# MULTILEVEL STRUCTURE-FUNCTION RELATIONSHIPS IN IMPAIRED STREAM ECOSYSTEMS

- FROM THEORY TO MANAGEMENT APPLICATIONS -

RICCARDO CABRINI

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UNIVERSITA' DEGLI STUDI DI MILANO
BICOCCA

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**: the multilevel concept. At first glance, the figure represents a square base pyramid. If you look in more detail, the pyramid can be clearly described as a set of five overlapping floors and each floor as a set of adjacent cubes. The 55 cubic units combine together to form a precisely structure. The multilevel approach is useful to better understand the river ecological dynamics: a simultaneous evaluation of multiple stressors at multiple spatial scales allows to have a clearer overview of the impaired streams.

UNIVERSITA' DEGLI STUDI DI MILANO-BICOCCA

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XXV ciclo

# MULTILEVEL STRUCTURE-FUNCTION RELATIONSHIPS IN IMPAIRED STREAM ECOSYSTEMS

## - FROM THEORY TO MANAGEMENT APPLICATIONS -

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# 1. INTRODUCTION

## 1.1 Impaired stream in urban areas

Increasing urbanization across the world has led to increased research on ecology in urban settings in the last decade. Urban ecological studies have investigated both impacts of urban development on native ecosystems and the dynamics of urban environments themselves as ecosystems (Grimm et al., 2000). In both areas of research, streams of urban areas have an important part to play because their position in the landscape makes these ecosystems particularly vulnerable to impacts associated with landcover change.
Urban stream ecosystems are affected by multiple stressors (Fig. 1) and their effects are synthetized in the "urban stream syndrome" theorized in Walsh et al. 2005. Consistent symptoms of the urban stream include flashier hydrograph, elevated concentrations of nutrients and contaminants, altered channel morphology and stability and reduced biodiversity, with increased tolerant species. These ecological effects are often accompanied by other symptoms not observed in all urban areas, such as reduced baseflow and increased suspended solids.

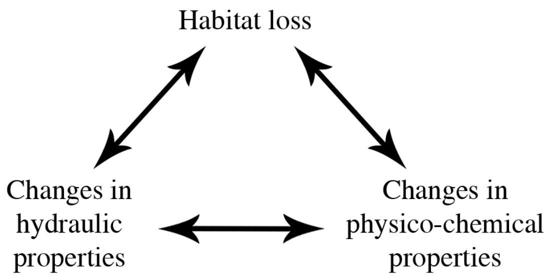


**Figure 1** The diagram shows the relations among the stressors acting on a urban stream

In impaired freshwater ecosystems, it is known that ecological integrity can be subdivided into two components, structural and functional integrity (Sandin *et al*., 2009; Minshall, 1996). Structural indicators of ecosystem health may be defined as the qualitative and quantitative composition of biological communities. Fish and macroinvertebrate assemblages have been the main focus for assessing structural integrity, although a variety of alternative targets such as benthic algal communities, protozoans, and macrophytes have also been used (e.g., Barbour et al., 1999, Norris and Thoms, 1999, Hill et al., 2000).
Macroinvertebrate assemblages play a central ecological role in many stream ecosystems and are among the most ubiquitous and diverse organisms in fresh waters. Macroinvertebrates are easily recognizable and classifiable and some *taxa* are representative of every different habitat and condition (sensibility or tollerance to pollution and environmental changes) and so it is easy to aggregate results of macrobenthos analyses into synthetic indeces (such as STAR_ICMi). Function indicators instead, that have a much shorter history, are complementary to structural indicators and refer to the autoecology of biological communities and ecological attributes within the ecosystem in which they are located. (Gessner et al., 2002).
In Water Framework Directive (WFD; European Union, 2000), develops by European Union to advance more comprehensive water legislation, the river basins with above mentioned characteristics are defined *heavily modified water bodies* (HMWBs).
HMWBs have unique water quality characteristics that, in most cases, are comparatively different from normal stream conditions upstream of the discharge or at regional reference sites (Taylor, 2002; Brooks et al., 2004). Reference sites are commonly used in bioassessment studies to identify undisturbed or pristine conditions and hence management targets (Hughes, 1995; Prins and Smith, 2007). The increase of urban development often results in the absence of reference sites in HMWBs (Chessman and Royal, 2004) and this leads in difficulties to define a target condition for restoring urban stream sites (Meyer et al., 2005).

