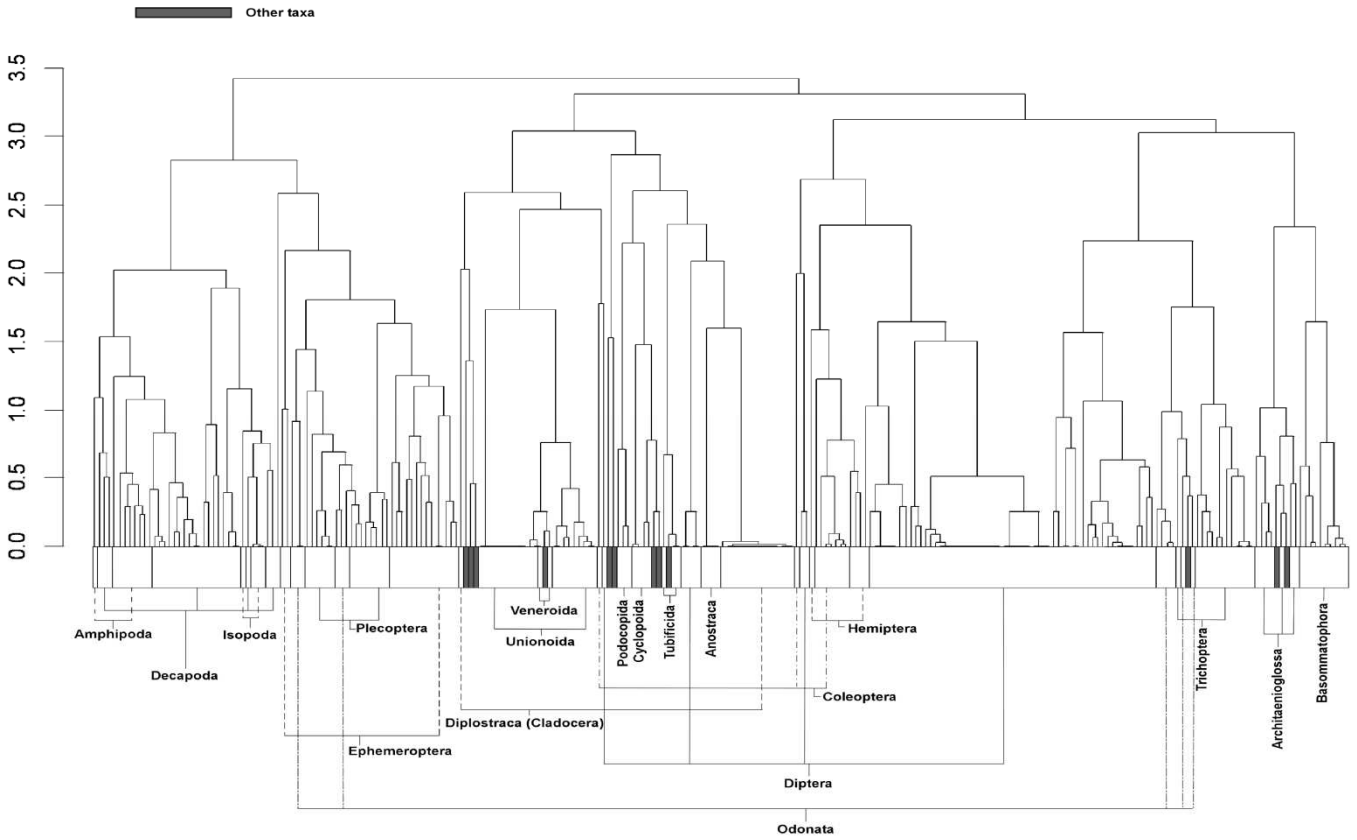

Figure 3.3 Dendrogram: euclidean distance between organisms calculated on the basis of traits quantification.

3.3.2 Selected substances

We made a preliminary evaluation of the total toxicity range of the substances (Figure 3.4).
Pyrethroids showed the highest values of both average and absolute toxicity, while carbaryl showed the lowest average value. Malathion (117 datapoints) presented the highest standard deviation (s = 1.65), while chlorpyrifos (94 datapoints) presented the lowest value (s = 0.88). Some statistics of the distribution of the datapoints for each substance are reported in Table 3.4.

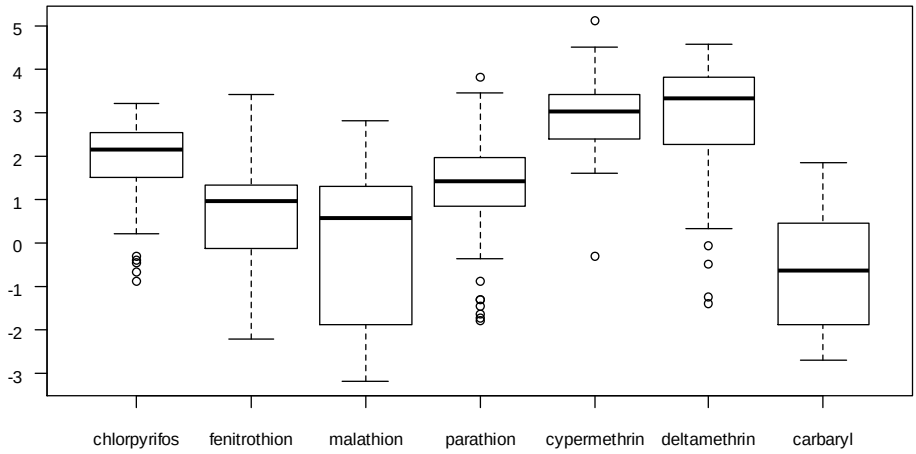

Figure 3.4 Evaluation of the toxicity range of the selected substances. Toxicity data are expressed as Tox (log(1/EC50) mmol/L). Solid line in the boxes represents the median. Circles represents outliers. Pyrethroids show the highest mean toxicity, while carbaryl show the lowest one.

Table 3.4 Statistical parameters of the distribution of toxicity data for each selected chemical. Toxicity data are expressed as Tox (log(1/EC50) mmol/L). chl=clorpyrifos; fen=fenitrothion; mal=malathion; par=parathion; cyp=cypermethrin; del=deltamethrin; car=carbaryl.

	chl	fen	mal	par	cyp	de	car
Objects	95	63	117	53	33	32	83
Mean	1.95	0.56	-0.02	1.24	2.92	2.71	-0.62
Median	2.16	0.98	0.57	1.44	3.03	3.35	-0.61
Std dev.	0.88	1.26	1.65	1.33	1.01	1.61	1.25
Min	-0.87	-2.20	-3.16	-1.79	-0.29	-1.38	-2.70
Max	3.23	3.44	2.84	3.82	5.13	4.59	1.87
Range	4.11	5.64	5.99	5.61	5.42	5.97	4.57

3.3.3 Regression models

The best prediction values (values of Q^2) obtained with the regression analysis at the species level for each compound are reported in Table 3.5. No significant models were found for cypermethrin, (boostrap validation gave $Q^2_{Boot} = 0$) probably due to the scarcity and variability of available information. Models with up to 5 variables have been produced and the best model for each dimension is reported. The 5-variables models give the best predictive values for all substances, with the exception of parathion and deltamethrin, where the introduction of the fifth variable caused a decrease of the predictive capacity. The best model for each substance was always able to predict more than 50% of the variance in toxicity (with the exception of parathion, where Q^2 was slightly < 0.5). For fenitrothion, malathion, carbaryl and deltamethrin the predictive power of the models exceeded 2/3 of the total variance. The best model was found for deltamethrin ($Q^2 > 0.8$). The average value of Q^2 for the OPs at species level is 0.61, while the cumulative mean for all compounds is 0.66.

Table 3.5 Q^2 values (%) for the best models obtained at the species level for each substance.

	Number of variables in the model				
	1	2	3	4	5
chlorpyrifos	39.4	45.8	52.2	54.4	56.9
fenitrothion	42.8	56.7	62.6	65.4	72.6
malathion	43.8	53.4	61.7	67.4	68.9
parathion	18.8	34.9	40.2	45.9	45.0
carbaryl	48.5	54.4	60.7	66.2	67.5
deltamethrin	68.3	75.4	82.1	83.6	83.6

Table 3.6 Q^2 values (%) for the best models obtained at the genus level for each substance.

	Number of variables in the model				
	1	2	3	4	5
chlorpyrifos	41.7	48.1	55.3	58.3	63.1
fenitrothion	44.9	56.3	60.4	64.4	70.7
malathion	42.0	55.0	62.4	66.3	67.1
parathion	24.1	43.5	54.5	61.4	62.7
carbaryl	52.0	56.5	63.1	68.4	69.3
deltamethrin	64.6	72.6	78.9	81.4	81.2

Variables involved and statistic evaluation (fitting, boostrap validation, etc) of the final selected models (three for each dimension, unless the final population of the 50 models contained less than three models for a certain dimension) for each substance is reported in the *Appendix 2*.
The best prediction values (values of Q^2) obtained with the regression analysis at the genus level for each compound are reported in Table 3.6. Once again no significant models were found for cypermethrin (boostrap validation gave $Q^2_{Boot} = 0$). In this case the best models were always able to predict more than 60% of the variance in toxicity, with an average Q^2 value of 0.66 for the OPs and an overall mean of 0.69 for all considered compounds.
For chlorpyrifos and malathion, due to the abundance of data, the same analysis was performed at the family level (data on traits and toxicity were just averaged as it was done for the genus level). Comparison of the best obtained Q^2 values between different taxonomic levels are summarized in Figure 3.5.
For carbaryl, deltamethrin and fenitrothion there are only slight differences in the predictive capacity of the best models at the species and the genus levels. Parathion instead showed a huge increase (more than 15% of the total) in the predictive capacity of models from the species to the genus level. For chlorpyrifos and malathion two opposite trends are highlighted. Power capacity of models increased with the taxonomic hierarchy in chlorpyrifos, while it decreased consistently in malathion, especially passing from the genus to the family order.
We investigated the role played by the single predicting variable (trait) and their relative importance in the models. The influence of the variable was assessed considering the frequency of appearance in the final selected models. The percentage values of appearance for the most frequent variables (at the species and at the genus levels) are reported in Table 3.7 (average values for OPs), Table 3.8 (carbaryl) and Table 3.9 (deltamethrin). In the tables whether the variable's coefficient was positive or negative is also reported. Positive values indicate that the traits is proportional to sensitivity, negative values determine an inverse correlation.
Body length, behavioural complexity and body shape seem to play a very important role as predictors for toxicity of OPs, but they generally have some effects on deltamethrin and carbaryl also. The ramification degree seems to have a certain importance for both deltamethrin and carbaryl. The life duration, which appears very frequently in predicting models for deltamethrin, has indeed a significant correlation with body length ($R^2 = 0.65$, $p<0.0001$): this is not surprising, since organisms with large body size usually have a longer lifespan. Probably, the correlation could also have been greater if all the organisms had been tested at their maturity.

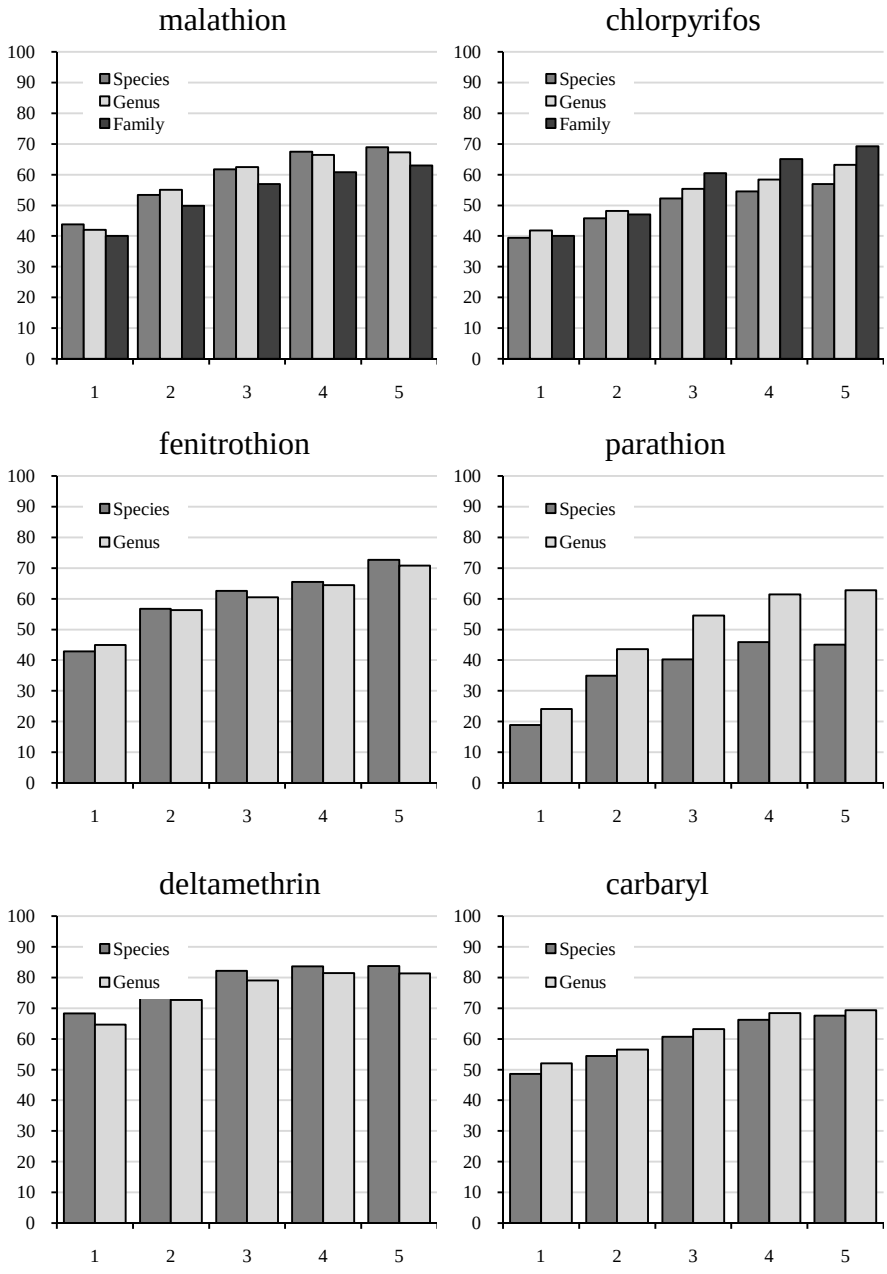

Figure 3.5 Best Q^2 values of regression models produced with genetic algorithms at the species and the genus level (for chlorpyrifos and malathion, the family level also). *x*-axis represents the model dimension (number of terms), while the *y*-axis reports the values of Q^2 in percentage.

Table 3.7 Frequency of the most significant variables in the regression models of OPs and positive/negative effects on the response (only values >25 % are reported).

Species			Genus		
Trait	Freq %	+/-	Trait	Freq %	+/-
BE	46.7	+	BE	54.0	+
BL	45.0	-	BL	51.1	-
BS_lf	27.3	+	FH_ff	32.7	+
BS_se	27.1	-	LD	26.5	-
FH_ff	25.6	+			

Table 3.8 Frequency of the most significant variables in the regression models of carbaryl and positive/negative effects on the response (only values >25 % are reported).

Species			Genus		
Trait	Freq %	+/-	Trait	Freq %	+/-
BS_se	92.9	-	BS_se	86.7	-
BL	71.4	-	RA	60.0	+
RA	50.0	+	FH_df	46.7	-
BS_lf	42.9	+	BL	46.7	-

Table 3.9 Frequency of the most significant variables in the regression models of deltamethrin and positive/negative effects on the response (only values >25 % are reported).

Species			Genus		
Trait	Freq %	+/-	Trait	Freq %	+/-
LD	66.7	-	LD	71.4	-
AR	60.0	+	AR	64.3	+
RA	53.3	+	RA	57.1	+
FH_df	33.3	-	FH_df	35.7	-
BE	26.7	+	BE	28.6	+

The main focus of our work was to identify traits that can have a certain influence on the response of macroinvertebrates to toxicants. Regression models indicated that body length (BL), behavioural complexity (BE) and body shape (BS) can play a major role in this process, especially for OPs.

For the first two traits, which are expressed by ordinal variables, we evaluated the correlation with the response variable Tox (Table 3.10). Behavioural complexity (BE) showed a significant correlation with Tox at the species and the genus level for all compounds, even though for chlorpyifos values of R^2 are extremely low.
Body length (BL) presents smaller a correlation with Tox, and generally decreases at the genus level (with some exceptions). Nevertheless, the influence played by the traits is pretty clear.
Body shape (BS) is not defined in an ordinal scale, so a simple correlation analysis is not feasible. Then we evaluated the trend of each trait category for each compound (results are represented in Figure 3.6 as box plots). Categories often show big variance in the correspondent sensitivity response, nevertheless, it appears quite clear that average values for linear, dorsoventrally flattened and laterally flattened animals are often pretty similar (chlorpyrifos is the only exception), while animals with more solid morphology (classified as solid ellipsoidal) show smaller average values of sensitivity.

Table 3.10 Correlation values (and relative statistical significance) of body length and behavioral complexity with toxicity at the species and the genus level for all the selected substances (values with p>0.05 are not reported).

Species				
	BL		BE	
	R^2	p value	R^2	p value
chlorpyrifos	0.42	<0.0001	0.07	0.0104
fenitrothion	0.23	<0.0001	0.46	<0.0001
malathion	0.30	<0.0001	0.45	<0.0001
parathion	0.14	0.0067	0.22	0.0004
cypermethrin	-	-	0.20	0.0081
deltamethrin	0.54	<0.0001	0.73	<0.0001
carbaryl	0.08	0.0103	0.26	<0.0001

Genus				
	BL		BE	
	R^2	p value	R^2	p value
chlorpyrifos	0.43	<0.0001	0.08	0.0350
fenitrothion	0.17	0.0055	0.49	<0.0001
malathion	0.38	<0.0001	0.45	<0.0001
parathion	0.10	0.0501	0.29	0.0005
cypermethrin	-	-	0.22	0.0233
deltamethrin	0.47	0.0001	0.70	<0.0001
carbaryl	0.07	0.0423	0.28	<0.0001

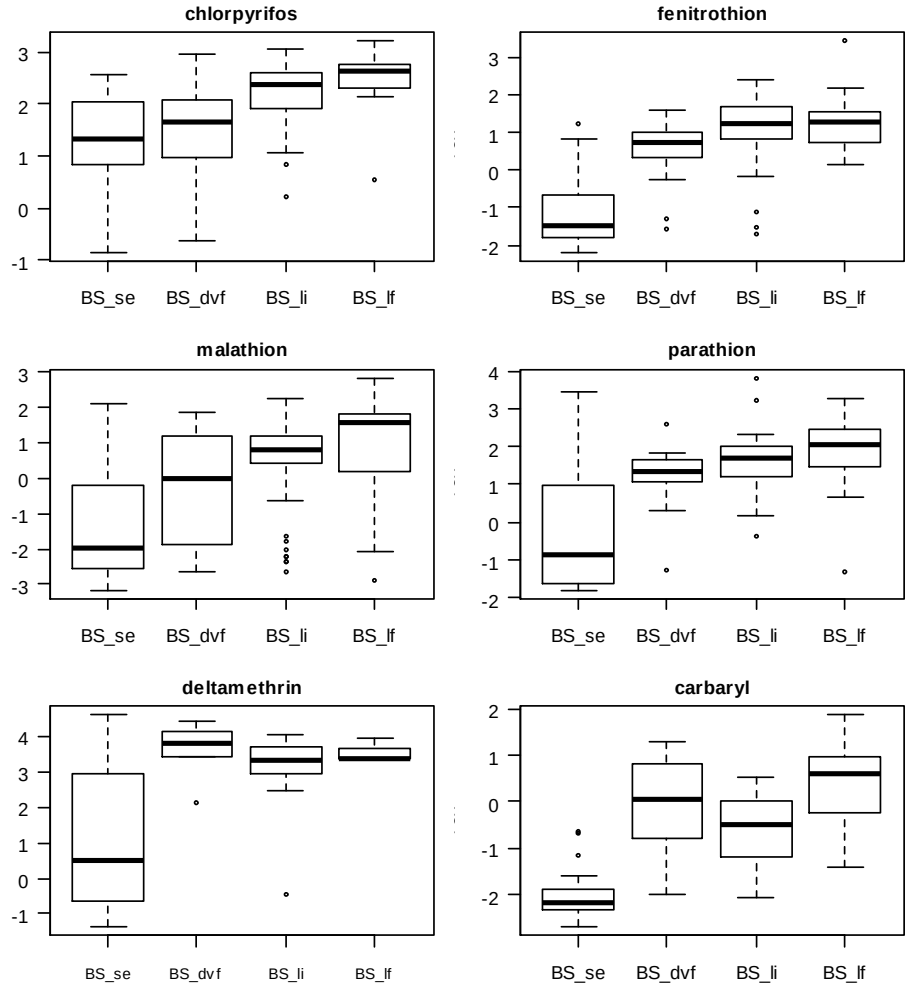

Figure 3.6 Box plots expressing the distributions of the sensitivity of species in relation to the categories of the "body shape" trait. Sensitivity data are expressed as Tox (log(1/EC50) mmol/L), then high values express high sensitivity.

3.4 Discussion

The use of chemometric methods for the development of trait-based models to predict sensitivity may represent an effective innovative approach. In particular, the application of the genetic algorithms technique proved to be a powerful tool providing predictive regression models, then highlighting the most predictive variables. The best regression models showed a good predictive power, being able to explain almost always more than a half of the total variability. This was especially true at the genus level, where probably the use of average decreased the influence of some non-aligned data.
The variables present in the best model were not the same for all the OPs, though a large overlap was observed. However, other studies (Hoekstra et al. 1994) demonstrated that substances of the same class often present different sensitivity rankings between organisms. We chose to evaluate each substance singularly to account for this variability, and evaluate only afterwards which variables had the greatest general influence.
However, the development and application of more effective statistical tools, capable to produce excellent empirical models, cannot be the solution for trait-based approaches without a sound mechanistic interpretation of the trait used. This is particularly important for chemicals with highly specific toxicological mode of action such as pesticides.
An important comparison can be made between the original formulation of the QSAR approach for predicting toxic effects of chemicals and the trait-based approach for predicting the response of living organisms. In the traditional Hansch analysis (Hansch, 1969), the toxic effect of chemicals may be explained by three kinds of molecular properties:

- the hydrophobic properties (e.g. Log Kow) may describe the capability of a chemical to cross biological membranes and to enter into the living organisms; these properties are sufficient to describe the baseline effect of non specific (narcotic) chemicals;
- the electronic properties may explain the reactivity of a chemical and its capability to move within and interfere with the biological structures; these properties are needed to describe the effect of less inert (e.g. polar narcotics) chemicals;
- the steric properties are needed to describe more specific interactions between a chemical and a biological receptor (e.g. the effect of enzymatic inhibitors).

A conceptually comparable approach may be applied for the selection of suitable traits for the prediction of the biological response of living organisms to different kinds of toxic chemicals:

- simple morphological and anatomic traits, for example related to the surface/volume ratio and to the presence of external skin protection, have been proved very effective to describe the capability of a chemical

to enter into the organism and, therefore, to explain a non-specific, narcotic type, toxicological effect;
- the effectiveness of mechanisms of internal transport (e.g. structure of respiratory and circulatory systems) may explain the circulation of the toxicant into the organism;
- more specific toxicological modes of action should be described by more detailed and specific knowledge of physiological and metabolic patterns of the exposed organisms.

An important attempt to apply a trait-based approach to chemicals with highly specific modes of action is reported by Rubach et al. (2010). These authors used several kinds of traits (morphological, anatomical, reproductive, and ecological) to explain the effect of different classes of insecticides. Even if they present their results as empirical relationships, the need for a mechanistic approach to understand some results is highlighted.

In fact, among the best predictors for the sensitivity ranking for OPs, these authors found the temperature preferendum and the current velocity preferendum. These ecological characteristics can certainly influence the response of an organisms which is tested at certain conditions. For example, the sensitivity of an organism which prefers cold and fast streams can be enhanced in a static test at 20°C; nevertheless:

- it is not clear whether this increase of sensitivity is related to the mode of action of the compound;
- this information is likely to be useless in a real environment, where each organism tends to stay in its ecological optimum;
- in the empirical relation presented by Rubach and colleagues, no information were collected about the gap between the ecological preferendum and the effective conditions of the tests

Our results probably represent an improvement of the work of Rubach et al (2010), not only because of the achievement of better statistical results, but even because we tried to use only biological traits, avoiding ecological characteristics that are hardly related to results obtained in laboratory tests (or hardly useful in the future to make predictions in the environment). Only two of the evaluated traits can be considered as ecological (feeding habits and voltinism), but both of them are strongly related to the biology of the organisms. In particular voltinism can be seen as a representation of the amount of energy that each organism invest in the reproduction, while the feeding type may play a role in the exposure routes, even when the food is missing as it is in standard tests. Although the approach used to develop models remains largely empirical, many of the used traits where selected, whenever possible, in order to cover the three main components of the toxic response described above: entering in the organism, internal transport, interaction with specific systems.

No specific comparisons were possible for the mode of action of chemicals, since the only AChE inhibitor apart from OPs was carbaryl, whose toxicity

however seems determined to a large extent by the same traits found for OPs.
Pyrethroids showed opposite results, probably determined by the paucity of available and reliable data. For cypermethrin no significant models were obtained, while for deltamethrin we found the overall best predictive models. In both cases results must be taken carefully.
Frequency of appearance in the best models suggested that "body length" (BL), "behavioural complexity" (BE) and "body shape" (BS) are important traits for the prediction of sensitivity. Further analysis (correlation with toxicity for the first two and distribution of sensitivity in each trait category for "body shape") confirmed the results.
"Body length" (used as a proxy of body size) and "body shape" are likely to influence chemical intake, determining the surface/volume ratio. In fact, large animals with massive body shape presented the lowest ratio, while small animals with other morphologies (linear, flattened) presented higher values of that parameter.
From our analysis, the effect of the respiration technique doesn't seem so strong, while it was found to be determinant both by Baird et al. (2007) and by Buchwalter et al. (2002). One possibile explanation for this difference may be that both of those papers consider arthropods only. It is possible that within arthropods the respiration mechanism plays a primary role, while considering more heterogeneous organisms (even in terms of the toxicological response) its effect is masked by other, more relevant characteristics. This suggests the possibility of a hierarchical approach for this kind of evaluation: the importance of each trait (or combination of traits) may depend on the focus of the analysis. If that was true, no absolute determination would be possible, and finding common patterns for real natural communities can be extremely complicated.
The attempt to include the oxygen transport mechanism in the analysis didn't bring any significative result. That can either mean that the internal transport of the chemical is not related with the internal transport of oxygen or that in such small organisms the internal transport of the chemical is much less important than the uptake to predict the sensitivity.
"Behavioural complexity", (here used as a proxy of structural nervous system complexity) represents an example of a specific trait, which is likely to be related to the mode of action of the toxicants. We are aware that the hypothesis that this trait can influence sensitivity to neurotoxicants needs further validation. This study was a first attempt to examine the possibility for a relationship, and results are indeed encouraging. Methods for the quantification of "Behavioural complexity" remain still largely arbitrary, though they are based on important behavioural aspects, and more focused studies are needed. With this attempt, however, we wanted to emphasize the need for research on less generic traits than what has been used in the past. The knowledge of the characteristic of the substances with specific toxicity,

and especially their mode of action, should be of primary importance for the identification of new traits related to sensitivity.
The inclusion of more ecological traits didn't bring any satisfactory results. In particular, no relationship was found between sensitivity and voltinism. This is in agreement with the findings of Brock et al. (2009), which already demonstrated by means of the SSD approach that voltinism does not affect the sensitivity of aquatic arthropods to several insecticides. No clear patterns were also found for feeding habits, although filter feeders were generally more sensitive to OPs. This seems reasonable, since this feeding mechanism can increase the contact between the organism and the medium, then enhancing the exposure.
The use of biological traits allows making realistic hypotheses about how a certain characteristic can influence the sensitivity of organisms, even if more detailed studies are needed to verify those hypotheses.
Unfortunately, as already stated by several authors, working with toxicity data whose origin is so diverse always creates problems. Despite the careful selection we made, inconsistency, unreliability and bias of data cannot be excluded. Different laboratory conditions, methodologies and tested (biological) material make data difficult to compare. Apart from toxicity data, the codification and quantification of the biological characteristics of organisms represents another huge source of uncertainness, for which we join the call of other authors for a complete, shared and well defined trait database for ecotoxicological purposes. Another possible origin of biases and uncertainty is related to the different possible responses in different life stages of the same species. Morphological and functional traits may also be substantially different in the different life stages.
Another, and even bigger, difficulty is the availability of suitable traits at the appropriate level of taxonomic hierarchy. This is particularly relevant for the physiological and metabolic traits required to explain the effects of specific modes of action.
Sometimes huge differences in sensitivity are present within the same family or even within the same genus, but this part of variability remains unexplained in our representation. However, it is worth noticing that this evaluation is based on actual characteristics of organisms, but it just considers a very small pool of possible traits. A further differentiation could probably derive from the inclusion of other traits which were not considered in the present study.
In this study we tried to identify which traits can have an influence on the intrinsic sensitivity of freshwater macroinvertebrates. It is worth highlighting that the effects experimented by animals in the real environment depends on the vulnerability of the species, which is a concepts that goes well beyond mere sensitivity. Nevertheless, intrinsic sensitivity is one of the key factors for the determination of the vulnerability to a stressor, and understanding

which are the parameters that influence the process of intoxication remains one of the principal challenges of ecotoxicology.
The trait based approach described in the paper is limited to the species level. For the development of more ecologically sound procedures for ecological risk assessment, the application of trait-based approaches at higher hierarchical level (community, ecosystem) is particularly important. In these cases, it would be important to develop tools for the description not only of the sensitivity but also of the other components of vulnerability, i.e. recovery capability and susceptibility to exposure (Ippolito et al. 2010). For this purpose, ecological behavioural and reproductive traits may be particularly relevant. However, one must be aware that the development of predictive models could not be based on relatively simple laboratory data but would require more complex higher tier information (mesocosms, field or semi-field data).
In this framework, the SPEAR index (Liess and Van der Ohe 2005) represents an important milestone towards a complete trait-based ecological risk assessment for pesticides. Nevertheless, one of the drawbacks of SPEAR is that the sensitivity of organisms is assigned on a taxonomic base, considering the information available in the ecotoxicological databases. Furthermore, no discrimination of the different mode of action of the pesticides is evaluated. For this reason one possible refinement of the index can be the inclusion of some other traits able to explain a major part of the physiological sensitivity for different chemical mode of action. Notwithstanding, more studies on the matter are needed to get a reliable estimation of this variable.
In a future perspective, another interesting application of traits could be the development of trait-based Species Sensitivity Distribution (SSD) approaches. It would allow developing SSD curves not dependent upon taxonomy but on structural and functional features which characterize biological communities likely to be present in specific ecosystem typologies.

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4. Evaluating pesticide effects on freshwater invertebrate communities in alpine environment: a model ecosystem experiment

Abstract

Pesticide loads in streams are potentially one of the most relevant stressors for macroinvertebrate communities. Nevertheless, real effects provoked at the community level are still largely unknown.

Model ecosystems are frequently used as tools for the risk assessment of pesticides, especially for their regulation, however, they can be also applied to site-specific risk assessment in order to gain better understanding of the responses of aquatic ecosystems to chemical stress. In the present work, an experimental system was composed of 5 artificial streams that reproduced a mountain lotic environment under controlled conditions. This study was aimed to better understand, whether (and how) the biological community was influenced by pesticides pulse exposures. 5 mixture load events were simulated over the productive season (March-July 2010): biological community was regularly sampled and nominal concentrations of water were tested.

The results were interpreted comparing the output of different metrics and statistical methodologies. The sensitivity of different metrics was analyzed considering single exposure events (maximum Toxic Units) as well as overall temporal trends. Results showed how some common taxonomic metrics (e.g. taxa richness, Shannon's index, total abundance of organisms, and the Extended Biotic Index) were not suitable to identify the effects of pesticides at community level. On the contrary EPT%, $SPEAR_{pesticide}$ and the Principal Response Curve methodology proved to be sensitive to this kind of stress, providing comparable results. Temporal trends of these metrics proved to be related to the concentration of chemicals. Remarkably, the first Principal Response Curve illustrates the trend followed by the most vulnerable species, while the second is more related to the trend of opportunistic species. A high potential risk for the invertebrate community was highlighted by a statistically significant decline of 40 points (comparison with the control) in both $SPEAR_{pesticide}$ and EPT%.

Keywords: pesticides; artificial streams; macroinvertebrates; SPEAR; EPT; PRC

Submitted manuscript: Ippolito A, Carolli M, Varolo E, Villa S, Vighi M. Evaluating pesticide effects on freshwater invertebrate communities in alpine environment: a model ecosystem experiment

4.1 Introduction

One of the key issues in modern ecotoxicology is to analyse the effects of chemicals at biological community level. Many researchers tried to interpret complicated patterns using different experimental and statistical techniques, mostly focussing on pesticide effects. This attention on pesticides is not unusual, since these compounds are one of the most widespread class of toxicants worldwide, especially for the aquatic compartment (Vörösmarty et al. 2010). Moreover, they are the only toxicants together with biocides, intentionally introduced into the environment. Their ecological relevance due to their efficacy and diffusion is very well acknowledged to cause both direct and indirect effects on natural ecosystems.

The historical transition in ecology from pure theoretical and observational studies to rigorous experiments and the following debate over the role of each approach, is well described in a book by Clements and Newman (2002). These authors highlighted how the introduction of manipulative experiments allowed to establish causal processes, while mere observation of natural systems didn't provide enough evidences to prove theories. In general terms, Popper (2002) divided true science from pseudoscience considering the ability to test theories with controlled experiments as discriminant criterion. This transition process in ecology strongly influenced community ecotoxicology: researchers started using scaled reproduction of natural systems to test the effects of chemicals on biological assemblages. Though, the use of model ecosystem in ecotoxicology was not the only direct consequence of the abovementioned transition in ecology. Some ecotoxicologists started to criticize the exclusive use of single-species tests for predicting the environmental effects (Cairns 1983). Furthermore, it became clear that the influence of biotic and abiotic factors on the response of population was relevant and could not be assessed by standard assays.

Despite some criticisms raised about the lack of criteria to interpret the results (see for example the debate originated by the paper of De Jong & Monforts (2006) and the related response of Van den Brink (2006)), the importance of model ecosystems (i.e. microcosms, mesocosms) in evaluating environmental effects has been acknowledged also for the pesticide regulation. The use of model ecosystems for pesticide regulation started in 1970s and developed until early 1990s mainly in the U.S. (Brock & Budde 1994) leading to at least three guidance documents (Touart 1988, SETAC 1992, SETAC Europe 1992). Later, studies based on model ecosystems started getting unpopular in the U.S. (U.S. Environmental Protection Agency stopped their requirement in 1992) though, became more frequent in Europe. During the last 15 years, at least five new guidance documents dealing with this topic were produced, often as outcome of scientific workshops: Higher Tier Aquatic Risk Assessment for Pesticide commonly known by its acronym, HARAP (Campbell et al. 1999); Community-Level

Aquatic System Studies-Interpretation Criteria (CLASSIC – Giddings et al. 2002); one EU guidance (European Union 2002); Effects of Pesticide In the Field (EPIF - Liess et al. 2005) and a RIVM (Dutch National Institute for Public Health and the Environment) guidance (de Jong et al. 2008).

Model ecosystems are not only useful for pesticide regulation but also to better understand the responses of aquatic ecosystems to chemical stress (Brock and Budde 1994). Though, this second aspect, that is more connected to chemical stress ecology (Van Straalen 2003, Van den Brink 2008), had often been neglected during the last years.

Furthermore, the introduction of Water Framework Directive (WFD) (European Union 2000) posed intriguing challenges to pesticide ecotoxicology and probably, it will influence the way Ecological Risk Assessment (ERA) is carried out. Attempts to harmonize WFD with the current regulations on pesticide registration (European Union Directive 91/414/EEC) are challenging (for example, refer to Brock et al. 2006) and this paper does not intend to present a detailed discussion about this subject. However, it is interesting to notice how the WFD shifts the focus of ERA from a chemical (to be placed on the market) to the ecosystem (to be protected). This is certainly an important change of perspective encouraging ecotoxicologists to better understand the effects of pesticides in real environment.

Furthermore, there are proofs that the same pesticide dosed at similar rate may cause different secondary effects in different freshwater model ecosystems (Brock & Budde 1994, De Noyelles et al. 1989, Brock et al. 1992) or even in the same model ecosystem if applied in different periods of the year (Hanazato & Yasuno 1990). This deals with the concept of Ecological Vulnerability (De Lange et al. 2010, Ippolito et al. 2010) and highlights the need for more sound methodologies for site-specific ERA.

The work presented here tries to follow this path, using an artificial ecosystem to focus on one particular biological community (i.e. freshwater invertebrate community in alpine environment) as a support for site-specific risk assessment. To do so, a realistic exposure pattern was modelled for a mountain stream of Northern Italy and was then applied to an artificial reproduction of the same environment.

Artificial streams have been used, even in recent past, to assess both fate (Beketov & Liess 2008) and effects (Stampfli et al. 2011) of the pesticides. Nevertheless, experiments with artificial streams have always been rare compared to those carried out in ponds (mainly due to practical reasons), moreover, the reproduction of mountain streams is even less common.

There had been a lot of ambiguity concerning the definitions of cosms (micro-, meso-, macro-) Thus, even if our experimental system appears closer to the definition of mesocoms given by Odum (1984) (“bounded and partially enclosed outdoor experimental setups”), we are not interested in

arguing about these definitions. Hereafter, we will simply refer to our system with the generic term of model streams.

4.2 Materials and methods

4.2.1Experimental system

The experimental system is located in Trentino-Alto Adige Region, Northern Italy. Several areas of this mountain region are well-known for the production of apples and other permanent crops. The surface occupied by apple orchards in some valleys is extremely relevant (e.g. Val di Non). This kind of cultivation requires huge external inputs to be maintained and that includes a relevant amount of plant protection products, especially fungicides and insecticides. Since most of the area is characterized by high slopes, the risk for pesticide surface runoff is potentially very high. In addition, orchards are usually grown in the lowest part of mountains slopes, very close to the streambeds.
Considering such situation, a realistic exposure scenario was mimicked in the presented experiment. In particular, the available information about hydrographic basin of the Novella stream was collected (Val di Non). Data about soil, precipitation and pesticide applications (products used, typical application rate and periods of application) were used as inputs for coupled SoilPlus-DynaNet models in order to simulate the exposure pattern during the productive season. Results and detailed descriptions of this simulation are described elsewhere (Morselli et al., in preparation).
The experimental setting (Figure 4.1) is represented by five flumes (20 m long, 30 cm wide), located on the riparian area of the Fersina stream (46° 04’32” N, 11° 16’ 24” E) at 577 m a.s.l.. The flumes had adjustable longitudinal slope and feeding discharge that allowed maintaining a water flow of 0.005 m^3/s and water velocity of 0.5 m/s during whole duration of the experiment.
The artificial streams were connected to a loading tank which is directly fed by water diverted from the Fersina stream. This water source was assumed to be of suitable quality, since the stream watershed upstream from the experimental setting was not affected by any relevant agricultural (or other human) activities, which made any pesticide load unlikely.
None of the problems listed by Crossland et al (1991) for flow-trough system affected our experiment: high flow rates were guaranteed by a pool built on the riverbed and directly connected to the loading tank; siltation was negligible due to the nature of the river substrate (mainly rocks and gravel). Furthermore, the abovementioned pool and the loading tank allowed some deposition of suspended solids. The flumes were made out of stainless steel, which, as reported by Mitchell et al. (1994) presents advantages because they are robust and can be easily cleaned (detoxified) after any experiment.

The substrate of artificial rivers was realized reproducing the natural substrate of the streams present in the area, excluding large rocks due to the reduced dimensions of the flumes. Each flume was filled with a 10-20 cm layer of gravel and sand collected from the riverbanks.

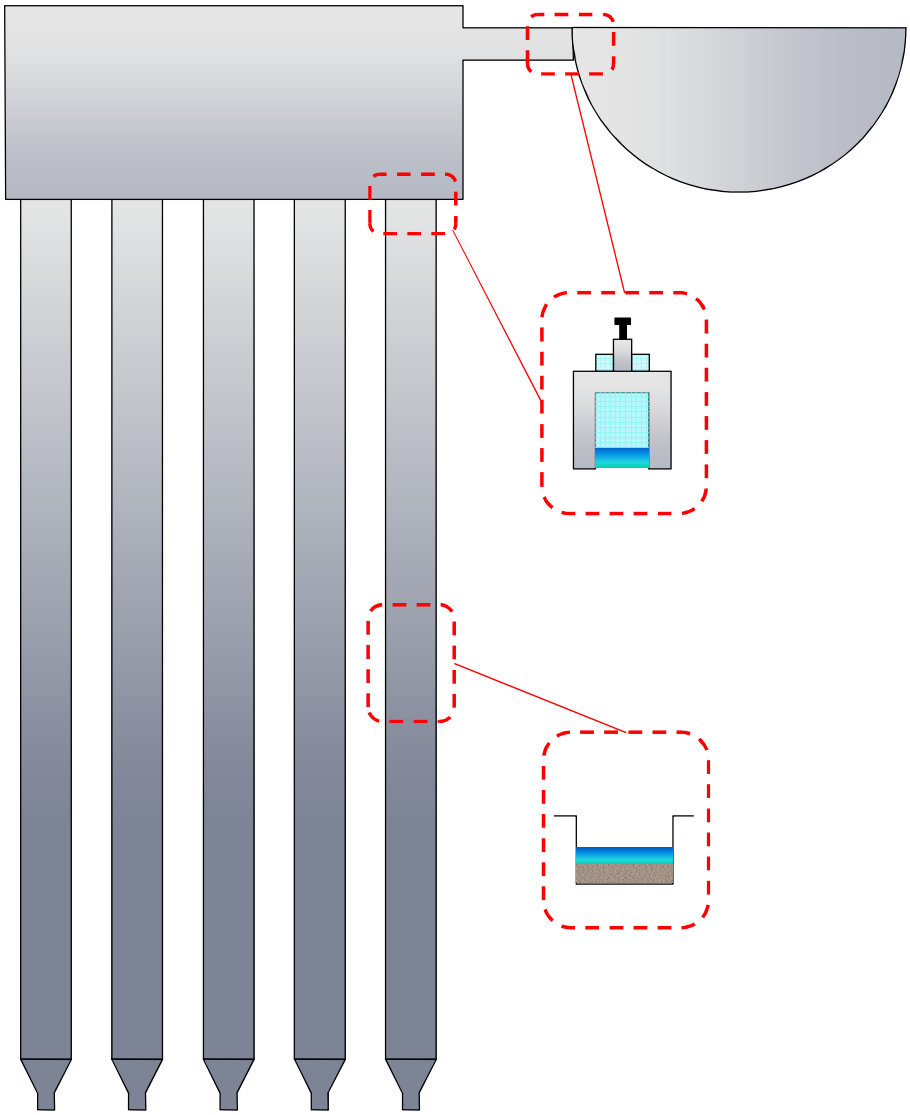

Figure 4.1 A schematic representation of the experimental system. 5 stainless steel flumes connected to a loading tank directly linked to a hemispheric pool placed in the riverbed of the Fersina stream. Flows are regulated at the entrance and at the exit of the loading tank.