## 1.2 Multiple-scale and quantile regression approach

The WFD requires that all waters achieve good ecological status and only slightly deviate from natural reference conditions, which has become the main objective of most restoration projects in Europe. The ecological status is quantified in many European member states using multi-metric indices, and good ecological status corresponds to a specific score value. However, there is little information on the limiting effects of large-scale pressures on the biological metrics.
As suggested by numerous research works (i.a. Donohue et al., 2004; Maddock, 1999), the scale to approach river investigations can be considered from the microhabitat level to basin scale. A river may be analysed across a variety of levels, which can be ordered into a hierarchy, with different degree of sensitivity and recovery time (Fig. 2; Maddock, 1999).

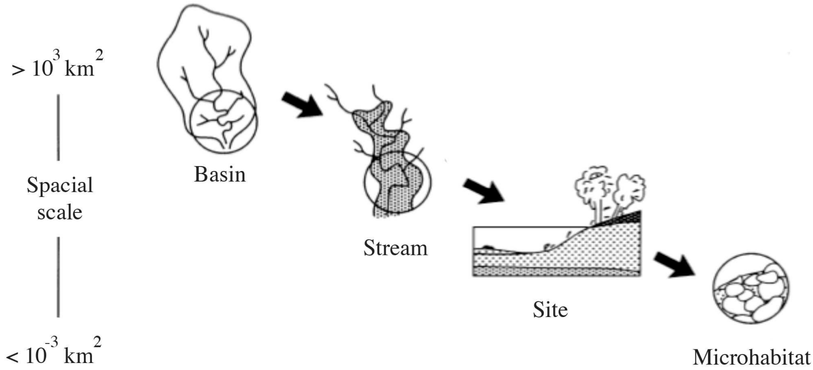


**Figure 2** Scale to approach river investigation (adapted from Maddock 1999)

Impacts of human activity are becoming increasingly unacceptable to a global community that focuses on environmental sustainability. Therefore, whole catchment approach management have been developed to preserve stream ecosystems or restore damaged ecosystems, and mitigate against further damage (e.g., Kreutzweiser et al., 2005).

The individuation of which factors set limits to biological community development and their respective values is of great interest for river managers and river restoration campaigns. In urban streams is usually hard to assess causal relationships among specific stressors and responses of biological communities using the most common statistical tools. Using macroinvertebrate assemblages as biological indicators in micro- and mesohabitat level works, applied statistics may be viewed as an elaboration of the linear regression model and associated estimation methods of least square (Koenker and Bassett, 1978).
In whole basin analyses, data variability is high and classic statistical approach may even become uninformative (Lancaster & Belyea, 2006). Moreover, the effects of many stressors (local and global) may influence simultaneously the response of biological community leading to a decrease of statistical model fit.
In this perspective, alternative statistical approaches are necessary. In 1978 Koenker and Bassett theorized the quantile regression in econometric sciences, a robust alternatives to the least squares estimator for the linear model. Thomson et al. (1996) and subsequently Cade et al. (1999) introduced this kind of regression in ecology declaring that quantile regression allows the various stressors to be considered as "constraints" to the distribution of biological communities, without compromising the model causal relationship.

## 1.3 Outline of the thesis

Aim of this work is to assess the overall pressure of human activities in river basins of Lombardy piedmont and floodplain area and to relate changes in the biological communities as a result of habitat loss and changes in both hydromorphological and physico-chemical properties. In this area, many rivers have a "channelized" nature with straight section, clear of river bank tree and uniform bed morphology. Flow regulation and modification have also been widespread. The quantity and timing of water availability have been altered for irrigation and industrial purposes, through the construction of dams and reservoir for water supply. Changes in water quality are also common, in particular in lowland areas where urbanization and agriculture are more strong.

For these reasons, the work is focusing on different scale (microhabitat, site, river reach and basin levels) to have a better resolution and understanding of existing dynamics among structural and functional indicators and pressures in impaired environments. These areas undergo different stresses (habitat loss, changes in physico-chemical properties and changes in flow) that affect the integrity of the ecosystems. Assessing the condition of ecosystems is a prerequisite to reduce the induced anthropogenic pressure. Decision-making in river restoration programs can also be helped by multilevel kind of information.

In particular, in *chapter II* the use of environmental gradients (water chemistry and hydromorfology) were used to test the structural and functional variability of the macroinvertebrate assemblages. To test the macroinvertebrate preferences to different leaf species, artificial leaf packs were used in sampling method. This work involved the analysis of six sites located in 3 different streams within Olona-Seveso-Lambro basin (OSL basin).

Leaf breakdown is an important ecosystem process and the recycling of nutrients during organic matter decomposition is an essential component of stream ecosystems. Leaf type directly affects the composition and abundance of the benthic macroinvertebrate assemblages that promote leaf degradation. Leaf breakdown is also influenced by the exposure time during which it is possible to find different functional units of macroinvertebrates. In *chapter III* these aspects (leaf degradation and macroinvertebrate diversity) have been explored in the same sampling sites mentioned above.

In *chapter IV* Lambro River (within OSL basin) was choose to analyse the response of macroinverterbrate assemblages to strong chemical impairment, due to a fuel oil spill into the sewage system north of the city of Milan, that causing the breakdown of the local treatment plant. 1000 tons of oil were spilled into the Lambro River, and wastewater was discharged therein for a month. The short-term effects on the benthic invertebrate communities were analysed in the following weeks, comparing data collected before/after, and upstream/downstream the spill

After short works at microhabitat and site levels, a whole basin analysis was conducted in *chapter V*. To have a better comprehension of large-scale pressure effects on the biological metrics, basin analyses were needed. We used a multivariate approach to focus on the characteristics of the streams and rivers in an urban district and to define which macroinvertebrate metrics should be used to assess the influence of the different kinds of alteration in a severely damaged environment.

The use of large datasets with high data variance and complex variable interactions has shown that to establish the relationship between pressures and biological responses, classic statistical approach leads to uninformative results. In *chapter VI* the usage of quantile regression was introduced and applied to large dataset; this statistical tool allows the various stressors to be considered as “constraints” to the distribution of biological communities and so to establish ecological potential useful for river restoration managers.

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# 2 LEAF PACKS IN IMPAIRED STREAMS: THE INFLUENCE OF LEAF TYPE AND ENVIRONMENTAL GRADIENTS ON BREAKDOWN RATE AND INVERTEBRATE ASSEMBLAGE COMPOSITION

**Abstract**

The presence of different kinds of leaf packs (native or alien) and environmental gradients can affect the composition and abundance of macroinvertebrate assemblages in freshwater ecosystems. Little is know about the interactive effects of both occurrences. So, we were interested in understanding (1) how leaf types and environmental gradients could influence each other and (2) which was the most important factor affecting macroinvertebrate assemblages in impaired streams.

Using Principal Component Analysis, we defined two environmental gradients: a water quality gradient, related to anthropogenic alteration, and a hydromorphological gradient, mostly related to the catchment features. Our results pointed out that, in impairment conditions, biological metrics were chiefly influenced by the water quality gradient, while different leaf types in packs influenced the total taxa richness, but did not cause significant variation in the distribution and abundance of macroinvertebrate functional groups. Mass loss, instead, differed among leaf types, in relation to the catchment features (mainly flow).

This work shows that, in impaired streams, water quality influences more than leaf types the macroinvertebrate assemblages colonizing leaf packs. Thus, water quality improvements should be the priority in restoration programs for impaired rivers and should be preliminary to restoration of native riparian vegetation.

**Keywords**

Macroinvertebrates, functional traits, leaf packs, environmental gradients

*Submitted manuscript*

Cabrini R., Canobbio S., Sartori L., Fornaroli R. & Mezzanotte V.

Leaf packs in impaired streams: the influence of leaf type and environmental gradients on breakdown rate and invertebrate assemblage composition.