Freshwater invertebrates naturally colonized the substrate, partly through oviposition and mainly entering the model streams via drift from upstream.

The invertebrate community had almost two years to establish, since substrate was placed at the beginning of 2008, while our experiment was carried out in the productive season of 2010. No intentional manipulation was done to the invertebrate assemblage, allowing to form the structure of the community in the most natural away, except for the influence of artificial habitat.

4.2.2 Experimental design

The experiment simulated 5 runoff events due to intense rainfall in the period between March and July 2010. During each simulated event, a different mixture of chemicals at a certain concentration was used as a result of the model simulation (thus, related to the typical amount and to the period when each chemical was applied in that area). 10 different active ingredients were used in the experiment including 3 fungicides (difeconazole, dithianon, pyrimethanil) and 7 insecticides (chlorpyrifos, methoxyfenozide, lufenuron, etofenprox, thiometoxam, phosmet and thiacloprid).
Four out of the five flumes were actively used for the experiment. The pesticide mixture was prepared in a 200 L tank placed at the head of the system. Formulated products were used to facilitate dissolution in the water and the mixture was poured continuously at constant rate in the flumes. The input flows of the chemicals were regulated by a peristaltic pump (Gilson Minipuls® 3, R4/HF high flow pump head) and the power supply was provided by the alternate use of a couple of car batteries (Bosch Silver S5 110 AH). Chemicals were dripped at half of the length of the flumes, so the first half of each flume was used as control. Two different concentration patterns were followed, with two replicates each (Figure 4.2). The exposure pattern in this area is characterized by pulses of extremely relevant concentrations which decrease very fast in the following hours. Since such a complicated profile was extremely difficult to reproduce, simplified exposure patterns were used (Figure 4.3). In treatment 2 (flumes E and D), the highest predicted concentrations were maintained for 24 hours and half of these concentrations for the following 24 hours (except for the first event that lasted only for 1 day). Treatment 2 represents the worst simulated scenario. Treatment 1 (flumes B and C) followed exactly the same pattern of Treatment 1, except that the input flow rate of the mixture (and thus the nominal concentrations of all the chemicals) was kept equal to half of Treatment 2. Flume A was kept as a further control in order to test for differences between the upstream and the downstream part. A complete overview of the simulated runoff events (dates, chemical used and their nominal concentration) is reported in Table 4.1.

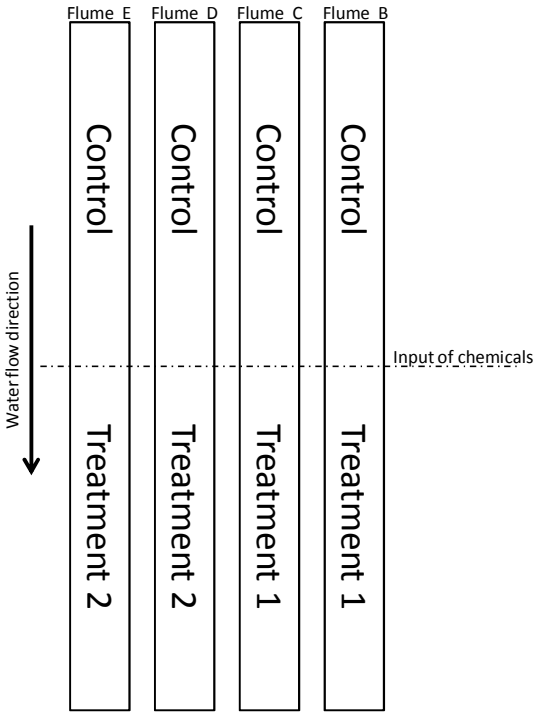

Figure 4.2 Spatial representation of the experimental design. Four flumes were actively used in the simulated events. Chemicals are trimmed at half of the flume length. The upstream part is kept as control. Flume E and D are the two replicates of the Treatment 2 (higher concentration exposure pattern), while flume C and B are the replicates of the Treatment 1 (lower concentration exposure pattern).

The invertebrate community was sampled upstream and downstream from the input point of the chemicals, immediately after each simulated event was concluded. Unfortunately, no inter-event sampling was possible due to the reduced size of the system: repetitive destructive sampling in fact, might have completely altered the community of the model streams, thus its limited use was preferred. Sampling was performed using a Hess Stream Bottom Sampler (0.0433 m^2). Some artificial substrates were also placed (and regularly sampled) in the model streams, however, results corresponding to this sampling technique are not reported in this paper. Organisms were fixed in the field in 75% ethanol and classified in the lab to the family level (except for Plecoptera and Ephemeroptera, which were classified to the genera). Only in some rare cases the classification stopped at higher taxonomic levels.

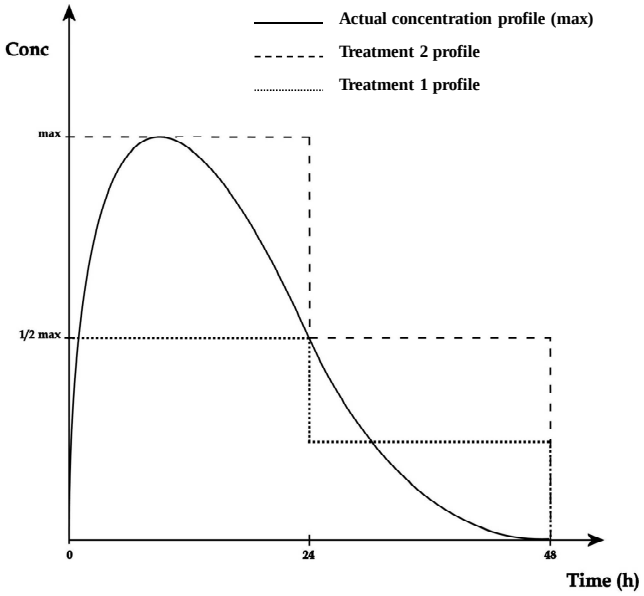

Figure 4.3 Comparison between the typical concentration pattern (max) of a generic chemical as simulated by the exposure model (solid line) and the approximations used in our experiment for Treatment 2 (dashed line) and Treatment 1 (dotted line).

Table 4.1 Nominal concentrations (µg/L) of the active ingredients applied in each simulated load event. 0-24 and 24-48 indicate the amount of A.I. applied during the first and the second day of each event, respectively. Events dates: I - 30.03.2010; II - 20.04.2010; III - 03.06.2010; IV - 30.06.2010; V - 29.07.2010.

		Treatment 2				Treatment 1			
		Flume E		Flume D		Flume C		Flume B	
Event	Active Ingredient	0-24	24-48	0-24	24-48	0-24	24-48	0-24	24-48
I	etofenprox	0.19	---	0.19	---	0.08	---	0.08	---
II [a]	pyrimethanil	84.80	84.80	84.80	84.80	40.00	40.00	40.00	40.00
	dithianon	12.86	12.86	12.86	12.86	6.07	6.07	6.07	6.07
III	pyrimethanil	67.20	36.80	72.00	39.20	36.80	20.00	35.20	19.20
	dithianon	8.40	4.60	9.00	4.90	4.60	2.50	4.40	2.40
	difeconazole	0.17	0.09	0.18	0.10	0.09	0.05	0.09	0.05
	lufenuron	0.06	0.03	0.06	0.03	0.03	0.02	0.03	0.02
	thiametoxam	16.80	9.20	18.00	9.80	9.20	5.00	8.80	4.80
	chlorpyriphos et.	0.67	0.37	0.72	0.39	0.37	0.20	0.35	0.19
IV	pyrimethanil	48.60	17.28	49.68	22.68	25.92	10.80	27.00	11.88
	dithianon	8.10	2.88	8.28	3.78	4.32	1.80	4.50	1.98
	difeconazole	0.36	0.13	0.37	0.17	0.19	0.08	0.20	0.09
	lufenuron	0.06	0.02	0.06	0.03	0.03	0.01	0.03	0.01
	thiametoxam	0.45	0.16	0.46	0.21	0.24	0.10	0.25	0.11
	chlorpyriphos et.	0.50	0.18	0.51	0.23	0.26	0.11	0.28	0.12
	methoxyfenozide	1.44	0.51	1.47	0.67	0.77	0.32	0.80	0.35
	thiacloprid	14.85	5.28	15.18	6.93	7.92	3.30	8.25	3.63
V	pyrimethanil	35.20	16.80	33.60	8.80	19.20	10.40	18.40	10.40
	dithianon	7.92	3.78	7.56	1.98	4.32	2.34	4.14	2.34
	difeconazole	0.31	0.15	0.29	0.08	0.17	0.09	0.16	0.09
	lufenuron	0.06	0.03	0.06	0.01	0.03	0.02	0.03	0.02
	fosmet	28.16	13.44	26.88	7.04	15.36	8.32	14.72	8.32
	chlorpyriphos et.	0.35	0.17	0.34	0.09	0.19	0.10	0.18	0.10
	methoxyfenozide	1.32	0.63	1.26	0.33	0.72	0.39	0.69	0.39

[a] No change of the input flow between the first and the second day.

4.2.3 Water analysis

Physico-chemical parameters (temperature, dissolved O_2, pH, turbidity and conductivity) were measured directly in the field for each experiment.
Chemicals concentration in the water was tested in the simulated events 3, 4, and 5 using three compounds as tracers (chlorpyrifos, phosmet and pyrimethanil). Chlorpyrifos and phosmet were opted since they account for more than 90% of overall nominal toxicity of the mixtures, while pyrimethanil was chosen to represent the behaviour of fungicides, which are usually more water soluble. Water was always collected during the lower exposure time (24-48 h), refrigerated immediately after collection and stocked at -20°C before analysis. Extraction was performed using 500 mg OASIS HLB cartridges (Waters, Hertfordshire, UK). Cartridges were conditioned with 5 mL of n-hexane, followed by 10 mL methanol and finally 10 mL of deionised water (Milli-Q). The samples (0.5 L) were drawn under vacuum through the cartridge at a regulated flow rate of 10 mL min^{-1}. After the extraction, the cartridges were dried using N_2 gas and subsequently eluted (under gravity) with 6 mL of ethyl acetate. Identification and quantification were performed by GC-MS (Agilent Technologies, Santa Clara, CA, USA), in SIM (Single Ion Monitoring). Samples (2 µl) were injected by automatic injector (Agilent Technologies 7683 Series Injector) and analyte separation was achieved using a 30 m Rxi – 5Sil MS capillary column (0.25 mm id, Restek, Bellefonte, PA, USA). Samples were run in splitless mode using helium as a carrier gas (flow 1 mL min^{-1}).

4.2.4 Endpoints measured

Main focus for this study was on the community structure. It is widely acknowledged that functional endpoints are rarely more sensitive than structural ones (Kersting 1994) due to functional redundancy. Moreover, functional endpoints are not suitable to protect biodiversity (Giddings et al. 2002). Different univariate endpoints and indices were measured evaluating their strengths and weaknesses. Some classical parameters were used: total density of organisms, taxa richness (family level), Shannon index (family level) and the EPT (Ephemeroptera, Plecoptera, Trichoptera) relative abundance (%). Other applied indices were: the pesticide-specific $SPEAR_{pesticide}$ (SPEcies At Risk) index (Liess and Von der Ohe 2005), which evaluates the abundance of vulnerable taxa (classified on the basis of an average value of sensitivity retrieved from the literature and some other ecological traits connected to the susceptibility to exposure and the recovery capability) and the EBI (Extended Biotic Index). The latter was used despite it is not conceived for this kind of contamination. The EBI was in fact originally designed to test the quality of water bodies in relation to anthropogenic oxygen depletion (Woodiwiss 1978, and Ghetti 1986 for the

Italian adaptation). Nevertheless, especially in Italy, it is commonly used to assess a generic quality of the water bodies. Significant differences between treatments and control (one way ANOVA followed by Dunnet's test) were calculated using the free software R, version 2.13.1 for Windows (http://www.r-project.org/).
The community was sampled in the upper part of each flume few weeks before the experiment was initiated, to check for significant undesired differences between replicates.
Multivariate analysis of the community structure was performed using the Principal Response Curve (PRC) technique (Van den Brink and Ter Braak, 1999). The PRC method was developed to analyze the data obtained in experimental community response studies with repeated sampling over time. It was based on the RDA (redundancy analysis) ordination technique, a constrained form of principal component analysis, but the scores of each sample are plotted against a "time-fixed" control, so that any alteration induced by treatments over time is immediately detectable. This method is regularly used for mesocosms studies. PCR was performed with CANOCO 4.5 for Windows (Wageningen, The Netherlands). Taxa abundance were ln(2x+1) transformed before analysis (Van den Brink and Ter Braak, 1999). Statistical significance of the PRC models was tested by Monte Carlo permutation test performed for the entire time series, using an F-type test statistic based on the eigenvalue of the components (Van den Brink and Ter Braak, 1999; Leps and Smilauer, 2003). The PRC incorporates an analysis of the responses of each taxon in the dataset by assigning a score (b_k) which reflects affinity of the taxa to the pattern followed in the principal response curves.
The results for different indices were related to an overall toxicity of each mixture, estimated using the concept of the Toxic Units (TU).

$$TU_{\textit{Daphnia magna}} = C_x / \text{ 48h } LC50_x \qquad (1)$$

Where x represents an individual component of the mixture. Values of 48 h acute toxicity referred to *Daphnia magna* were retrieved for each active ingredient from the Footprint database (FOOTPRINT 2006). Considering that chemical concentrations were variable during the experiment, C_x was calculated as time weighted average during the 48 hours of experimental exposure.
The authors of $SPEAR_{pesticide}$ index also established an empirical relationship between $SPEAR_{pesticide}$ values and the highest single-substance (Log-transformed) TU of the mixture that the community was exposed to (Schäfer et al. 2007). Predicted TU with this method had been compared with measured TU for the simulated events 3, 4 and 5. The regression for sites "with recovery area" was used, since organisms from upstream (unpolluted) were free to enter the contaminated part of the model streams.

4.3 Results

4.3.1 Water analysis

The stability of the system in this study was confirmed by the results derived measuring various physicochemical parameters (Table 4.2), since only minor variations were recorded. Temperature was the only variable showing a relevant increase from the first to the last simulated event. Dissolved oxygen was very high, always remaining around the 100% of the saturation except during the fourth simulated event (66.12%), when a surprisingly low value for this kind of environment was observed. However, even in that case the saturation level was enough not to cause a significant alteration to life conditions in the habitat. pH remained quite constant during the whole experiment, with values typical for surface waters in that region. Water usually remained transparent, with the highest average turbidity value being less than 5 NTU (Nephelometric Turbidity Units). Finally, the conductivity was always measured between 120-145 µS/cm.

Pesticide analysis performed on the water collected during the simulated events 3, 4 and 5 highlighted serious efficiency problems of the system. Concentrations were found usually lower than the nominal. Furthermore, a serious inter-event and inter-substance variability was observed. On the contrary, replicates within the same simulated event presented a good agreement. A complete overview of the nominal/measured concentration and their ratio is reported in Table 4.3. As a general pattern, it seems that pyrimethanil always presented the highest measured/nominal ratio, while chlorpyrifos had the lowest. It is interesting to note that this rank reflected the values of water solubility (pyirimethanil = 121 mg/L, phosmet = 15.2 mg/L. chlorpyrifos = 1.05 mg/L, data retrieved from FOOTPRINT 2006, see Online Resource 1). This is likely to be due to chemical adsorption on the tank walls or inside the tubes connecting the tank, pump and taps above the flumes. Concerning the inter-event variability, event 3 (03/06/2010) showed the lowest measured/nominal concentration ratio (chlorpyrifos ≈ 6 %, pyrimethanil ≈ 30 %), while the event 4 (30/06/2010) presented the highest (chlorpyrifos ≈ 42 %, pyrimethanil ≈ 60 %). Treatment 2 always presented higher measured/nominal concentration ratios than Treatment 1 thus, enhancing the differences between two treatments with respect to the experimental design.

Table 4.2 Physical-chemical parameters of the water measured during each simulated event.

Date	Temperature	Dissolved O_2(%)	Dissolved O_2 (mg/l)	pH 20°C	Turbidity (NTU)	Conductivity (µS/cm)
30/03/2010	5.60 (±0.00)	101.74 (±0.74)	11.90 (±0.08)	7.80 (±0.04)	2.24 (±0.67)	133.56 (±0.36)
20/04/2010	5.32 (±0.08)	110.64 (±21.54)	13.17 (±2.58)	7.82 (±0.07)	0.78 (±0.12)	128.62(±0.32)
03/06/2010	10.18 (±0.08)	n.a	n.a	7.92 (±0.15)	0.48 (±0.51)	120.70 (±0.10)
30/06/2010	14.36 (±0.06)	66.12 (±3.88)	6.32 (±0.37)	7.95 (±0.12)	4.30 (±0.84)	127.54 (±1.14)
29/07/2010	14.42 (±0.08)	101.56 (±1.74)	9.64 (±0.14)	7.95 (±0.03)	2.33 (±0.52)	141.30 (±0.20)

Table 4.3 Tracers (chlorpirifos, pyrimethanil, phosmet) measured - nominal concentrations and correspondent ratio (%) during the simulated events 3, 4, 5.

	Chlorpyrifos (µg/L)			Pyrimethanil (µg/L)			Phosmet (µg/L)		
Sample	Nominal	Measured	%	Nominal	Measured	%	Nominal	Measured	%
03/06 B	0.19	0.007	3.70	19.20	3.81	19.82			
03/06 C	0.20	0.009	4.50	20.00	3.52	17.61			
03/06 D	0.39	0.02	6.10	39.20	14.38	36.68			
03/06 E	0.37	0.04	10.88	36.80	17.98	48.85			
30/06 B	0.12	0.04	33.95	11.88	4.67	39.34			
30/06 C	0.11	0.03	27.78	10.80	5.31	49.17			
30/06 D	0.23	0.10	44.71	22.68	16.17	71.29			
30/06 E	0.18	0.11	62.65	17.28	14.36	83.13			
29/07 B	0.10	0.005	4.60	10.40	4.29	41.23	8.32	0.73	8.77
29/07 C	0.10	0.004	3.77	10.40	4.06	39.06	8.32	0.76	9.14
29/07 D	0.09	0.01	11.01	8.80	10.26	116.57	7.04	2.71	38.52
29/07 E	0.17	0.01	6.61	16.80	10.56	62.86	13.44	2.35	17.45

4.3.2 Univariate Community endpoints

Six different structural community endpoints were considered.
No relevant variation was found between flumes in the pre-treatment sampling and the highest CV was found for EPT% (0.27). Differences between upstream and downstream not due to treatments were tested and thus, the variance between upstream and downstream in flume A (entirely not treated) was compared with the variance between the upstream part of the other flumes (Levene's test). No significant difference was found for the results of all considered metrics ($p > 0.05$).
Values referring to the samples that were collected after the 5 simulated events are shown in Figure 4.4.

1) *Total abundance*: The total number of individuals/m^2 sampled (disregarding any classification) didn't give any appreciable results (Figure 4.4a). No effects for the treatment were recorded, since from the beginning till the end of the experiment, in most of the cases the number of individuals in the treated parts of the flumes exceeded the number of organisms found in the controls. This difference was likely to be due to an unequal distribution between "upstream" and "downstream" which was already present before the beginning of the experiment. The same pattern was always recorded for flume A (entirely used as control), confirming this hypothesis. Nevertheless, differences between upstream and downstream were never statistically significant. A considerable reduction over time recorded for both Treatment 1 (passed from about 24000 individuals/m^2 in March to 5000 individuals/m^2 in July) and Treatment 2 (from 15000 to 3000 individuals/m^2) could not be ascribed to the chemical exposure, since a comparable reduction was recorded for the control also (passed from 12000 to 3000 individuals/m^2), but is likely to be a natural process due to seasonal emergence of organisms (mainly insects, that account for the largest part of this community).

2) *Taxa richness*: Taxa richness was evaluated at family level. For Acari, Harpacticoida, Ostracoda, Nematoda, Hirudinea and Oligochaeta (usually present in low abundance), classification to family was not achieved and thus, they were considered as homogeneous groups. The same procedure was followed for all those Plecoptera and Diptera that were too young to be further classified. Also in this case, no effect of the chemical exposure was recorded. The number of taxa in the treated areas (both Treatment 1 and Treatment 2) always exceeded (with one exception after the second simulated event) the number of taxa sampled in the control (Figure 4.4b). Difference between Treatment 1 and control was significant for the first and the last simulated events (Dunnet's test, $p < 0.05$). No clear time trend was observed.

3) *Shannon's diversity index*: Results given by this index were not immediately interpretable (Figure 4.4c). During the first two simulated events, only certain effects could be detected, with alteration in Treatment 2 being much more evident than in Treatment 1. After the second simulated event, in particular the difference between Treatment 2 and the control was statistically significant ($p < 0.01$). However, after this date the Shannon's index highlighted how taxonomic diversity between both treatments and the control was not relevant and usually the values of the index were higher in Treatment 1.

4) *EBI*: The extended biotic index was originally designed to test the quality of water bodies in relation to anthropogenic oxygen depletion. Our results (Figure 4.4d) clearly show how this index was completely insensitive to pesticide contamination. Despite the stability of the control, no information about community alteration induced by pesticide exposure could be retrieved from the application of this index. According to EBI values, water quality always remained high (Class I-II). However, this was not surprising, since oxygen saturation was usually high during our experiment.

5) *EPT%*: The relative abundance of EPT (Ephemeroptera, Plecoptera, Trichoptera) was shown to be sensitive to pesticide contamination (Figure 4.4e). Values of this parameter remained always lower in the treated parts of the flumes with respect to the control (with the only exception of Treatment 1 after the second simulated event). Furthermore, this index was able to clearly highlight a difference between the two treatments, being always lower for Treatment 2 when compared to Treatment 1. Particularly, the effects with respect to the control were relevant after the fourth simulated event, close to significance for Treatment 1 ($p = 0.053$) and significant for Treatment 2 ($p < 0.05$). A sort of time trend was also detected, with a constant decrease in EPT% from the second to the fourth simulated event, with a relevant recovery after the final event.

6) *$SPEAR_{pesticide}$*: This index, specifically designed for the stressor considered in this study, showed a good sensitivity to pesticide as well as EPT% (Figure 4.4f). $SPEAR_{pesticide}$ values were always lower in the treatments with respect to the control. Furthermore, due to a scarce variability within groups, the effects in the treatments were often statistically significant (events 1, 3 and 4). In accordance with what was found for EPT%, the effect was extremely evident after the fourth treatment, both for Treatment 1 and Treatment 2 ($p < 0.01$). Nevertheless, the highest concentration treatment (2) caused a greater alteration than the low dose treatment (1) only with respect to the second and the fourth simulated events. Remarkably, the average value of the control remained extremely stable over time (CV = 0.05). It was

interesting to note that, after the first simulated event, $SPEAR_{pesticide}$ values decreased significantly in both treatments with respect to the control. However, both of them stayed above 50%, which is usually considered a rather high value for this parameter. $SPEAR_{pesticide}$ followed the same time trend found with EPT%, which was even more clearer in this case.

Correlation between all metrics were evaluated (Table 4.4). EBI correlated very well with taxa richness (which was logical, since the value of former depends on the latter) and probably due to external correlation with total abundance. Interestingly, the indices that have shown the highest sensitivity for pesticide contamination (EPT% and $SPEAR_{pesticide}$) were strongly correlated ($p < 0.0001$, Pearson's correlation test). In fact, both indices were based on the relative abundance of particular taxa, that in many cases were both EPT and SPEcies At Risk (SPEAR). Several EPT organisms in the model streams were classified as SPEAR in the online database provided by the authors of the index (http://www.systemecology.eu/SPEAR/index.php). Notwithstanding a clear similarity between the two indices, some important differences should be highlighted. First of all, a small variability within groups and stability of the control over time found with $SPEAR_{pesticide}$, made easier to detect significant alteration of the community due to pesticide exposure. For the same reason, time trends were also clearer. However, $SPEAR_{pesticide}$ couldn't relate the magnitude of the effects with the two different concentration levels induced in the model streams (except for two cases, after simulated event 2 and 4), while, on the contrary, EPT% did.

Table 4.4 Pearson's correlation matrix between structure endpoints. Values in bold represent statistical significant correlation ($p < 0.01$).

	$SPEAR_{pesticide}$	Shannon	Taxa richness	Tot. Abundance	EPT%	EBI
$SPEAR_{pesticide}$	1.000	0.012	-0.268	0.228	**0.680**	0.332
Shannon		1.000	0.299	-0.160	**0.556**	0.191
Taxa richness			1.000	**0.582**	-0.054	**0.715**
Tot. Abundance				1.000	0.034	**0.737**
EPT%					1.000	0.267
EBI						1.000

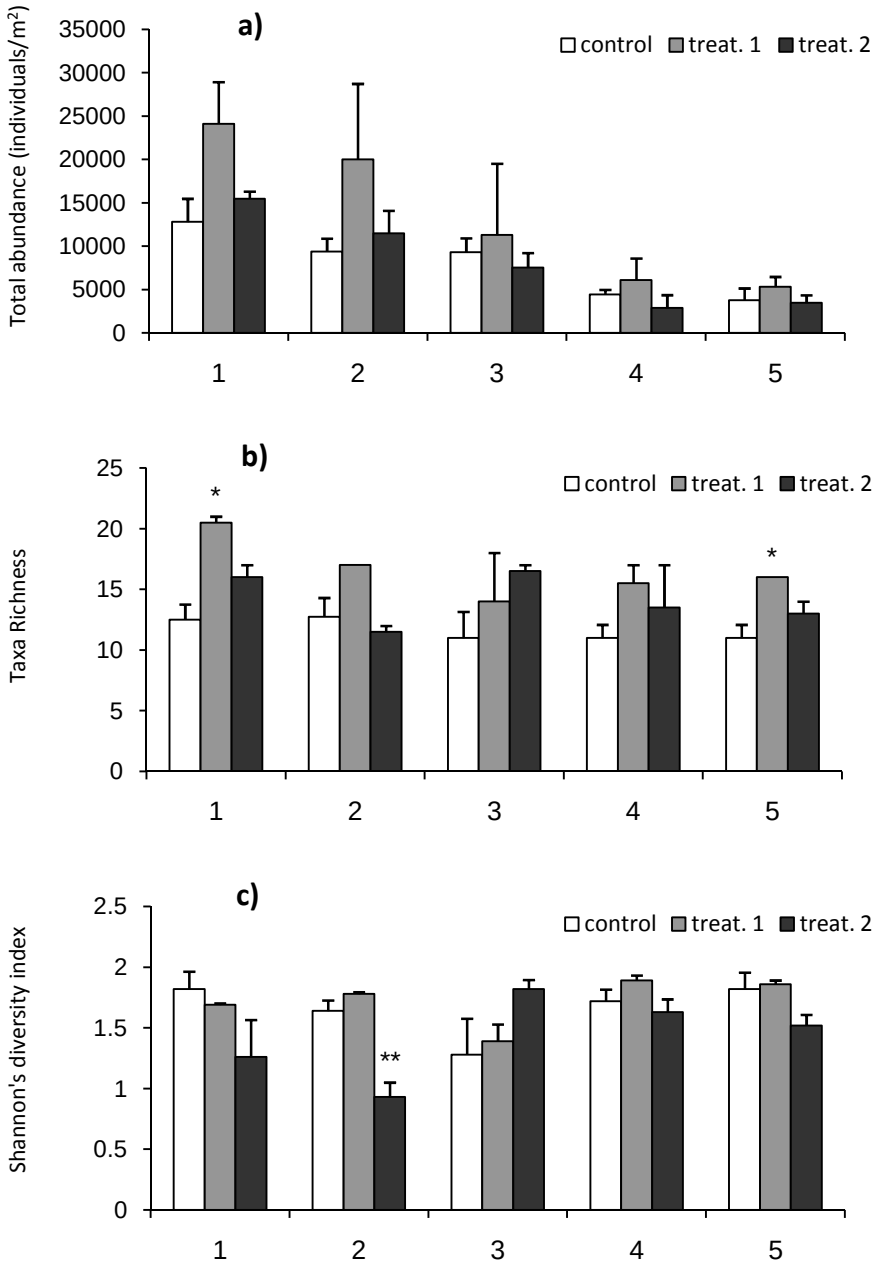

Figure 4.4 Comparison between means (+ SE) of different metrics after 5 pulses (*x*-axis) of lower (treat.1) and higher (treat.2) concentration of pesticides mixture in relation to the control. Significance between treatments and control (ANOVA, Dunnet's test) is reported (* = $p < 0.05$; ** = $p < 0.01$). (a) Total abundance (organisms/m^2); (b) Taxa richness (family level); (c) Shannon's diversity index.

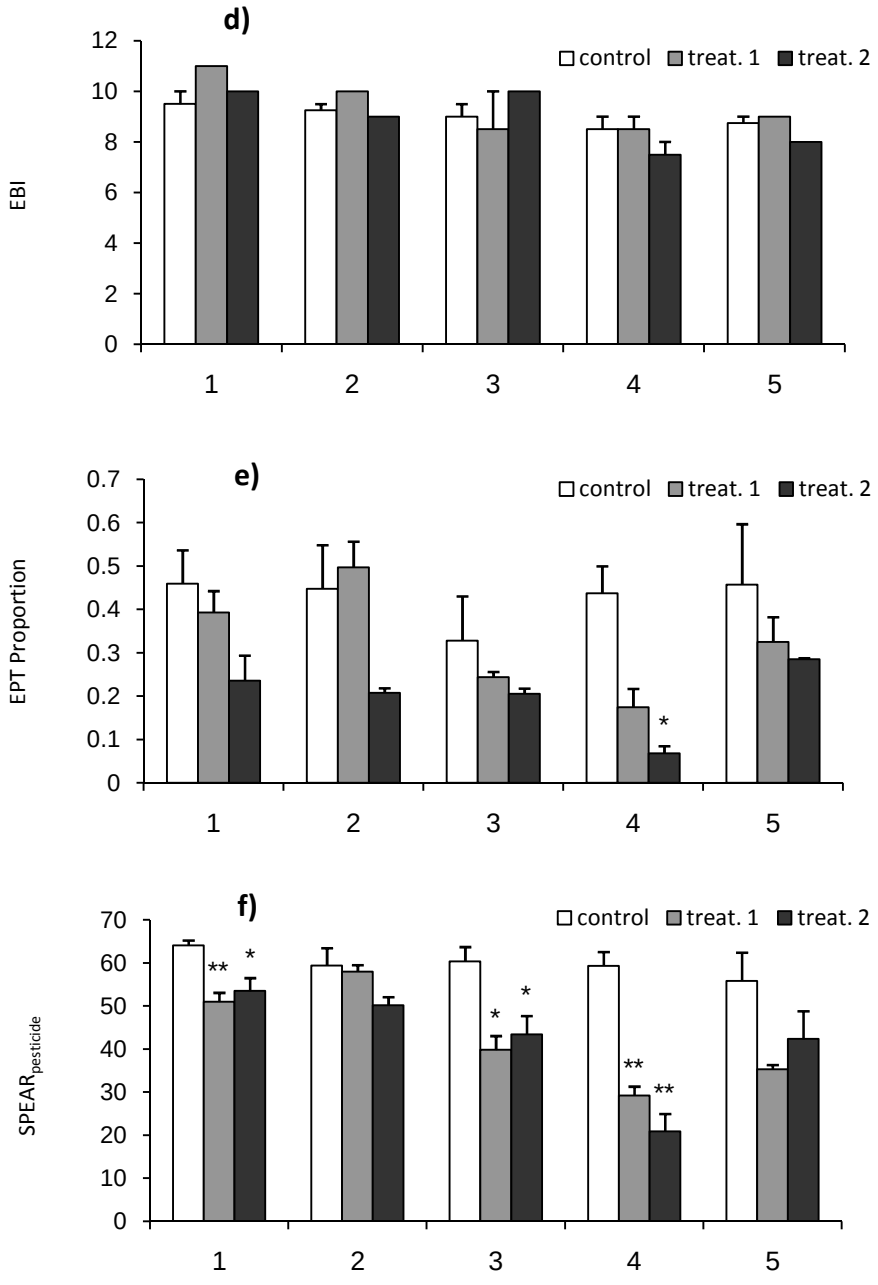

Figure 4.4 *(continues from previous page)* (d) Extended Biotic Index; (e) EPT relative abundance (fraction); (f) $SPEAR_{pesticide}$.

4.3.3 Principal Response Curves (PRCs)

Statistical significance of the first PRC (Figure 4.5) was confirmed by the Monte-Carlo permutation test (p = 0.002, 499 permutations). The first PCR showed how Treatment 2 in spite of higher concentrations, caused less alteration than Treatment 1 with respect to the control. The differences between two treatments were not so relevant, except for the community sampled after the last simulated event. This was very much in agreement with what was already found from the application of $SPEAR_{pesticide}$. Both treatments showed a relevant deviance of the community from the control already after the first simulated event, with a certain recovery in the community sampled after the second. In both the treatments, alteration increased from second until the fourth simulated event. This was also in agreement with the findings derived by the application of $SPEAR_{pesticide}$ and the EPT%. The first PRC showed that after the last simulated event, there was a recovery of the community in Treatment 2, while there was a further alteration in Treatment 1. Taxa scores (b_k) showed that Heptagenidae, Leuctridae and Nemouridae were the three most affected families. Remarkably, all of them were also counted as EPT and SPEcies At Risk. On the other hand, taxa that resulted as being less affected by the treatments were not counted in the EPT and were Species not at risk (SPEnotAR) (Oligochaeta, Planariidae and Elmidae). In general, within the organisms that showed a score > 0.2 (in Figure 4.5 only taxa with values > 0.5 and < - 0 5 are reported, due to reasons of clarity), nine out of eleven were classified as SPEAR, while eight were counted as EPT. Conversely, among 18 taxa presenting a score < -0.2, only four were classified as SPEAR and only three belonged to EPT.

The second PRC (Figure 4.6) was found to be statistically significant (p = 0.004), while the third PRC was not (p > 0.05) and thus, was not reported. The interpretation of the second PRC is probably more complicated. Despite the trends of the two treatment regimes were comparable, the curve corresponding to Treatment 2 always presented a little deviance from the control. On the contrary, the curve for Treatment 1 provided important information, especially on the taxa presenting the highest correlation with this trend (taxa with the highest b_k). In fact, according to this diagram, there was a relevant alteration after the first simulated event, followed by a slight further alteration after the second (where the first PRC presented a slight recovery) and a strong recovery after the third and the fourth events (where the first PRC presented the most important alteration). The last sample point show another relevant alteration (where the first PRC showed only a slight alteration in Treatment 1 and a recovery in Treatment 2). Basically, this second PRC showed a trend that was almost the opposite to what was presented in the first PRC.

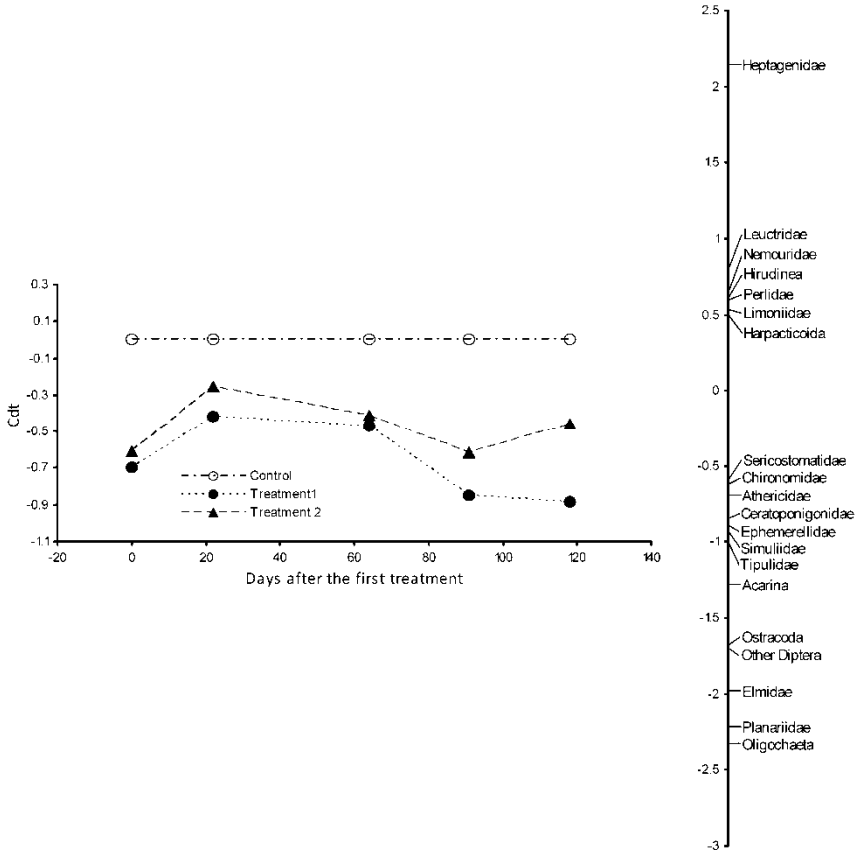

Figure 4.5 First Principal Response Curve (PRC) diagram indicating the effect of the simulated events (pesticides loads) on the macroinvertebrate community. The vertical axis represents the difference in the community structure between treatments (solid symbols) and the control (empty symbol) expressed as regression coefficient (C_{dt}) of the PRC model. The taxa score (b_k) on the right can be interpreted as a correlation of each taxon with the response given in the diagram. Only taxa with values > 0.5 and < -0.5 are showed.

Remarkably, most of the taxa that exhibited greater correlation with this diagram (Harpacticoida, Ostracoda, Ceratoponigonidae, Canthocamptidae) were characterized by relatively short life cycle and great biotic potential: they can be classified as opportunistic species. Conversely, many vulnerable species presenting negative values of b_k for this second PRC were classified as SPEAR. This may be an example of secondary effect: during the period of maximum decrease of vulnerable species, opportunistic taxa increased. In presence of even slight recovery of vulnerable taxa (e.g. after the second and the last simulated events), opportunistic taxa decreased.

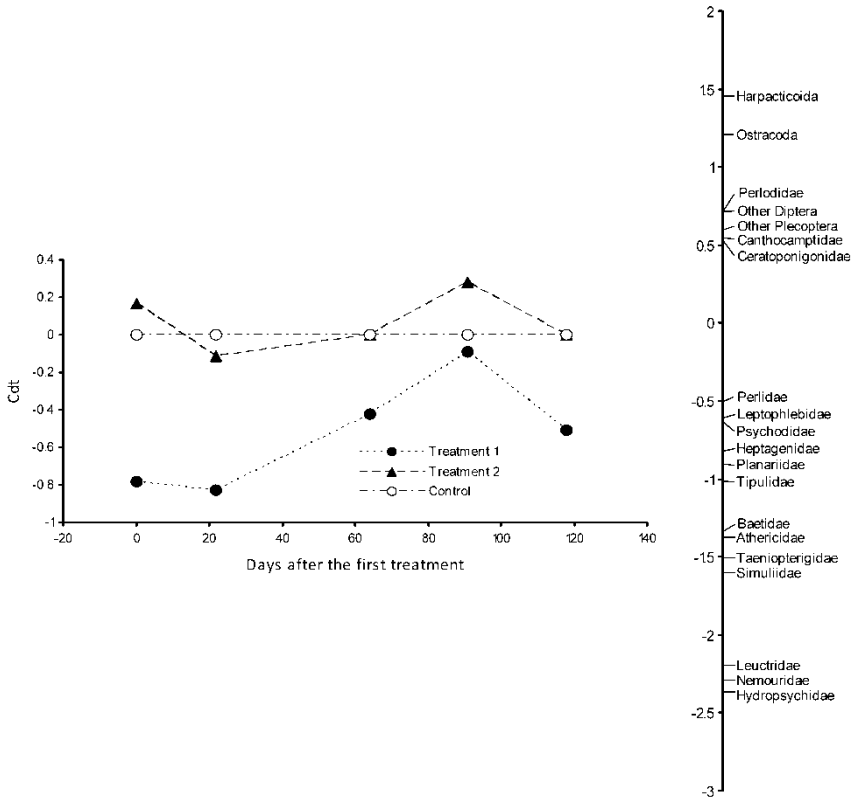

Figure 4.6 Second Principal Response Curves (PRC) diagram indicating the effect of the simulated events (pesticides loads) on the macroinvertebrate community.

4.3.4 Community response to the toxicity of the mixture

A precise assessment of the actual exposure to the mixture was impossible because the available analytical data indicated that actual concentrations were substantially lower than the nominal ones. Moreover, only three chemicals where analytically tested. However, the three chemicals selected as tracers (chlorpyrifos, pyrimethanil and phosmet) accounted for most of the toxicity (between 96% and 99%) of nominal mixture used in the simulated runoff events 3, 4, and 5 (the ones from which water samples were collected).

An approximated exposure range was calculated on the basis of the available analytical data. Details of the approximation procedure are described in the *Appendix 3*. In Table 4.5, maximum and minimum estimates of TUs are shown. It is worth noting that, for events 3, 4 and 5, the difference between minimum and maximum TU values were low or negligible.

Table 4.5 Estimated maximum and minimum potencies of the mixtures (expressed as $TU_{Daphnia\ magna}$; ± values reflects variation between replicates) used in the simulated events and relative proportion (%) covered by measured concentrations of the tracers (chlorpyrifos, pyrimethanil and phosmet). Explanations in the text.

	Treatment 1			Treatment 2		
Event	TU max	TU min	%	TU max	TU min	%
1	0.07	0	0.0	0.16	0	0.0
2	0.04	0	0.0	0.08	0	0.0
3	0.15 (±0.01)	0.12 (±0.01)	75.7-100	0.52 (±0.11)	0.46 (±0.11)	85.0-100
4	0.62 (±0.08)	0.59 (±0.08)	94.8-100	1.91 (±0.23)	1.85 (±0.23)	96.5-100
5	0.62 (±0.01)	0.59 (±0.01)	94.7-100	2.80 (±0.75)	2.75 (±0.76)	97.2-100

Both multivariate and univariate analysis of the community structure were in agreement with the calculation of total TUs of the mixture, even if some exceptions were found.

PCR, $SPEAR_{pesticide}$ and EPT% showed a relevant alteration of the community after the first simulated event (although the values of $SPEAR_{pesticide}$ remained above 50), however, the TU range of the only chemical used (etofenprox) was 1-2 orders of magnitude below the acute LC50 of *Daphnia magna*. One possible reason could be due to the contamination period. This simulated event took place at the end of march, when a lot of organisms (especially insects) were still in their earlier larval stages. Of course, this could have greatly enhanced the sensitivity of a large part of the community. The second simulated event involved only fungicides, with the lowest level of TU. In fact, scarce alteration was detected by the principal metrics used. The community response to the third, fourth and fifth simulated events seemed to be highly driven by chlorpyrifos concentrations rather than total TU. Moreover, the total mixture toxicity of the third and fourth events relied mainly on chlorpyrifos. Despite the overall toxicity of the last mixture was nominally the highest, the response of the community was less relevant. Indeed the largest part of the toxicity of this last mixture depended on phosmet (only used in this event), while chlorpyrifos concentrations (TWA, see *Appendix 3*) were the lowest. This can be explained considering that in our evaluation, TUs were calculated using *Daphnia magna* as reference species. Phosmet is highly toxic for *Daphnia* (FOOTPRINT reports a 48h LC_{50} of 2 µg/L) and in general for crustaceans (e.g. 48h LC_{50} *Gammarus fasciatus* = 5-5.2 µg/L, Mayer & Ellersieck 1986; 24h LC_{50} *Gammarus pseudolimnaeus* = 2.4 µg/L, Julin & Sanders 1977). This was also confirmed by the results of the second PRC. The only crustaceans present in our model streams (Harpacticoida, Ostracoda and Canthocamptidae) showed a strong decrease after the last simulated event (see trend of the PRC and b_k values of Figure 4.6).

However, phosmet presents much lower toxicity on insects (e.g 48h LC_{50} *Chironomus plumosus* = 3150-10000 µg/L, Mayer & Ellersieck 1986; 24h LC_{50} *Culex pipiens* = 600 µg/L, Mulla et al. 1962; 48h LC_{50} *Cloeon dipterum* = 130 µg/L; Nishiuchi & Asano 1979) that are the major component of the community in the model streams. Thus, it seemed reasonable that the community response was more related to chlorpyrifos concentrations.

The relationship between $SPEAR_{pesticide}$ and the Log-transformed highest measured value of single-substance TU was tested (Figure 4.7). The highest single-substance TU of the mixture were determined by chlorpyrifos for the events 3 and 4 and by phosmet for the event 5. Predictions of the regression highly underestimated measured values in event 3 and 5, while they were in the same order of magnitude for event 4. However, a significant correlation was found between $SPEAR_{pesticide}$ values and maximum single-substance Log(TU) (calculated as TWA, see *Appendix 3* for values and regression plots) (R^2 = 0.62, p = 0.007, Pearson's correlation test) using chlorpyrifos instead of phosmet for event 5. A comparable though better correlation was found between maximum single-substance Log(TU) and EPT% (R^2 = 0.72, p = 0.002, Pearson's correlation test).

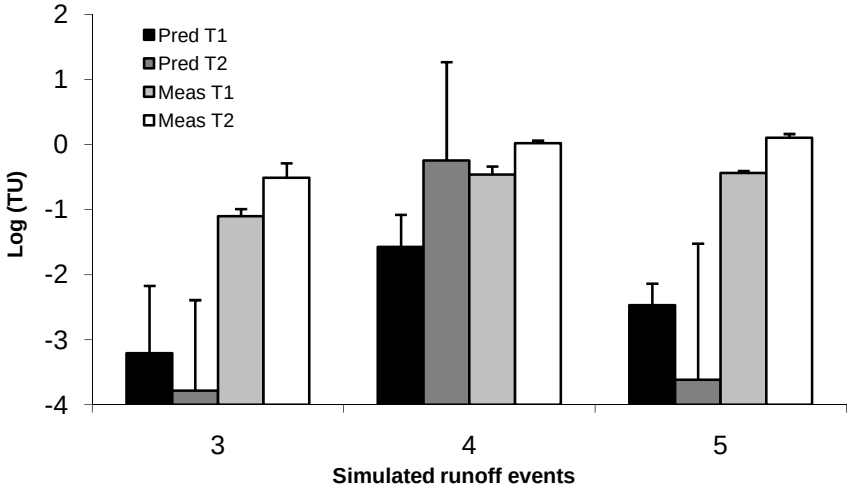

Figure 4.7 Maximum single-substance log-transformed TU (mean + SE) as predicted by the regression based on SPEAR values (Schäfer et al. 2007) and comparison with values calculated from the measured concentrations (mean + SE). Comparison is possible for the simulated events 3, 4 and 5 (no available samples from the events 1 and 2). T1 indicates treatment 1 (lowest concentrations pattern), while T2 indicates Treatment 2 (higher concentrations pattern).

4.4 Discussion

In designing and in realizing this model ecosystem experiment, we considered most of the indications given by mesocosms guidance (e.g. CLASSIC – Giddings et al. 2002). However, this study was not aimed at determining NOEC or LOEC for a particular chemical, thus defining a precise exposure-response relationship. Mesocosms study executed to determine such Ecologically Acceptable Concentrations (EAC) are usually designed to test the response of the community exposed to a broad spectrum of concentrations, often with differences of orders of magnitude (Beketov et al. 2008; Colville et al. 2008; Pestana et al. 2009). For this study, experiment was conceived as a tool to support site specific ecological risk assessment by reproducing a realistic exposure scenario for a well defined region of Northern Italy. Pesticide application followed the exposure pattern resulting from a preliminary model simulation, with just two different exposure levels, differentiated by a factor 2. Model ecosystems like the one used for this work guaranteed a good protection from many disturbing factors, and a direct causal relationship could be established between the changes in the community (in relation to the control) and the applied stressor.

The chemical analysis performed on the water samples clearly reported a problem in the efficacy of the experimental system, with actual concentrations of chemicals being even 30 times lower than the nominal, probably depending on the water solubility of the chemicals. However, those problems didn't affect the value of the results obtained: on the contrary, this work showed that relevant alterations of the community can occur even at much lower concentrations than those predicted by the exposure model for the area of interest.

Several metrics were used and evaluated to describe the results of the experiment. The total number of organisms showed a constant decrease over time, but likely to be caused by natural factors (e.g. natural emergence of insects) rather than by the treatments. Indeed, treated flumes always presented higher density of organisms compared to the control (though this difference is never statistically significant), probably because of an uneven distribution at the beginning of the experiment. Other model streams experiments performed with single pesticides (Beketov et al 2008; Colville et al. 2008; Pestana et al. 2009) already showed that this parameter is significantly altered only with very high concentrations applied, which was not the case in this study. Furthermore, all the cited experiments presented a lower degree of exchange with the external environment (i.e. input of new organisms) compared to this study.

Taxa richness has sometimes been indicated as an appropriate tool for studying the impact of pesticides on freshwater model ecosystem (Brock & Budde 1994). However for this study, this metric was shown to be scarcely

related to pesticide contamination. Moreover, this fact was in agreement with the results published by other authors for micro-mesocosms (Beketov et al 2008; Colville et al. 2008; Flemer et al. 1997) and field studies (Castillo et al. 2006; Maltby & Hills 2008). This is probably because the populations within a (model) ecosystem are hardly driven to a complete extinction by a pesticide contamination in such a short time: only very high concentrations can cause certain species to disappear, while much lower concentrations can easily alter the equilibrium and the structure of the overall community.

Diversity indices (e.g. Shannon's Index) were already acknowledged to be unreliable descriptors of pesticides impacts on freshwater communities (Brock & Budde 1994; Ford 1989) and the theory was confirmed with this study. This is especially true for mid/short-term experiments like the one related to this study. In case of chronic exposure, the alteration of the macroinvertebrate community can evolve following a very different path, favouring much more resistant species in spite of their ecological traits connected to the after-stress recovery (e.g. biotic potential, voltinism, etc.) and thus, inducing a small number of taxa to dominate the community.

The choice to include the EBI for evaluation of this study was related to the wide usage of this index in Italy to assess the quality of the water bodies. It was important to know what kind of information this index could give about pesticide contamination, which is not the stressor that it was conceived for. In fact, no examples showing this relationship were found in the literature. The results from this study clearly showed that the EBI was totally insensitive to the community alterations induced by pesticide contamination.

Within the univariate metrics that were applied, the two most efficient in detecting any pesticide-induced community alteration were $SPEAR_{pesticide}$ and EPT%, being strongly correlated to each other. SPEAR was developed specifically to detect pesticide effects and its sensitivity to test the contamination levels is well documented (e.g. Schäfer et al. 2007). EPT% on the other hand has already shown a certain sensitivity to this kind of stressor (Castillo et al. 2006, Liess et al. 2008). In studies evaluating the effect of pesticides on the benthic invertebrate community, the number of EPT taxa is probably more frequently used (e.g. Beketov and Liess 2008) than the EPT% (referring to the proportion of EPT individuals within the entire community, not to the number of taxa). The results of this and other studies confirmed that quantitative indices considering the abundances within groups (taxonomic-based or trait-based) are in general, more suitable to detect pesticide-induced alteration at the community level, since complete disappearance of taxa (or functional groups) is usually less frequent. Due to the nature of this study, no general conclusions were reached concerning the suitability of EPT% as a metric to detect pesticide contamination. It is surely possible that vulnerable species in this case showed a relevant overlap with EPT (as confirmed by the PRC analysis), but in a different ecosystem, or with different chemicals, this relationship could have been completely

altered. Rasmussen et al. 2011, focusing on pesticide contamination of Danish lowland rivers, showed how EPT% was correlated with some environmental variables (as well as with $SPEAR_{pesticide}$ values).
$SPEAR_{pesticide}$ confirmed its value as index for pesticide contamination. A very significant correlation was found with the maximum Log-transformed single-substance TU of the applied mixture. However, due to experimental design of this study that was not focused on establishing this kind of relationship, problems related to temporal pseudoreplication could not be excluded. Nevertheless, the high dynamicity of our system, together with a relevant time span between successive sampling dates (usually one month), should have minimized the temporal intercorrelation between samples. Moreover, treated areas of channels were directly below untreated areas. Hence, recolonisation within stream may have quickly buffered the effects induced by pesticide loads.
The linear regression model that was found (see *Appendix 3*) in this study was extremely different from those found by Schäfer et al (2007). Particularly, the slope of the regression seemed to be very different. Even if not explicitly reported in the paper, the slopes of the regressions found by Schäfer and colleagues for French and German streams should be around -7 (inferred from figure 3 of the same paper), while in this study, steeper slope of -15 (data not shown) was found. There are several possible explanations for these differences. First of all, data used for this work, being resulted from a model ecosystem experiment, were not entirely comparable with field data. The same community was sampled to test how it was varying over time, but inevitably each sampling was not independent from the previous one. However, another possible cause for such difference could be linked to the ecosystems typology. Schäfer et al (2007) only considered lowland streams in different biogeographical regions, while the experiment performed for this study mimicked an alpine stream scenario. Apart from the obvious differences in environmental conditions (higher current velocity, different substrate, etc), there were important differences in terms of the exposure path. Alpine catchments, due to high slopes, may present a very sudden response to rain events. Thus, pesticides concentrations in alpine streams might be characterized by extremely relevant peaks for very short periods, while in lowland rivers this exposure could be distributed over a longer timespan. If that was true, the applicability of the $SPEAR_{pesticide}$ - maximum TU relationship would be limited, but it wouldn't affect the efficacy of $SPEAR_{pesticide}$ index to verify the effects at community level caused by pesticides load.
Recently, a rather heated debate began about the efficacy of the $SPEAR_{pesticide}$-derived index $SPEAR_{mesocosm}$ to detect alterations in the communities exposed to pesticide in comparison with PRC. Particularly, Liess & Beketov (2011) reported the results of a mesocosm study in which they showed how $SPEAR_{mesocosm}$ was able to detect a significant alteration in

the community at much lower concentrations (and for longer periods) than what found with the PRC method. Furthermore, the authors found a discrepancy between the most affected species according to the PRC and the most vulnerable according to a priori classification that the $SPEAR_{mesocosm}$ is built on (sensitive uni/semivoltine).
For this experiment, classical $SPEAR_{pesticide}$ index was applied instead of $SPEAR_{mesocosm}$, since in the study preferred to understand the in-stream "migration ability", given the structure of system and experimental design. Drift, recolonization from upstream and movements against water direction were important processes in model streams, but they are not considered in the $SPEAR_{mesocosm}$, conceived for closed system. Unlike Liess & Beketov (2011) a good agreement was found between the results of $SPEAR_{pesticide}$ and those of the PRC. The temporal trends of the community in the treated flumes with respect to the control was absolutely comparable for the two methods; moreover, many of the most affected taxa indicated by the first PRC were classified as SPEAR. Similarly, many of the less affected taxa in the first PRC were classified as SPEnotAR.
A recent paper by Van den Brink & Ter Braak (2011) critically analyzed the findings of the abovementioned paper by Liess & Beketov (2011), highlighting some methodological criticisms (not relevant in this discussion) as well as some interesting conceptual arguments. Particularly, they pointed out how SPEAR indices uses a sensitivity ranking based on averaged data retrieved from the EPA AQUIRE database. They also highlighted how this database is biased towards a few classes of pesticides (e.g. organophosphates). This observation is particularly relevant since $SPEAR_{pesticide}$ had been successfully applied several times in the field, where toxic effects are more likely to be caused by exposure to mixtures (thus the "average" approach is somehow justified) rather than to single substances. On the contrary, mesocosm experiments are frequently run to test the effects of a single compound (unlike the experiment presented here), thus the sensitivity ranking normally used in the SPEAR approach might need several adjustments each time, since it has been proven that pesticide with different mode of action are characterized by different sensitivity ranking (Rubach et al. 2010). Carrying out all these corrections each time might result in a difficult and lumbering process, not justified in cost-efficiency terms.
The success experimented by the $SPEAR_{pesticide}$ index over other metrics is likely to be related to its high ecological content. However, the sensitivity component is still empirically-based and causes the index to be less suitable for specific evaluations. The usage of this tool within the Ecological Risk Asessment can be widened, strengthening the trait-based framework of the index and focusing on a way to mechanistically relate biological traits and sensitivity to different mode of actions (a promising start has already been made with the works of Rubach et al. 2010 and Ippolito et al. 2011). In fact,

one of the greatest strengths of the SPEAR approach is the possibility to easily compare the results of manipulative studies (like the one presented here) with field studies and biomonitoring campaigns. On the contrary, this linkage is much more complicated using multivariate approaches, due to the natural variability of the taxonomic structure of the communities and the difficulties to find a proper reference (control) in the real environment.
A quantitative relationship between the estimate of mixture risk based on laboratory toxicity tests (expressed as TUs calculated for *Daphnia*) and the effects on aquatic community estimated with the applied metrics, may be biased due to different issues:

- the sensitivity of the community may substantially change during the seasonal cycle;
- the concentration addition (CA) approach for calculating the TUs of a mixture is generally accepted as a reasonable worst case in aquatic toxicology (Junghans et al. 2006; Vighi et al. 2003); this is fully justified for the toxicity on single organism, but it is not generally applicable for assessing the effects on a complex community, where the sensitivity of individual species to each chemical may be substantially different;
- *Daphnia magna* is generally a very sensitive organisms and, for many chemicals, it falls in the lowest 10 percentile in the species sensitivity distribution (SSD); however, in some cases, particularly for insecticides, many insect larvae may be substantially more sensitive (or extremely less) than *Daphnia*. Unfortunately, acute ecotoxicological data for insect larvae are often not available (4 out of 10 investigated chemicals in our case) or extremely inconsistent. Therefore, even with all evident limitations, the use of *Daphnia magna* as reference species is the only possible choice.

In spite of these difficulties, results from this study showed that a good relationship can be established between exposure and effects at community level.
It was also clearly shown that realistic (or even lower) patterns of pesticide concentrations can induce relevant alteration in the benthic invertebrate community for the streams of the studied area. Unfortunately, no inference about the recovery can be done. In the original study design, more samplings were planned after the last treatment (to be simulated in August), in order to monitor the community recovery. However, a violent flood of the Fersina stream on August 15th 2010 seriously damaged this experimental system, forcing to terminate this experiment.

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5 Site-specific pesticide risk assessment in a small Alpine catchment: a multi-level approach

Abstract
With the introduction of the Water Framework Directive, the approach used so far to assess pesticide risk in surface waters revealed to be insufficient to be applied on actual situations and protect real ecosystems. A site-specific approach is required to evaluate the characteristic of particular exposure patterns and induced effects, taking into account the vulnerability of the ecological system. In this work the preliminary results of a study on a small alpine catchment of Northern Italy are reported. The final goal is to present a detailed site-specific risk assessment, with high temporal resolution, for an alpine stream (Novella River), focusing on surface runoff events. To do so, a nested multi-level approach was used, in order to compare different evaluations of the exposure (models and water samples), and of the effects (biomarkers and invertebrate community responses). Furthermore, a comparison between exposure and effects was made 1) on theoretical basis, using the official standard procedures, and 2) using actual community data. Results highlight the presence of a relevant potential risk for the invertebrate community, causing significant alterations at the community level. Nevertheless, the establishment of a clear causal relationship between exposure and effects is difficult. The ecological system proved to have good resilience, with a complete recovery achieved within one year.

Keywords: site-specific risk assessment; modeling; exposure-effects link; SPEAR; resilience

Unpublished manuscript

5.1 Introduction

The characterization of chemical risk to aquatic ecosystems is performed by comparing potential effects, usually estimated by measuring toxicological endpoint for standard indicator species, and expected exposure concentrations. Within the EU, the guidance document regulating the risk characterization for plant protection products (SANCO/3268/2001 revision 4 – EC 2002) provides for a toxicity-exposure ratio (TER), in which effect endpoints are divided by the exposure concentrations. Effect endpoints and TER triggers for tier 1 assessment (acute and chronic) for indicator species are defined in the Annex VI of Directive 91/414/EEC and in the aquatic guidance document (EC 2002).
Due to the extremely wide spectrum of possible environmental scenarios, current regulatory procedures as described in the abovementioned documents, necessarily adopt a simplified approach. This allows to speed up the evaluative process, which would be otherwise too complex, but introduces a relevant uncertainty in the outcome of the assessment. The extent of this uncertainty is usually expressed by the magnitude of the so-called “safety factors” or “TER triggers”, that are assumed to be protective enough for any possible scenario.
One of the most important source of uncertainty in plant protection products (PPP) risk assessment regards the characterization of the exposure, and the relative link with effects. Particularly, time-variable surface water exposure profiles are not correctly addressed in current procedures (Brock et al. 2010). Most of the potential sources of pesticide load in water bodies are usually discontinuous (runoff, drift, many point sources, etc), thus this issue is pivotal in PPP risk assessment. Disregarding the process originating the load, pesticide presence in surface waters is usually characterized by concentration peaks, which may be single (as in the case of spray drift) or repeated over time (very common for runoff events). A precise knowledge of the shape of these peaks is required for a proper evaluation of the exposure. In the “executive summary and recommendation” drawn up after the ELINK EU Workshop (Brock et al. 2010) some guidelines are given about which parameters should be studied when characterizing a time-variable exposure profile:

- Height of peak concentrations
- Area-under-the-curve concentrations
- Duration of peak exposure
- Interval between peaks
- Height of a possible long-term background concentration
- Frequency of peaks

This kind of evaluation for regulatory purpose in EU context is carried out through the use of FOCUS (Forum for the Co-ordination of Pesticide Fate Models and Their Use) models and standard scenarios.
Predictive exposure models are probably the only way to standardize the evaluation of PECs (Predicted Environmental Concentrations) within risk assessment procedures. After the introduction of the Water Framework Directive (WFD, EC 2000), they have been used for site-specific studies also, since they allow to reduce massively the cost (in terms of sampling effort and analysis), especially when the evaluation is carried out over a big spatio-temporal scale. Particularly, the application of geographical information systems (GIS) in pesticide transport modeling has gained importance in site-specific risk assessment. Several examples of GIS-based procedures for predicting pesticide distribution and fate are already available in the literature (Verro et al. 2002, Verro et al. 2009a, Sood et al. 2005, Schriever et al. 2007). However, models only consider a finite number of variables, while processes in environment (transport, adsorption, volatilization, deposition, degradation, etc) are determined by a huge number of possible factors. Hence, the use of predictive models should always be associated with suitable concentration measurements.
While pesticide effects on aquatic organisms are relatively easy to be measured in laboratory conditions, their extrapolation to the real environment is much more critical. For this reason, the importance of site-specific field studies is not only relevant for a specific validation of the effectiveness of current risk management procedures, but even to give more general information about the process of extrapolation from the single-species (and single chemical) approach to the real environment.
The importance of field studies, especially monitoring studies, has been highlighted in the outcome of the workshop “Effect of Pesticides in the Field” (Liess et al. 2005), where, among other themes, some of the most relevant issues concerning the detection of the effects were listed. First of all it is necessary to find a proper reference system, which needs to incorporate “the natural variability characteristics of the ecosystem under consideration” and minimize “the importance of environmental parameters and confounding factors by avoiding differences other than pesticides between reference and tested sites”. Another important issue regards the choice of the endpoint(s) to be measured, and particularly the choice of the level of biological organization to be studied. One must be aware that the final goal of any Ecological Risk Assessment procedure is the protection of communities and ecosystems, and thus a study at these levels is certainly more informative. Nevertheless, analysis at such high levels may be extremely complicated, thus lower level studies (from sub-individual to population) may help with the interpretation of data obtained at higher levels. Structural endpoints have proved to be more reliable indicators of pesticide contamination (Kersting

1994), but information from functional endpoints is also very important in understanding the stability of the ecological system.
Last but not least, the evaluation of effects in the field must consider the time for recovery. Some authors challenged the classic concept of recovery (Wiens 1996) at higher level of biological organization, arguing that this implies a previous equilibrium state which is uncommon in ecological systems. Nevertheless, many studies dealing with pesticides have proven, using different endpoints, that recovery processes are the common rule in communities and populations after pesticide exposure. Certainly, space and time are important parameters to consider in the recovery process. Recovery may depend on life history traits of the species, on the magnitude and of the length of the exposure, and on the presence of unpolluted areas that may act as "source", to use a typical landscape ecology term.
The uncertainties regarding both the characterization of the exposure and of the detection of the effects concur in complicating the establishment of a proper causal link between the two terms. In fact, a review carried out some years ago about pesticide field studies (Schulz 2004) highlighted how only few studies were able to establish a causal relationship between the measured concentrations in surface waters and the observed effects.
In this work the preliminary results of a study on a small alpine catchment of Northern Italy are reported. The final goal of this work is to present a detailed site-specific risk assessment for an alpine stream (Novella River), focusing on surface runoff events. To do so, a nested multi-level approach was used, in order to compare different evaluations of the exposure (modelled and measured), and of the effects (at different level of biological organization). Furthermore, a comparison between exposure and effects was made 1) on theoretical basis, using the official standard procedures, and 2) using actual community data.

5.2 Materials and methods

5.2.1 Experimental area

The object of the study is the Novella River, whose alpine catchment (132 km^2) is sited in Trentino-Alto Adige region, Northern Italy (Figure 5.1). Like most of the valleys in the area, the basin of the Novella River is intensely cultivated with apple orchard, which occupy a relevant portion of the entire surface of the watershed. This kind of cultivation requires huge external inputs to be maintained, and that includes a relevant amount of plant protection products, especially fungicides and insecticides. Since most of the area is characterized by high slopes, the risk for pesticide surface runoff is potentially very high. In addition, orchards are usually grown in the lowest part of mountains slopes, very close to the streambed, often without proper buffer strips, enhancing the risk for pesticide load due to surface runoff.

However, a forested area is present in the upstream part of the basin, where no relevant human activities are present.
The pesticide application scheme (substances, rates and dates) in the basin is largely determined by two consortia, each one operating on a well determined sub-basin. Particularly, the extent of the area regulated by the SASA consortium is 250 ha, while the SABAC consortium determines the application pattern for an area of 230 ha. Precise data of Plant Protection Products applied (active ingredient and formulation), rate and dates of application were obtained from both consortia for the productive season 2010 and 2011. A detailed resume of this information is reported in the *Appendix 4*.

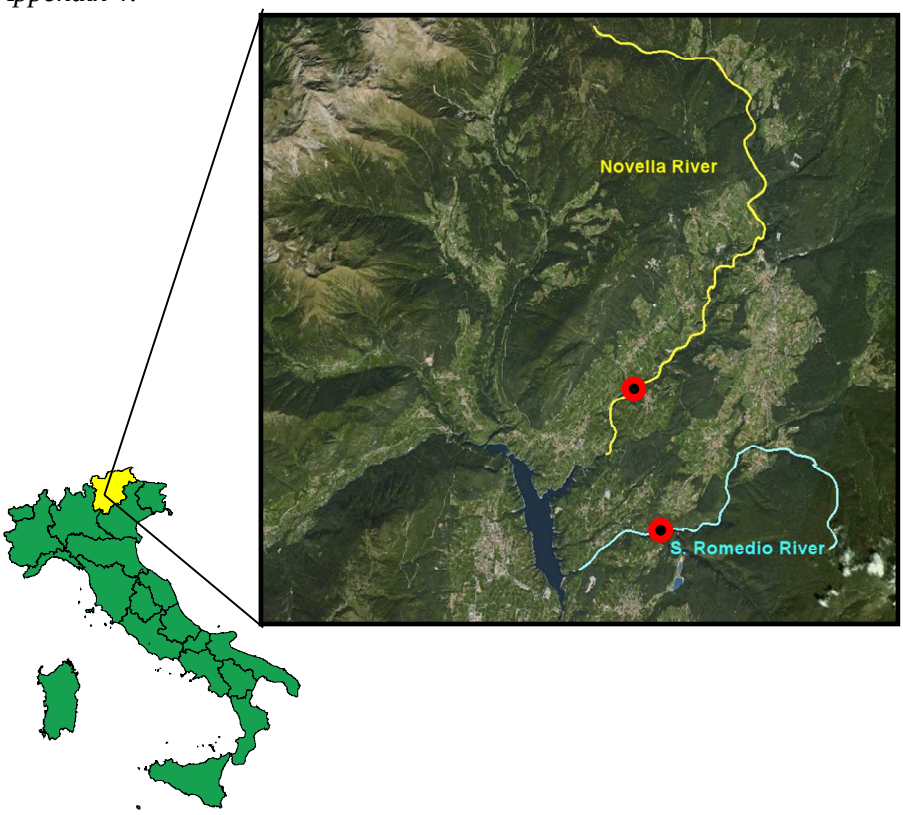

Figure 5.1 Location of the studied rivers. Novella River is likely to be impacted by pesticides loads, while S. Romedio River is used ad reference. Red circles indicate sampling stations.

Surface runoff is one of the most relevant processes that determine pesticide loads in surface waters (Wauchope 1978, Larson et al. 1995, Schriever et al. 2007, Schriever and Liess 2007) and thus our analysis concentrated on this entry route. To pursue this goal, rainfall data (mm per hour), relative to the closest meteorological station (Romeno, sited in the same basin) were

retrieved from an on-line database (www.meteotrentino.it). Data were collected for the studied period (2010-2011) and for previous years. Temperature data were also collected from the same station.

5.2.1.1 Reference site

To detect effects induced by pesticide loads, a suitable reference site was selected following the guidelines provided by Liess et al. (2005). The S. Romedio River flows only a few kilometers apart from the Novella River (Figure 5.1), which ensure the presence of a common geological substrate. The two streams are characterized by a comparable discharge and average slope. However, the S. Romedio River flows in a natural park, thus anthropogenic influence is minimized, and relevant pesticide input can be reasonably excluded since no agriculture is present in the watershed. Physical-chemical parameters (mean velocity, temperature, dissolved O_2, pH, turbidity and conductivity) were measured in both rivers during the entire productive season to exclude natural confounding factors.

5.2.2 Experimental design

A nested multi-level approach was followed to address the most complete evaluation of both exposure and effects. A resume of the experimental scheme is reported in Figure 5.2. The first core of the scheme is represented by the exposure assessment. The first level of this evaluation is represented by a model simulation of the runoff events during the productive season 2011, while the second level, used as calibration for the model, is a direct measurement of chemicals in the stream water. Thus, the comparison of this two levels was used to make an estimation of the actual exposure (node #1 in Figure 5.2). The outcome was used to make a preliminary "theoretical" risk assessment (first level of risk assessment, node #2 in Figure 5.2), using standard laboratory effect endpoints (EC_{50} for the three standard trophic levels). Another core of the evaluation was the effects assessment: this was done for benthic macroinvertebrates at two levels of biological organization. The first level was the measurement of sub-individual response (using some biomarkers), while the second one was the measurement of endpoints at the community level. Keeping in mind that the aim of any risk assessment is the protection of communities and ecosystems, the first level of the effect evaluation (biomarkers) was used to get, whether it was possible, a better understanding of the response at the community level. The comparison of the effects at different levels of biological organization is indicated by node #3 in Figure 5.2. Since the evaluation of the effects is only possible with a suitable reference, several parameters were recorded (an then compared) for the Novella River and for the S. Romedio River. The last part of the experimental design is the comparison of the exposure pattern (expressed as

Toxic Units) with the recorded effects, in order to perform an "actual" risk assessment (second level of risk assessment; node #4 in Figure 5.2).

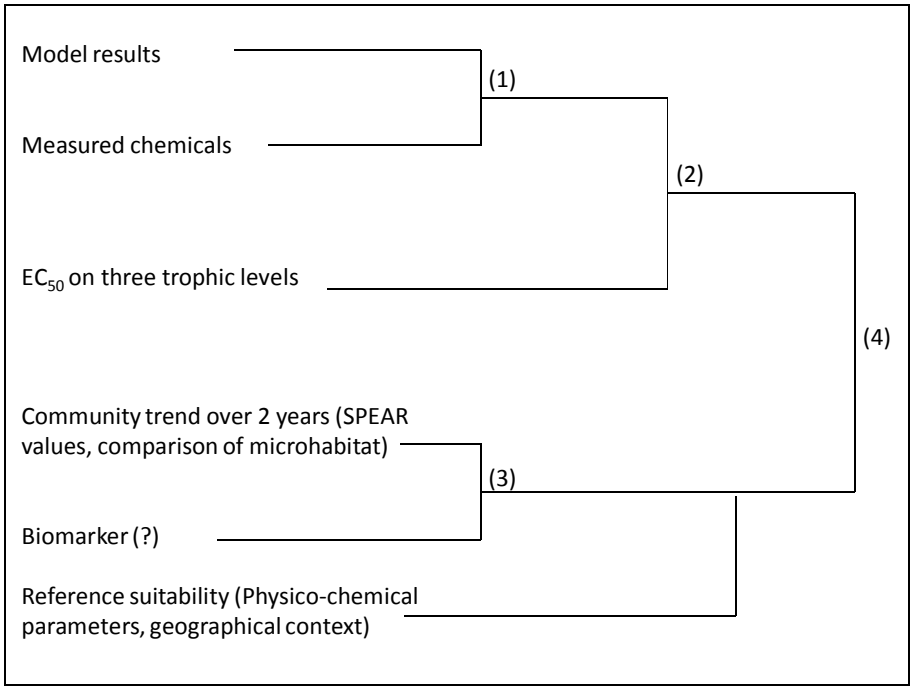

Figure 5.2 Resume of the multi-level approach followed in the study design (see text for explanation). (1) Exposure assessment; (2) Tier 1 risk assessment; (3) Effects assessment; (4) Final risk assessment.

5.2.2.1 Modelling

The concentration of chemicals in the stream (due to surface runoff) over time was estimated using two coupled models. SoilPlus (Ghirardello et al. 2010) is a dynamic fugacity model which allows to calculate the mass balance of water, organic carbon and a certain molecule in a stratified soil. This model was used to calculate the runoff of each single modelled chemical after the application and the following rainfall events. The output of the simulation obtained with SoilPlus was used as input for the DynANet model. DynANet is a dynamic fugacity model developed for the calculation of the concentration of a certain molecule within the hydrological network.
Some assumption and approximations were made for the application of these models.

- DynANet cannot simulate the runoff from different sub-basin, thus the total runoff has been distributed over the entire basin area, without considering the actual position of the application areas.
- Any stream segment was considered to receive an amount of load proportional to its length.
- SoilPlus doesn't consider the slope of the watershed (possible underestimation).
- SoilPlus doesn't consider solid runoff (possible underestimation).
- DynANet doesn't consider the input of runoff water to change the volume of the different stream segments (no dilution factor – possible overestimation).
- To simulate a worst-case scenario, the chemicals were considered to be applied directly on the bare soil (great possible overestimation).

The principal characteristics of the simulation scenario are listed below:

- Simulation period: 25 March 2011 – 14 September 2011.
- Soil: 10 cm deep, loamy, no litter; divided in 20 layers of 5 mm.
- Temperature and precipitation were retrieved from the Romeno meteorological station (see *Appendix 4*).
- Global radiation (average monthly values) calculated with the ENEA SOLTERM model (http://www.solaritaly.enea.it/CalcRggmmOrizz/Calcola1.php).
- Any segment of the hydrological network (Figure 5.3) was parameterized on the basis of the respective Stralher order (see Table 5.1).

Table 5.1 Assumed characteristics of any stream segment on the basis of the Stralher order.

Stralher order	Width (m)	Height (m)	Discharge (m^3/h)
1	1	0.5	175
2	2.5	1	350
3	3.5	1.5	700

Simulations were performed for 8 out of the 31 active ingredients applied on the basin: six insecticides (imidacloprid, chlorpyrifos, methoxyfenozide, etofenprox, flonicamid, pirimcarb) and two fungicides (difenoconazole and dithianon). These chemicals were selected because from a preliminary analysis they resulted to be responsible for a large part of the overall toxicity of the mixtures. Details about properties of these chemicals and their application (rates and dates) are listed in the *Appendix 4*. Further details about the model simulation are given elsewhere (Morselli et al., in preparation).

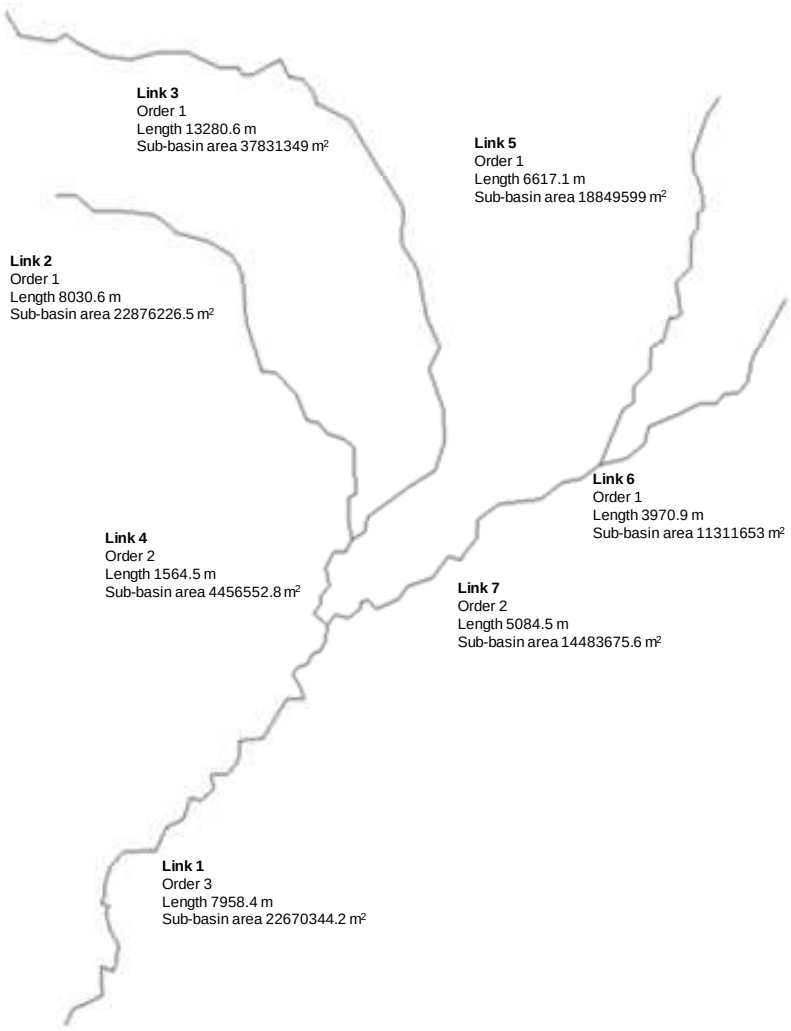

Figure 5.3 Hydrological network used for the model simulations.

5.2.2.2 Water samples

Water samples were collected in different moments of the year, always in the same station (see Figure 5.1 for the location). Samples (1.5 L) were grabbed manually. Three samples were collected on random dates (17 May, 3 June, 28 June), while other 8 samples were collected during an intense rain event (13-14 July, about 40 mm of rain in 7 hours), in order to follow the concentration trend of the chemicals due to surface runoff. The first 7 samples were collected hourly, while the last one was collected the following morning (18 hours after the beginning of the rain event).

Chlorpyrifos was selected as tracer since previous simulations (see Ippolito et al. 2012) revealed that a large amount of the total toxicity of the mixture for benthic invertebrates relies on this chemical.
Samples were refrigerated immediately after collection and stocked at -20°C before analysis. Extraction was performed using 500 mg OASIS HLB cartridges (Waters, Hertfordshire, UK). Cartridges were conditioned with 5 mL of n-hexane, followed by 10 mL of methanol and finally 10 mL of demonized water (Milli-Q). A fixed volume (0.5 L) of any sample was drawn, under vacuum, through the cartridge at a regulated flow rate of 10 mL min^{-1}. After the extraction, the cartridges were dried using N_2 gas and subsequently eluted (under gravity) with 6 mL of ethyl acetate. Identification and quantification were performed by GC-MS (Agilent Technologies, Santa Clara, CA, USA), in SIM (Single Ion Monitoring). Samples (2 µl) were injected by automatic injector (Agilent Technologies 7683 Series Injector) and analyte separation achieved using a 30 m Rxi – 5Sil MS capillary column (0.25 mm id, Restek, Bellefonte, PA, USA). Samples were run in splitless mode using helium as a carrier gas (flow 1 mL min^{-1}).

5.2.2.3 Theoretical risk assessment
The first level of risk assessment was performed by comparing an estimated exposure with values of standard laboratory endpoints. The actual exposure in the stream was estimated considering both the outcome of the model simulations and the results of the sample analysis. Values of acute EC50 for any chemical were retrieved from the Footprint database (FOOTPRINT 2006). EC50 values were searched for standard organisms, representative of three trophic levels: algae (*Scenedemus subspicatus* or *Pseudokirchneriella subcapitata*) as primary producer, *Daphnia magna* as primary consumer and fish (*Oncorhynchus mykiss*) as representative of higher consumer levels.
Concentration peaks were used to calculate the Toxic Unit (TU) of each chemical:

$$TU_i = C_x / LC50_x$$

Where i represent one standard organism and x represents a certain chemical. TUs (referring to the same trophic level) of different chemicals of the same mixture was summed following the principle of the Concentration Addition (Deneer 2000).
Maximum Cumulative Ratios (Price and Han, 2011) were also calculated according to the formula:

$$MCR = TU \text{ of the total mixture} / \text{Maximum TU from one chemical}$$

This technique was originally conceived to test the necessity of performing cumulative risk assessment i.e. risk assessment on mixtures. Minimum value

of this index is 1 (the toxicity of the mixture entirely relies on one single chemical), while maximum value corresponds to the number of chemicals in the mixture (all chemicals contribute equally to the overall toxicity of the mixture).

5.2.2.4 Invertebrate community monitoring

The benthic invertebrate community has been monitored over the entire productive season 2011. Two samples per month were collected from the studied stream (the Novella River) and the reference (the S. Romedio River). A Surber sampler (area ≈ 0.05 m^2) was used, organisms were fixed in the field in 75% ethanol and classified in the lab to the lowest possible taxonomic level. Three separate samples were collected on each river, one for every kind of microhabitat (riffle, run, pool).

A preliminary study using a model ecosystem to mimic realistically the scenario of the Novella River (Ippolito et al. 2012) suggested that $SPEAR_{pesticide}$ (SPEcies At Risk) index (Liess and Von de Ohe 2005) is an appropriate endpoint to test alteration at the community level due to pesticide contamination. $SPEAR_{pesticide}$ evaluates the abundance of vulnerable taxa - using a conceptual definition of vulnerability very close to the one given by Ippolito et al (2010) and De Lange et al. (2010) - classified on the basis of an average value of sensitivity retrieved from the literature and some other ecological traits connected to the susceptibility to exposure and the recovery potential.

Values of $SPEAR_{pesticide}$ were calculated for each subsample (representative of microhabitats) as well as pooled samples (one per river for each sampling date) using the online platform provided by the creators of the index (http://www.systemecology.eu/SPEAR/index.php). Statistical significance of difference between microhabitats was tested with a standard ANOVA performed with the free software R, version 2.13.1 for Windows (http://www.r-project.org/).

A previous monitoring campaign was carried out on both rivers the previous year (March-October 2010, one sample per month), in order to compare the eventual occurrence of repetitive patterns in the communities and to test if any initial alteration was due to some previous influence.

5.2.2.5 Biomarkers

Four different enzymes (alcaline phosphatase, glutathione-S-transferase, acetylcholinesterase (AChE) and catalase) were measures in several taxa. Samples were collected between March and June 2011. The choice of the taxa depended on the abundance of available biomass. Baetidae were always sampled, while other taxa (Simuliidae, Rhyacophiliidae, Heptageniidae and Hydropsichidae) were sampled only in some occasions. A resume of the sampling dates and correspondent taxa sampled is reported in Table 5.2

Table 5.2 Sampling dates and correspondent taxa sampled for biochemical analysis.

Sampling	Taxa Sampled
29/03/2011	Baetidae, Simuliidae, Rhyacophiliidae, Heptageniidae
20/04/2011	Baetidae, Simuliidae, Rhyacophiliidae, Hydropsichidae
17/05/2011	Baetidae, Rhyacophiliidae
03/06/2011	Baetidae, Hydropsichidae

All organisms were frozen in liquid nitrogen immediately after sampling. Detailed procedure for the enzyme determination are described in Bonzini et al. (2008) and Forcella et al. (2007). Since pesticide with several mode of action (often fungicide, exerting a narcotic effects on animals) were used in the basin, biomarker response was related to a generic chemical stress using the concept of Integrated Biomarker Response (IBR). Details about the concept and the methodology to calculate the index are reported in Beliaeff and Burgeot (2002).

5.3 Results

5.3.1 Exposure characterization

The model simulations highlighted a different behaviour for the selected compounds. Particularly, two different clusters can be identified. Imidacloprid, flonicamid and pirimcarb (Figure 5.4a), characterized by a $Log(K_{ow}) < 2$ and thus not tightly retained in the soil, generated only 2 concentration peaks in the stream during the first two relevant rain events after the application, on 16/05/2011 (14 mm) and on 28/05/2011 (37 mm). Methoxyfenozide, chlorpyrifos, etofenprox and difenoconazole (Figure 5.4b) on the other hand, showed a higher number of peaks in the concentration profile. This depends on the higher values of $Log(K_{ow})$ for these chemicals, which causes a longer retention in the application soil layer and thus may cause peaks even many days after the application date. Concentration peaks corresponded to relevant rainfall events and their width wass determined by the sediment uptake and the speed of the following release into the water. A particular behaviour was observed for dithianon (Figure 5.4c), whose multimodal trend was similar to those of the abovementioned molecules ($Log(K_{ow}) > 2$); nevertheless, the peaks width was very small (2-4 days) due to the brief DT_{50} of the compound in the water and in the sediment (0.05 days).

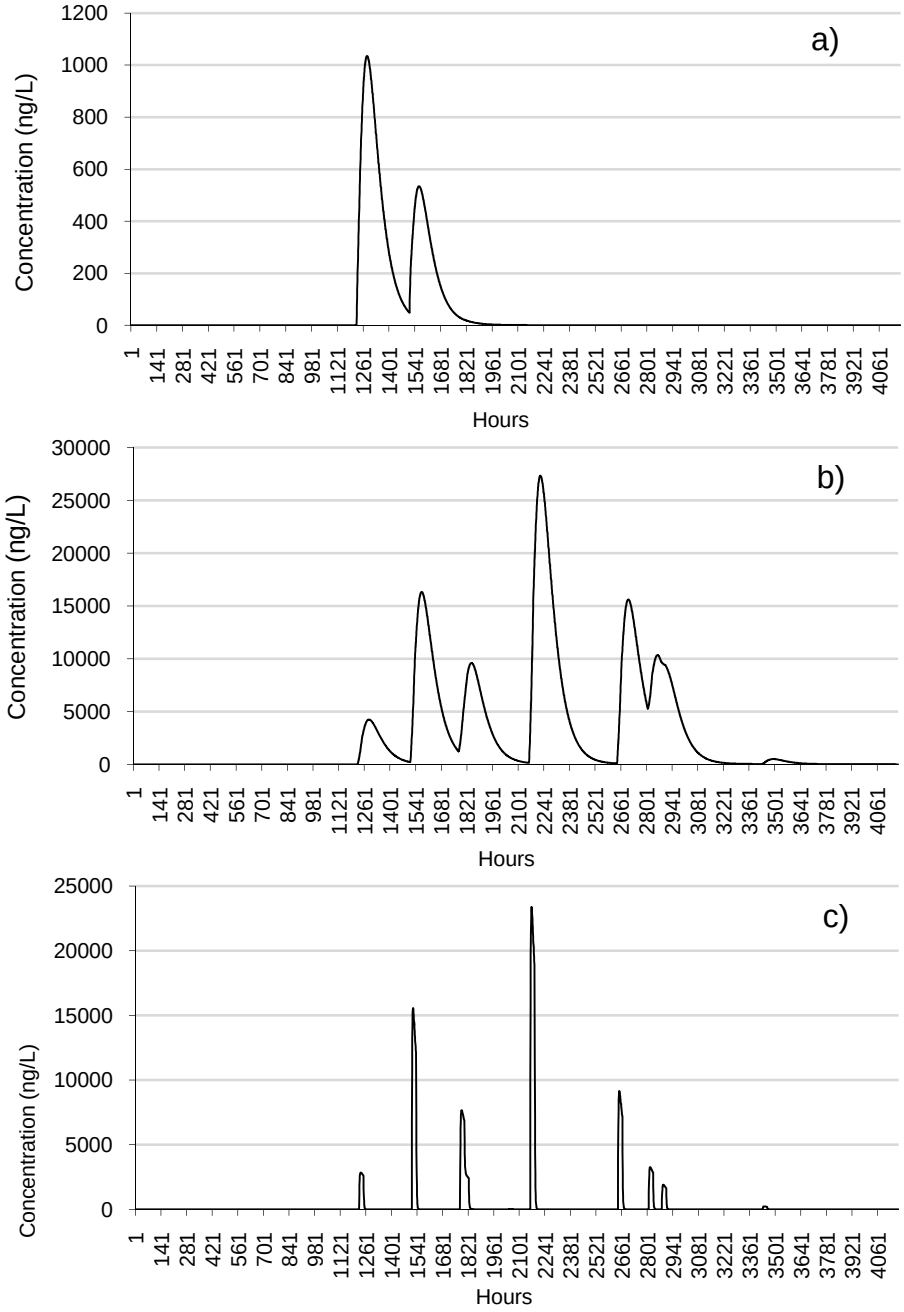

Figure 5.4 Water concentration profiles of different chemicals as simulated by the modeling approach. a) example (pirimicarb) of concentration pattern followed by chemicals characterized by a Log(K_{ow}) < 2; b) example (methoxyfenozide) of concentration pattern followed by chemicals characterized by a higher values of Log(K_{ow}); c) Concentration profile of dithianon, with very narrow peaks due to the brief DT50 of the compound in the water and in the sediment.

Measured concentration of chlorpyrifos in the water varied between 4 and 50 ng/L. The comparison with these measured values clearly show a massive overestimation of the model predictions. In fact, chlorpyrifos concentration peaks as predicted by the simulations ranged between 1000 and 10000 ng/L. However, the magnitude of this overestimation seems to be quite constant over the entire period: a plot of measured and predicted concentrations with scales staggered by two orders of magnitude (Figure 5.5) highlights a good overall agreement. Much more measurements would be necessary to obtain a sound interpretation of the concentration pattern, but it seems that both timing and proportion between peaks are respected in the model simulation. Thus model results, despite a necessary change of the scale, are expected to provide a reasonable estimation of actual concentrations in the water.

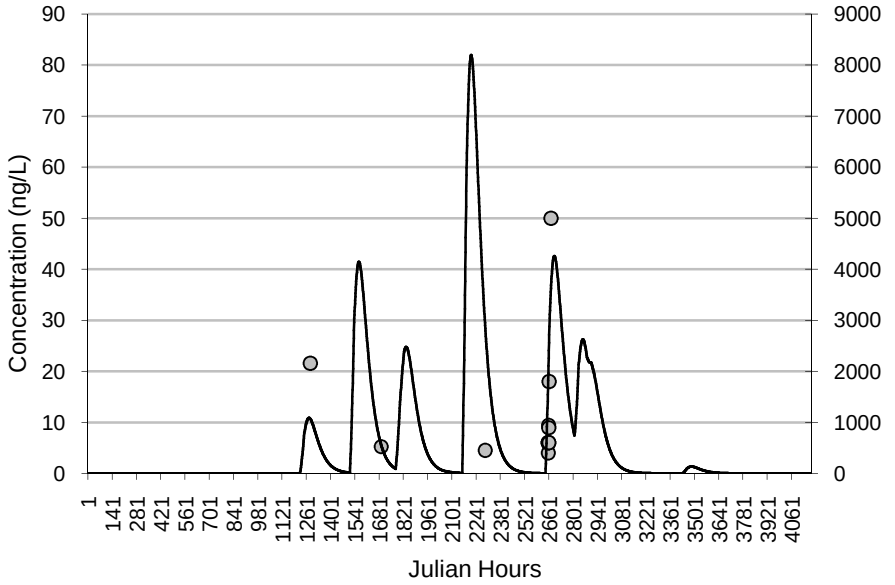

Figure 5.5 Comparison between chlorpyrifos water concentration profile as predicted by the model simulation (right scale) and analytical measurement of grab water samples (left scale).

However, some further consideration should be made. First of all, a similar level of concentration has been found in the second (5.3 ng/L – collected 03/06/2011) and in the third (4.5 ng/L – collected 28/06/2011) measured sample. This is not in accordance with the model results, which predicted a much higher concentration of chlorpyrifos in the third sample. Nevertheless, one relevant issue should be considered. Peaks as predicted by the model are quite wide (even more than 300 hours), as consequence of processes of chemical absorption on the sediment and subsequent slow release. However, the effect related to this process was not calibrated on the sediment typology present in the study area. Being an alpine stream, the sediment of the

Novella River is composed almost exclusively by rocks and gravel; sand is only in a minor fraction and finer particles are almost absent. Thus the importance of retention processes is extremely reduced and concentration peaks are likely to be considerably narrower than what predicted by the model. Considering this, the third sample was probably collected when the peak was already passed, and the found level of chlorpyrifos is a representation of a background concentration. The same concentration level was indeed found not only in the second sample, but even at the beginning of the monitored rain event (13/07/2011).

The extremely fast response of the studied system is also confirmed by the results obtained from the only complete monitored rain event. In this occasion detailed information was collected about precipitation (data referring to two different stations, one at high altitude and one at low altitude, available on the entire event), water level and chlorpyrifos concentration. Data are resumed in Figure 5.6. Water level responded almost immediately to rainfall intensity (about 1 hour gap) and so did chlorpyrifos concentration. According to the model simulation a sample was collected almost exactly when the concentration reached the maximum value for the peak. Unfortunately, no measured data are available to test the decreasing rate of the concentration.

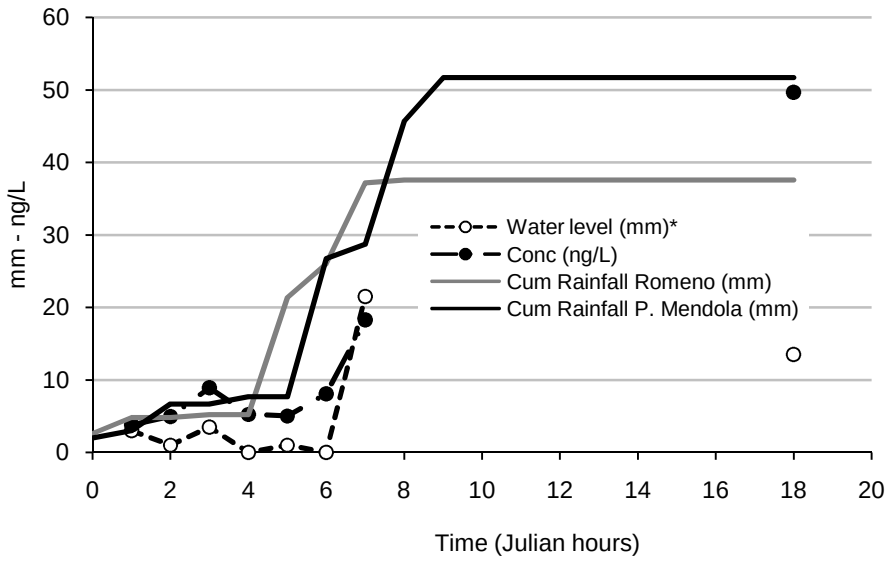

Figure 5.6 Chlorpyrifos concentration trend during a monitored storm event (13/07/2011). Water level of Novella River (zero is set to the height of the lowest registered value) and cumulative precipitations (mm) measured in the two closest meteorological stations are reported.

5.3.2 Theoretical risk assessment

According to the comparison of model simulations and experimental measurements, peak concentrations of pesticides were estimated by decreasing by two orders of magnitude the value found with the model simulations. The comparison of these values with laboratory acute toxicity endpoints for fish, *Daphnia magna* and algae is expressed in terms of TUs in Table 5.3. The trend of the overall toxicity of the mixture, calculated according to the concentration addition principle is expressed in Figure 5.7. The theoretical risk associated to the estimated toxicity of the mixture is very low for algae with value of TU that never exceeded $3x10^{-3}$. TU values for fish ranged between 0.01 and 0.1 highlighting a situation of a certain concern. Nevertheless the most relevant results regard TUs referred to *Daphnia magna*: values are always above 0.01, with a maximum value of 0.82 (close to acute toxicity) during the fourth runoff event.

Table 5.3 EC_{50} values for each modelled chemical, estimated peak concentrations during each runoff event and corresponding values of Toxic Units. Total TUs are calculated on the basis of the Concentration Addition principle.

Runoff event			Chlorpyrifos (insec)	Difenoconazole (fung)	Dithianon (fung)	Etofenprox (insec)	Flonicamid (insec)	Imidacloprid (insec)	Methoxyfenozide (insec)	Pirimicarb	**Total TU**
	EC50 (µg/L)	Fish	1.3	1100	70	2.7	100000	211000	4200	100000	
		Daphnia	0.1	770	260	1.2	100000	85000	3700	17	
		Algae	480	1200	90	150	100000	10000	3400	140000	
1	Conc.	µg/L	0.0109	0.004	0.03	2.50E-06	0.000047	0.157	0.04	0.0125	
	TU	Fish	0.008385	3.64E-06	0.000429	9.26E-07	4.70E-10	7.44E-07	9.52E-06	1.25E-07	0.009
		Daphnia	0.109	5.19E-06	0.000115	2.08E-06	4.70E-10	1.85E-06	1.08E-05	0.000735	0.110
		Algae	2.27E-05	3.33E-06	0.000333	1.67E-08	4.70E-10	1.57E-05	1.18E-05	8.93E-08	3.87E-04
2	Conc.	µg/L	0.0415	0.03	0.15	0.000009	0.000018	0.06	0.17	0.0057	
	TU	Fish	0.031923	2.73E-05	0.002143	3.33E-06	1.80E-10	2.84E-07	4.05E-05	5.70E-08	0.034
		Daphnia	0.415	3.90E-05	0.000577	7.50E-06	1.80E-10	7.06E-07	4.59E-05	0.000335	0.416
		Algae	8.65E-05	0.000025	0.001667	6.00E-08	1.80E-10	0.000006	0.00005	4.07E-08	0.002
3	Conc.	µg/L	0.0248	0.02	0.075	4.50E-06			0.09		
	TU	Fish	0.019077	1.82E-05	0.001071	1.67E-06	0	0	2.14E-05	0	0.020
		Daphnia	0.248	2.60E-05	0.000288	3.75E-06	0	0	2.43E-05	0	0.248
		Algae	5.17E-05	1.67E-05	0.000833	3.00E-08	0	0	2.65E-05	0	0.001
4	Conc.	µg/L	0.082	0.075	0.23	1.25E-05			0.275		
	TU	Fish	0.063077	6.82E-05	0.003286	4.63E-06	0	0	6.55E-05	0	0.067
		Daphnia	0.82	9.74E-05	0.000885	1.04E-05	0	0	7.43E-05	0	0.821
		Algae	0.000171	6.25E-05	0.002556	8.33E-08	0	0	8.09E-05	0	0.003
5	Conc.	µg/L	0.0426	0.05	0.09	0.000005			0.16		
	TU	Fish	0.032769	4.55E-05	0.001286	1.85E-06	0	0	3.81E-05	0	0.034
		Daphnia	0.426	6.49E-05	0.000346	4.17E-06	0	0	4.32E-05	0	0.426
		Algae	8.88E-05	4.17E-05	0.001	3.33E-08	0	0	4.71E-05	0	0.001
6	Conc.	µg/L	0.0263	0.03	0.03	0.000003			0.105		
	TU	Fish	0.020231	2.73E-05	0.000429	1.11E-06	0	0	0.000025	0	0.021
		Daphnia	0.263	3.90E-05	0.000115	2.50E-06	0	0	2.84E-05	0	0.263
		Algae	5.48E-05	0.000025	0.000333	2.00E-08	0	0	3.09E-05	0	4.44E-04

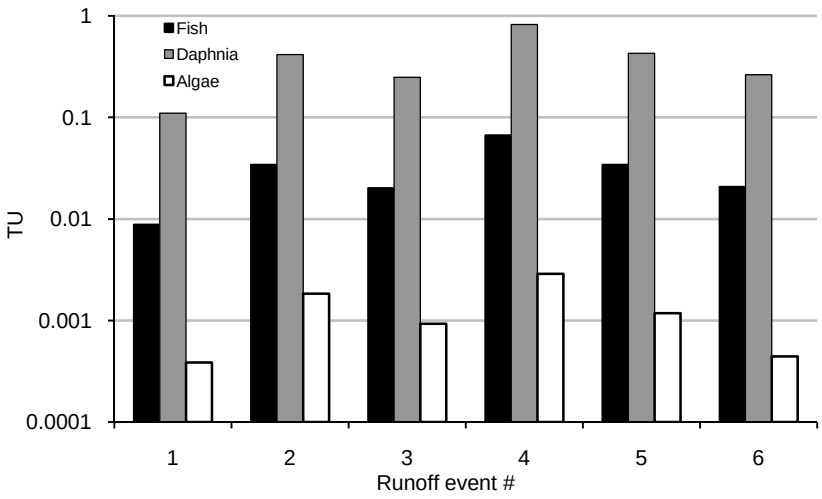

Figure 5.7 Predicted max toxic units of pesticide mixture in the Novella River during any runoff event (estimation based on the comparison of model simulations and analytical measurements). Values refers to each considered standard species.

Values of MCR are reported in Table 5.4. MCR for *Daphnia magna* is always very close to 1, in fact chlorpyrifos is always responsible for more than 99% of the nominal toxicity of the overall mixture. A very similar scenario is found for fish and, even if MCR values are slightly higher, chlorpyrifos accounts at least for more than 93% of the total toxicity. Dithianon is always responsible for the larger part of toxicity to algae (75-90%) thus MCR values are still very low.

Table 5.4 MCR values for the three trophic levels for each runoff event.

	Runoff event					
	1	2	3	4	5	6
# chemicals	8	8	6	6	6	6
Fish	1.05	1.07	1.06	1.05	1.04	1.02
Daphnia	1.01	1.00	1.00	1.00	1.00	1.00
Algae	1.16	1.10	1.11	1.12	1.18	1.33

5.3.3 Suitability of Reference system

Physical-chemical parameters were measured in the Novella River and in S. Romedio River to test the suitability of the latter as reference system. Mean values are reported in Table 5.5. No significant difference (ANOVA) were found between the two rivers for temperature, turbidity (characterized by

great variance within sites), oxygen saturation and mean velocity. Oxygen values are unexpectedly low, considering that both streams don't receive relevant organic wastes and present a continuous exchange with the atmosphere due to turbulence caused by the speed and rough streambed surface. These value are likely to be the result of a malfunctioning of the used probe, and must be considered with extreme care.

Table 5.5 Mean values of the physical-chemical parameters measured in the Novella River and in S. Romedio River.

	Novella	S. Romedio
O_2%	66.1	71.4
pH	8.4	8.7
Conductivity (µs/cm)	367.4	452.8
Temperature (°C)	11.8	10.2
Turbidity (NTU)	11.9	3.5
Mean velocity (m/s)	0.4	0.4

The only parameters that showed a statistical significant difference were pH (ANOVA, $p < 0.001$) and conductivity (ANOVA, $p < 0.05$). Nevertheless, registered differences are unlikely to cause any alteration in the biological community.

5.3.4 Biological community

No significant difference in $SPEAR_{pesticide}$ values was found between microhabitats in both rivers, confirming the independence of the index from this kind of natural variability. Thus, samples from different microhabitats were pooled for further analysis.
The $SPEAR_{pesticide}$ trend of the 2 years monitoring campaign is reported in Figure 5.8. The suitability of the S. Romedio River as reference system is testified by the abundance of vulnerable species in the invertebrate community: values of $SPEAR_{pesticide}$ always remained ≥ 50 (except one case in June 2010, with a value of 48), which is often assumed as trigger value. In addition, $SPEAR_{pesticide}$ in the S. Romedio River showed a good stability during the productive season 2011, while the previous year it presented higher variability. On the other hand, this endpoint shows relevant alterations in the community structure of the Novella River, with values around 25 reached during both years.
However, patterns in the Novella River were very different in the two years. During 2010, $SPEAR_{pesticide}$ values decreased almost constantly from March to late September, with a quick alteration from late June to July. A strong recovery was observed from September to October. During 2011 instead a

sudden alteration of the community was observed already at the end of April, followed by a constant recovery until late June, when a new consistent decrease of $SPEAR_{pesticide}$ values was recorded.
Remarkably, despite all relevant alterations registered during the productive season, $SPEAR_{pesticide}$ values were extremely similar for the two rivers in the pre-treatment samplings of both 2010 and 2011, and a clear recovery trend was observed also in the last samplings of 2011.

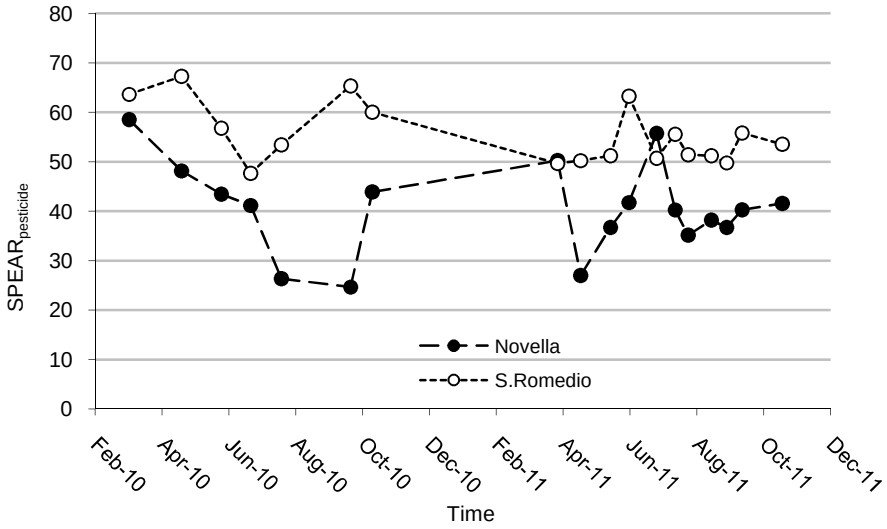

Figure 5.8 $SPEAR_{pesticide}$ trends in the Novella River and in the S. Romedio River during years 2010-2011.

5.3.5 Biomarkers

Results of the biomarker analysis are reported in Table 5.6 as IBR values. High values of this index should correspond to a generic chemical stress for the organisms.
The information contained in the results of the biomarker assays is hardly interpretable. Baetidae are the only taxon for which a temporally complete information is available. A consistent temporal variation is observed, with March values being much higher than those of the following months in both rivers. Despite a seasonal variation can be acceptable, the scenario described by these numbers is hardly believable, especially for the S. Romedio River, which doesn't receive any chemical input. Furthermore, values registered for the S. Romedio River are higher in half of the sampling dates. No significant patterns were found for the other taxa. In addition, variability within site is often much greater than variability between sites.

Table 5.6 IBR values for the analyzed taxa.

		Novella	S.Romedio
Baetidae	March	8.87	11.14
	April	2.11	3.07
	May	2.47	0.53
	June	4.62	1.40
Simuliidae	March	0.23	3.86
	April	3.38	1.86
Rhyacophiliidae	March	5.74	5.81
	April	4.78	4.70
	May	0.14	0.02
Heptageniidae	March	0.00	2.00
Hydropsichidae	April	6.81	2.55

The comparison between taxa is extremely unclear as well, since opposite response (considering the two rivers) is often present for the same date. Due to the absence of clear patterns, no link is possible with the results obtained at the community level.

5.3.6 Linking exposure and effects

The relationship between estimated exposure and observed effects is not immediately interpretable. Figure 5.9 presents the temporal relationship between measured $SPEAR_{pesticide}$ values and pesticide load peaks due to runoff events, whose magnitude is represented by the estimated $TU_{Daphnia\ magna}$ (2^{nd} *y* axis).

The sudden alteration suffered by the invertebrate community between March and April in the Novella River ($SPEAR_{pesticide}$ value decreases from 50 to 27) cannot be explained by any predicted runoff event, since the first one occurred about one month later. As already described in paragraph 5.3.4, the community of the Novella River constantly recovers from the first initial alteration until late June. However, in this period (April-June) 3 relevant runoff events were predicted by the model simulations, so a recovery is quite unexpected. On the other hand the highest predicted value of TU due to pesticide runoff corresponds to the beginning of a relevant alteration of the community in the Novella River in the first 15 days of July ($SPEAR_{pesticide}$ value decreases from 55 to 40) and then again two other runoff events are predicted in correspondence with a further alteration of the community in the following 12 days. Finally, the absence of other predicted runoff events corresponds to a slight but constant recovery of the community.

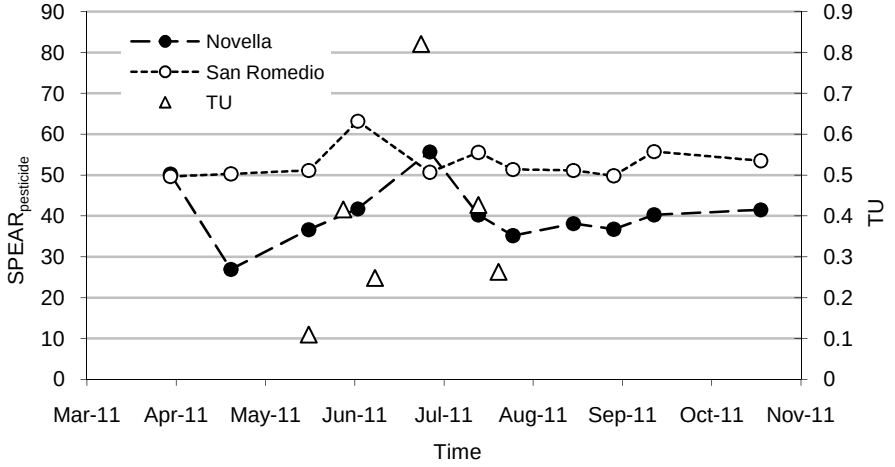

Figure 5.9 Temporal relationship (2011) between measured $SPEAR_{pesticide}$ values (left scale) and pesticide load peaks due to runoff events, whose magnitude is represented by the estimated $TU_{Daphnia\ magna}$ (right scale).

5.4 Discussion

Temporal resolution plays a pivotal role in the characterization of exposure and related effects, especially at site-specific scale (Rabiet et al. 2010). In fact, pesticide transfer dynamics and loads in small catchments are largely unknown, due to relevant and sudden variation of hydrological conditions. Pesticide concentration patterns in streams are well acknowledged to be extremely dynamic, with relevant variations within hours (Holvoet et al. 2007): this is particularly true for small catchments, like the one we studied. Several authors (Bach et al. 2001, Dabrowski and Schulz 2003) have demonstrated, by means of modelling and monitoring data, that runoff is the most important entry route of pesticides in streams, especially in small catchments. Despite the relevance in terms of cumulative load, runoff processes are usually extremely limited in time. Rabiet et al. (2010) showed that in the small watershed of the Morcille stream (France), the 89 % of the total diuron load occurred in only 5 days (15 % of the total monitoring time of the study). The Morcille watershed is characterized by high slopes, exactly like the one of the Novella River; thus, since slope plays a very important role in runoff processes, comparable timing might be expected.
Furthermore, Schulz (2004) performing a review of all available previous insecticide-related field studies, was able to find a very significant negative correlation between (log-transformed) catchment size and (log-transformed) maximum insecticide concentration detected. One possible explanation is that rapid flow processes like those occurring in small catchments, may

drastically reduce the time available for reactions, such as sorption and degradation, and may lead to a direct transport of pesticide toward surface waters (Müller et al. 2003).
To overcome the problems connected with the characterization of such dynamic and variable concentration patterns, Rabiet et al. (2010) suggested that monitoring program should consider a representative range of flow conditions rather than performing time-fixed grab sampling which could highly underestimate peak concentrations. Similar observations are reported in Bonzini et al. (2006).
In this work we tried to prevent this underestimation in two ways. First of all, we tried to monitor chlorpyrifos concentration in the stream, with high temporal detail, during a relevant storm event. Furthermore, we adopt a combined strategy of modelling and monitoring data, as advised by Holvoet et al. 2007, to identify the timing and the relevance of concentration peaks.
However, from the comparison with monitoring data, a huge overestimation of model predictions was found (around two orders of magnitude). The causes of this overestimation are likely to be in the extreme worst-case assumptions made during the modelling processes. Particularly, chemicals were assumed to be applied directly on the bare soil rather than on the orchards canopy. This assumption is likely to introduce a huge systematic bias in the model results, since plant interception in apple orchards may be extremely relevant (Linders et al. 2000 report a fraction of 0.7 of the total product applied in case of full foliage). Nevertheless this systematic error is probably not compromising the global pattern predicted by the model, as already stated in the results paragraph.
One of the recommendations for linking exposure and effects reported in the executive summary of the ELINK workshop (Brock et al. 2010) regards some metrics to be evaluated when characterizing time-variable exposure profiles of pesticides in surface water. In the present study, some of these parameters can be evaluated with reasonable confidence from the combined approach of monitoring data and modelling (at least for chlorpyrifos), while others are more uncertain.

- Height of peak concentrations: according to timing predicted by model simulations two out of the six peaks were represented in the collected samples. Proportions between different peaks are reasonably correctly represented by the model, thus this parameter can be estimated (at least for chlorpyrifos) with a sufficient confidence.
- Duration of the peak exposure: as already discussed in the results section, this parameter is likely to be overestimated by the model predictions, due mainly to the nature of the sediment in the Novella River. Nevertheless, the magnitude of this overestimation is unknown, since no samples were collected in the hours following the concentration peaks.

- Interval between peaks/frequency of peaks: timing of peaks was estimated by model simulations and confirmed, at least in two cases, by direct measurement of samples. Furthermore, surface runoff processes are primarily caused by relevant rain events. Since rain data were collected with great temporal resolution, the estimation performed for this parameter can be considered reliable.
- Height of a possible long-term background concentration: a possible level of background concentration was found for chlorpyrifos from three off-peak samples, collected in very different moments of the productive season. Concentrations in these samples vary from 3.8 to 5.3 ng/L, but more samples would probably be necessary to confirm this hypothesis.

Measured peak concentrations for chlorpyrifos range from 20 to 50 ng/L, while according to the pattern predicted by the model the highest peak is estimated to be around 80 ng/L.
Previous works carried out in catchments of comparable extent reported heterogeneous values of chlorpyrifos peak concentrations due to runoff events. Battaglin and Fairchild (2002) working on different streams in the U.S. Midwest, found concentrations between 4 and 869 ng/L; Schulz (2001) found a range of 30-200 ng/L on the Lourens River and tributaries (South Africa); Hunt et al. (2003) found concentrations between 30 and 3200 ng/L in California; Jergentz et al. (2005) on the Brown Stream (Argentina) measured a range of 210-450 ng/L, while Castillo et al. 2000 reported values between 50 and 100 ng/L on some Suerte River tributaries (Costa Rica). Verro et al. (2009b), in a completely different area (Meolo River catchment, Northern Italy) characterized by plain surface, reported chlorpyrifos peak concentrations well above our findings (up to 157 ng/L) in relation to drift processes, while values were one order of magnitude below those presented in this work for what concern runoff processes (< 2 ng/L).
Another recommendation reported in Brock et al. (2010), concerning the characterization of the exposure, regards the toxicological and ecological independence of different peaks. To test the toxicological dependence of different peaks, the knowledge of toxicokinetic / toxicodynamic (TD/TK) models is needed. Testing such hypothesis is extremely difficult in the present study, since many chemicals and many species are involved. Nevertheless chlorpyrifos is predicted to play a major role in the overall toxicity of the mixtures to the invertebrate community, thus models referring to this chemical may give important indications. Rubach et al. (2010) studied the toxicokinetic variation in 15 freshwater arthropod species exposed to chlorpyrifos and found that elimination rate (k_{out}) was extremely variable between taxa. The highest value (0.546 day^{-1}), reported for *Daphnia magna*, would certainly lead to the conclusion that concentration peaks are toxicologically independent (considering that the peak duration is likely to

be shorter than what predict by the model) except maybe for the last two peaks. However, the lowest reported value of elimination rate (0.021 day^{-1}) reported for *Sialis lutaria* would lead to a complete different scenario. Hence, no general statements can be done at community level. On the contrary, ecological independence of peaks can be easily excluded from the analysis performed of the community data, even if a clear relationship between exposure and effects pattern cannot be established.

Despite the effort in monitoring and modelling, a better characterization of the exposure is needed in the catchment to reduce all the uncertainties listed above. Particularly, a better calibration of the models is urgent, and more samples with high temporal resolution can certainly be helpful. Nevertheless, future efforts should not be addressed only to improve predictions about runoff, but even to evaluate the contribution of other entry routes. In fact, point sources were demonstrated to be extremely important across Europe. Mismanagement of spray equipments is reported in some cases to be responsible for high percentages of the total pesticides loads in streams (Holvoet et al. 2007). In addition, drift processes may have significant effects on freshwater ecosystems, especially in orchard regions (Bach et al. 2001; Ritter 2001), like the one investigated in this study.

Poor temporal resolution may cause significant problems in characterization of the exposure as well as in the evaluation of the effects. The results of the present study demonstrate that in dynamic systems such as an alpine stream, not only the exposure but also the community is subject to fast changes. This is, to our knowledge, the first application of the $SPEAR_{pesticide}$ index to an alpine environment and it is also the most temporally detailed study using this endpoint. Only two other studies (Liess and Von der Ohe 2005; Schäfer et al. 2007) reported some data about temporal trend of $SPEAR_{pesticide}$, but both performed only two-three sampling per site, with the first sampling date in the pre-application period. Hence, comparison of the present results with available literature is not possible.

Remarkably, $SPEAR_{pesticide}$ did not show significant variation between different microhabitats. This is an important finding to assess the suitability of the index to be applied for large scale monitoring programs. Furthermore, this result confirms what reported by Beketov and Liess (2008), which demonstrated the independence of $SPEAR_{organic}$ from confounding environmental factors over an extensive river continuum.

In this work, an attempt was done to establish a link between subindividual endpoints (biomarkers) and responses at the community level. The seek for easily measurable ecologically relevant endpoints is not new, but it still has great importance within the field of ecotoxicology, as testified by the presence of a specific session at the 21st SETAC Europe Meeting (Milan). In the present study, no links between biomarkers (summarized into the IBR values) and responses at the community level ($SPEAR_{pesticide}$) were found. The unsuitability of biomarkers as early predictors of responses has been

already criticized in the past (Forbes et al. 2006), with several arguments. Nevertheless, the extreme variability found in this study within and between taxa also in the reference system poses serious doubts about the reliability of these results.

The application of classical methodologies for ecological risk assessment (comparison of environmental chemical concentrations and toxicity values on standard species) would suggest the presence of unacceptable acute risk in the studied site. The guidance document regulating the risk characterisation for plant protection products (SANCO/3268/2001 revision 4 – EC 2002) establishes a TER trigger of 100 for Tier 1 assessment on *Daphnia magna*, while measured concentrations of chlorpyrifos alone always exceeded this threshold, with values up to 50 times higher. The same trigger was exceed in two occasions also for fish (*Oncorhynchus mykiss*). In fact, significant alterations were recorded at the community level during both years of monitoring (2010 – 2011). $SPEAR_{pesticide}$ values decreased dramatically in the Novella River, while no similar pattern was recorded for the S. Romedio River, thus excluding the influence of any process due to normal natural dynamics. According to the regression model obtained in a preliminary experiment with a model ecosystem (Ippolito et al. 2012) the lowest observed level of $SPEAR_{pesticide}$ during 2011 (26.97) should correspond to a maximum $TU_{Daphnia\ magna}$ of 0.62, which is in the range of $TU_{Daphnia\ magna}$ reported in Table 5.3 (0.1-0.8). Conversely, the regression model proposed by Schäfer et al. (2007) leads to values of $TU_{Daphnia\ magna}$ of 0.07, well below maximum measured concentrations.

The alteration-recovery pattern of the community is very different during the two investigated years. Many possible explanations are possible, and meteorological conditions in relation to treatments may have played a pivotal role. Martin and Owens (2003) showed than the timing of rainfall can have a much greater effect on yearly losses of atrazine than the agronomic management processes.

This is one of the few studies available in the literature in which the aim was to find a clear link between exposure and effects with high temporal resolution and not only general seasonal observations. This link between exposure and effects was not always established with the sufficient robustness; however, in his review of insecticide field investigations, Schulz (2004) counted very few studies able to establish such a clear link. One possible reason is the insufficient characterization of the exposure. For example, overlooking any possible entry route of pesticides other than surface runoff, may have consistently biased the overall assessment. Particularly, drift processes and point sources might be the cause of the sudden alteration of the community of the Novella River at the beginning of the productive season 2011. In fact, during this period two consistent insecticide treatments (pirimicarb, etofenprox) were applied on the orchards.

According to $SPEAR_{pesticide}$, complete recovery of the community was achieved within one year, probably due to the presence of an unpolluted forested area upstream. The important role played by in-stream recolonization processes in water bodies subjected to pesticide pressures was already highlighted by Liess et al. (2005), and here is another confirmation.
Orchard cultivation is one of the main activity in the area since a very long time (the foundation of the first consortium dates back to the beginning of the 20th century). Hence, from the comparison with the reference river at the beginning of both investigated productive seasons, long-terms effects can be excluded. In this context the Pollution Induced Community Tolerance (PICT) approach (Blanck and Wangberg, 1988) cannot be applied: the patchy land use with relevant forested areas and the extreme dynamicity of the hydrological conditions enhance greatly the resilience of the ecosystem, thus decreasing the overall vulnerability towards this kind of stressor. Nevertheless, no measurements of functional activity were done: relevant alterations of the community composition may cause significant changes in the functionality of the overall system, whose effects may become visible outside of the studied stream (e.g. accumulation of organic matter in the lake at the end of the stream due to reduced leaf breakdown) rather than inside the investigated catchment.

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6 Pesticide risk in surface waters: a global map[2]

Abstract
Pesticides are one of the most widespread class of toxicants worldwide. Their global diffusion and the efficacy of their action makes them one of the most ecologically relevant class of chemicals, particularly for surface water ecosystems.
A spatially-explicit model was applied at global scale to model to calculate the insecticide runoff potential (RP), as a generic indicator of the magnitude of insecticide inputs into streams via runoff. In addition, the contributions of natural (vulnerability) and of human (hazard) variables were separated in order to better characterize the overall risk and thus individuate areas where correct management of cropland may more effectively reduce insecticide risk for surface waters. We found that more than 40% of emerged lands are interested by phenomena of insecticide surface runoff and almost 15% of the whole continental surface presents RP values classified from high to very high. The RP map testifies the presence of a clear geographical gradient in the northern hemisphere. Values of RP increase from north to south. This geographical tendency is mostly due to the amount of insecticide used per area, which is strongly dependent on the average temperature. For this reason climate change is likely to have effect on RP patterns in the next future. Contribution of both vulnerability and hazard proved to be relevant in determining the overall risk.

Keywords: pesticide runoff; maps; vulnerability; hazard; ecological risk

Unpublished manuscript

[2] This chapter presents a particular outline due to the requirements of the journal that it is going to be submitted to.

6.1 Main text

Pesticides are applied all over the world on cultivated land summing up to 2.3 billions of kilograms (Grube et al. 2010) of yearly application on agro-ecosystems. This widespread and intentional introduction of substances designed to kill unwanted populations of animals and plant makes the crucial difference in the environmental effects compared to other toxicants. It has been shown several times that pesticides are a major threat for terrestrial (Barmaz et al. 2010, Boatman et al. 2007, Newton 1995) and aquatic biodiversity (Verro et al. 2009, Vonesh and Kraus 2009, Relyea 2005, Beketov et al. 2008). Particularly, insecticide may have direct and indirect effects (Fleeger et al. 2003, Wendt-Rasch et al. 2004) on animal biodiversity. However, the global extent of this phenomenon is still largely unknown. Here we applied at global scale a spatially-explicit model to estimate the insecticide runoff into streams (RP) (Schriever et al. 2007, Schriever and Liess 2007, Kattwinkel et al. 2011), and to assess the magnitude of potential ecological effects. We found that streams in more than 40% of land surface are affected by insecticide surface runoff. U.S. Midwest, Central America, the Mediterranean area, India and some countries of East Asia present the highest estimated risk. In most of these areas the ecological effects on aquatic communities will also be significant, as no unsprayed recovery areas are present. Additionally, we separated the influence of natural variables from those under human control and thereby identified areas where correct management of cropland may reduce the input of insecticides in surface waters most efficiently. This is the first estimate for a global mapping of agricultural pesticide runoff and related effects on biodiversity.

We used previously published raster maps (FAO&IIASA 2006, Batjes 2006) and spatial databases (NOAA, GHNC daily database; FAO, global spatial database of agricultural land-use statistics) as input data for the RP model to estimate pesticide input to streams via surface runoff. The RP model was split into two parts in order to separate environmental factors from human-controlled variables determining runoff. The vulnerability map (Figure 6.1) expresses the potential magnitude of insecticide runoff due to the environmental conditions regardless of the actual agricultural activity. A previous sensitivity assessment of the model (Schriever et al. 2007) showed that such natural factors, particularly slope and intensity of rainfall, have a strong influence on the overall process of runoff. This is consistent with the results illustrated in the map (Figure 6.1). Central America, almost all India, Southern China and a very large part of South-East Asia present high values of vulnerability due to their climatic regime, which is characterized by intense precipitation events during the productive season.

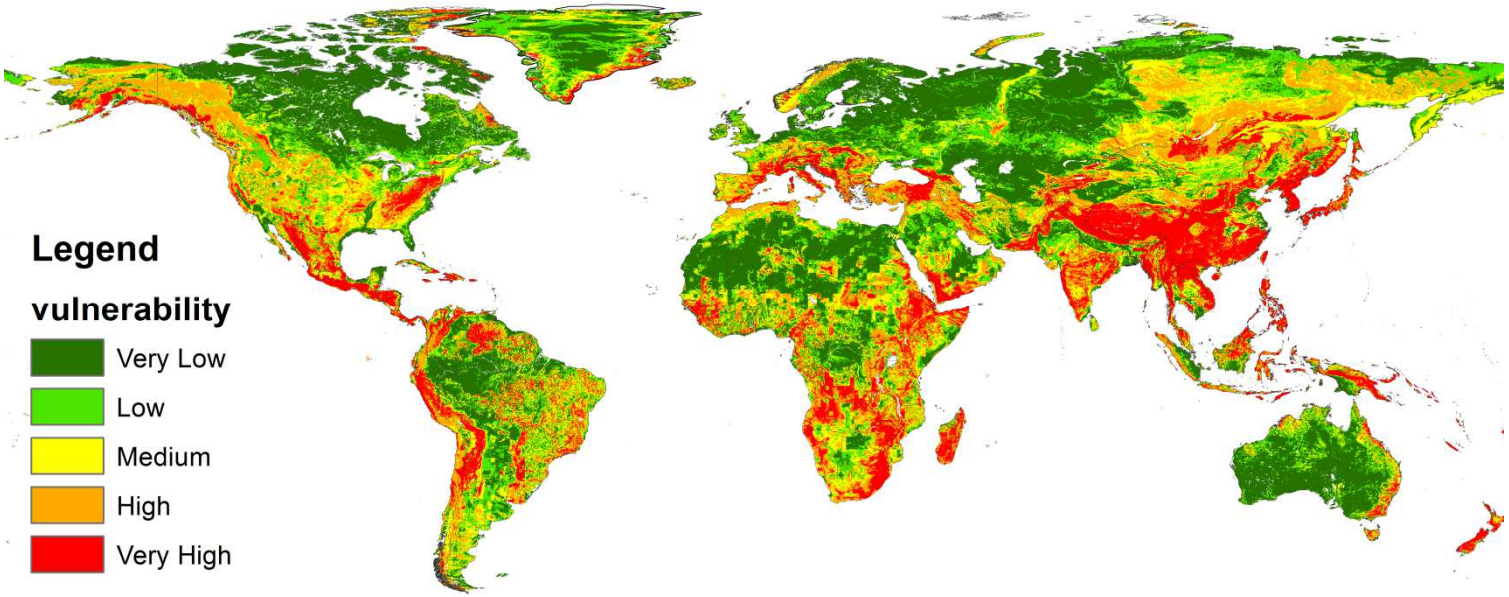

Figure 6.1 Global insecticide runoff vulnerability map. The map expresses the potential magnitude of insecticide runoff regardless of the actual agricultural activity. It takes into account all the natural variables included in the RP model. Classes boundaries have been assigned ex-post according to the distribution of the values (see Section 6.3).

High values of vulnerability found in other areas are mainly due to high slopes (Appalachian Mountains in the U.S., the Mediterranean basin, The Andes, The Himalaya, etc). It is worth noticing that a large part of those areas with steep slopes (especially high mountains) is unsuitable for agricultural activities. Nevertheless, some others regions, disregarding the high values of slopes, may have important cultivations like orchards and vineyards, which often require consistent amounts of insecticides.

The hazard map (Figure 6.2) considers the anthropogenic factors pesticide application rate, percentage of land actually used for agriculture, and plant interception, which depends on the crop typology grown. No official data of pesticide usage were found for more than 30 major countries, some of which (e.g. China) are of primary importance for what concern agricultural production and overall extension. A strong influence of temperature on the insecticide rate has been already shown for European countries (Kattwinkel et al. 2011). Additionally, we hypothesized that in some part of the world the amount of applied pesticides is constrained by economic factor, so that the effective quantity results to be lower than what simply predicted by climatic variables. Therefore, we estimated a country-based insecticide application rate for those countries lacking information using as predictors (1) the average of the accumulated temperature weighted by the proportion of area occupied by croplands, (2) the median GDP (values from 1990 to 2009), and the interaction of these two. The linear regression model (based on the data from countries where the application rate was recorded) was highly significant (F-test, $p < 0.001$) and R^2 was 0.314. The available data of insecticide use indicates great differences between countries, up to 4 orders of magnitude in the application rate [kg A.I./ha].

High values of hazard, mainly due to high application rates, are found in the United States, Mexico and other Central America countries, Caribbean, Colombia and Chile (Figure 6.2). Only small values were estimated for Africa, except the Nile River area and the Mediterranean coast. High hazard values were also found for the European side of the Mediterranean Sea, for countries such as Italy, Spain, Turkey, and Greece. The Netherlands and Belgium represent the only hotspot in Central Europe, but these results may be biased by a relevant presence of greenhouses, that are known for particularly high application rates. Japan and South Korea have the highest values of application rate worldwide resulting in high values of hazard, but other Asiatic countries (India, Viet Nam, Malaysia, Thailand) present also high hazard values. Sharp contrasts that are sometime present in the hazard map reflect the data resolution of the application rate, which is country based.

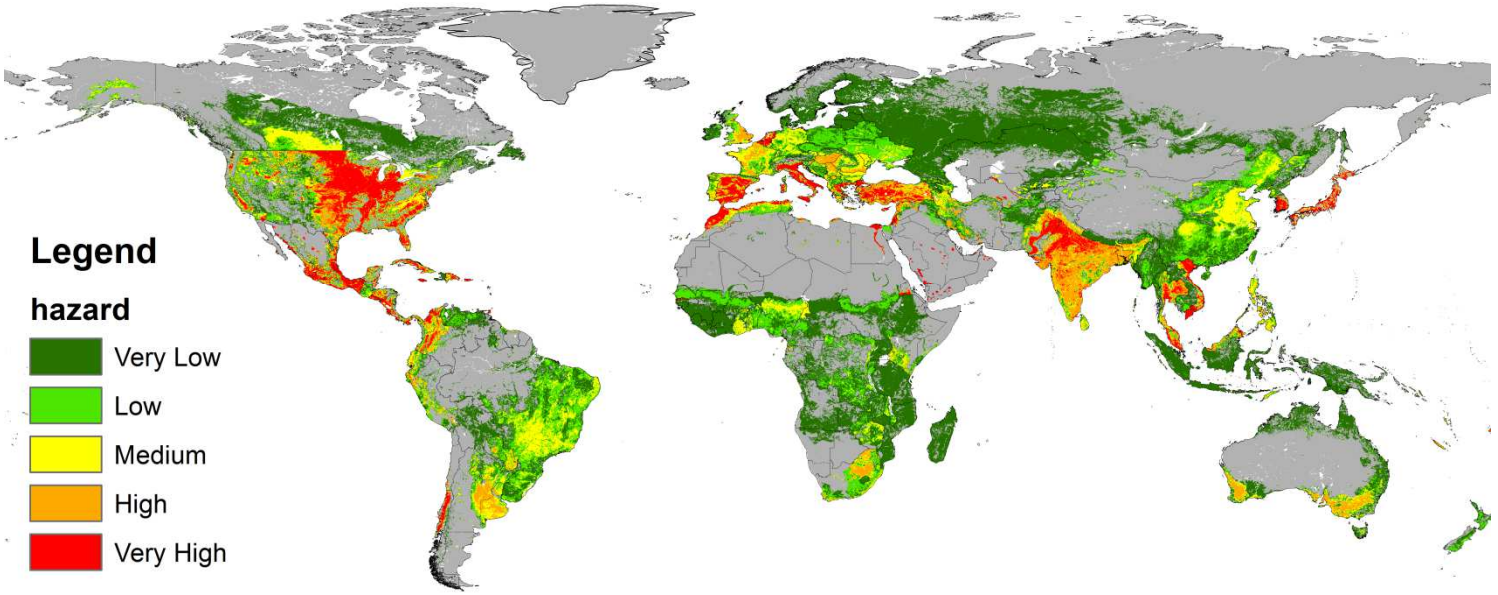

Figure 6.2 Global insecticide hazard map. The map deals with the estimated use of insecticide worldwide. It considers all variables of the RP model under human control and connected to the agricultural activities. Classes boundaries have been assigned ex-post according to the distribution of the values (see Section 6.3), while grey areas indicate absence of any relevant agricultural activity.

The runoff potential map (RP, Figure 6.3), resulting from the product of vulnerability and hazard, gives indications about the likelihood of actual insecticide contamination in rivers. We found that more than 40% of emerged lands are subject to insecticide surface runoff and, remarkably, more than 30% of that fraction (almost 15% of the whole continental surface) presents RP values classified as high to very high. This is consistent with previous findings (Vörösmarty et al. 2010), which already highlighted how pesticides are one of the dominant pollutants of rivers at global scale.

Most of the hotspots in the RP map correspond to those found in the hazard map. Nevertheless, some important differences must be considered. A large part of Western United States, disregarding only low to medium levels of hazard, presents medium to high levels of runoff potential: in this area, despite the low percentage of surface dedicated to agriculture, the risk associated to insecticide runoff potential is not negligible. On the other hand, in Florida, where hazard is very high, the overall RP is buffered by a low vulnerability. Central America presents very high RP values almost everywhere, due to concurrent high values of both vulnerability and hazard. South America is divided into two parts by an ideal line represented by the Amazons and the Andes. West of this line values of RP are generally very high, while east values range from low to high. Europe presents RP values that range from medium to very high, with the exception of the Northern countries. The Mediterranean basin is almost entirely classified as having a very high RP. The only RP hotspots in Africa are in South Africa, around the city of Johannesburg, and in Eritrea. In our representation, India is a primary issue: almost 87 % of its enormous area is classified as featuring high or very high RP values. A huge fraction of China is also characterized by high RP; however, areas classified as at very high risk are extremely scarce in this country. Other Asiatic countries, already mentioned as hazard hotspots (Japan, South Korea, Viet Nam, Malaysia, Thailand), are also of extreme concern in the RP map.

The RP map shows the presence of a clear geographical gradient in the northern hemisphere. Values of RP increase in all Eurasia from north to south. The same trend is observable from North to Central America, where nevertheless the Midwest area in the U.S. represents an exception. This geographical tendency is mostly due to the amount of insecticide used per area. As already stated, where no other constraints are present, insecticide rate is strongly dependent on the average temperature. For this reason climate change is likely to have effect on RP patterns in the next future (Kattwinkel et al. 2011). At least in the northern hemisphere, areas classified as subject to high or very high RP are likely to expand from south to north. In the southern hemisphere, geographical gradients of RP are hardly present. This means that the main driver in insecticide application in those countries is not climate. The general lower level of insecticide use in the southern hemisphere may be due to constraints in pesticide availability, probably

linkable to economic factors as confirmed in our regression model for pesticide application.
In terms of ecological risk resulting from pesticide input to rivers a significant change in the aquatic invertebrate community was reported compared to reference sites with no measured pesticide contamination at several areas in Europe (Schäfer et al. 2007). Moreover, estimated runoff potential likewise corresponds to derivations in community composition (Schriever and Liess 2007). In particular, at sites without upstream recovery area like forests and unsprayed pastures the community was shown to be shifted towards species tolerant to pesticide contamination (Schriever and Liess 2007). On the global scale, 60% of the area with high and very high RP values feature less than 20% forested area (data obtained by the comparison with the relative FGGD map on global forest occurrence, FAO&IIASA 2006). Hence, it is very likely for a significant part of the continental surface that aquatic communities is degraded in comparison to reference sites.
The present work identified a high variability in the available quality of model input data. For some (environmental data, occurrence of croplands), the quality of the information was good in terms of both spatial resolution and reliability. For others, such as plant interception, the quality of the input information was rather jeopardized: that forced us to use temporal and spatial averaged values, thus slightly smoothing the overall influence of this variable. However, the main issue of concern is related to the insecticide consumption data. To our knowledge, the only available database comprehensive of worldwide data is implemented by FAO (Food and Agriculture Organization of the United Nations, FAOSTAT online database). In this database the geographical unit is country-based. For some large countries, this resolution is not high enough for reliable estimation of the actual scenario. Furthermore, some huge differences between neighbouring countries are hardly explainable. An accurate estimation of application rate is a hard task. There is just one study that attempts to estimate the pesticide application rate of those countries for which FAO data are not available (Esty et al. 2005), but the methodology used is not clearly expressed and no distinction between different categories of chemicals (insecticides, herbicides, fungicides, etc.) is made. In our estimation we used average temperature and GDP, but other factors may be involved. This is especially true for those countries where the crop management is not homogeneous, i.e. tropical countries where locally developed subsidence agriculture and multinational companies coexist. In addition, even in those countries where subsistence agriculture is very relevant, the tendency of an increasing use of pesticides is documented (Satapornvanit et al. 2004, Van den Brink et al. 2003).
Despite all the drawbacks, the present work still gives very important input for the environmental management of agricultural activities. Pesticides

remain one of the most important threats for biodiversity and functioning (Schäfer et al. 2007) of freshwater ecosystems, but so far the large majority of studies focused on small scales (usually single catchments, e.g. Verro et al. 2002). Only recently some works have started to extend the focus on larger areas like regions (Liess and Von der Ohe 2005), countries (Huber et al. 2000), or continents (Schriever et al. 2007, Schriever and Liess 2007, Kattwinkel et al. 2011).

The main goal of mapping is usually to help decision makers to address their policies. That is why we produced a vulnerability map representing natural factors that cannot be changed (at least at large scale) and a hazard map on which instead it is possible to intervene with integrated strategies. Environmental management should be operatively performed at local scale, but investment policies can be addressed at continental or even global scale by international agencies and authorities (OECD, FAO, UN, etc). Hence, based on our findings, we suggest directing management measures to areas featuring high values of runoff potential due to high hazard. There, buffer strips along water bodies could reduce pesticide input while the creation of additional unsprayed recovery areas could mitigate negative ecological effects.

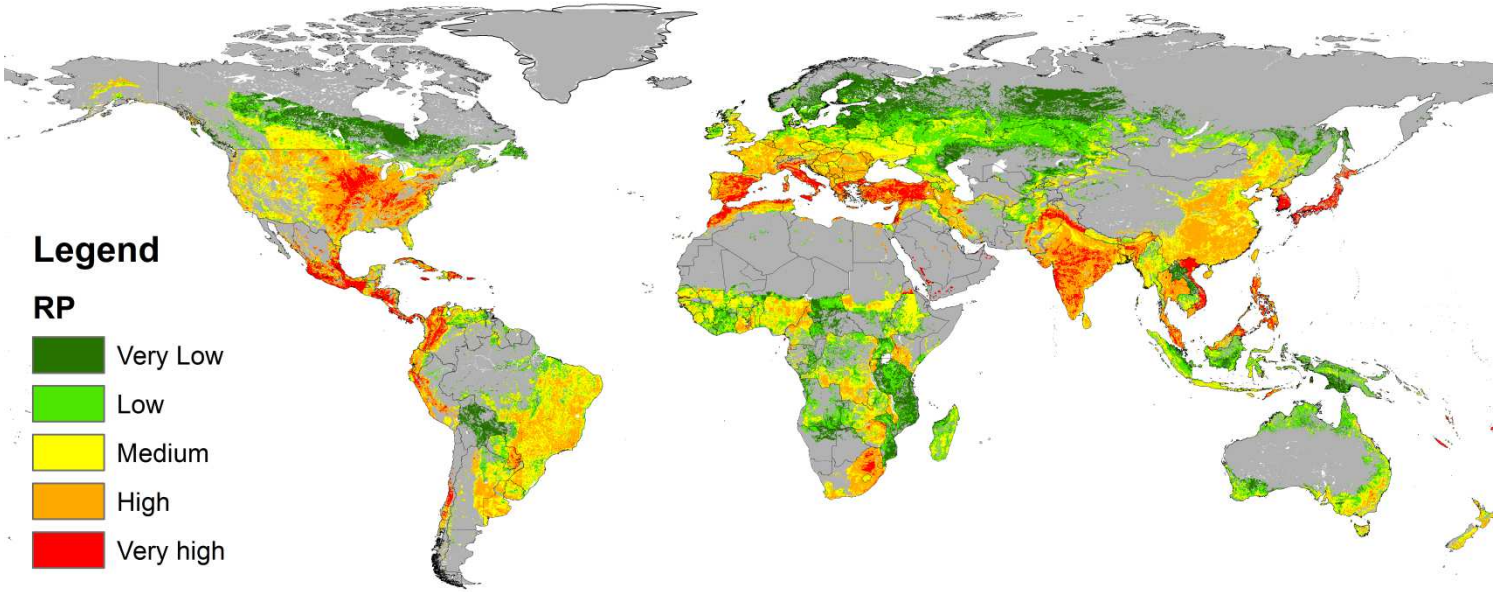

Figure 6.3 Global insecticide runoff potential map. The map shows the spatial distribution of potential risk to freshwater ecosystems due to insecticide runoff. According to this estimation, surface waters in 43% of total continental areas are potentially subject to insecticide load as a consequence of present agricultural practises. Classes boundaries (reported in Table 6.2, section 6.3) are the same used in previous works (Schriever and Liess 2007, Kattwinkel et al. 2011). Grey areas indicate absence of any relevant agricultural activity.

6.2 Methods summary

The RP model (Schriever et al. 2007, Schriever and Liess 2007, details about the equation and parameters can be found in section 6.3) was used to estimate insecticide load in rivers at global scale. According to a previously used simplification (Schriever and Liess 2007), the model was not applied to real stream courses but to 5 x 5 arc minute grid cells. Hence, we substituted the real courses of streams in each grid cell with a generic stream segment. The RP model is based on seven spatial variables, elaborated with GIS. four of them (organic carbon content in soil, slope, soil texture and occurrence of cropland) were directly derived by existing maps, while for the remaining three variables (rain intensity, insecticide application rate and plant interception), raster maps were implemented starting from numeric databases. Particularly, for rain events we started from GHNC daily database (http://www.ncdc.noaa.gov/oa/climate/ghcn-daily/), extracting the average of the maximum values during the productive season for each of the 77468 stations. Point values were then interpolated by linear kriging. Missing values of insecticide application rate were estimated on the basis of a linear model. As predictive variables of the model we used GDP values (1990-2009) and an average temperature weighted on the basis of the occurrence of cropland for each grid cell. Further details about data elaboration and map resolution are given in the next section. The vulnerability map was built considering natural factors only (rainfall intensity, slope, soil texture and organic carbon content), while the other anthropogenic variables were used to built the hazard map. Break values used for the classification of these maps are based on quintiles. Break values in the final RP map (-3; -2; 1; 0) are derived from previous work (Kattwinkel et al. 2011) to facilitate the comparison.

6.3 Supplementary methods

For our evaluation we used a generic indicator (RP - runoff potential) which distinguishes stream sites using key environmental characteristics of the near-stream environment to assess the potential for insecticide runoff inputs.
The RP is based on a mathematical model (Eq.6.1) that calculates the dissolved amount (gLOAD [g]) of a generic substance that was applied in the near-stream environment and that is likely to reach a certain stream site during one rainfall event. No real stream courses were considered in this study. In accordance with previous works (Schriever et al. 2007, Schriever and Liess 2007, Kattwinkel et al. 2011), we considered for each grid cell a generic stream segment with one bifurcation. The near-stream environment was set to an area of 0.45 km^2, deriving from a two-sided 100-m stream corridor extending for 1500 m upstream of the site (bifurcation is placed

midway of the upstream corridor). All spatial calculations were performed with ArcGis 10 (ESRI, Redlands, CA, USA). Grid cell size in this study was 5 x 5 arc-minutes.

$$gLOAD_i = A_i \cdot D \cdot \frac{1}{1+\frac{Koc \cdot OC_i}{100}} \cdot f(s_i) \cdot \frac{f(P_i,T_i)}{P_i} \cdot P_i \cdot \left(1-\frac{I_{i,j}}{100}\right) \quad (6.1)$$

Table 6.1 Explanation of the variables of the gLOAD model

Variable	Explanation
gLOAD	Generic insecticide runoff that reach the stream site
i	Grid cell index
j	Crop typology index
A_i	Area of the stream corridor (fixed to 0.45 km^2, see text for rationale)
D	Insecticide application rate (g/ha)
Koc	Sorption coefficient of a certain substance to organic carbon; since we didn't consider specific substances, this value was set to 100 in order to maximize distinction of sites due to differences in soil organic carbon content
OC_i	Soil organic carbon content (%)
s_i	Mean slope (%)
$f(s_i)$	Influence of slope on runoff (OECD, 1998) $\begin{cases} 0.001423 \cdot s_i^{\,2} + 0.02153 \cdot s_i, & \text{if } s_i \le 20\% \\ 1, & \text{if } s_i > 20\% \end{cases}$
P_i	Precipitation (mm)
T_i	Soil texture (sand/loam)
$f(P_i,T_i)$	Volume of surface runoff (mm) (OECD, 1998) $= \begin{cases} -5.86 \cdot 10^{-6} \cdot P_i^3 + 2.63 \cdot 10^{-3} \cdot P_i^2 - 1.14 \cdot 10^{-2} \cdot P_i - 1.64 \cdot 10^{-2} & \text{if } T = \text{sand} \\ -9.04 \cdot 10^{-6} \cdot P_i^3 + 4.04 \cdot 10^{-3} \cdot P_i^2 - 4.16 \cdot 10^{-3} \cdot P_i - 6.11 \cdot 10^{-2} & \text{if } T = \text{loam} \end{cases}$
P_i	Proportion of croplands in cell *i*
$I_{i,j}$	Average of crop-specific plant interception in cell *i* (%)

As a result, gLOAD reflected the mean generic exposure of a stream section, which is located in cell and has the same environmental characteristics as the grid cell. To distinguish between stream sites with respect to their potential exposure, the RP of an individual grid cell was derived as the log10-transformed gLOAD and classified into five order-of-magnitude categories (Table 6.2).

Table 6.2 RP categories

Class	RP Value
Very Low	≤ -3
Low	≤ -2
Medium	≤ -1
High	≤ 0
Very High	> 0

Additionally, the model has been split into 2 sub-model: one includes all natural variables (Eq.6.2), while the other one regards all the variables that are under human control (Eq.6.3). In analogy with the classical risk equation, the first sub-model has been used to built a vulnerability map, being vulnerability the natural degree of susceptibility to runoff in each grid cell. The second sub-model has been used to represents the hazard connected to the human management of insecticides in agriculture. Vulnerability and Hazard maps present fully different range of variation, so the classes boundaries for these map have been assigned ex-post according to the distribution function of the values (quintiles**).**

$$Vulnerability_i = \frac{1}{1+\frac{Koc \cdot OC_i}{100}} \cdot f(s_i) \cdot \frac{f(P_i, T_i)}{P_i} \qquad (6.2)$$

$$Hazard_i = D \cdot P_i \cdot \left(1 - \frac{I_{i,j}}{100}\right) \qquad (6.3)$$

6.3.1 Variables

6.3.1.1 Insecticide application rate (D)

Country-based data on insecticide application rate were retrieved from FAOSTAT Database. All available data for each country were averaged over the entire period (1990-2009) reported in the database. For missing countries in the database, a typical insecticide application rate was estimated. Since a strong influence of the temperature on the insecticide rate has been already proven (Kattwinkel et al. 2011) for European countries, we calculated an average accumulated temperature for each country, weighting the value of each grid cell ($AccT_i$) by the correspondent percentage of cultivated land (Eq.6.4). A global accumulated temperatures (Tmean > 0° C) (FAO & IIASA, 2000) was used. The same map (FAO & IIASA 2006) considered for the estimation of the variable *P*, was here used as weighting factor.

$$\textit{Country-specific weighted temperature} = \frac{\sum_{i=1}^{n}(AccT_i \cdot P_i)}{\sum_{i=1}^{n} P_i} \qquad (6.4)$$

Nevertheless, in some parts of the world the availability of insecticides is likely to be constrained by economic factor, so that the effective quantity results to be lower than what simply predicted by climatic variables. Hence we also included in our evaluation the country specific GDP (Gross Domestic Products). Although this is probably not the most suitable indicator for our purpose, it is certainly the most easily accessible and reliable. GDP values (expressed in 2005 dollars) per capita per country per year were retrieved from ERS International Macroeconomic Data Set.
Median values of GDP were calculated for all countries over the period 1990-2009. Particularly, for those countries with some gaps in the insecticide application rate, correspondent values of GDP were excluded from the median. Calculation of the best regression model was performed with [R] using all the available data at country level.
All country-based insecticide application rates were scaled by dividing them by the application rate of Germany in order to make them comparable to those used in previous studies (Schriever and Liess 2007, Kattwinkel et al. 2011).

6.3.1.2 Precipitation (P)
Runoff Potential is determined by singles rainfall events, hence yearly average values are not suitable for this model. We calculated the median value of the monthly maximum precipitation for each of the 76687 station (over the entire available period) collected in the GHCN daily database. Then we assigned to each station the maximum value within the productive season.
Productive seasons were assessed using two maps implemented within the FGGD project (FAO & IIASA 2006). At first, we considered the areas where lack of water is the principal constraint for agriculture according to the “Hierarchical distribution of severe environmental constraints map”. In those areas, no period limitations were established, since the most rainy month is likely to be the period of maximum productivity. In all other areas, the length of the growing period was assigned on the basis of the correspondent FGGD map(FAO & IIASA 2006), while the temporal collocation of the growing season was differentiated between northern and southern hemisphere (Table 6.3).

Table 6.2 Temporal limitation of the growing period in the Northern and in the Southern hemispheres imputed on the basis of the Length of the Growing Period (LGP) as estimated in the FGGD map (FAO&IIASA 2006).

LGP (FGGD map)	Months	Northern hemisphere	Southern hemisphere
0 day	12	All year	All year
1-29 days	12	All year	All year
30-59 days	2	June-July	December-January
60-89 days	3	June-August	December-February
90-119 days	4	May-August	November-February
120-149 days	5	May-September	November-March
150-179 days	6	April-September	October-March
180-209 days	7	April-October	October-April
210-239 days	8	March-October	September-April
240-269 days	9	March-November	September-May
270-299 days	10	February-November	August-May
300-329 days	11	February-December	August-June
330-364 days	12	All year	All year
365- days	12	All year	All year
365 days	12	All year	All year
365+ days	12	All year	All year

With this methodology, we assigned one value to each of the GHCN stations, whose coordinates were known. Hence, the points were interpolated (ordinary kriging – spherical semivariogram, output cell size 5 x 5 arc minutes) over the global continental surface.
The function *f(T,P)/P* proposed by the OECD (1998) is based on empirical values, and it has a maximum around the value of 224 mm. Nevertheless, it doesn't seem reasonable that the runoff would decrease when precipitation exceeds this value. Thus, we substituted all values above 224 with 224.

6.3.1.3 Plant interception (I)
When pesticide are applied, especially if sprayed, a consistent fraction is intercepted by the vegetation of the crops and does not reach the soil. Plant interception is plant specific and growth phase specific. Nevertheless, agricultural pattern (growth phases in which plant are treated) are too complex and spatially heterogeneous to be taken into account in this elaboration. Hence, average values ((minimum P.I. + maximum P.I)/2) for each crop typology were used. Minimum and maximum values of plant interception were retrieved from literature (Linders et al. 2000) when available. Missing values were imputed on the basis of the foliar shape and density.

Spatial distribution of crops was derived from the FAO database Agro-MAPS (FAO, global spatial database of agricultural land-use statistics) . The typology of data retrieved was area harvested [Ha] per crop typology. Data were aggregated at two different administrative level. The primary level (coarser) divides the global continental surface in 2963 polygons, while the secondary (finer) is composed of 22622 polygons. The resolution of these data is extremely heterogeneous, since polygons range from small municipalities to extremely large regions. Information was not complete nor at the first nor at the second administrative level. Thus, a standard procedure was followed.

- We chose to use polygons of the secondary administrative level.
- Gaps at this level were filled using the information of the overhanging primary level polygon.
- If no information was available at the primary and secondary level a national average was used
- If no information was available for the entire country a default value of 50 % was used for plant interception

For each secondary administrative level polygon an average plant interception was calculated (on the basis of the relative occurrence of each crop type).

6.3.1.4 Soil variables (T, OC)

Values of soil variables were retrieved from the ISRIC-WISE derived soil properties on a 5 by 5 arc-minutes global grid (Batjes, 2006). Organic carbon content of the first soil layer (0-20 cm) was considered. In the ISRIC-WISE map the organic carbon is expressed in 5 classes + rocks and glaciers (OC% = 0). In our elaboration each cell took the average rounded value of the class it belonged. Texture was derived from the Access Database related to the ISRIC-WISE map. The % content of clay, silt and sand in the first soil layer (0-20 cm) was used to classify the texture as sandy or loamy. Criteria for the classification were retrieved from literature (Finnern et al. 2005).

6.3.1.5 Other variables (p, s)

Occurrence of cropland (proportion of croplands in each grid cell) and average slope were taken from correspondent maps compiled in the FGGD project (FAO & IIASA 2006). Cell size of the maps is 5 x 5 arc minutes. In the FGGD map the slope is expressed in 7 classes. In our elaboration each cell took the average rounded value of the class it belonged. No major elaborations were necessary for the occurrence of cropland map.

6.4 Supplementary discussion

The reliability of data is a central issue in our elaboration. Particularly, data of insecticide application rate present strong limitation. The FAOSTAT database is implemented with very heterogeneous data provided by each state. Data are usually expressed as active ingredients [ton] applied, nevertheless sometimes they refer to consumption in formulated product, sales, distribution or imports for use in the agricultural sector.
Notwithstanding, this is the only database of such type on a global scale, so we had little chance to estimate its reliability at the scale of our study. However, a similar database (reporting insecticide sales per country) has been made available by EUROSTAT for European Union. A comparison of this database with data of European countries of the FAOSTAT database was performed (Table 6.4), enhancing a strongly jeopardized scenario. Data referring to the period from 1990 to 2008 were compared. A simple correlation coefficient (R^2) was used to evaluate the relationship between the two databases. For some countries there is a good agreement, with just slight differences or, in two cases absolutely no difference (Belgium and Spain). 10 out of 20 countries present $R^2 \geq 70$, nevertheless for at least 7 countries almost no correspondence has been found between the two databases.

Table 6.3 Correlation coefficient (R^2) and relative statistical significance between data of insecticide application rate as reported in the FAOSTAT and in the EUROSTAT datasets.

Country	R^2	p value	Notes
Austria	0.99	<0.0001	
Belgium	1.00	<0.0001	
Denmark	0.94	<0.0001	
Estonia	0.99	<0.0001	
Finland	0.95	<0.0001	
France	0.92	<0.0001	
Germany	0.28	0.0198	
Greece	0.07	0.4280	
Hungary	0.14	0.1204	
Ireland	0.70	0.0003	
Italy	0.01	0.7333	
Latvia	---	---	Inverse correlation
Netherlands	---	---	Inverse correlation
Norway	0.92	<0.0001	
Poland	0.61	0.0002	
Portugal	0.78	<0.0001	
Slovenia	---	---	Inverse correlation
Spain	1.00	<0.0001	
Sweden	0.53	0.0009	
United Kingdom	0.65	<0.0001	

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7 General conclusions

7.1 Ecotoxicology has come to a turning point.

Decades of compiling data about environmental concentrations of thousands of chemicals and evaluating their toxicity with laboratory assays were fundamental to answer some pressing needs, due to an increasing concern about chemical effects, in order to have standardized methodologies for risk assessment (Van den Brink 2008). But now the research in ecotoxicology must overcome this approach: the challenge is to go out to the real environment and decrease the huge uncertainty derived from extrapolations processes (from the lab to the field). To do so, a deep knowledge of ecological processes should be achieved, and this knowledge should be integrated in the procedures to evaluate chemical environmental risk (Van Straalen 2003). To pursue this unique goal, multiple challenges have to be addressed, as testified by the recent activity of a Working Group of the European Commission (DG SANCO) on "Addressing the New Challenges for Risk Assessment".

The aim of this work was to deal with some of these challenges, with a focus on plant protection products (PPP) and, particularly, on their effects on freshwater communities and ecosystems.

Ecosystem responses to diffuse pollution is highly variable, due to a huge number of possible combinations of source type, pathways and receptors (Posthuma et al. 2008). Hence, actual effects of chemicals in the environment can be assessed with a sufficient accuracy only with a site-oriented approach, used as integration of the chemical-oriented approach currently applied in any risk assessment procedure. The characterization of the ecological system exposed to a certain chemical (or mixture of chemicals) is probably the most overlooked aspect in current risk assessment procedures. This work started from this consideration to propose a defined framework for the evaluation of the ecosystem vulnerability, supported by a qualitative case study. A simple index was implemented, which can be a useful tool applicable at several levels of biological organization (see for example Vaj 2011). Three main components were identified to assess vulnerability: susceptibility to exposure, direct sensitivity, and resilience.

Recent literature (Baird et al. 2008; Rubach et al. 2011; De Lange et al. 2009; Beketov and Liess 2012) suggest how the use of biological and ecological species traits can be a useful tool for vulnerability analysis. In the present work a trait-based approach was used to predict species sensitivity of some freshwater macroinvertebrates toward three classes of insecticides (organophosphates, carbamates, and pyrethroids). Despite relevant

drawbacks need to be solved, like the lack of available data on most relevant traits for ecotoxicological studies, the results obtained confirmed the promising role of Trait-based Ecologically Risk Assessment (TERA). One of the most important insight (the significant correlation between a proxy of nervous system complexity and the sensitivity toward neurotoxicants) emphasizes the need for research on less generic traits than those used in the past, in order to achieve a mechanistic comprehension of the link between different exposures (e.g. different modes of action) and effects (e.g. different sensitivity).

The same attempt to establish a sound mechanistic link between exposure and effects was made with another case study, in which a clear site-oriented approach was applied. A two-year monitoring campaign of the invertebrate community was carried out on an alpine stream, characterized by relevant loads of PPP. Contemporary, a characterization of the exposure was performed, using a model approach coupled with chemical analysis. A particular attention was posed to the time resolution of the analysis, since many studies (i.e. Bonzini et al. 2006) already highlight that complex exposure profiles such those of PPP in running waters may lead to great underestimations of predicted environmental concentrations (PECs), thus biasing the results of the risk assessment procedure. Nevertheless, in the present work another source of concern was identified, since it was shown how not only exposure, but even effects at the community level, expressed by mean of the $SPEAR_{pesticide}$ index (Liess and Von der Ohe 2005), may present sudden alterations within a short time range.

The existence of a potential risk in the studied catchment was confirmed by a model ecosystem study. In agreement with Posthuma et al. 2008, which highlighted the importance of higher tier assessment when performing site-oriented risk assessment, the studied scenario was reproduced (in terms of chemical concentrations and peak timing as well as for what concern the community structure) in a system composed by five artificial streams, where influence of any confounding factor was minimized. The use of the model ecosystem proved to be extremely useful for another relevant issue, that is the selection of the endpoints to be measured in the field. The choice of the endpoint is a matter of major concern, since it should be sensitive to the stressor under investigation, but contemporarily unaffected by any other confounding factor, both natural or anthropic. In addition, it should be quantitative in order to make precise comparison, and it should be suitable to be effective at the largest possible scale, without further adjustments. According to our results $SPEAR_{pesticide}$ proved to be the best metric within those that were tested, confirming the importance of the ecological vulnerability paradigm that this index is based on.

Finally, the concept of vulnerability was upscaled to the maximum possible extent in order to produce a global map of vulnerability to pesticide runoff, which was combined to an hazard map to produce a map of runoff potential

risk. Contribution of both vulnerability (natural abiotic factors) and hazard (factors connected to human activity) proved to be relevant in determining the overall risk, but results highlighted that correct local policies and agriculture management may play a pivotal role in reducing pesticide potential impact on freshwater environments in many areas of the world. However, this part of the work has been intended as an exercise to show how the integration of vulnerability and hazard assessments to evaluate the risk is possible at any scale.
Concluding, this work tried to deal with some of the most relevant challenges of modern ecotoxicology, in the view of a more ecological realism in PPP risk assessment, providing new tools and insights that may trace important patterns in the future research of ecotoxicology.

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Appendix 1

Supplementary material of Chapter 2

Ecological vulnerability analysis: a river basin case study

Table I.I Some features of River Serio and River Trebbia.

	Length	Average flow rate	Basin extent	Altitude
R.Serio	124 km	40 m^3/s	1256 km^2	From 2583 to 48 m a.s.l.
R.Trebbia	115 km	24 m^3/s	1000 km^2	From 1406 to 60 m a.s.l.

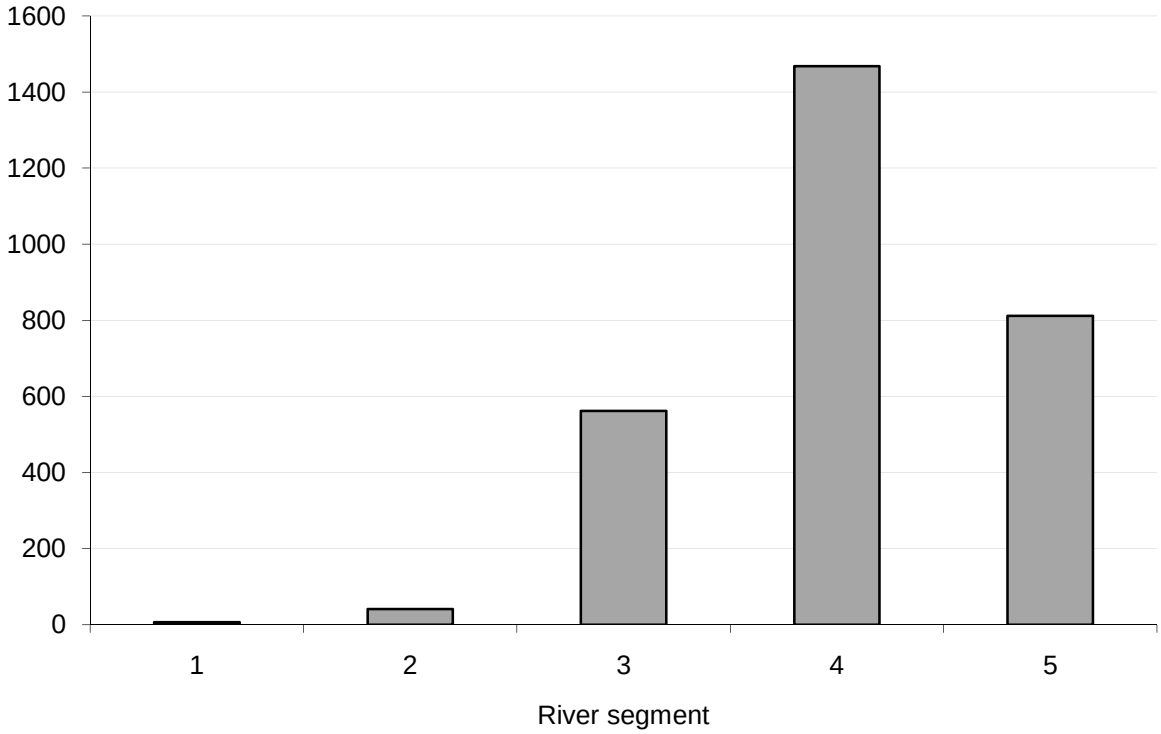


Figure I.I Characterisation of stressors on Serio River - number of agricultural enterprises in the 5 river segments.

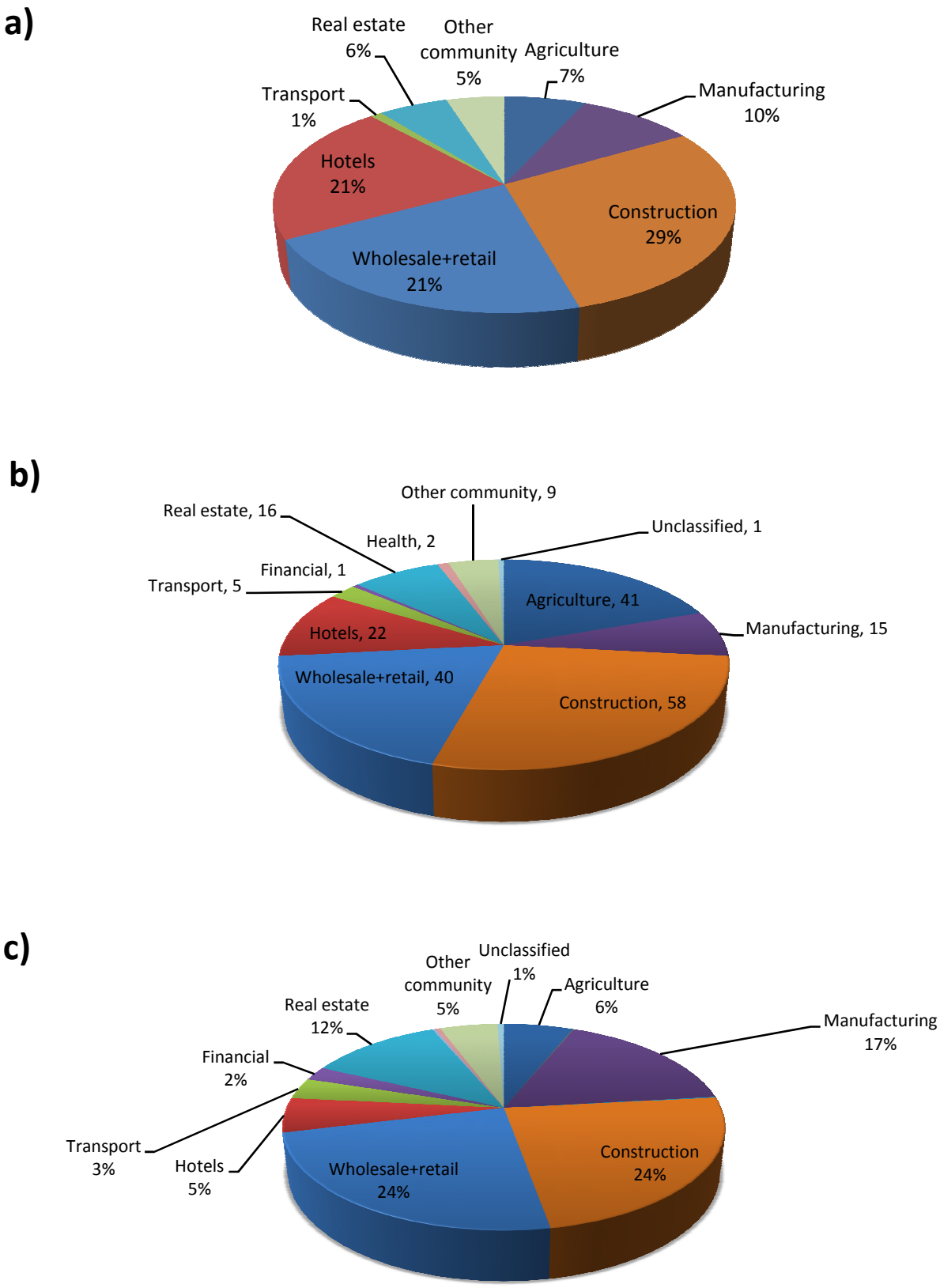
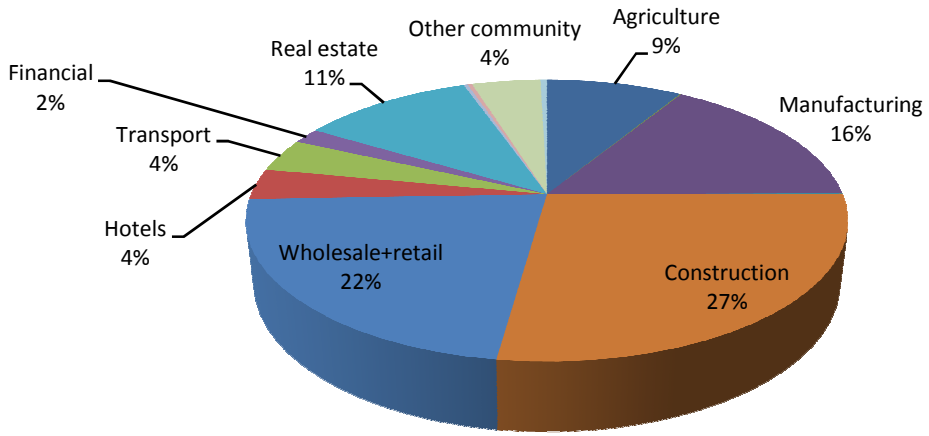


Figure II.I Characterization of stressors on Serio River: distribution of enterprise typology according to national statistic categories on each river segment; a) r.s. 1; b) r.s. 2; c) r.s. 3. Source: Infocamere, 2009. Imprese attive presenti nel Registro delle Imprese al 31.12 per sezione di attività economica. Totale forme giuridiche. Comunale. Anno 2008. www.ring.lombardia.it/asrnew/index.html (accessed July 2009)

d)



e)

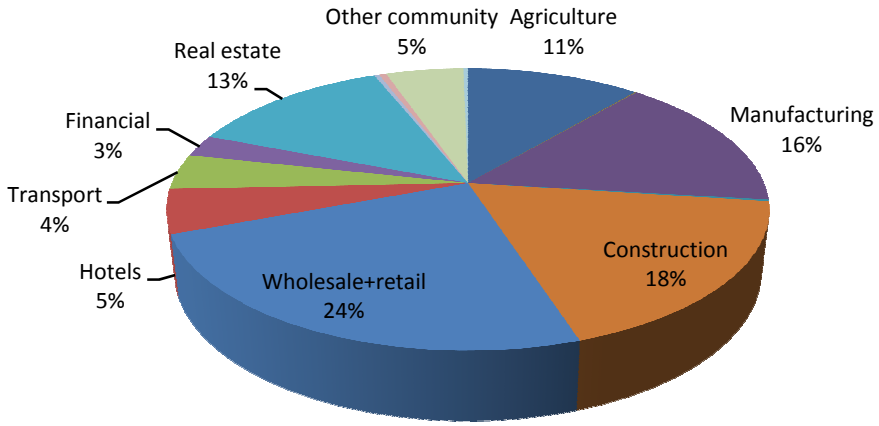


Figure II.I *(continues from previous page)* d) r.s. 4; e) r.s. 5.

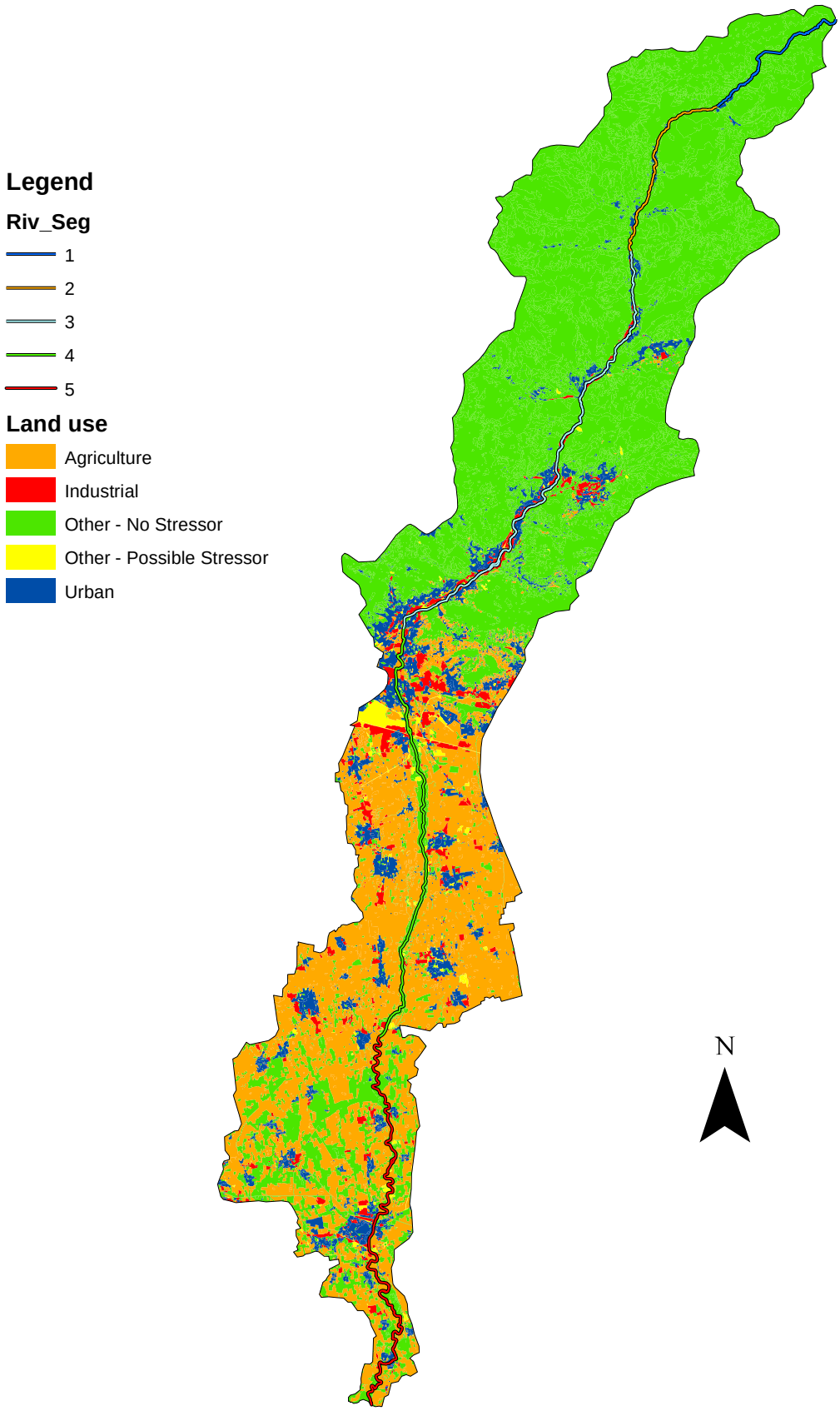


Figure III.I Land use of watersheds referred to each river segment.

Appendix 2

Supplementary material of Chapter 3
Sensitivity assessment of freshwater macroinvertebrates to pesticides using biological traits

1. Trait database

See text for coding and meaning of values

Species	BL	LD	VL	RT_te	RT_gi	RT_pl	RT_ae	FH_df	FH_sh	FH_sc	FH_ff	FH_pr	FH_pa	OT_tc	OT_to	OT_hcy	OT_hgl	OT_nopg	AR	BS_li	BS_dvf	BS_lf	BS_se	RA	BE
Acroneuria sp	0.25	1.00	0.25	0.40	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.77
Aedes aegypti	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes albopictus	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes atropalpus	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes canadensis	0.08	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes cantans	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes caspius	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes communis	0.08	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes excrucians	0.08	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes hendersoni	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes nigromaculis	0.08	0.00	0.75	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes punctor	0.08	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes sticticus	0.08	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes stimulans	0.08	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes taeniorhynchus	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes triseriatus	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes trivittatus	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Aedes vexans	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.10	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Alonella sp.	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Ameletus sp.	0.12	0.50	0.50	0.40	0.60	0.00	0.00	0.50	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.72
Anisops sardeus	0.08	0.50	0.75	0.25	0.00	0.00	0.75	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.80	0.00	0.20	0.00	0.50	0.00	0.00	0.00	1.00	0.30	1.00
Anodonta anatina	0.75	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Anodonta anatina larvae	0.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Anodonta cygnea	0.92	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Anodonta cygnea larvae	0.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Anodonta sp.	0.86	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Anopheles albimanus	0.07	0.00	0.75	0.00	0.00	0.00	1.00	0.33	0.00	0.17	0.00	0.50	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.87
Anopheles culicifacies	0.07	0.00	0.75	0.00	0.00	0.00	1.00	0.33	0.00	0.17	0.00	0.50	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.87
Anopheles freeborni	0.07	0.00	0.75	0.00	0.00	0.00	1.00	0.33	0.00	0.17	0.00	0.50	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.87
Anopheles gambiae	0.07	0.00	0.75	0.00	0.00	0.00	1.00	0.33	0.00	0.17	0.00	0.50	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.87
Anopheles quadrimaculatus	0.06	0.00	1.00	0.00	0.00	0.00	1.00	0.33	0.00	0.17	0.00	0.50	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.87
Anopheles stephensi	0.07	0.00	0.75	0.00	0.00	0.00	1.00	0.33	0.00	0.17	0.00	0.50	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.87
Aplexa hypnorum	0.10	0.65	0.50	0.00	0.00	0.00	1.00	0.20	0.20	0.60	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Arctopsyche grandis	0.35	1.00	0.25	0.40	0.60	0.00	0.00	0.25	0.25	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.77
Asellus aquaticus	0.12	0.75	1.00	0.00	1.00	0.00	0.00	0.40	0.30	0.30	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.60
Asellus brevicaudus	0.13	0.75	1.00	0.00	1.00	0.00	0.00	0.40	0.30	0.30	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.60
Asellus hilgendorfi	0.12	0.75	1.00	0.00	1.00	0.00	0.00	0.40	0.30	0.30	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.60
Astacopsis gouldi	0.05	1.00	1.00	0.00	1.00	0.00	0.00	0.70	0.30	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.60
Atherix sp.	0.30	0.25	0.50	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.30	0.77
Atherix variegata	0.30	0.50	0.50	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.30	0.77
Austrolestes colensonis	0.30	0.65	0.25	0.33	0.67	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	0.80	0.77
Baetis sp.	0.09	0.25	0.75	0.40	0.60	0.00	0.00	0.50	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	1.00	0.00	1.00	0.83
Barytelphusa cunicularis	0.60	1.00	0.50	0.00	1.00	0.00	0.00	0.33	0.34	0.00	0.00	0.33	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.60
Bellamia bengalensis	0.35	1.00	0.25	0.00	1.00	0.00	0.00	0.50	0.00	0.50	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Biomphalaria glabrata	0.14	0.75	0.50	0.00	0.00	0.00	1.00	0.20	0.20	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27

Species	BL	LD	VL	RT_te	RT_gi	RT_pl	RT_ae	FH_df	FH_sh	FH_sc	FH_ff	FH_pr	FH_pa	OT_tc	OT_to	OT_hcy	OT_hgl	OT_nopg	AR	BS_li	BS_dvf	BS_lf	BS_se	RA	BE
Biomphalaria havanensis	0.05	0.65	0.50	0.00	0.00	0.00	1.00	0.20	0.00	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Bosmina fatalis	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Bosmina longirostris	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Branchiura sowerbyi	0.75	0.75	1.00	0.60	0.40	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.30	0.19
Brachycentrus americanus	0.08	0.25	0.50	0.40	0.60	0.00	0.00	0.00	0.00	0.20	0.50	0.30	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.60
Caenis horaria	0.07	0.25	0.87	0.40	0.60	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.60	0.60
Caenis miliaria	0.07	0.25	0.87	0.40	0.60	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.60	0.60
Calineuria californica	0.08	0.00	0.00	0.40	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.77
Caridina rajadhari	0.28	0.75	0.50	0.00	1.00	0.00	0.00	0.30	0.00	0.70	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.60
Ceriodaphnia dubia	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Chaoborus obscuripes	0.15	0.75	0.75	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.87
Chaoborus punctipennis	0.15	0.75	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.87
Chauliodes sp.	0.45	1.00	0.00	0.40	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	0.60	0.77
Cheumatopsyche sp.	0.10	0.25	0.75	0.40	0.60	0.00	0.00	0.00	0.00	0.20	0.50	0.30	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.60
Chironomus crassicaudatus	0.11	0.00	0.75	1.00	0.00	0.00	0.00	0.70	0.00	0.00	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Chironomus decorus	0.12	0.00	1.00	1.00	0.00	0.00	0.00	0.70	0.00	0.00	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Chironomus plumosus	0.20	0.00	0.75	1.00	0.00	0.00	0.00	0.60	0.00	0.00	0.40	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Chironomus riparius	0.11	0.00	0.75	1.00	0.00	0.00	0.00	0.70	0.00	0.00	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Chironomus salinarius	0.11	0.00	1.00	1.00	0.00	0.00	0.00	0.40	0.00	0.30	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Chironomus tentans	0.12	0.00	1.00	1.00	0.00	0.00	0.00	0.60	0.00	0.00	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Chironomus tepperi	0.18	0.00	0.75	1.00	0.00	0.00	0.00	0.70	0.00	0.00	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Chironomus thummi	0.13	0.00	0.75	1.00	0.00	0.00	0.00	0.70	0.00	0.00	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Chironomus utahensis	0.11	0.00	0.75	1.00	0.00	0.00	0.00	0.70	0.00	0.00	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Chironomus yoshimatsui	0.11	0.00	0.75	1.00	0.00	0.00	0.00	0.70	0.00	0.00	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Cinygma sp.	0.09	0.50	0.50	0.40	0.60	0.00	0.00	0.20	0.00	0.80	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.60
Cipangopaludina malleata	0.50	1.00	0.50	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.50	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Claassenia sabulosa	0.23	1.00	0.00	0.40	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.77
Claassenia sp.	0.30	1.00	0.00	0.40	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.77
Cloeon dipterum	0.06	0.25	1.00	0.40	0.60	0.00	0.00	0.43	0.00	0.43	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.83
Cloeon sp.	0.09	0.25	1.00	0.40	0.60	0.00	0.00	0.50	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.83
Corbicula manilensis	0.50	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Cordulia aenea	0.25	0.75	0.33	0.25	0.75	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.60	0.77
Corixa punctata	0.13	0.00	0.75	0.00	0.00	0.00	1.00	0.70	0.00	0.30	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.30	0.83
Cricotopus sp.	0.06	0.00	1.00	1.00	0.00	0.00	0.00	0.20	0.00	0.80	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Crocothemis erythraea	0.20	0.25	0.83	0.25	0.75	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.60	0.77
Culex fuscocephala	0.08	0.00	0.75	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Culex melanurus	0.02	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Culex peus	0.08	0.00	0.75	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Culex pipiens	0.10	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Culex quinquefasciatus	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Culex restuans	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Culex salinarius	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Culex tarsalis	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Culex tritaeniorhynchus	0.08	0.00	0.75	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70
Culicoides sp.	0.04	0.65	1.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.77
Culicoides variipennis	0.04	0.65	1.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.77
Culiseta annulata	0.08	0.00	0.75	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.70

Species	BL	LD	VL	RT_te	RT_gi	RT_pl	RT_ae	FH_df	FH_sh	FH_sc	FH_ff	FH_pr	FH_pa	OT_tc	OT_to	OT_hcy	OT_hgl	OT_nopg	AR	BS_li	BS_dvf	BS_lf	BS_se	RA	BE
Culiseta incidens	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	1.00	0.00	0.00	0.00	0.70
Culiseta logiareolata	0.08	0.00	0.75	0.00	0.00	0.00	1.00	0.00	0.00	0.90	0.00	0.10	0.00	0.00	1.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.70
Cypretta kawatai	0.00	0.00	0.50	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.40	0.10	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.83
Cypria sp.	0.01	0.00	0.50	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.83
Cypridopsis vidua	0.01	0.00	0.50	1.00	0.00	0.00	0.00	0.50	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.83
Daphnia carinata	0.02	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Daphnia cucullata	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Daphnia longispina	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Daphnia magna	0.02	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Daphnia obtusa	0.02	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Daphnia pulex	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Diaptomus sp.	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.30	0.53
Dicrotendipes californicus	0.08	0.00	1.00	1.00	0.00	0.00	0.00	0.30	0.00	0.40	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.60
Drunella grandis	0.13	0.25	0.50	0.40	0.60	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.60
Dugesia tigrina	0.20	1.00	0.50	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.43
Echinogammarus tibaldii	0.10	0.65	1.00	0.00	1.00	0.00	0.00	0.20	0.40	0.10	0.20	0.30	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.83
Elliptio icterina	0.65	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.00	0.02
Enallagma sp.	0.23	0.65	0.75	0.25	0.75	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.80	0.77
Ephemerella sp.	0.10	0.25	0.50	0.40	0.60	0.00	0.00	0.15	0.29	0.42	0.00	0.15	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.60	0.60
Ephemerella subvaria	0.12	0.25	0.50	0.40	0.60	0.00	0.00	0.50	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.60	0.60
Eretes stiticus	0.13	0.75	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.30	0.88
Eriocheir sinensis	0.60	1.00	0.50	0.00	1.00	0.00	0.00	0.20	0.20	0.00	0.00	0.60	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.77
Erpobdella octoculata	0.50	0.75	0.38	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.43
Eucyclops sp.	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.30	0.53
Eudiaptomus graciloides	0.01	0.00	0.75	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.30	0.53
Gammarus fasciatus	0.14	0.75	0.84	0.00	1.00	0.00	0.00	0.00	0.75	0.15	0.00	0.10	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.83
Gammarus fossarum	0.08	0.65	1.00	0.00	1.00	0.00	0.00	0.20	0.70	0.10	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.83
Gammarus lacustris	0.15	0.75	0.50	0.00	1.00	0.00	0.00	0.20	0.50	0.10	0.00	0.20	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.83
Gammarus palustris	0.11	0.65	1.00	0.00	1.00	0.00	0.00	0.15	0.60	0.00	0.00	0.25	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.83
Gammarus pseudolimnaeus	0.15	0.75	0.75	0.00	1.00	0.00	0.00	0.00	0.50	0.10	0.00	0.20	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.83
Gammarus pulex	0.12	0.75	1.00	0.00	1.00	0.00	0.00	0.10	0.60	0.20	0.00	0.10	0.00	0.00	0.00	1.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00	1.00	0.83
Glossiphonia sp.	0.30	0.75	0.50	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.25	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.35
Glyptotendipes paripes	0.09	0.00	0.50	1.00	0.00	0.00	0.00	0.50	0.00	0.20	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Goeldichironomus holoprasinus	0.06	0.00	1.00	1.00	0.00	0.00	0.00	0.70	0.00	0.00	0.30	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.60
Helisoma trivolvis	0.09	0.65	0.50	0.00	0.00	0.00	1.00	0.20	0.20	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Heptagenia spp.	0.11	0.75	0.37	0.40	0.60	0.00	0.00	0.17	0.33	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.60
Hesperoperla pacifica	0.23	1.00	0.00	0.40	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.77
Hexagenia bilineata	0.25	0.75	0.50	0.40	0.60	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.83
Hexagenia sp	0.22	0.75	0.25	0.40	0.60	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.83
Hirudo nipponia	0.45	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.10
Hyalella azteca	0.02	0.00	0.75	0.00	1.00	0.00	0.00	0.60	0.20	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.60
Hydrophilus sp.(adult)	0.45	0.75	0.62	0.00	0.00	1.00	0.00	0.00	0.75	0.00	0.00	0.25	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.30	0.88
Hydrophilus triangularis (larva)	0.50	0.75	0.50	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	1.00	0.30	0.88
Hydropsyche californica	0.35	0.25	0.75	0.40	0.60	0.00	0.00	0.00	0.00	0.20	0.50	0.30	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.60
Hydropsyche sp.	0.20	0.25	0.75	0.40	0.60	0.00	0.00	0.00	0.00	0.20	0.50	0.30	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	1.00	0.60
Hygrotus sp. (adult)	0.04	0.75	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.30	0.88

Species	BL	LD	VL	RT_te	RT_gi	RT_pl	RT_ae	FH_df	FH_sh	FH_sc	FH_ff	FH_pr	FH_pa	OT_tc	OT_to	OT_hcy	OT_hgl	OT_nopg	AR	BS_li	BS_dvf	BS_lf	BS_se	RA	BE
Indoplanorbis exustus	0.15	0.00	0.50	0.00	0.00	0.00	1.00	0.20	0.20	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.27
Ischnura verticalis	0.23	0.65	0.75	0.25	0.75	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.60	0.77
Isogenus sp.	0.18	0.75	0.50	0.40	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.77
Isonychia sp.	0.16	0.25	0.75	0.40	0.60	0.00	0.00	0.00	0.00	0.00	0.50	0.50	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	1.00	0.00	1.00	0.88
Isoperla sp.	0.13	0.75	0.50	0.60	0.40	0.00	0.00	0.10	0.10	0.10	0.00	0.70	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.77
Laccophilus fasciatus (adult)	0.05	0.75	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.30	0.88
Laccophilus maculosus maculosus (adult)	0.05	0.75	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.30	0.88
Lampsilis cardium larvae	0.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Lampsillis siliquoidea	1.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Lampsillis siliquoidea larvae	0.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Lampsillis straminea claibornen	0.62	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Lampsillis subangulata	0.62	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Lanistes carinatus	0.40	0.75	0.75	0.00	0.50	0.00	0.50	0.15	0.00	0.80	0.05	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Lepidostoma unicolor	0.09	0.50	0.50	0.40	0.60	0.00	0.00	0.00	0.50	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.60
Leptodea fragilis larvae	0.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Leptodora kindtii	0.13	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.60	0.53
Lestes congener	0.27	0.65	0.62	0.33	0.67	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	0.80	0.77
Lestes sponsa	0.25	0.65	0.62	0.33	0.67	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	0.80	0.77
Ligumia subrostrata larvae	0.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Limnephilus bipunctatus	0.17	0.25	0.50	0.40	0.60	0.00	0.00	0.00	0.50	0.20	0.00	0.30	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.60
Limnephilus indivisus	0.20	0.25	0.50	0.40	0.60	0.00	0.00	0.00	0.50	0.20	0.00	0.30	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.60
Limnephilus lunatus	0.17	0.25	0.50	0.40	0.60	0.00	0.00	0.00	0.50	0.20	0.00	0.30	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.60
Limnephilus sp.	0.20	0.25	0.50	0.40	0.60	0.00	0.00	0.00	0.50	0.20	0.00	0.30	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.60
Limnodrilus hoffmeisteri	0.60	0.75	1.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.19
Lumbriculus variegatus	0.60	0.75	1.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.27
Lymnaea acuminata	0.26	0.65	0.70	0.00	0.00	0.00	1.00	0.00	0.24	0.73	0.00	0.03	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Lymnaea stagnalis	0.52	0.65	0.50	0.00	0.00	0.00	1.00	0.00	0.50	0.50	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Macrobrachium dayanum	0.53	1.00	0.50	0.00	1.00	0.00	0.00	0.20	0.30	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.77
Macrobrachium kistnensis	0.25	0.75	0.50	0.00	1.00	0.00	0.00	0.20	0.30	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.77
Macrobrachium lamarrei	0.63	1.00	0.50	0.00	1.00	0.00	0.00	0.20	0.30	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.77
Macrocyclops albidus	0.02	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.30	0.87
Megalonaias nervosa larvae	0.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Melanopsis dufouri	0.16	1.00	0.25	0.00	1.00	0.00	0.00	0.40	0.00	0.60	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Mesocyclops sp.	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.30	0.87
Metapenaeus monoceros	0.75	1.00	0.75	0.00	1.00	0.00	0.00	0.20	0.30	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.60
Moina macrocopa	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Moina micrura	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Moina sp.	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Mysis relicta	0.20	1.00	0.50	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.60	0.00	0.00	0.00	0.00	0.00	1.00	0.50	0.00	1.00	0.00	0.00	1.00	0.87
Neoplea striola	0.03	0.65	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.30	1.00
Notonecta undulata	0.14	0.65	0.75	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.80	0.00	0.20	0.00	0.50	0.00	0.00	0.00	1.00	0.30	1.00
Ophiogomphus rupinsulensis	0.30	1.00	0.75	0.25	0.75	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.60	0.77
Ophiogomphus_sp	0.30	1.00	0.75	0.25	0.75	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.60	0.77
Orconectes immunis	0.21	1.00	1.00	0.00	1.00	0.00	0.00	0.30	0.40	0.00	0.00	0.30	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	1.00	0.60
Orconectes nais	0.70	1.00	0.50	0.00	1.00	0.00	0.00	0.30	0.40	0.00	0.00	0.30	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	1.00	0.60
Orconectes nais juv	0.10	1.00	0.50	0.00	1.00	0.00	0.00	0.30	0.40	0.00	0.00	0.30	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	1.00	0.60
Orconectes propinquus	0.90	1.00	0.50	0.00	1.00	0.00	0.00	0.40	0.20	0.00	0.00	0.40	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	1.00	0.60

Species	BL	LD	VL	RT_te	RT_gi	RT_pl	RT_ae	FH_df	FH_sh	FH_sc	FH_ff	FH_pr	FH_pa	OT_tc	OT_to	OT_hcy	OT_hgl	OT_nopg	AR	BS_li	BS_dvf	BS_lf	BS_se	RA	BE
Orthetrum albistylum speciosum	0.22	1.00	0.25	0.25	0.75	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.60	0.77
Oziotelphusa senex senex	0.40	1.00	0.50	0.00	1.00	0.00	0.00	0.50	0.50	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.77
Palaemonetes argentinus	0.30	0.70	0.50	0.00	1.00	0.00	0.00	0.20	0.30	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.77
Palaemonetes kadiakensis	0.28	0.75	0.50	0.00	1.00	0.00	0.00	0.20	0.30	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.77
Paratelphusa jacquemontii	0.70	1.00	0.50	0.00	1.00	0.00	0.00	0.33	0.34	0.00	0.00	0.33	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.60
Paratelphusa masoniana	0.70	1.00	0.50	0.00	1.00	0.00	0.00	0.33	0.34	0.00	0.00	0.33	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.60
Paratya australianensis	0.17	1.00	1.00	0.00	1.00	0.00	0.00	0.40	0.10	0.40	0.10	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.60
Paratya compressa improvisa	0.11	0.50	1.00	0.00	1.00	0.00	0.00	0.40	0.10	0.40	0.10	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.60
Pecten yessoensis	0.35	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.05
Peltodytes sp.(adult)	0.04	0.75	0.87	0.25	0.38	0.00	0.38	0.00	0.50	0.00	0.00	0.50	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.30	0.88
Phasganophora sp.	0.17	0.75	0.25	0.40	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.77
Physella acuta	0.08	0.65	0.50	0.00	0.00	0.00	1.00	0.20	0.20	0.60	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Pila globosa	0.35	1.00	0.25	0.00	0.50	0.00	0.50	0.15	0.00	0.80	0.05	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Planorbis corneus	0.15	0.75	0.50	0.00	0.00	0.00	1.00	0.20	0.20	0.60	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Pomacea canaliculata	0.50	0.75	0.75	0.00	0.50	0.00	0.50	0.20	0.20	0.60	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Pomacea patula	0.40	0.75	0.75	0.00	0.50	0.00	0.50	0.20	0.20	0.60	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Pontoporeia hoyi	0.09	1.00	0.00	0.00	1.00	0.00	0.00	0.20	0.80	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.83
Proasellus coxalis	0.10	0.75	1.00	0.00	1.00	0.00	0.00	0.10	0.80	0.10	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.60
Procambarus acutus acutus	0.55	1.00	0.50	0.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.77
Procambarus clarkii	0.74	1.00	0.50	0.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.77
Procambarus simulans simulans	0.65	1.00	0.50	0.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.77
Procambarus sp.	0.65	1.00	0.50	0.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.77
Procladius sp.	0.09	0.00	0.50	1.00	0.00	0.00	0.00	0.40	0.00	0.00	0.00	0.60	0.00	1.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	1.00	0.00	0.77
Procloeon sp.	0.07	0.25	0.87	0.40	0.60	0.00	0.00	0.33	0.00	0.67	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.83
Pseudagrion sp.	0.20	0.65	0.50	0.25	0.75	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.80	0.77
Psorophora columbiae	0.08	0.00	1.00	0.00	0.00	0.00	1.00	0.20	0.00	0.00	0.20	0.60	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.87
Psychoglypha sp. Stage 1	0.10	0.00	0.50	0.40	0.60	0.00	0.00	0.50	0.50	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.60
Psychoglypha sp. Stage 2	0.16	0.00	0.50	0.40	0.60	0.00	0.00	0.50	0.50	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.60
Pteronarcella badia	0.18	1.00	0.25	0.40	0.60	0.00	0.00	0.00	0.80	0.10	0.00	0.10	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.60
Pteronarcis sp.	0.42	1.00	0.25	0.40	0.60	0.00	0.00	0.00	0.70	0.20	0.00	0.10	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.60
Pteronarcys californicus stage 1	0.33	1.00	0.25	0.40	0.60	0.00	0.00	0.00	0.70	0.20	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.60
Pteronarcys californicus stage 2	0.50	1.00	0.25	0.40	0.60	0.00	0.00	0.00	0.70	0.20	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.60
Pteronarcys dorsata	0.39	1.00	0.35	0.40	0.60	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.60
Pycnopsyche sp.	0.20	0.25	0.50	0.40	0.60	0.00	0.00	0.00	0.70	0.30	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	0.60	0.60
Ranatra elongata	0.40	0.00	0.65	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	0.60	0.77
Semisulcospira libertina	0.22	1.00	0.25	0.00	1.00	0.00	0.00	0.20	0.00	0.80	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Simocephalus serrulatus	0.04	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Simocephalus vetulus	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.30	0.53
Simulium latigonium	0.07	0.00	0.75	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.17
Simulium sp.	0.07	0.00	0.75	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.17
Simulium venustum	0.07	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.17
Simulium vittatum	0.07	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.17
Skwala sp.	0.25	0.75	0.50	0.40	0.60	0.00	0.00	0.00	0.00	0.10	0.00	0.90	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.77
Stenacron sp.	0.10	0.65	0.75	0.40	0.60	0.00	0.00	0.50	0.00	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	0.00	0.00	1.00	0.60
Streptocephalus proboscideus	0.25	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.53
Streptocephalus rubricaudatus	0.25	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.53
Streptocephalus sudanicus	0.25	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.53

Species	BL	LD	VL	RT_te	RT_gi	RT_pl	RT_ae	FH_df	FH_sh	FH_sc	FH_ff	FH_pr	FH_pa	OT_tc	OT_to	OT_hcy	OT_hgl	OT_nopg	AR	BS_li	BS_dvf	BS_lf	BS_se	RA	BE
Streptocephalus texanus	0.25	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.53
Tanypus grodhausi	0.11	0.00	1.00	1.00	0.00	0.00	0.00	0.30	0.00	0.00	0.00	0.70	0.00	1.00	0.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.77
Tanypus nubifer	0.11	0.00	1.00	1.00	0.00	0.00	0.00	0.30	0.00	0.00	0.00	0.70	0.00	1.00	0.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.77
Tanytarsus sp.	0.04	0.00	0.75	1.00	0.00	0.00	0.00	0.60	0.00	0.30	0.10	0.00	0.00	0.80	0.00	0.00	0.20	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.60
Tapes philippinarum	0.25	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Thermocyclops oblongatus	0.01	0.00	1.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.30	0.70
Thermonectus basillaris (adult)	0.10	0.75	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.30	0.88
Toxorhynchites splendens	0.05	0.00	1.00	0.00	0.00	0.00	1.00	0.20	0.00	0.00	0.00	0.80	0.00	0.00	1.00	0.00	0.00	0.00	0.25	1.00	0.00	0.00	0.00	0.00	0.87
Trichodactylus borellianus	0.07	1.00	0.50	0.00	1.00	0.00	0.00	0.33	0.34	0.00	0.00	0.33	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.60
Triops longicaudatus	0.50	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.60
Tropisternus lateralis (adult)	0.09	0.75	0.50	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.30	0.88
Tubifex tubifex	0.60	0.75	1.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.19
Unio elongatulus	1.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Utterbackia imbecilis	0.58	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Utterbackia imbecilis larvae	0.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Villosa lienosa	0.46	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Villosa lienosa larvae	0.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Villosa villosa	0.50	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.02
Villosa villosa larvae	0.00	1.00	0.50	0.80	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Viviparus bengalensis	0.25	1.00	0.75	0.00	1.00	0.00	0.00	0.00	0.00	0.70	0.30	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.27
Xanthocnemis zealandica	0.30	1.00	0.25	0.25	0.75	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	1.00	0.00	0.00	0.00	0.60	0.77

2. Trait database references

Note for the reader
Here follows a organisms vs. traits matrix. In each cell there are acronyms which indicate the references for the correspondent data.

No reference were collected for the body shape and degree of ramification, since they're only observational traits. Some references are missing for the "degree of sclerification" (armor), which is also pretty easy to evaluate.
Scores attributed for the behavioural complexity are shown in part 3 of this *Appendix*.

The list of the references follows the matrix and it is organized in three different sections, each one alphabetically ordered. The first one includes references retrieved in databases (ordered by first author's name), the second one comprehends all the papers and books (ordered by first author's name), while the last one collects all the websites (ordered by internet address).

Species	BL	LD	VL	RT	FH	OT	AR
Acroneuria sp	WS.bug	WDB.Hen.1999	P.Mer.1995	P.Tho.1933	WDB.Vie.2006; WS.wat	WS.res	WDB.Vie.2006
Aedes aegypti	WS.ent	WS.ent	WS.ent	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes albopictus	P.Cam.1999	WS.ent.ubo	WS.ent.ubo; P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes atropalpus	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes canadensis	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes cantans	P.Cam.1999	P.Ser.1977	P.Ser.1977	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes caspius	P.Cam.1999	WS.wik.mos	P.Wal.1980	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes communis	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes excrucians	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes hendersoni	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes nigromaculis	P.Cam.1999	WS.wik.mos	WDB.Hen.1999	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes punctor	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes sticticus	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes stimulans	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes taeniorhynchus	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes triseriatus	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes trivittatus	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Aedes vexans	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Alonella sp.	WS.gle	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Ter.2001	
Ameletus sp.	P.Pet.2001	WDB.Buf.2009	WDB.Buf.2009; WS.gun; WDB.Vie.2006	WDB.Buf.2009; P.Tho.1933	WDB.Vie.2006; WDB.AQE.2002; WS.ilm	WS.res	WDB.Vie.2006
Anisops sardeus	WDB.Tac.2002; P.Cam.1994	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WS.res	
Anodonta anatina	P.Var.1987	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	WDB.Nes.1995	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Anodonta anatina larvae	P.Bro.1968; P.Ken.2005; P.Lim.2006	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Ken.2005	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Anodonta cygnea	P.Var.1987	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	WDB.Nes.1995	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Anodonta cygnea larvae	P.Bro.1968; P.Ken.2005; P.Lim.2006	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Ken.2005	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Anodonta sp.	P.Ern.1989	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	WDB.Nes.1995	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Anopheles albimanus	WS.wik.ano	WS.wik.ano	WDB.Tac.2002	WS.res	WDB.Tac.2002	WS.res	WS.wat
Anopheles culicifacies	WS.wik.ano	WS.wik.ano	WDB.Tac.2002	WS.res	WDB.Tac.2002	WS.res	WS.wat
Anopheles freeborni	WS.wik.ano	WS.wik.ano	WDB.Tac.2002	WS.res	WDB.Tac.2002	WS.res	WS.wat
Anopheles gambiae	WS.wik.ano	WS.wik.ano	WDB.Tac.2002	WS.res	WDB.Tac.2002	WS.res	WS.wat
Anopheles quadrimaculatus	P.Kno.1943	WS.wik.ano	P.Cra.2004	WS.res	WDB.Tac.2002	WS.res	WS.wat
Anopheles stephensi	WS.wik.ano	WS.wik.ano	WDB.Tac.2002	WS.res	WDB.Tac.2002	WS.res	WS.wat
Aplexa hypnorum	P.Cam.1994	WDB.Hen.1999; P.Cam.1994	WDB.Hen.1999; WDB.Ani.2005	WDB.Hen.1999	WDB.Nes.1995	P.Lad.1991	
Arctopsyche grandis	P.Gau.1965	WDB.Hen.1999; WS.zeb	P.Mer.1995	WDB.Gra.2008	WDB.Hen.1999; WDB.Vie.2006; WS.zeb	P.Axp.2000	WDB.Vie.2006
Asellus aquaticus	P.Cam.1994	WS.wik.ase	WDB.Hen.1999	P.Wad.2004	WDB.Ede.1995	P.Wad.2004	

Species	BL	LD	VL	RT	FH	OT	AR
Asellus brevicaudus	WS.wik.ase	WDB.Hen.1999; WS.wik.ase	WDB.Hen.1999	P.Wad.2004	WDB.Ede.1995	P.Wad.2004	
Asellus hilgendorfi	P.Wil.1970	WDB.Hen.1999; WS.wik.ase	WDB.Hen.1999	P.Wad.2004	WDB.Ede.1995	P.Wad.2004	
Astacopsis gouldi	P.Dav.2005	WDB.Dep.2010	WDB.Dep.2010	P.Wad.2004	WDB.Dep.2010	P.Wad.2004	
Atherix sp.	WDB.Tac.2002	WDB.Tac.2002	WDB.Vie.2006	P.Kri.2005	WDB.Tac.2002	WS.res	WS.wat
Atherix variegata	WDB.Tac.2002	WDB.Tac.2002	WDB.Vie.2006	P.Kri.2005	WDB.Tac.2002	WS.res	WS.wat
Austrolestes colensonis	P.The.2006	WDB.Hen.1999	P.Cor.2006; WDB.hen.1999	WDB.Tac.2002; P.Tho.1933	P.Cru.1979	WS.res	
Baetis sp.	P.Cam.1994	WDB.Buf.2009	WDB.Buf.2009; P.Mer.1995	WDB.Buf.2009; P.Tho.1933	WDB.Vie.2006; WDB.Bau.2002	WS.res	WDB.Vie.2006
Barytelphusa cunicularis	P.Bah.2007	WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	WS.wik.cra; P.Yeo.2008	P.Wad.2004	
Bellamia bengalensis	WS.bio; P.Gho.2004	P.Gho.2004	WDB.Hen.1999	WS.che	WDB.Hen.1999; P.Wad.2004	P.Lad.1991	
Biomphalaria glabrata	P.Bel.1984	WS.wik.bio	WDB.Hen.1999	WDB.Hen.1999	WDB.Nes.1995	WS.wik.bio	
Biomphalaria havanensis	P.Tch.1991	WDB.Hen.1999	WDB.Hen.1999	WDB.Hen.1999	WDB.Nes.1995	WS.wik.bio	
Bosmina fatalis	WS.wik.bos	P.Wad.2004	P.Wad.2004	P.Wad.2004	WS.wik.bos	P.Wad.2004; P.Ter.2001	
Bosmina longirostris	WS.wik.bos; WDB.Ali.xxxx	P.Wad.2004	P.Wad.2004	P.Wad.2004	WS.wik.bos; WDB.Ali.xxxx	P.Wad.2004; P.Ter.2001	
Brachycentrus americanus	P.Pet.2001	WDB.Gra.2008	WDB.Gra.2008; WDB.Vie.2006	WDB.Gra.2008	WDB.Gra.2002	P.Axp.2000	WDB.Vie.2006
Branchiura sowerbyi	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	P.Cam.1994	WS.nas	WDB.Vie.2006	
Caenis horaria	P.Cam.1994	WDB.Buf.2009	WDB.Tac.2002	WDB.Buf.2009; P.Tho.1933	WDB.Tac.2002	WS.res	WDB.Vie.2006
Caenis miliaria	P.Cam.1994	WDB.Buf.2009	WDB.Tac.2002	WDB.Buf.2009; P.Tho.1933	WDB.Tac.2002	WS.res	WDB.Vie.2006
Calineuria californica	P.Pet.2001	WS.wes	WDB.Vie.2006	P.Tho.1933	WDB.Vie.2006	WS.res	WDB.Vie.2006
Caridina rajadhari	P.Nag.1994	WS.car; WS.cru; WS.pva	WDB.Hen.1999	P.Wad.2004	WS.wik.car	P.Wad.2004	
Ceriodaphnia dubia	WS.mbl	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004; P.Ter.2001	
Chaoborus obscuripes	P.Fed.1972	P.Pon.2009	WDB.Hen.1999	P.Cam.1999	WDB.Vie.2006; WDB.Ali.xxxx	WS.res	
Chaoborus punctipennis	P.Fed.1972	P.Pon.2009	WDB.Ali.xxxx; P.Mer.1995	P.Cam.1999	WDB.Vie.2006; WDB.Ali.xxxx	WS.res	
Chauliodes sp.	WS.wat	WS.wik.cor2	WDB.Vie.2006	P.Tho.1933	WDB.Vie.2006	WS.res	
Cheumatopsyche sp.	WDB.Vie.2006; WS.wat	WDB.Hen.1999; WDB.Gra.2008	P.Mer.1995	WDB.Gra.2008	WDB.Gra.2008	P.Axp.2000	WDB.Vie.2006
Chironomus crassicaudatus	P.Fro.2002	P.Fro.2002	WDB.Hen.1999	WS.res	WDB.Jan.2002; P.Ali.1990	WS.res; P.Arm.1995	WS.wat
Chironomus decorus	P.Kne.1988	P.Kne.1988	P.Utb.1982	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Chironomus plumosus	P.Mor.2010	P.Wat.2000	WDB.Vie.2006	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Chironomus riparius	P.Dom.2007; P.Per.2006; P.Wat.1998	P.Wat.1998; P.Wat.2000	P.Mer.1995; P.Ras.1984	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Chironomus salinarius	WS.wat; P.Per.2006; P.Wat.1998	P.Wat.2000	P.Dra.1995	WS.res	WDB.AQE.2002	WS.res; P.Arm.1995	WS.wat
Chironomus tentans	P.Wat.2000	P.Ben.1997; P.Wat.2000	P.Wru.1990	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Chironomus tepperi	P.Bai.2007	P.Wat.2000	WDB.Hen.1999	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Chironomus thummi	P.Bat.2001	P.Wat.2000	WDB.Hen.1999	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Chironomus utahensis	WS.wat	P.Wat.2000	WDB.Hen.1999	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Chironomus yoshimatsui	WS.wat	P.Wat.2000	WDB.Hen.1999	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Cinygma sp.	P.Pet.2001	P.Per.1982	WDB.Vie.2006; P.Per.1982	WDB.Buf.2009; P.Tho.1933	P.Per.1982	WS.res	WDB.Vie.2006

Species	BL	LD	VL	RT	FH	OT	AR
Cipangopaludina malleata	WDB.Vie.2006	WDB.Dil.2006	WDB.Vie.2006	WS.che	WDB.Dil.2006	P.Lad.1991	
Claassenia sabulosa	P.San.1965	WS.ent.ual	WS.gun	P.Tho.1933	WDB.Vie.2006; WS.gun	WS.res; P.Arm.1995	WDB.Vie.2006
Claassenia sp.	P.San.1965	WS.ent.ual	WS.gun	P.Tho.1933	WDB.Vie.2006; WS.gun	WS.res	WDB.Vie.2006
Cloeon dipterum	P.Dor.1980	WDB.Buf.2009	WDB.Buf.2009	WDB.Buf.2009; P.Tho.1933	WDB.Tac.2002; WDB.Bau.2002	WS.res	
Cloeon sp.	P.Cam.1994	WDB.Buf.2009	WDB.Buf.2009	WDB.Buf.2009; P.Tho.1933	WDB.Bau.2002	WS.res	
Corbicula manilensis	WS.wik.cor	WS.iss	P.Mou.2001	P.Bee.1998	WS.wik.cor	P.Wad.2004	
Cordulia aenea	P.Cam.1994	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002; P.Tho.1933	WDB.Tac.2002	WS.res	
Corixa punctata	WS.abs	P.Bai2.2007	WDB.Hen.1999	WS.res	WDB.AQE.2002	WS.res	WDB.Vie.2006
Cricotopus sp.	P.Bro.1960	WS.ent	P.Her.1987; P.Mer.1996	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Crocothemis erythraea	P.Cam.1994	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002; P.Tho.1933	WDB.Tac.2002	WS.res	
Culex fuscocephala	P.Cam.1999	WS.wik.mos	WDB.Hen.1999	WS.res	WDB.Car.1995	WS.res	WS.wat
Culex melanurus	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Culex peus	P.Cam.1999	WS.wik.mos	WDB.Hen.1999	WS.res	WDB.Car.1995	WS.res	WS.wat
Culex pipiens	P.Bra.2001	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Culex quinquefasciatus	P.Cam.1999	WS.wik.mos	WS.ent	WS.res	WDB.Car.1995	WS.res	WS.wat
Culex restuans	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Culex salinarius	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Culex tarsalis	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Culex tritaeniorhynchus	P.Cam.1999	WS.wik.mos	WDB.Hen.1999	WS.res	WDB.Car.1995	WS.res	WS.wat
Culicoides sp.	WS.ipm	WS.ani	P.Mer.1995	P.Mul.2009	WS.wat; P.Bec.2009; P.Mul.2009	P.Whi.1959	
Culicoides variipennis	WS.ipm	WS.ani	P.Mer.1995	P.Mul.2009	WS.wat; P.Bec.2009; P.Mul.2009	P.Whi.1959	
Culiseta annulata	P.Cam.1999	WS.wik.mos	WDB.Hen.1999	WS.res	WDB.Car.1995	WS.res	WS.wat
Culiseta incidens	P.Cam.1999	WS.wik.mos	P.Cra.2004	WS.res	WDB.Car.1995	WS.res	WS.wat
Culiseta logiareolata	P.Cam.1999	WS.wik.mos	WDB.Hen.1999	WS.res	WDB.Car.1995; P.Bla.1994	WS.res	WS.wat
Cypretta kawatai	P.Soh.1973	P.Smi.2001; P.Soh.1973	P.Smi.2001	P.Wad.2004	P.Soh.1973; P.Van.1998	P.Ter.2001	
Cypria sp.	P.Kar.2001	P.Smi.2001	P.Smi.2001	P.Wad.2004	P.Van.1998	P.Ter.2001	
Cypridopsis vidua	WS.drr	P.Fer.1944; P.Smi.2001	P.Smi.2001	P.Wad.2004	P.Roc.1993; P.Van.1998	P.Ter.2001	
Daphnia carinata	P.San.1976	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004; P.Ter.2001	
Daphnia cucullata	P.Sma.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004; P.Ter.2001	
Daphnia longispina	P.Ran.1993	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004; P.Ter.2001	
Daphnia magna	Personal Observation	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004; P.Ter.2001	
Daphnia obtusa	WS.cla	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004; P.Ter.2001	
Daphnia pulex	P.Ran.1993	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004; P.Ter.2001	
Diaptomus sp.	WS.cna	WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	P.Can.1928	P.Ter.2001	
Dicrotendipes californicus	P.Noc.1985	P.Wat.2000	P.Mer.1995	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat

Species	BL	LD	VL	RT	FH	OT	AR
Drunella grandis	WS.wes;	WDB.Buf.2009	P.Mer.1995; P.Rad.1989	WDB.Buf.2009; P.Tho.1933	P.Rad.1989	WS.res	WDB.Vie.2006
Dugesia tigrina	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WS.ani	WDB.Eur.???	WS.ani	
Echinogammarus tibaldii	WS.nlb	WDB.hen.1999	WDB.hen.1999	P.Wad.2004	WDB.Ede.1995	P.Wad.2004	
Elliptio icterina	P.Hea.1979	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Cam.1994	P.Bee.1998	
Enallagma sp.	P.Cam.1994	WDB.Hen.1999	WDB.Vie.2006; P.Mer.1995	WDB.Tac.2002; WDB.Vie.2006; P.Tho.1933	WDB.Tac.2002; WDB.Vie.2006	WS.res	WDB.Vie.2006
Ephemerella sp.	WDB.Vie.2006	WDB.Buf.2009	WDB.Buf.2009; P.Mer.1995	WDB.Buf.2009; P.Tho.1933	WDB.Tac.2002	WS.res	WDB.Vie.2006
Ephemerella subvaria	WS.win	WDB.Buf.2009	WDB.Buf.2009	WDB.Buf.2009; P.Tho.1933	WDB.Vie.2006; WDB.Bau.2002	WS.res	WDB.Vie.2006
Eretes stiticus	WS.bug	WDB.Hen.1999	WDB.Hen.1999	WS.res	WDB.Hen.1999; P.Kin.1985	WS.res	
Eriocheir sinensis	WS.iss	WS.iss	WDB.Hen.1999	P.Wad.2004	WDB.Sch.1996; Ws.iss	P.Wad.2004	
Erpobdella octoculata	WDB.Tac.2002; P.Cam.1994	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WS.acc	
Eucyclops sp.	WS.luc	WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	P.Mus.2008	P.Ter.2001	
Eudiaptomus graciloides	P.Cze.2002	WDB.Hen.1999	P.San.1998	P.Wad.2004	P.Wad.2004	P.Ter.2001	
Gammarus fasciatus	WDB.Vie.2006	WDB.Hen.1999; WDB.Tac.2002; WDB.Vie.2006	WDB.Hen.1999; WDB.Tac.2002; WDB.Vie.2006	P.Wad.2004	WDB.Tac.2002; WDB.Vie.2006	P.Wad.2004	WDB.Vie.2006
Gammarus fossarum	P.Gef.2010	WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	WDB.Ede.1995	P.Wad.2004	
Gammarus lacustris	WDB.Vie.2006; P.Ber.2009; P.Yem.2002	WDB.Hen.1999	WDB.Vie.2006	P.Wad.2004	WDB.Ede.1995	P.Wad.2004	WDB.Vie.2006
Gammarus palustris	P.Van.1978	WDB.Hen.1999	P.Gab.1977; P.Res.1975	P.Wad.2004	P.Gab.1977; P.Mac.1997	P.Wad.2004	
Gammarus pseudolimnaeus	WDB.Vie.2006; P.Rya.2010	WDB.Tac.2002	WDB.Tac.2002; WDB.Vie.2006	P.Wad.2004	WDB.Ede.1995	P.Wad.2004	WDB.Vie.2006
Gammarus pulex	P.Van.1993	WDB.Hen.1999; WDB.Tac.2002	WDB.Hen.1999; WDB.Tac.2002	P.Wad.2004	WDB.Ede.1995	P.Wad.2004	
Glossiphonia sp.	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WS.acc	
Glyptotendipes paripes	P.Fro.2002	P.Fro.2002	P.Mer.1995	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Goeldichironomus holoprasinus	P.Zil.2009	P.Zil.2009	P.Mer.1996	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Helisoma trivolvis	P.Tch.1991	WDB.Hen.1999	WDB.Hen.1999	WDB.Hen.1999	WDB.Nes.1995	WDB.Dil.2006	
Heptagenia spp.	P.Cam.1994	WDB.Tac.2002; P.Cli.1982	WDB.Tac.2002; P.Cli.1982	WDB.Buf.2009; P.Tho.1933	WDB.Tac.2002	WS.res	
Hesperoperla pacifica	P.Gau.1965; P.Jen.1964; P.Gau.1961	WS.gun	WS.gun	P.Tho.1933	WS.gun	WS.res	WDB.Vie.2006
Hexagenia bilineata	P.Fre.1973	P.Cli.1982	P.Cli.1982	P.Tho.1933	P.Der.1981	WS.res	WDB.Vie.2006
Hexagenia sp	WS.wat; P.Edm.1976	WS.bug; P.Cli.1982	P.Cli.1982	P.Tho.1933	P.Der.1981	WS.res	WDB.Vie.2006
Hirudo nipponia	P.Kim.1966	WDB.Vie.2006	WDB.Vie.2006	WS.web	WDB.Moo.1995	WS.web	
Hyalella azteca	P.Mcn.1999	WS.ele	P.Edw.1992; P.Fra.1993	P.Wad.2004	WS.fcp	P.Wad.2004	WDB.Vie.2006
Hydrophilus sp.(adult)	WDB.Tac.2002; P.Bro.1963	WDB.Tac.2002	WDB.Tac.2002	WS.res	WDB.Tac.2002	WS.res	WDB.Vie.2006
Hydrophilus triangularis (larva)	WS.dli	WDB.Hen.1999	WDB.Hen.1999; WS.dli	P.Mer.1995	WS.dli	P.Axp.2000	
Hydropsyche californica	P.Gau.1961	WDB.Hen.1999; WDB.Tac.2002; WDB.Gra.2008	WDB.Tac.2002; WDB.Vie.2006; WDB.Gra.2008; P.Mer.1995	WDB.Gra.2008	WDB.Gra.2008	P.Axp.2000	WDB.Vie.2006
Hydropsyche sp.	P.Cam.1999	WDB.Hen.1999; WDB.Tac.2002; WDB.Gra.2008	WDB.Tac.2002; WDB.Vie.2006; WDB.Gra.2008; P.Mer.1995	WDB.Gra.2008	WDB.Gra.2008	WS.res	WDB.Vie.2006
Hygrotus sp. (adult)	WS.wat	WDB.Hen.1999	WDB.Hen.1999	WS.res	WDB.Hen.1999; WS.cre	WS.res	WDB.Vie.2006
Indoplanorbis exustus	WS.wik.ind	WS.wik.ind	WS.wik.ind	WDB.Hen.1999	WDB.Nes.1995	WS.wik.bio	
Ischnura verticalis	P.Cam.1994	WDB.Hen.1999	P.Cam.1994	WDB.Vie.2006; P.Tho.1933	WDB.Vie.2006	WS.res	WDB.Vie.2006

Species	BL	LD	VL	RT	FH	OT	AR
Isogenus sp.	P.Cam.1994	WDB.Gra.2009	WDB.Gra.2009	P.Tho.1933	WDB.Gra.2002	WS.res	
Isonychia sp.	WS.wes	WDB.Buf.2009	WDB.Vie.2006; P.Mer.1995	P.Tho.1933	WDB.Vie.2006; WS.wat	WS.res	WDB.Vie.2006
Isoperla sp.	P.Cam.1994	WDB.Hen.1999	WDB.Hen.1999; WDB.Vie.2006; P.Mer.1995	P.Tho.1933	WDB.Vie.2006; WDB.Gra.2009; WS.gun	WS.res	WDB.Vie.2006
Laccophilus fasciatus (adult)	WS.wat	WDB.Hen.1999	WDB.Hen.1999	WS.res	WDB.Hen.1999; WS.cre	WS.res	
Laccophilus maculosus maculosus (adult)	WS.wat	WDB.Hen.1999	P.Mer.1995	WS.res	WDB.Hen.1999; WS.cre	WS.res	
Lampsilis cardium larvae	P.Ken.2005	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Ken.2005	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Lampsillis siliquoidea	WS.ani	WDB.Hen.1999; WS.ani	WDB.Hen.1999	P.Bee.1998	WS.ani; P.Bee.1998	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Lampsillis siliquoidea larvae	P.Ken.2005	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Ken.2005	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Lampsillis straminea claibornen	WS.aub	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Bee.1998	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Lampsillis subangulata	WS.jax	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Bee.1998	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Lanistes carinatus	WS.arn	WS.app	WS.app	WS.app	WS.app	P.Lad.1991	
Lepidostoma unicolor	P.Pet.2001	WS.ent.ual	WDB.Vie.2006	WDB.Gra.2008	WDB.Gra.2002; WDB.Vie.2006	P.Axp.2000	WDB.Vie.2006
Leptodea fragilis larvae	P.Ken.2005	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Ken.2005	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Leptodora kindtii	WS.gle	WDB.Hen.1999	WS.wik.lep	P.Wad.2004	WS.wik.lep	WS.wik.lep	
Lestes congener	P.Cam.1994	WDB.Hen.1999	WDB.Tac.2002	WDB.Tac.2002; P.Tho.1933	WDB.Tac.2002; WDB.Vie.2006	WS.res	WDB.Vie.2006
Lestes sponsa	WDB.Tac.2002	WDB.Hen.1999	WDB.Tac.2002	WDB.Tac.2002; P.Tho.1933	WDB.Tac.2002; WDB.Vie.2006	WS.res	WDB.Vie.2006
Ligumia subrostrata larvae	P.Ken.2005	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Ken.2005	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Limnephilus bipunctatus	P.Nor.1967	WDB.Hen.1999	WDB.Hen.1999	WDB.Gra.2008	WDB.Gra.2008	P.Axp.2000	WDB.Vie.2006
Limnephilus indivisus	WS.gun	WDB.Hen.1999	P.Mer.1995	WDB.Gra.2008	WDB.Gra.2008	P.Axp.2000	WDB.Vie.2006
Limnephilus lunatus	P.Nor.1967	WDB.Hen.1999	WDB.Gra.2008	WDB.Gra.2008	WDB.Gra.2008	P.Axp.2000	WDB.Vie.2006
Limnephilus sp.	P.Cam.1999	WDB.Hen.1999	WDB.Hen.1999; WDB.Tac.2002; WDB.Buf.2009; WDB.Gra.2008	WDB.Gra.2008	WDB.Gra.2008	P.Axp.2000	WDB.Vie.2006
Limnodrilus hoffmeisteri	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	P.Kas.1982	WDB.Hor.1996	WS.mar	
Lumbriculus variegatus	P.Cam.1994; WDB.Vie.2006; WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WS.eeo	WS.eeo	WS.eeo	
Lymnaea acuminata	P.Sin.1986	WDB.Hen.1999	WDB.Tac.2002	WDB.Hen.1999	WDB.Tac.2002	P.Lad.1991	
Lymnaea stagnalis	WS.ver	WDB.Hen.1999	WDB.Hen.1999	WDB.Hen.1999	WDB.Nes.1995	P.Lad.1991	WDB.Vie.2006
Macrobrachium dayanum	P.Omk.1985	WS.aqu2; WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	WDB.Vie.2006	P.Wad.2004	WDB.Vie.2006
Macrobrachium kistnensis	P.Kha.2009	WDB.Hen.1999; WDB.Vie.2006	WDB.Hen.1999	P.Wad.2004	WDB.Vie.2006	P.Wad.2004	
Macrobrachium lamarrei	P.Shu.1984	WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	WDB.Vie.2007	P.Wad.2005	WDB.Vie.2006
Macrocyclops albidus	WDB.Hud.2003	WDB.Hud.2003	WDB.Hen.1999; WDB.Hud.2003	P.Wad.2004	WDB.Hud.2003; P.Wad.2004	P.Ter.2001	
Megalonaias nervosa larvae	P.Kel.1997	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Tho.2001	P.Bee.1998	
Melanopsis dufouri	P.Alm.1988	WS.all	WDB.Hen.1999	WS.wik.mel	WS.all	P.Lad.1991	
Mesocyclops sp.	P.Sua.2005	WDB.Hen.1999	WDB.Hen.2000	P.Wad.2004	P.Sin.1999	P.Ter.2001	
Metapenaeus monoceros	P.Red.1992	WDB.Hen.1999; P.Din.2006	P.Yil.2009	P.Wad.2004	P.Col.1999	P.Wad.2004	
Moina macrocopa	P.Bur.1997; P.Rot.2003	P.Rot.2003	P.Rot.2003	P.Wad.2004	P.Wad.2004	P.Ter.2001	
Moina micrura	WS.gle	P.Rot.2003	P.Rot.2003	P.Wad.2004	P.Wad.2004	P.Ter.2001	

Species	BL	LD	VL	RT	FH	OT	AR
Moina sp.	P.Rot.2003	P.Rot.2003	P.Rot.2003	P.Wad.2004	P.Wad.2004	P.Ter.2001	
Mysis relicta	WS.wik.mys	WS.nas	WS.nas	P.Mak.1977	WS.nas; WDB.Vie.2006	WS.nas	
Neoplea striola	WS.bug; P.Cam.1994	WDB.Hen.1999	WDB.Hen.1999; P.Mer.1995	WS.wik.ple	WS.nat	WS.res	WDB.Vie.2006
Notonecta undulata	P.Cam.1994	WDB.Hen.1999; WDB.Vie.2006	P.Mer.1995	WS.res	WS.wik.not	WS.res	WDB.Vie.2006
Ophiogomphus rupinsulensis	P.Cam.1994	WDB.Vie.2006; WDB.Lee.2007	WDB.Hen.1999; WDB.Vie.2006	WDB.Vie.2006; P.Tho.1933	WDB.Vie.2006	WS.res	WDB.Vie.2006
Ophiogomphus_sp	P.Cam.1994	WDB.Vie.2006; WDB.Lee.2007	WDB.Hen.1999; WDB.Vie.2006	WDB.Vie.2006; P.Tho.1933	WDB.Vie.2006	WS.res	WDB.Vie.2006
Orconectes immunis	P.Bah.2005; P.Phi.1985	WS.pin	WDB.Hen.1999	P.Wad.2004	WDB.Vie.2006; WS.fie	P.Wad.2004	WDB.Vie.2006
Orconectes nais	WS.pin	WDB.Hen.1999; WS.pin	WDB.Hen.1999	P.Wad.2004	WDB.Vie.2006; WS.fie	P.Wad.2004	
Orconectes nais juv	WS.pin	WDB.Hen.1999; WS.pin	WDB.Hen.1999	P.Wad.2004	WDB.Vie.2006; WS.fie	P.Wad.2004	WDB.Vie.2006
Orconectes propinquus	WS.bki	WDB.Vie.2006; WS.pin	WDB.Hen.1999	P.Wad.2004	WDB.Vie.2006; WS.fie	P.Wad.2004	WDB.Vie.2006
Orthetrum albistylum speciosum	P.Cam.1994	WDB.Hen.1999	WDB.Hen.1999; P.Cam.1994	WDB.Tac.2002; P.Tho.1933	WDB.Jan.1995	WS.res	
Oziotelphusa senex senex	P.Bah.2007	WS.pan	WDB.Hen.1999	P.Wad.2004	WS.pan	P.Wad.2004	
Palaemonetes argentinus	P.Cha.1999	P.Spi.1977	WDB.Hen.1999	P.Wad.2004	P.Col.1999	P.Wad.2004	WDB.Vie.2006
Palaemonetes kadiakensis	P.Cha.1975	WS.chi	WDB.Hen.1999	P.Wad.2004	WS.chi; P.Col.1999	P.Wad.2004	WDB.Vie.2006
Paratelphusa jacquemontii	WS.san	WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	WS.wik.cra; P.Yeo.2008	P.Wad.2004	
Paratelphusa masoniana	P.Kau.1993	WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	WS.wik.cra; P.Yeo.2008	P.Wad.2004	
Paratya australianensis	P.Tho.2008	P.Han.1997	WS.aqu	P.Wad.2004	WS.wik.new; P.Pio.2008	P.Wad.2004	
Paratya compressa improvisa	P.Tad.2006	P.Tad.2006	P.Tad.2006	P.Wad.2004	WS.wik.new; P.Pio.2008	P.Wad.2004	
Pecten yessoensis	P.Nis.1977	WS.fao	WS.fao	P.Bee.1998	P.Wad.2004	P.Wad.2004	
Peltodytes sp.(adult)	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WDB.Tac.2002	WS.res	WDB.Vie.2006
Phasganophora sp.	WDB.Vie.2006	WDB.Hen.1999	WDB.Hen.1999; P.Mer.1995	P.Tho.1933	WDB.Vie.2006	WS.res	WDB.Vie.2006
Physella acuta	P.Muñ.2001	WDB.Hen.1999	WDB.Hen.1999; P.Lar.1992	WDB.Hen.1999	WDB.Nes.1995	P.Lad.1991	
Pila globosa	P.Sin.1981	WDB.Hen.1999	WDB.Hen.1999	WS.app	WS.app	P.Lad.1991	
Planorbis corneus	WS.ver	WDB.Hen.1999	WDB.Hen.1999	WDB.Hen.1999	WDB.Nes.1995; WS.ver	WS.ver	
Pomacea canaliculata	WS.app	WS.app	WS.app	WS.app	WS.wik.pom	P.Bar.1993	
Pomacea patula	P.Car.2003	WS.app	WS.app	WS.app	WS.wik.pom	P.Bar.1993	
Pontoporeia hoyi	WDB.Vie.2006	WDB.Vie.2006	WDB.Vie.2006	P.Wad.2004	WDB.Vie.2006	P.Wad.2004	WDB.Vie.2006
Proasellus coxalis	WS.nrm	WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	WDB.Sch.1996	P.Wad.2004	
Procambarus acutus acutus	P.Maz.2004	WDB.Hen.1999; WDB.Vie.2006	WDB.Hen.1999	P.Wad.2004	WDB.Tac.2002	P.Wad.2004	WDB.Vie.2006
Procambarus clarkii	P.Can.1999; P.Mor.2006	WDB.Hen.1999; WDB.Tac.2002; WDB.Vie.2006	WDB.Hen.1999; WDB.Tac.2002	P.Wad.2004	WDB.Tac.2002	P.Wad.2004	WDB.Vie.2006
Procambarus simulans simulans	P.Cha.1975	WDB.Hen.1999; WDB.Vie.2006	WDB.Hen.1999	P.Wad.2004	WDB.Tac.2002	P.Wad.2004	WDB.Vie.2006
Procambarus sp.	P.Can.1999; P.Mor.2006; P.Maz.2004; P.Cha.1975	WDB.Hen.1999; WDB.Vie.2006	WDB.Hen.1999	P.Wad.2004	WDB.Tac.2002	P.Wad.2004	WDB.Vie.2006
Procladius sp.	P.Bak.1979; P.Fer.1983	P.Wat.2000	P.Mer.1996	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Procloeon sp.	WDB.Vie.2006; P.Cam.1994	WDB.Buf.2009	WDB.Tac.2002	WDB.Buf.2009; P.Tho.1933	WDB.Tac.2002	WS.res	WDB.Vie.2006
Pseudagrion sp.	WS.bri	WDB.Hen.1999	P.Mer.1995	P.Tho.1933	WDB.Hen.1999; WS.bri	WS.res	

Species	BL	LD	VL	RT	FH	OT	AR
Psorophora columbiae	P.Cam.1994	WS.wik.mos	P.Cra.2004	WS.res	WDB.Vie.2006	WS.res	WS.wat
Psychoglypha sp. Stage 1	P.Pet.2001	WDB.Vie.2006	WDB.Vie.2006; WDB.Hen.1999	WDB.Gra.2008	WDB.Vie.2006	P.Axp.2000	WDB.Vie.2006
Psychoglypha sp. Stage 2	P.Pet.2001	WDB.Vie.2006	WDB.Vie.2006; WDB.Hen.1999	WDB.Gra.2008	WDB.Vie.2006	P.Axp.2000	WDB.Vie.2006
Pteronarcella badia	P.San.1965	WS.wik.pte	WDB.Vie.2006; WS.gun	P.Tho.1933	WDB.Vie.2006	WS.res	WDB.Vie.2006
Pteronarcis sp.	WDB.Vie.2006	WS.wik.pte	WDB.Vie.2006; P.Mer.1995	P.Tho.1933	WDB.Vie.2006	WS.res	WDB.Vie.2006
Pteronarcys californicus stage 1	P.San.1965	WDB.Vie.2006; WS.wik.pte; P.Tow.1998	P.Kru.1983; P.Mer.1995	P.Tho.1933	WDB.Vie.2006; P.Sho.1977	WS.res	WDB.Vie.2006
Pteronarcys californicus stage 2	P.Gau.1965	WDB.Vie.2006; WS.wik.pte; P.Tow.1998	P.Kru.1983; P.Mer.1995	P.Tho.1933	WDB.Vie.2006; P.Sho.1977	WS.res	WDB.Vie.2006
Pteronarcys dorsata	WDB.Vie.2006	WS.wik.pte	WDB.Vie.2006; P.Kru.1983; P.Mer.1995; P.Pes.1997	P.Tho.1933	WDB.Vie.2006	WS.res	WDB.Vie.2006
Pycnopsyche sp.	WDB.Vie.2006; WS.bug; WS.wat	WDB.Hen.1999	WDB.Hen.1999; WDB.Vie.2006; P.Mer.1995	WDB.Gra.2008	WDB.Vie.2006	P.Axp.2000	WDB.Vie.2006
Ranatra elongata	WDB.Vie.2006; P.Cam.1994	WDB.Vie.2006	WDB.Vie.2006; WDB.Hen.1999	WDB.Vie.2006; WDB.Hen.1999	WDB.Vie.2006; WDB.Hen.1999	WS.res	WDB.Vie.2006
Semisulcospira libertina	WS.con	WS.wik.sem	WDB.Hen.1999	WS.con	WDB.Hen.1999; P.Shi.2001	P.Wad.2004	
Simocephalus serrulatus	WS.cst	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Ter.2001	
Simocephalus vetulus	WS.drr	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Wad.2004	P.Ter.2001	
Simulium latigonium	P.Zha.1996	WS.the	WDB.Hen.1999	WDB.Vie.2006	WDB.Car.1995	WS.res	P.Mer.1995
Simulium sp.	WDB.Vie.2006	WS.the	WDB.Vie.2006	WDB.Vie.2006	WDB.Car.1995	WS.res	WS.wat; P.Mer.1995
Simulium venustum	WDB.Vie.2006	WS.the	WDB.Vie.2006	WDB.Vie.2006	WDB.Car.1995	WS.res	WS.wat; P.Mer.1995
Simulium vittatum	WDB.Vie.2006; WS.wat	WS.the	WDB.Vie.2006	WDB.Vie.2006	WDB.Car.1995	WS.res	P.Mer.1995
Skwala sp.	WDB.Vie.2006; WS.wes	WDB.Hen.1999	WDB.Hen.1999; WDB.Vie.2006	P.Tho.1933	WDB.Vie.2006	WS.res	
Stenacron sp.	P.Tan.1995	P.Cli.1982	WDB.Vie.2006; P.Cli.1982; P.Mer.1995	WDB.Buf.2009; P.Tho.1933	WDB.Vie.2006; WDB.Vie.2006	WS.res	WDB.Vie.2006
Streptocephalus proboscideus	WS.eco; WS.wik.sud	WDB.Hen.1999; WS.wik.sud	WDB.Hen.1999; WS.wik.sud	P.Wad.2004	WS.wik.sud	P.Ter.2001	
Streptocephalus rubricaudatus	WS.eco	WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	WS.wik.sud	P.Ter.2001	
Streptocephalus sudanicus	WS.eco	WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	WS.wik.sud	P.Ter.2001	
Streptocephalus texanus	WS.eco	WDB.Hen.1999	WDB.Hen.1999	P.Wad.2004	WS.wik.sud	P.Ter.2001	
Tanypus grodhausi	P.Fer.1983	P.Wat.2000	P.Mer.1996	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Tanypus nubifer	P.Fer.1983	P.Wat.2000	P.Mer.1996	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Tanytarsus sp.	P.Neb.1973	P.Wat.2000	WDB.Vie.2006; WDB.Jan.2002; P.Mer.1995; P.Mer.1996	WS.res	WDB.Jan.2002	WS.res; P.Arm.1995	WS.wat
Tapes philippinarum	P.Nis.1977	WS.wik.ven	WS.wik.ven	P.Bee.1998	P.Wad.2004	P.Wad.2004	
Thermocyclops oblongatus	P.Hop.1997; P.Mir.2001; P.Vel.1978	P.Hop.1997	P.Hop.1997	P.Wad.2004	P.Hop.1997	P.Ter.2001	
Thermonectus basillaris (adult)	WS.iss	WDB.Hen.1999	WDB.Hen.1999; P.Mer.1995	WS.res	WDB.Hen.1999; WS.cre	WS.res	
Toxorhynchites splendens	WS.rci	WS.wik.mos	WS.rci	WS.res	WS.wik.mos; WS.rci	WS.res	WS.wat
Trichodactylus borellianus	P.Mon.2008; P.Ver.2003	P.Pin.2005	WDB.Hen.1999	P.Wad.2004	P.Yeo.2008	P.Wad.2004	
Triops longicaudatus	WS.wik.tri	WS.wik.tri	WDB.Hen.1999	WS.wik.tri; P.Wad.2004	WS.wik.tri	WS.wik.tri	
Tropisternus lateralis (adult)	WS.bug	WDB.Hen.1999	WDB.Hen.1999	WS.res	WDB.Hen.1999; WS.cre	WS.res	
Tubifex tubifex	WDB.Vie.2006	WDB.Tac.2002	WDB.Tac.2002	WDB.Vie.2006	WDB.Hor.1995; WDB.Vie.2006	WDB.Vie.2006	
Unio elongatulus	P.Cam.1994	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Bee.1998	P.Bee.1998; P.Bon.1983; P.Tho.2001	

Species	BL	LD	VL	RT	FH	OT	AR
Utterbackia imbecilis	WS.inh; WS.jax	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Bee.1998	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Utterbackia imbecilis larvae	P.Kel.1997	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Ken.2005	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Villosa lienosa	WS.inh	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Bee.1998	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Villosa lienosa larvae	P.Kel.1997	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Ken.2005	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Villosa villosa	WS.aub	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Bee.1998	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Villosa villosa larvae	P.Kel.1997	WDB.Hen.1999	WDB.Hen.1999	P.Bee.1998	P.Ken.2005	P.Bee.1998; P.Bon.1983; P.Tho.2001	
Viviparus bengalensis	P.Gup.1981	P.Gho.2004	WDB.Hen.1999; WDB.Vie.2006	WS.che	WDB.Nes.1995	P.Lad.1991	
Xanthocnemis zealandica	P.Moo.1989	P.Har.1996	P.Cor.2006	P.Tho.1933	WDB.Hen.1999	WS.res	

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3. Resume of the scores attributed for the assessment of behavioural complexity

Note for the reader

Multiplicative coefficient per category are in brackets, see main text for further details

	Main movement					Main feeding habit				Predation avoidance			
Species	sessile (0)	crawler (0.3)	poor swimmer (0.6)	skilled swimmer (1)		passive consumer (0)	passive material (0.5)	active consumer on active material (1)		passive (0)	active (1)		total
Acroneuria sp		1			0.3			1	1		1	1	0.77
Aedes aegypti			1		0.6		1		0.5		1	1	0.70
Aedes albopictus			1		0.6		1		0.5		1	1	0.70
Aedes atropalpus			1		0.6		1		0.5		1	1	0.70
Aedes canadensis			1		0.6		1		0.5		1	1	0.70
Aedes cantans			1		0.6		1		0.5		1	1	0.70
Aedes caspius			1		0.6		1		0.5		1	1	0.70
Aedes communis			1		0.6		1		0.5		1	1	0.70
Aedes excrucians			1		0.6		1		0.5		1	1	0.70
Aedes hendersoni			1		0.6		1		0.5		1	1	0.70
Aedes nigromaculis			1		0.6		1		0.5		1	1	0.70
Aedes punctor			1		0.6		1		0.5		1	1	0.70
Aedes sticticus			1		0.6		1		0.5		1	1	0.70
Aedes stimulans			1		0.6		1		0.5		1	1	0.70
Aedes taeniorhynchus			1		0.6		1		0.5		1	1	0.70
Aedes triseriatus			1		0.6		1		0.5		1	1	0.70
Aedes trivittatus			1		0.6		1		0.5		1	1	0.70
Aedes vexans			1		0.6		1		0.5		1	1	0.70
Alonella sp.			1		0.6	1			0		1	1	0.53
Ameletus sp.		0.5		0.5	0.7		1		0.5		1	1	0.72
Anisops sardeus				1	1			1	1		1	1	1.00
Anodonta anatina	0.8	0.2			0.1	1			0	1		0	0.02
Anodonta anatina larvae	1				0	1			0	1		0	0.00
Anodonta cygnea	0.8	0.2			0.1	1			0	1		0	0.02
Anodonta cygnea larvae	1				0	1			0	1		0	0.00
Anodonta sp.	0.8	0.2			0.1	1			0	1		0	0.02
Anopheles albimanus			1		0.6			1	1		1	1	0.87
Anopheles culicifacies			1		0.6			1	1		1	1	0.87
Anopheles freeborni			1		0.6			1	1		1	1	0.87
Anopheles gambiae			1		0.6			1	1		1	1	0.87
Anopheles quadrimaculatus			1		0.6			1	1		1	1	0.87

	Main movement					Main feeding habit				Predation avoidance			
Species	sessile (0)	crawler (0.3)	poor swimmer (0.6)	skilled swimmer (1)		passive consumer (0)	passive material (0.5)	active consumer on active material (1)		passive (0)	active (1)		total
Anopheles stephensi			1		0.6			1	1		1	1	0.87
Aplexa hypnorum		1			0.3		1		0.5	1		0	0.27
Arctopsyche grandis		1			0.3			1	1		1	1	0.77
Asellus aquaticus		1			0.3		1		0.5		1	1	0.60
Asellus brevicaudus		1			0.3		1		0.5		1	1	0.60
Asellus hilgendorfi		1			0.3		1		0.5		1	1	0.60
Astacopsis gouldi		1			0.3		1		0.5		1	1	0.60
Atherix sp.		1			0.3			1	1		1	1	0.77
Atherix variegata		1			0.3			1	1		1	1	0.77
Austrolestes colensonis		1			0.3			1	1		1	1	0.77
Baetis sp.				1	1		1		0.5		1	1	0.83
Barytelphusa cunicularis		1			0.3		1		0.5		1	1	0.60
Bellamia bengalensis		1			0.3		1		0.5	1		0	0.27
Biomphalaria glabrata		1			0.3		1		0.5	1		0	0.27
Biomphalaria havanensis		1			0.3		1		0.5	1		0	0.27
Bosmina fatalis			1		0.6	1			0		1	1	0.53
Bosmina longirostris			1		0.6	1			0		1	1	0.53
Brachycentrus americanus		1			0.3		1		0.5		1	1	0.60
Branchiura sowerbyi	0.8	0.2			0.1		1		0.5	1			0.19
Caenis horaria		1			0.3		1		0.5		1	1	0.60
Caenis miliaria		1			0.3		1		0.5		1	1	0.60
Calineuria californica		1			0.3			1	1		1	1	0.77
Caridina rajadhari		1			0.3		1		0.5		1	1	0.60
Ceriodaphnia dubia			1		0.6	1			0		1	1	0.53
Chaoborus obscuripes			1		0.6			1	1		1	1	0.87
Chaoborus punctipennis			1		0.6			1	1		1	1	0.87
Chauliodes sp.		1			0.3			1	1		1	1	0.77
Cheumatopsyche sp.		1			0.3		1		0.5		1	1	0.60
Chironomus crassicaudatus		1			0.3		1		0.5		1	1	0.60
Chironomus decorus		1			0.3		1		0.5		1	1	0.60
Chironomus plumosus		1			0.3		1		0.5		1	1	0.60

	Main movement					Main feeding habit				Predation avoidance			
Species	sessile (0)	crawler (0.3)	poor swimmer (0.6)	skilled swimmer (1)		passive consumer (0)	passive material (0.5)	active consumer on active material (1)		passive (0)	active (1)		total
Chironomus riparius		1			0.3		1		0.5		1	1	0.60
Chironomus salinarius		1			0.3		1		0.5		1	1	0.60
Chironomus tentans		1			0.3		1		0.5		1	1	0.60
Chironomus tepperi		1			0.3		1		0.5		1	1	0.60
Chironomus thummi		1			0.3		1		0.5		1	1	0.60
Chironomus utahensis		1			0.3		1		0.5		1	1	0.60
Chironomus yoshimatsui		1			0.3		1		0.5		1	1	0.60
Cinygma sp.		1			0.3		1		0.5		1	1	0.60
Cipangopaludina malleata		1			0.3		1		0.5	1		0	0.27
Claassenia sabulosa		1			0.3			1	1		1	1	0.77
Claassenia sp.		1			0.3			1	1		1	1	0.77
Cloeon dipterum				1	1		1		0.5		1	1	0.83
Cloeon sp.				1	1		1		0.5		1	1	0.83
Corbicula manilensis	0.8	0.2			0.1	1			0	1		0	0.02
Cordulia aenea		1			0.3			1	1		1	1	0.77
Corixa punctata				1	1		1		0.5		1	1	0.83
Cricotopus sp.		1			0.3		1		0.5		1	1	0.60
Crocothemis erythraea		1			0.3			1	1		1	1	0.77
Culex fuscocephala			1		0.6		1		0.5		1	1	0.70
Culex melanurus			1		0.6		1		0.5		1	1	0.70
Culex peus			1		0.6		1		0.5		1	1	0.70
Culex pipiens			1		0.6		1		0.5		1	1	0.70
Culex quinquefasciatus			1		0.6		1		0.5		1	1	0.70
Culex restuans			1		0.6		1		0.5		1	1	0.70
Culex salinarius			1		0.6		1		0.5		1	1	0.70
Culex tarsalis			1		0.6		1		0.5		1	1	0.70
Culex tritaeniorhynchus			1		0.6		1		0.5		1	1	0.70
Culicoides sp.		1			0.3			1	1		1	1	0.77
Culicoides variipennis		1			0.3			1	1		1	1	0.77
Culiseta annulata			1		0.6		1		0.5		1	1	0.70
Culiseta incidens			1		0.6		1		0.5		1	1	0.70

	Main movement					Main feeding habit					Predation avoidance			
Species	sessile (0)	crawler (0.3)	poor swimmer (0.6)	skilled swimmer (1)		passive consumer (0)	passive material (0.5)	active consumer on active material (1)			passive (0)	active (1)		total
Culiseta logiareolata			1		0.6		1		0.5			1	1	0.70
Cypretta kawatai				1	1		1		0.5			1	1	0.83
Cypria sp.				1	1		1		0.5			1	1	0.83
Cypridopsis vidua				1	1		1		0.5			1	1	0.83
Daphnia carinata			1		0.6	1			0			1	1	0.53
Daphnia cucullata			1		0.6	1			0			1	1	0.53
Daphnia longispina			1		0.6	1			0			1	1	0.53
Daphnia magna			1		0.6	1			0			1	1	0.53
Daphnia obtusa			1		0.6	1			0			1	1	0.53
Daphnia pulex			1		0.6	1			0			1	1	0.53
Diaptomus sp.			1		0.6	1			0			1	1	0.53
Dicrotendipes californicus		1			0.3		1		0.5			1	1	0.60
Drunella grandis		1			0.3		1		0.5			1	1	0.60
Dugesia tigrina		1			0.3			1	1			1		0.43
Echinogammarus tibaldii				1	1		1		0.5			1	1	0.83
Elliptio icterina	0.8	0.2			0.1	1			0		1		0	0.02
Enallagma sp.		1			0.3			1	1			1	1	0.77
Ephemerella sp.		1			0.3		1		0.5			1	1	0.60
Ephemerella subvaria		1			0.3		1		0.5			1	1	0.60
Eretes stiticus		0.5		0.5	0.7			1	1			1	1	0.88
Eriocheir sinensis		1			0.3			1	1			1	1	0.77
Erpobdella octoculata		1			0.3			1	1		0.8	0.2		0.43
Eucyclops sp.			1		0.6	1			0			1	1	0.53
Eudiaptomus graciloides			1		0.6	1			0			1	1	0.53
Gammarus fasciatus				1	1		1		0.5			1	1	0.83
Gammarus fossarum				1	1		1		0.5			1	1	0.83
Gammarus lacustris				1	1		1		0.5			1	1	0.83
Gammarus palustris				1	1		1		0.5			1	1	0.83
Gammarus pseudolimnaeus				1	1		1		0.5			1	1	0.83
Gammarus pulex				1	1		1		0.5			1	1	0.83
Glossiphonia sp.		1			0.3	0.25		0.75	0.75		0.8	0.2		0.35

	Main movement					Main feeding habit				Predation avoidance			
Species	sessile (0)	crawler (0.3)	poor swimmer (0.6)	skilled swimmer (1)		passive consumer (0)	passive material (0.5)	active consumer on active material (1)		passive (0)	active (1)		total
Glyptotendipes paripes		1			0.3		1		0.5		1	1	0.60
Goeldichironomus holoprasinus		1			0.3		1		0.5		1	1	0.60
Helisoma trivolvis		1			0.3		1		0.5	1		0	0.27
Heptagenia spp.		1			0.3		1		0.5		1	1	0.60
Hesperoperla pacifica		1			0.3			1	1		1	1	0.77
Hexagenia bilineata				1	1		1		0.5		1	1	0.83
Hexagenia sp				1	1		1		0.5		1	1	0.83
Hirudo nipponia		1			0.3	1			0	0.8	0.2		0.10
Hyalella azteca		1			0.3		1		0.5		1	1	0.60
Hydrophilus sp.(adult)		0.5		0.5	0.7			1	1		1	1	0.88
Hydrophilus triangularis (larva)		0.5		0.5	0.7			1	1		1	1	0.88
Hydropsyche californica		1			0.3		1		0.5		1	1	0.60
Hydropsyche sp.		1			0.3		1		0.5		1	1	0.60
Hygrotus sp. (adult)		0.5		0.5	0.7			1	1		1	1	0.88
Indoplanorbis exustus		1			0.3		1		0.5	1		0	0.27
Ischnura verticalis		1			0.3			1	1		1	1	0.77
Isogenus sp.		1			0.3			1	1		1	1	0.77
Isonychia sp.		0.5		0.5	0.7			1	1		1	1	0.88
Isoperla sp.		1			0.3			1	1		1	1	0.77
Laccophilus fasciatus (adult)		0.5		0.5	0.7			1	1		1	1	0.88
Laccophilus maculosus maculosus (adult)		0.5		0.5	0.7			1	1		1	1	0.88
Lampsilis cardium larvae	1				0	1			0	1		0	0.00
Lampsillis siliquoidea	0.8	0.2			0.1	1			0	1		0	0.02
Lampsillis siliquoidea larvae	1				0	1			0	1		0	0.00
Lampsillis straminea claibornen	0.8	0.2			0.1	1			0	1		0	0.02
Lampsillis subangulata	0.8	0.2			0.1	1			0	1		0	0.02
Lanistes carinatus		1			0.3		1		0.5	1		0	0.27
Lepidostoma unicolor		1			0.3		1		0.5		1	1	0.60
Leptodea fragilis larvae	1				0	1			0	1		0	0.00
Leptodora kindtii			1		0.6	1			0		1	1	0.53
Lestes congener		1			0.3			1	1		1	1	0.77

	Main movement					Main feeding habit				Predation avoidance			
Species	sessile (0)	crawler (0.3)	poor swimmer (0.6)	skilled swimmer (1)		passive consumer (0)	passive material (0.5)	active consumer on active material (1)		passive (0)	active (1)		total
Lestes sponsa		1			0.3			1	1		1	1	0.77
Ligumia subrostrata larvae	1				0	1			0	1		0	0.00
Limnephilus bipunctatus		1			0.3		1		0.5		1	1	0.60
Limnephilus indivisus		1			0.3		1		0.5		1	1	0.60
Limnephilus lunatus		1			0.3		1		0.5		1	1	0.60
Limnephilus sp.		1			0.3		1		0.5		1	1	0.60
Limnodrilus hoffmeisteri	0.8	0.2			0.1		1		0.5	1			0.19
Lumbriculus variegatus		1			0.3		1		0.5	1			0.27
Lymnaea acuminata		1			0.3		1		0.5	1		0	0.27
Lymnaea stagnalis		1			0.3		1		0.5	1		0	0.27
Macrobrachium dayanum		1			0.3			1	1		1	1	0.77
Macrobrachium kistnensis		1			0.3			1	1		1	1	0.77
Macrobrachium lamarrei		1			0.3			1	1		1	1	0.77
Macrocyclops albidus			1		0.6			1	1		1	1	0.87
Megalonaias nervosa larvae	1				0	1			0	1		0	0.00
Melanopsis dufouri		1			0.3		1		0.5	1		0	0.27
Mesocyclops sp.			1		0.6			1	1		1	1	0.87
Metapenaeus monoceros		1			0.3		1		0.5		1	1	0.60
Moina macrocopa			1		0.6	1			0		1	1	0.53
Moina micrura			1		0.6	1			0		1	1	0.53
Moina sp.			1		0.6	1			0		1	1	0.53
Mysis relicta			1		0.6			1	1		1	1	0.87
Neoplea striola				1	1			1	1		1	1	1.00
Notonecta undulata				1	1			1	1		1	1	1.00
Ophiogomphus rupinsulensis		1			0.3			1	1		1	1	0.77
Ophiogomphus_sp		1			0.3			1	1		1	1	0.77
Orconectes immunis		1			0.3		1		0.5		1	1	0.60
Orconectes nais		1			0.3		1		0.5		1	1	0.60
Orconectes nais juv		1			0.3		1		0.5		1	1	0.60
Orconectes propinquus		1			0.3		1		0.5		1	1	0.60
Orthetrum albistylum speciosum		1			0.3			1	1		1	1	0.77

	Main movement					Main feeding habit				Predation avoidance			
Species	sessile (0)	crawler (0.3)	poor swimmer (0.6)	skilled swimmer (1)		passive consumer (0)	passive material (0.5)	active consumer on active material (1)		passive (0)	active (1)		total
Oziotelphusa senex senex		1			0.3			1	1		1	1	0.77
Palaemonetes argentinus		1			0.3			1	1		1	1	0.77
Palaemonetes kadiakensis		1			0.3			1	1		1	1	0.77
Paratelphusa jacquemontii		1			0.3		1		0.5		1	1	0.60
Paratelphusa masoniana		1			0.3		1		0.5		1	1	0.60
Paratya australianensis		1			0.3		1		0.5		1	1	0.60
Paratya compressa improvisa		1			0.3		1		0.5		1	1	0.60
Pecten yessoensis	0.5	0.5			0.2	1			0	1		0	0.05
Peltodytes sp.(adult)		0.5		0.5	0.7			1	1		1	1	0.88
Phasganophora sp.		1			0.3			1	1		1	1	0.77
Physella acuta		1			0.3		1		0.5	1		0	0.27
Pila globosa		1			0.3		1		0.5	1		0	0.27
Planorbis corneus		1			0.3		1		0.5	1		0	0.27
Pomacea canaliculata		1			0.3		1		0.5	1		0	0.27
Pomacea patula		1			0.3		1		0.5	1		0	0.27
Pontoporeia hoyi				1	1		1		0.5		1	1	0.83
Proasellus coxalis		1			0.3		1		0.5		1	1	0.60
Procambarus acutus acutus		1			0.3			1	1		1	1	0.77
Procambarus clarkii		1			0.3			1	1		1	1	0.77
Procambarus simulans simulans		1			0.3			1	1		1	1	0.77
Procambarus sp.		1			0.3			1	1		1	1	0.77
Procladius sp.		1			0.3			1	1		1	1	0.77
Procloeon sp.				1	1		1		0.5		1	1	0.83
Pseudagrion sp.		1			0.3			1	1		1	1	0.77
Psorophora columbiae			1		0.6			1	1		1	1	0.87
Psychoglypha sp. Stage 1		1			0.3		1		0.5		1	1	0.60
Psychoglypha sp. Stage 2		1			0.3		1		0.5		1	1	0.60
Pteronarcella badia		1			0.3		1		0.5		1	1	0.60
Pteronarcis sp.		1			0.3		1		0.5		1	1	0.60
Pteronarcys californicus stage 1		1			0.3		1		0.5		1	1	0.60
Pteronarcys californicus stage 2		1			0.3		1		0.5		1	1	0.60

	Main movement					Main feeding habit				Predation avoidance			
Species	sessile (0)	crawler (0.3)	poor swimmer (0.6)	skilled swimmer (1)		passive consumer (0)	active consumer on passive material (0.5)	active consumer on active material (1)		passive (0)	active (1)		total
Pteronarcys dorsata		1			0.3		1		0.5		1	1	0.60
Pycnopsyche sp.		1			0.3		1		0.5		1	1	0.60
Ranatra elongata		1			0.3			1	1		1	1	0.77
Semisulcospira libertina		1			0.3		1		0.5	1		0	0.27
Simocephalus serrulatus			1		0.6	1			0		1	1	0.53
Simocephalus vetulus			1		0.6	1			0		1	1	0.53
Simulium latigonium	1				0		1		0.5	1		0	0.17
Simulium sp.	1				0		1		0.5	1		0	0.17
Simulium venustum	1				0		1		0.5	1		0	0.17
Simulium vittatum	1				0		1		0.5	1		0	0.17
Skwala sp.		1			0.3			1	1		1	1	0.77
Stenacron sp.		1			0.3		1		0.5		1	1	0.60
Streptocephalus proboscideus			1		0.6	1			0		1	1	0.53
Streptocephalus rubricaudatus			1		0.6	1			0		1	1	0.53
Streptocephalus sudanicus			1		0.6	1			0		1	1	0.53
Streptocephalus texanus			1		0.6	1			0		1	1	0.53
Tanypus grodhausi		1			0.3			1	1		1	1	0.77
Tanypus nubifer		1			0.3			1	1		1	1	0.77
Tanytarsus sp.		1			0.3		1		0.5		1	1	0.60
Tapes philippinarum	0.8	0.2			0.1	1			0	1		0	0.02
Thermocyclops oblongatus			1		0.6	1	1		0.5		1	1	0.70
Thermonectus basillaris (adult)		0.5		0.5	0.7			1	1		1	1	0.88
Toxorhynchites splendens			1		0.6			1	1		1	1	0.87
Trichodactylus borellianus		1			0.3		1		0.5		1	1	0.60
Triops longicaudatus		1			0.3		1		0.5		1	1	0.60
Tropisternus lateralis (adult)		0.5		0.5	0.7			1	1		1	1	0.88
Tubifex tubifex	0.8	0.2			0.1		1		0.5	1			0.19
Unio elongatulus	0.8	0.2			0.1	1			0	1		0	0.02

	Main movement				Main feeding habit				Predation avoidance			
Species	sessile (0)	crawler (0.3)	poor swimmer (0.6)	skilled swimmer (1)	passive consumer (0)	active consumer on passive material (0.5)	active consumer on active material (1)		passive (0)	active (1)		total
Utterbackia imbecilis	0.8	0.2		0.1	1			0	1		0	0.02
Utterbackia imbecilis larvae	1			0	1			0	1		0	0.00
Villosa lienosa	0.8	0.2		0.1	1			0	1		0	0.02
Villosa lienosa larvae	1			0	1			0	1		0	0.00
Villosa villosa	0.8	0.2		0.1	1			0	1		0	0.02
Villosa villosa larvae	1			0	1			0	1		0	0.00
Viviparus bengalensis		1		0.3		1		0.5	1		0	0.27
Xanthocnemis zealandica		1		0.3			1	1		1	1	0.77

4. Complete report of regression models

Note for the reader
Complete reports of the selected regression models (variables used, evaluation of predictive capacity, fitting and boostrap validation). See text for further details.

Carbaryl Regression Models

Carbaryl_species

Retained variables: 8
Final selected models (14)

Size	Models	R2	R2adj	Q2	Q2boot
5	BL BS_li BS_se FH_ff RA	71.82	69.99	67.52	66.5
5	BL BS_lf BS_se FH_ff RA	71.69	69.85	67.21	66.04
5	AR BL BS_lf BS_se RA	71.81	69.97	66.65	65.29
4	BL BS_se FH_ff RA	69.82	68.27	66.24	65.63
4	BL BS_lf BS_se RA	67.81	66.16	63.36	62.27
4	BL BS_se FH_df RA	67.58	65.92	63.24	62.27
3	BL BS_se RA	64.3	62.94	60.72	60.33
3	BL BS_li BS_se	64.05	62.68	60.21	59.84
3	BL BS_lf BS_se	61.96	60.52	57.82	57.51
2	BL BS_se	57.64	56.58	54.45	54.69
2	BS_li BS_se	56.13	55.03	53.15	53.36
2	BS_lf BS_se	55.52	54.41	52.24	52.31
1	BS_se	50.54	49.93	48.54	49.53
1	BS_lf	26.51	25.6	23.03	24.15

Carbaryl_genus

Retained variables: 10
Final selected models (15)

Size	Models	R2	R2adj	Q2	Q2boot
5	BL BS_se FH_df RA VL	74.49	72.21	69.36	68.04
5	BL BS_se FH_df FH_pa RA	73.8	71.47	69.05	67.57
5	BL BS_se FH_df FH_ff RA	73.91	71.58	69.02	67.59
4	BL BS_se FH_ff RA	72.4	70.46	68.41	67.81
4	BL BS_se FH_df RA	71.31	69.3	66.72	66.03
4	BS_se FH_df LD RA	70.18	68.09	65.35	64.25
3	BL BS_se RA	67.32	65.63	63.18	62.76
3	BS_se FH_ff RA	65.55	63.77	60.97	60.25
3	BS_li BS_se FH_df	65.43	63.64	60.94	60.27
2	BS_li BS_se	60.34	59	56.55	56.73
2	BS_se FH_df	60.35	59.01	56.41	56.78
2	BL BS_se	60.22	58.87	56.26	56.32
1	BS_se	54.62	53.87	52	52.93
1	RA	36.93	35.88	33.27	34.49
1	BE	28.04	26.84	24.02	25.73

Chlorpyrifos Regression Models

Chlorpyrifos_species

Retained variables: 10
Final selected models (13)

Size	Models	R2	R2adj	Q2	Q2boot
5	BL BS_lf BS_li OT_hcy RT_gi	64.06	62.04	56.94	54.51
5	BL BS_lf BS_se OT_hcy OT_to	63.37	61.31	56.45	53.84
5	BL BS_lf LD OT_hcy OT_hgl	62.59	60.49	55.45	52.13
4	BL BS_lf BS_li OT_hcy	60.19	58.42	54.48	52.29
4	BL BS_lf BS_se OT_hcy	60.37	58.61	54.07	51.78
4	BL BS_lf BS_li LD	57.92	56.05	53.12	52.16
3	BL BS_lf LD	56.16	54.72	52.29	51.45
3	BL BS_lf BS_li	55.25	53.77	51.21	50.47
3	BL BS_lf OT_hcy	55.07	53.59	49.7	47.75
2	BL LD	49.35	48.25	45.83	45.13
2	BL BS_se	50.67	49.6	45.75	45.16
2	BL FH_ff	47.96	46.83	45.08	44.82
1	BL	42.23	41.61	39.45	39.67

Chlorpyrifos_genus

Retained variables: 9
Final selected models (14)

Size	Models	R2	R2adj	Q2	Q2boot
5	BL BS_lf OT_hcy RT_gi VL	71.8	69.03	63.16	59.44
5	BL BS_lf LD OT_hcy RT_gi	71.27	68.45	62.49	59.28
5	BL BS_lf BS_li OT_hcy RT_gi	69.45	66.45	60.2	57.08
4	BL BS_lf OT_hcy VL	67.33	64.81	58.35	54.61
4	BL BS_lf OT_hcy RT_gi	66.41	63.82	58.07	55.61
4	BL BS_lf BS_se OT_hcy	66.3	63.71	57.66	54.64
3	BL BS_lf OT_hcy	62.73	60.62	55.35	52.7
3	BL BS_lf LD	59.92	57.65	54.38	53.27
3	BL LD RT_ae	57.36	54.94	50.27	48.79
2	BL LD	53.16	51.43	48.17	47.7
2	BL BS_lf	50.1	48.25	45.62	45.6
2	BL VL	49.26	47.38	42.82	41.96
1	BL	42.83	41.79	38.64	38.59
1	LD	35.21	34.03	30.23	31.69

Chlorpyrifos_family

Retained variables: 9
Final selected models (13)

Size	Models	R2	R2adj	Q2	Q2boot
5	BL BS_lf OT_hcy RT_gi VL	82.45	79.53	69.18	65.29
5	BL BS_lf LD OT_hcy RT_gi	80.28	76.99	65.86	61.29
5	AR BL BS_lf OT_hcy VL	80.88	77.69	65.58	60.35
4	BL BS_lf OT_hcy RT_gi	78.14	75.32	65.06	61.83
4	BL BS_lf OT_hcy RT_ae	77.72	74.85	63.89	60.29
4	BL BS_lf BS_se OT_hcy	77.26	74.33	63.29	59.59
3	BL BS_lf OT_hcy	74.01	71.57	60.47	57.88
3	BL BS_lf LD	62.86	59.37	54	52.51
3	BL LD RT_ae	63.4	59.97	50.84	47.97
2	BL BS_lf	53.9	51.11	47.06	46.3
2	BL LD	54.79	52.05	46.69	46.25
2	BL RT_ae	56.79	54.17	44.65	42.62
1	BL	46.84	45.28	40.1	40.84

Cypermethrin Regression Models

Cypermethrin_species

Retained variables: 12
Final selected models (13)

Size	Models	R2	R2adj	Q2	Q2boot
5	BL FH_sh LD RA RT_gi	60.32	52.97	46.43	0.00
5	AR BE BS_li BS_se FH_sh	55.62	47.40	36.41	0.00
5	AR BS_dvf BS_lf FH_sh LD	54.69	46.30	35.94	0.00
4	AR BE BS_li FH_sh	51.06	44.06	31.39	0.00
4	AR BS_dvf LD RT_gi	49.98	42.83	30.60	0.00
3	BE LD RT_gi	47.39	41.95	28.55	3.05
3	AR BS_li FH_sh	43.68	37.85	27.30	3.10
4	BE LD OT_tc RT_gi	49.00	41.72	27.21	0.00
3	AR LD RT_gi	46.83	41.33	25.86	0.00
2	AR FH_sh	35.62	31.33	16.64	3.65
2	BE BS_se	34.07	29.68	9.18	5.35
2	LD RT_gi	33.89	29.48	5.94	0.00
1	BE	20.55	17.99	1.71	0.30

Cypermethrin_genus

Retained variables: 14
Final selected models (12)

Size	Models	R2	R2adj	Q2	Q2boot
5	BL FH_sh LD RA RT_gi	68.47	59.20	53.36	0.00
5	AR BS_li FH_sh RT_ae RT_te	63.45	52.70	38.05	0.08
5	AR BL LD OT_hgl RT_pl	66.70	56.91	37.53	0.00
4	AR BE BS_li FH_sh	57.74	48.35	34.22	0.00
3	AR BS_li FH_sh	51.90	44.31	32.74	13.97
4	AR BS_dvf LD RT_gi	57.69	48.29	32.36	0.00
4	AR BS_dvf BS_li FH_sh	52.01	41.34	31.67	0.00
3	BE LD RT_gi	52.46	44.95	30.20	5.30
3	AR FH_sh OT_hgl	51.21	43.51	29.99	0.00
2	AR FH_sh	43.21	37.53	21.59	10.31
2	BE BS_se	37.94	31.73	8.11	3.89
2	LD RT_gi	38.78	32.65	6.60	0.00

Deltamethrin Regression Models

Deltamethrin_species

Retained variables: 11
Final selected models (15)

Size	Models	R2	R2adj	Q2	Q2boot
5	AR FH_df FH_sh LD RA	88.29	86.04	83.65	79.51
4	AR FH_df LD RA	87.74	85.92	83.64	81.39
5	AR FH_df FH_pr LD RA	88.41	86.18	83.38	80.65
5	AR FH_df LD OT_tc RA	87.92	85.6	83.08	79.64
4	AR FH_pr LD RA	87.32	85.44	82.62	80.81
4	AR FH_sh LD RA	86.79	84.83	82.33	78.09
3	AR LD RA	86.03	84.53	82.1	80.18
3	BE LD RA	84.4	82.73	79.41	78.39
3	AR FH_df LD	82.23	80.32	77.47	75.97
2	AR LD	79.76	78.36	75.48	74.44
2	BE VL	77	75.41	71.46	71.42
2	BE FH_pr	75.78	74.11	70.71	70.08
1	BE	72.34	71.42	68.31	68.52
1	BL	54.12	52.59	44.01	43.5
1	OT_nopg	47.64	45.9	41.24	41.92

Deltamethrin_genus

Retained variables: 11
Final selected models (14)

Size	Models	R2	R2adj	Q2	Q2boot
4	AR FH_df LD RA	86.99	84.52	81.4	78.65
5	AR FH_df FH_sh LD RA	87.77	84.72	81.26	74.5
5	AR FH_df FH_pr LD RA	87.71	84.63	80.93	77.48
5	AR FH_df LD OT_tc RA	87.07	83.84	80.16	74.91
4	AR FH_pr LD RA	86.27	83.66	79.88	77.48
4	AR FH_sh LD RA	85.54	82.79	79.33	74.62
3	AR LD RA	84.44	82.32	78.97	76.66
3	BE LD RA	83.11	80.8	76.28	75.35
3	AR FH_df LD	82.24	79.82	76.05	74.03
2	AR LD	78.39	76.51	72.64	71.75
2	BE FH_ff	74.98	72.81	68.01	67.42
2	BE VL	75.17	73.01	66.9	67.17
1	BE	70.11	68.86	64.67	65.21
1	BL	46.47	44.23	32.3	29.43

Fenitrothion Regression Models

Fenitrothion_species

Retained variables: 10
Final selected models (14)

Size	Models	R2	R2adj	Q2	Q2boot
5	AR BE BS_se FH_ff FH_sc	77.42	75.44	72.67	71.11
5	AR BE BS_se FH_pr FH_sh	75.34	73.18	69.56	67.75
5	BE BS_se FH_pr FH_sh OT_hgl	74.11	71.84	68.83	66.71
4	BE BS_se FH_ff FH_sc	71.11	69.12	65.47	63.36
4	BE BS_se FH_pr FH_sh	70.02	67.95	65.37	64.81
4	BE BS_se FH_pr OT_to	69.29	67.17	65.18	64.43
3	BE BS_se FH_pr	66.6	64.9	62.6	62.2
3	BE BS_se OT_to	64.67	62.88	61.06	60.79
3	BE BS_se FH_ff	64.85	63.07	60.85	60.38
2	BE BS_se	60.23	58.91	56.73	56.85
2	BE FH_pr	59.4	58.04	55.77	56.12
2	AR LD	58	56.6	53.94	54.06
1	BE	46.2	45.32	42.89	43.65
1	BS_se	33.92	32.84	29.08	30.47

Fenitrothion_genus

Retained variables: 12
Final selected models (14)

Size	Models	R2	R2adj	Q2	Q2boot
5	AR BE BS_se FH_ff FH_sc	77.99	75.02	70.78	68.32
5	AR BL BS_lf BS_se OT_to	75.66	72.37	67.51	64.55
5	BE BS_lf BS_se OT_hgl OT_to	75.06	71.69	67.43	64.36
4	AR BL BS_se LD	71.08	68.03	64.46	62.28
4	BE BS_lf BS_se OT_to	71.58	68.59	64.29	62.6
4	AR BL BS_dvf BS_se	71.05	68	63.62	61.3
3	BE BS_se FH_pr	66.28	63.69	60.48	60.14
3	AR BS_se LD	67.16	64.63	60.46	59.89
3	AR BL BS_se	67.02	64.48	60.22	58.68
2	BE BS_se	61.1	59.16	56.32	57.08
2	BE FH_pr	59.05	57	53.8	54.07
2	AR LD	59.86	57.85	53.67	54.01
1	BE	49.06	47.82	44.93	46.44
1	AR	38.23	36.72	31.68	32.92

Malathion Regression Models

Malathion_species

Retained variables: 10
Final selected models (15)

Size	Models	R2	R2adj	Q2	Q2boot
5	BL BS_lf LD OT_tc OT_to	73.14	71.93	68.93	67.56
5	BS_lf BS_li LD OT_tc OT_to	73.39	72.19	68.81	67.4
5	BL BS_lf FH_pa OT_tc OT_to	72.83	71.61	68.62	67.29
4	BS_lf LD OT_tc OT_to	71.43	70.41	67.49	66.41
4	BL BS_lf OT_tc OT_to	69.55	68.46	65.56	64.48
4	BE BL FH_ff OT_tc	68.07	66.93	65.49	65.11
3	BE LD OT_tc	64.12	63.17	61.77	61.6
3	BS_lf OT_tc OT_to	64.24	63.29	60.84	60.22
3	BE BL OT_tc	61.35	60.32	58.7	58.22
2	BE LD	55.77	55	53.41	53.47
2	BE BL	54.1	53.29	51.62	51.54
2	LD OT_tc	53.64	52.82	51.46	51.66
1	BE	45.22	44.74	43.8	44.32
1	LD	38.8	38.27	36.63	37.05
1	OT_nopg	31.94	31.35	30.43	31.51

Malathion_genus

Retained variables: 8
Final selected models (14)

Size	Models	R2	R2adj	Q2	Q2boot
5	BE BL FH_ff OT_tc VL	72.78	70.62	67.19	65.34
5	BE BL FH_ff OT_nopg OT_tc	72.61	70.44	66.98	65.18
5	BE BL FH_ff LD OT_tc	72.56	70.38	66.89	65.27
4	BE BL FH_ff OT_tc	71.43	69.65	66.39	65.03
4	BE FH_ff LD OT_tc	70.6	68.76	65.86	64.99
4	BE FH_ff OT_tc VL	69.02	67.08	64.05	63
3	BE FH_ff OT_tc	66.84	65.31	62.42	61.97
3	BE LD OT_tc	65.9	64.33	61.9	61.52
3	BE LD RT_ae	64.21	62.55	60.35	60.3
2	BE LD	58.77	57.52	55.05	55.34
2	BE BL	56.88	55.57	52.76	52.6
2	BE FH_ff	56.43	55.11	52.24	52
1	BE	44.64	43.81	42.04	43.1
1	BL	37.45	36.51	34.52	36.12

Malathion_family

Retained variables: 9
Final selected models (14)

Size	Models	R2	R2adj	Q2	Q2boot
5	BL BS_lf FH_sc OT_nopg OT_tc	73.36	69.33	62.94	59.8
5	BL BS_lf FH_df OT_nopg OT_tc	70.29	65.79	61.01	57.51
5	BL BS_lf FH_sc FH_sh OT_tc	72.19	67.98	61	57.57
4	BL BS_lf OT_nopg OT_tc	68.18	64.44	60.83	59.62
4	BE BL BS_lf OT_tc	67.39	63.55	58.06	56.01
4	BL BS_lf OT_tc OT_to	69.38	65.78	58.06	55.76
3	BL BS_lf OT_tc	64.55	61.52	56.93	56.83
3	BL OT_nopg OT_tc	61.82	58.54	56.13	56.08
3	BE BL OT_tc	61.68	58.4	53.62	52.78
2	BL OT_tc	56.05	53.61	49.85	50.49
2	BL OT_nopg	53.29	50.69	49.25	50.15
2	BE BL	55.96	53.52	49.22	49.53
1	BL	44.61	43.11	40.1	42.26
1	BE	35.59	33.85	29.9	31.94

Parathion Regression Models

Parathion_species

Retained variables: 11
Final selected models (15)

Size	Models	R2	R2adj	Q2	Q2boot
4	BE BL FH_ff RT_ae	54.14	50.32	45.9	43.44
5	BE BL FH_ff FH_sc RT_ae	55.69	50.98	45.06	40.61
5	BE BL FH_ff FH_pr RT_ae	54.14	49.26	44.78	41.9
5	BE BL BS_li FH_ff RT_ae	54.38	49.52	44.56	41.58
4	BE FH_ff LD RT_ae	52.16	48.18	44.13	42.7
4	BE BL FH_ff OT_to	51.23	47.16	42.26	40.11
3	BE FH_ff RT_ae	46.4	43.12	40.21	39.57
3	BE FH_ff RT_te	45.93	42.62	38.26	36.84
3	BE FH_ff OT_to	44.65	41.26	38.13	36.92
2	BL FH_sc	43.97	41.73	34.99	34.83
2	BE FH_ff	37.97	35.49	30.88	30.78
2	FH_sc LD	38.68	36.22	29.07	28.79
1	FH_sc	27.32	25.9	18.89	19.57
1	BE	21.44	19.9	14.63	15.47
1	BS_se	22.61	21.1	12.77	12.84

Parathion_genus

Retained variables: 10
Final selected models (15)

Size	Models	R2	R2adj	Q2	Q2boot
5	BE FH_ff LD RT_ae VL	73.58	69.32	62.75	59.73
4	BE FH_ff LD RT_ae	69.26	65.42	61.44	59.68
5	BE FH_ff FH_sc LD RT_ae	69.94	65.09	60.24	55.62
5	BE FH_ff LD OT_to RT_ae	69.33	64.39	60.15	55.92
4	BE FH_sc LD RT_ae	67.05	62.93	58.07	54.08
4	BE BL FH_ff RT_ae	66.51	62.32	57.12	53.75
3	BE LD RT_ae	62.93	59.56	54.58	53.11
3	BE FH_ff RT_te	59.97	56.33	53.27	52.44
3	BE FH_ff RT_ae	58.74	54.99	52.13	51.53
2	BE RT_te	51.01	48.13	43.56	42.93
2	BE FH_ff	49.43	46.46	41.23	40.37
2	BE LD	50.43	47.52	40.12	38.91
1	BS_se	34.49	32.61	24.11	25.73
1	FH_sc	32.04	30.1	21	21.97
1	BE	29.13	27.11	20.66	21.7

Appendix 3

Supplementary material of Chapter 4

Evaluating pesticide effects on freshwater invertebrate communities in alpine environment: a model ecosystem experiment

Tab.1 Physical-chemical and toxicological properties of the tested chemicals (FOOTPRINT, 2006).

	water solubility mg/L	Kow	*Daphnia magna* 48hEC50 mg/L
chlorpyriphos	1.05	4.7	0.0001
pyrimethanil	121	2.84	2.9
phosmet	15.2	2.96	0.002
etofenprox	0.023	6.9	0.0012
dithianon	0.2	3.2	0.26
difeconazole	15	4.4	0.77
lufenuron	0.046	5.12	0.0013
thiametoxam	4100	-0.13	>100
methoxyfenozide	3.3	3.7	3.7
thiacloprid	184	1.26	85.1

Estimation of exposure and toxic potency of the mixtures (TUs)

A range of TU values was calculated for each chemical on the basis of the max/min Time Weighted Average (TWA).
of the two experimental days (except for simulated event 1, which lasted 24 hours).
In detail, the procedures have been the following:

1. ***Tracers (measured chemicals):*** experimentally measured data are available for three chemicals (pendimethalin, chlorpyriphos and phosmet). Data refer to the second day of experiment (lowest concentrations). Concentrations for the first experimental day (0-24 h) have been estimated multiplying the respective nominal concentration (0-24 h) times the specific measured/nominal (24-48h) ratio. TWA have been calculated as a mean (the time interval is the same for both concentrations) of the first day concentration (estimated) and the second day concentration (measured). In this case $TWA_{min} = TWA_{max}$

$$TWA_{\min} = TWA_{\max} = \frac{\left(\frac{measured_{24-48h}}{nominal_{24\text{-}48h}} \times nominal_{0\text{-}24h} \right) + measured_{24-48h}}{2} \qquad (1)$$

2. ***Not measured chemicals:*** for not measured chemicals, TWA_{max} was calculated as a mean of the nominal concentrations (0-24 h and 24-48 h), while TWA_{min} has been set to zero.

$$TWA_{\min} = 0$$
$$TWA_{\max} = \frac{nominal_{0-24h} + nominal_{24-48h}}{2} \qquad (2)$$

Tab.2 minimum and maximum TWA (see previous paragraph for details of the calculations) and correspondent TUs for each chemical used in the 5 simulated events

		B				C				D				E			
Event #	Compound	TWA_{min}	TWA_{max}	TU_{min}	TU_{max}	TWA_{min}	TWA_{max}	TU_{min}	TU_{max}	TWA_{min}	TWA_{max}	TU_{min}	TU_{max}	TWA_{min}	TWA_{max}	TU_{min}	TU_{max}
1	etofenprox	0.00	0.08	0.00	0.07	0.00	0.08	0.00	0.07	0.00	0.19	0.00	0.16	0.00	0.19	0.00	0.16
2	pyrimethanil	0.00	40.00	0.00	0.01	0.00	40.00	0.00	0.01	0.00	84.80	0.00	0.03	0.00	84.80	0.00	0.03
	dithianon	0.00	6.07	0.00	0.02	0.00	6.07	0.00	0.02	0.00	12.86	0.00	0.05	0.00	12.86	0.00	0.05
3	pyrimethanil	5.40	5.40	0.00	0.00	5.00	5.00	0.00	0.00	20.40	20.40	0.01	0.01	25.41	25.41	0.01	0.01
	dithianon	0.00	3.40	0.00	0.01	0.00	3.55	0.00	0.01	0.00	6.95	0.00	0.03	0.00	6.50	0.00	0.03
	difenoconazole	0.00	0.07	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.13	0.00	0.00
	lufenuron	0.00	0.03	0.00	0.02	0.00	0.03	0.00	0.02	0.00	0.05	0.00	0.03	0.00	0.05	0.00	0.03
	thiametoxam	0.00	6.80	0.00	0.00	0.00	7.10	0.00	0.00	0.00	13.90	0.00	0.00	0.00	13.00	0.00	0.00
	chlorpyrifos	0.01	0.01	0.10	0.10	0.01	0.01	0.13	0.13	0.03	0.03	0.34	0.34	0.06	0.06	0.56	0.56
4	pyrimethanil	7.64	7.64	0.00	0.00	9.03	9.03	0.00	0.00	25.80	25.80	0.01	0.01	27.37	27.37	0.01	0.01
	dithianon	0.00	3.24	0.00	0.01	0.00	3.06	0.00	0.01	0.00	6.03	0.00	0.02	0.00	5.49	0.00	0.02
	difenoconazole	0.00	0.15	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.27	0.00	0.00	0.00	0.25	0.00	0.00
	lufenuron	0.00	0.02	0.00	0.02	0.00	0.02	0.00	0.02	0.00	0.05	0.00	0.03	0.00	0.04	0.00	0.03
	thiametoxam	0.00	0.18	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.34	0.00	0.00	0.00	0.31	0.00	0.00
	chlorpyrifos	0.07	0.07	0.67	0.67	0.05	0.05	0.50	0.50	0.16	0.16	1.61	1.61	0.21	0.21	2.08	2.08
	methoxyfenozide	0.00	0.58	0.00	0.00	0.00	0.55	0.00	0.00	0.00	1.07	0.00	0.00	0.00	0.98	0.00	0.00
	thiacloprid	0.00	5.94	0.00	0.00	0.00	5.61	0.00	0.00	0.00	11.06	0.00	0.00	0.00	10.07	0.00	0.00
5	pyrimethanil	5.94	5.94	0.00	0.00	5.78	5.78	0.00	0.00	24.72	24.72	0.01	0.01	16.34	16.34	0.01	0.01
	dithianon	0.00	3.24	0.00	0.01	0.00	3.33	0.00	0.01	0.00	4.77	0.00	0.02	0.00	5.85	0.00	0.02
	difenoconazole	0.00	0.13	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.23	0.00	0.00
	lufenuron	0.00	0.03	0.00	0.02	0.00	0.03	0.00	0.02	0.00	0.04	0.00	0.03	0.00	0.05	0.00	0.03
	fosmet	1.01	1.01	0.51	0.51	1.08	1.08	0.54	0.54	6.53	6.53	3.26	3.26	3.64	3.64	1.82	1.82
	chlorpyriphos et.	0.01	0.01	0.07	0.07	0.01	0.01	0.06	0.06	0.02	0.02	0.24	0.24	0.02	0.02	0.17	0.17
	methoxyfenozide	0.00	0.54	0.00	0.00	0.00	0.56	0.00	0.00	0.00	0.80	0.00	0.00	0.00	0.98	0.00	0.00

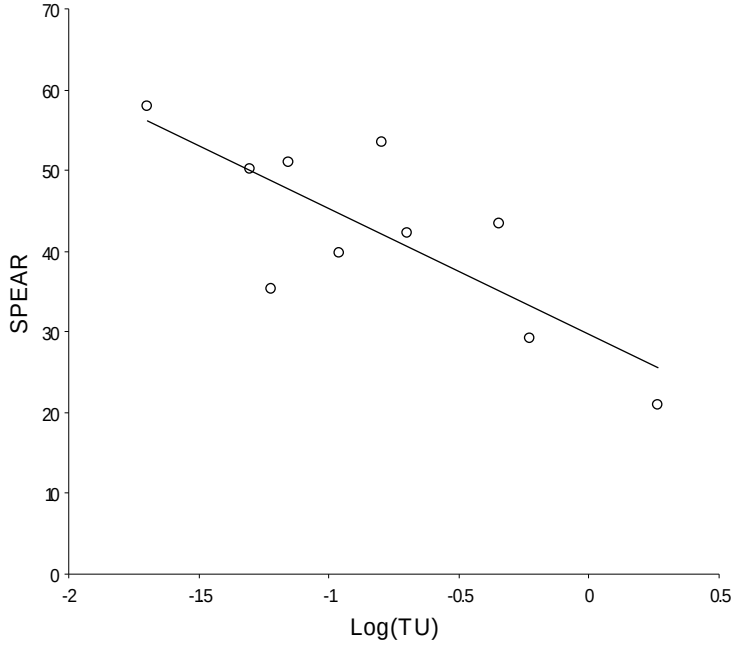

Fig.1 regression between SPEAR values and Log(TU). TUs are calculated from the highest single substance TWA from each simulated events (See table 2 SI), except for the last sampling event in which chlorpyrifos TWA was considered instead of phosmet (see main text for rationale). $R^2 = 0.62$, $p = 0.007$

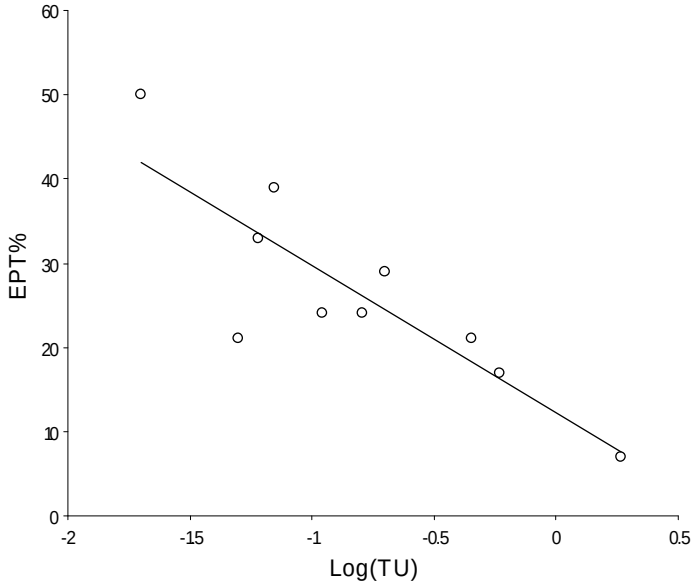

Fig.2 regression between EPT% values and Log(TU). TUs are calculated from the highest single substance TWA from each simulated events (See table 2 SI), except for the last sampling event in which chlorpyrifos TWA was considered instead of phosmet (see main text for rationale). $R^2 = 0.72$, $p = 0.002$

Appendix 4

Supplementary material of Chapter 5

Site-specific pesticide risk assessment in a small Alpine catchment: a multi-level approach

Table I Plant Protection Products applied (formulation and active ingredient), rate (cc/hl for liquid and g/hl for solid compounds) and dates of application for SABAC consortium (230 ha – 15 hl/ha) relative to productive season 2011.

DATE	PRODUCT	DOSE	A.I.	TIPOLOGY
25-Mar	Duke	200	Copper chloride	fung
2-Apr	Eko Oil Spray	2000	Mineral oil	insec, herb
	Polyram Df	200	Metiram	fung
9-Apr	Trebon	30	Etofenprox	insec
	Aphox	200	Pirimicarb	aficid
	Delan 70 WG	30	Dithianon	fung
	Nimrod 250 EW	40	Bupirimate	fung
	Vector	50	Alkyl Alcohol ethoxylates	addit
23-Apr	Delan 70 WG	30	Dithianon	fung
	Vector	50	Alkyl Alcohol ethoxylates	addit
24-May	Amid thin W	20	NAD	dira
28-Apr	Score 10 WG	37	Difenoconazole	fung
	Delan 70 WG	40	Dithianon	fung
	Microthiol D	200	Copper	fung
4-May	Kohinor 200 SL	30	Imidacloprid	insec
	Prodigy	40	Methoxyfenozide	insec
	Score 10 WG	37	Difenoconazole	fung
	Delan 70 WG	40	Dithianon	fung
11-May	Dursban 75 wg	75	Chlorpyrifos	insec
	Delan 70 WG	50	Dithianon	fung
	Scudex	25	Penconazole	fung
12-May	Brancher dir	80	BAR	fitoreg
	Dirager	10	NAA	dira
17-May	Score 10 WG	37	Difenoconazole	fung
	Delan 70 WG	50	Dithianon	fung
	Fitogold	150	Fertilizer	
	Vector	50	Alkyl Alcohol ethoxylates	addit
24-May	Score 10 WG	37	Difenoconazole	fung
	Delan 70 WG	50	Dithianon	fung
	Arius	25	Quinoxyfen	fung
	Vector	50	Alkyl Alcohol ethoxylates	addit
30-May	Delan 70 WG	50	Dithianon	fung
	Vector	50	Alkyl Alcohol ethoxylates	addit
7-Jun	Calypso	25	Thiacloprid	insec
	Delan 70 WG	50	Dithianon	fung
	Vector	50	Alkyl Alcohol ethoxylates	addit
13-Jun	Delan 70 WG	50	Dithianon	fung
29-Jun	Banjo	70	Fluazinam	fung
	Nimrod 250 EW	40	Bupirimate	fung
21-Jul	Grado 66 WG	50	Dithianon	fung
	Stopit	200	???	
6-Aug	Merpan 80 WDG	130	Captan	fung
	Stopit	200	???	
23-Aug	Grado 66 WG	50	Dithianon	fung
	Vector	50	Alkyl Alcohol ethoxylates	addit
2-Sep	Dodil WG	150	Dodine	fung

Table II Plant Protection Products applied (formulation and active ingredient), rate (cc/hl for liquid and g/hl for solid compounds) and dates of application for SASA consortium (250 ha – 15 hl/ha) relative to productive season 2011.

DATE	PRODUCT	DOSE	A.I.	TIPOLOGY
25-Mar	trebon	10	Etofenprox	insec
26-Mar	rame	200	Copper	fung
	white oil	300	Mineral oil	insec
1-Apr	vernoil	2200	Mineral oil	insec
2-Apr	mancozeb	200	Mancozeb	fung
8-Apr	delan	40	Dithianon	fung
9-Apr	nimrod	40	Bupirimate	fung
	trebon	25	Etofenprox	insec
	teppeki	8	Flonicamid	insec
	bortrac	50	Fertilizer	
27-Apr	delan	40	Dithianon	fung
28-Apr	scala	50	Pyrimethanil	fung
	tiopron	200	Sulfur	fung
2-May	delan	40	Dithianon	fung
3-May	nuprid	30	Imidacloprid	insec
	topas	12	Penconazole	fung
	prodigi	37	Methoxyfenozide	insec
11-May	delan	40	Dithianon	fung
12-May	tiopron	200	Sulfur	fung
	alisè	50	Chlorpyrifos	insec
17-May	delan	40	Dithianon	fung
18-May	score	37	Difenoconazole	fung
	tiopron	200	Sulfur	fung
	kaolin	100	Caolino	repel
24-May	delan	40	Dithianon	fung
25-May	score	37	Difenoconazole	fung
	idromag	150	Fertilizer	
	mantrac	30	Fertilizer	
	kaolin	100	Caolino	repel
30-May	delan	40	Dithianon	fung
31-May	coragen	37	Chlorantraniliprole	insec
7-Jun	delan	40	Dithianon	fung
15-Jun	delan	40	Dithianon	fung
17-Jun	envidor	25	Spirodiclofen	insec
28-Jun	ohayo	60	Fluazinam	fung
29-Jun	mantrac	30	Fertilizer	
	nimrod	40	Bupirimate	fung
	caltrac	150	Fertilizer	
15-Jul	flint	14	Trifloxistrobina	fung
	caltrac	150	Fertilizer	
27-Jul	dodine	100	Dodine	fung
9-Aug	captan	130	Captan	fung
10-Aug	calsol	400	Fertilizer	
25-Aug	delan	50	Dithianon	fung
	calsol	400	Fertilizer	
7-Sep	dodine	100	Dodine	fung
	etravon	50	Sorbitan	addit

Table III Properties of molecules modelled in the runoff simulations. Values with no apex are retrieved from The Pesticide Manual (Tomlin 2003). Values with apex "2" are retrieved from the Footprint Database (FOOTPRINT 2006), while apex "3" indicates that no values where available in the existing literature, thus properties were derived making several assumptions.

	Imidacloprid	Methoxyfenozide	Chlorpyrifos	Etofenprox	Flonicamid	Pirimicarb	Difenoconazole	Dithianon
Molecular mass (g/mol)	255.7	368.5	350.6	376.5	229.16 [2]	238.3	406.3	296.3
Melting point (°C)	144	204.5	42.75	37.2	157.5 [2]	90.5	78.6	225
Vapor pressure (Pa)	4E-10 (20 °C)	1.48E-6 (25 °C)	2.7E-3 (25 °C)	8.13E-7 (25 °C) [2]	9.43E-7 (25 °C) [2]	9.7E-4 (25 °C)	3.3E-8 (25 °C)	2.7E-9 (25 °C)
Solubility (mg/L)	610 (20 °C)	3.3 (20 °C)	1.4 (25 °C)	0.0225 (25 °C) [2]	5200 (20 °C) [2]	3000 (20 °C)	15 (25 °C)	0.14 (20 °C)
Log(K_{OW})	0.57 (22 °C)	3.7	4.7	7.05	0.3 (20 °C) [2]	1.7	4.2 (25 °C)	3.2
Soil DT50 (days)	174 [2]	68 [2]	21 [2]	11 [2]	33.8 [3]	86 [2]	85 [2]	35 [2]
Sediment DT50 (days)	129 [2]	68 [3]	36.5 [2]	13.3 [2]	40 [2]	195 [2]	1053 [2]	0.05 [2]
Water DT50 (days)	30 [2]	68 [3]	5 [2]	5.7 [2]	33.8 [2]	33.3 [2]	3 [2]	0.05 [2]

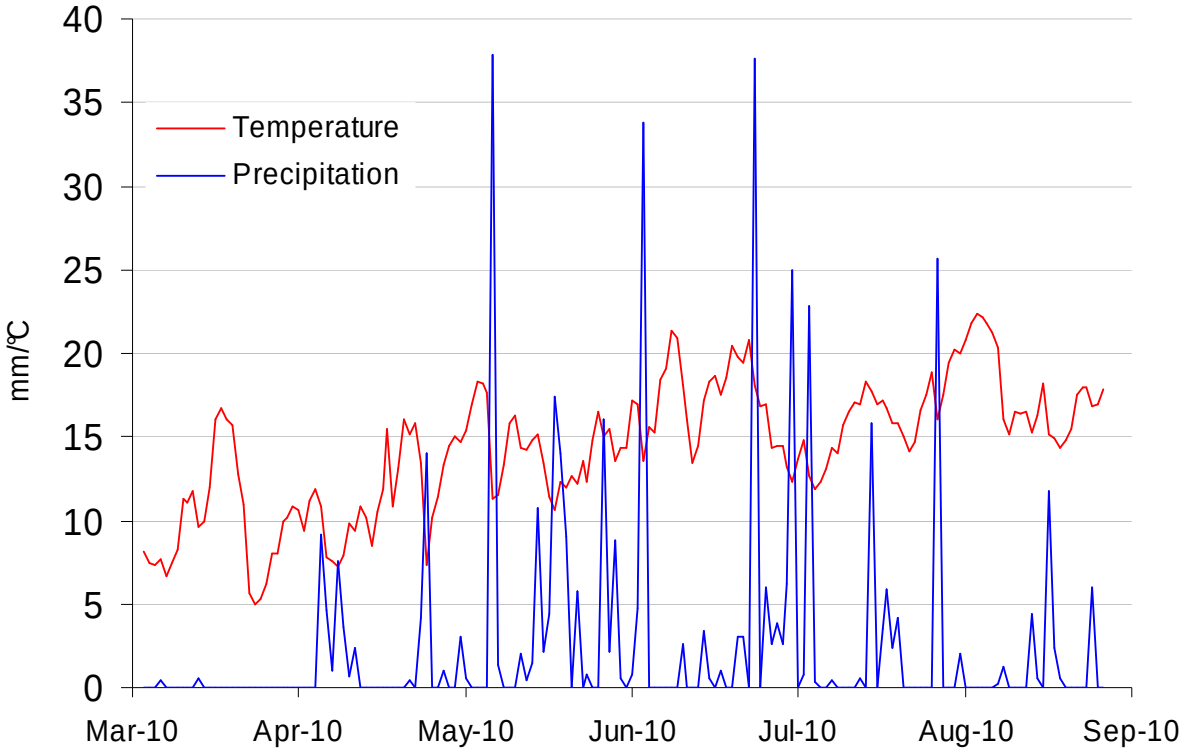

Figure I Daily temperature (°C) and precipitation (mm) data for the period 25/03/2011-14/09/2011 retrieved from the meteorological station of Romeno (sited in the Novella watershed).