## 2.1 Introduction

Small streams are considered to be mainly heterotrophic ecosystems that are energetically dependent on the input of external organic matter, primarily abscissed leaves, bark and branches (Cummins and Klug 1979; Allan 1995). Leaf breakdown is an important ecosystem process and the recycling of nutrients during organic matter decomposition is an essential component of stream ecosystems (Irons et al. 1988; Cummins et al. 1989; Murphy et al. 1998), and studies about it have appeared in literature for a long time (e.g. Cummins et al. 1973; Short and Maslin 1977). Shed leaves coming from riparian vegetation (Wallace et al. 1997; Power and Dietrich 2002) form leaf packs, which are then degraded by a combination of physical and biological processes (Richardson 1992; Carlisle and Clements 2005). Through the production of faecal pellets and orts, invertebrate shredders convert coarse particulate organic matter (CPOM) into fine particulate organic matter (FPOM), which is then distributed downstream and ingested by many other consumers, such as collector gatherers and filterers.
Shed leaves break down at different rates, according to their initial structural and physico-chemical properties. Species-specific breakdown rates may vary with stream, location in the stream, time of year, microbial activities, presence of shredders, water quality and hydromorphological characteristics. Leaf type directly affects the composition and abundance of the benthic macroinvertebrate assemblages (Cummins 1986; Cummins et al. 1989; Ormerod et al. 1993): several studies have shown that macroinvertebrates prefer leaf litter of some species to others (i.a. Graça 2001; Schulze and Walker 1997). These preferences are related to different leaf properties, such as toughness, nitrogen content, microorganism preconditioning of leaves and presence of secondary metabolites, all of which determine leaf palatability (Webster and Benfield 1986; Graça 2001). Thus, the introduction of alien leaves in streams, which is likely to happen in basins with a high level of anthropogenic modification, can produce effects on the biological communities (Kominoski and Pringle 2009).
Densities of macroinvertebrates are usually much higher on leaf packs than in the surrounding substratum (Mackay and Kalff 1969) and this

seems to be related to the nutritional value of leaf tissues. However, as many taxa do not directly consume leaf tissues, it is possible that leaf packs are colonized not only for their nutritional value (Egglishaw 1964), but also because they provide shelter from direct current, space and attachment site for settlement, and perhaps refuge from predators (i.e. Davies and Boulton 2009).

Macroinvertebrate assemblages in leaf packs can be modified by the stream water quality, its hydromorphology and, in general, by the surrounding environmental factors (Davies et al. 2010). The discharge of untreated or inadequately treated wastewater in streams may cause water pollution and the input of large amounts of FPOM (Chang 2005). Thus, invertebrate communities downstream the discharge of wastewater are often impoverished and dominated by pollution-tolerant species (Canobbio et al. 2009; Prenda and Gallaro-Mayenco 1996; Wright et al. 1995), and show shifts in the composition of feeding groups (Rawer-Jost et al. 2000). Nevertheless, the advances in sewage treatment technology, the upgrading of the existing wastewater treatment plants (WWTPs) and the construction of new ones are improving the quality of effluents and, consequently, of the receiving water (Daniel et al. 2002; Gücker et al. 2006; Mladenov et al. 2005). Hence, impaired streams, even within the same basin, can present a gradient of water quality deriving from various levels of pollution, which can influence in different ways leaf packs colonization by invertebrates and leaf breakdown rates (Spanhoff et al. 2007).

In such streams, the macroinvertebrate relationship with leaf litter can be simultaneously influenced by other factors, both natural and anthropogenic. For example, changes in land use can modify the amount of CPOM and FPOM entering the streams, the habitat availability, and the flow regimes (Dyer et al. 2003; Tillman 2003), while natural differences in basin size, stream morphology and dimensions, even in streams of the same order, can determine another environmental gradient causing shifts in the ecosystem functions (Johnson et al. 2006).

So, the presence of different kinds of leaf packs and the environmental gradients can both affect the composition and abundance of the colonizing biological communities. Many studies have analyzed the effects of the presence of different kinds of leaves in packs (i.a. Lacan et al. 2010; Graça 2001), *or* of different environmental conditions, such as increasing alteration (i.a Canobbio et al. 2010; Coimbra et al. 1996), on invertebrates. No one has evaluated the interactive effects of both occurrences, which are likely to happen simultaneously in impaired streams. Thus, we wanted to test hypotheses about the combined effects of different kinds of leaves (native and alien) and environmental gradients on the macroinvertebrate assemblages colonizing leaf packs. Few ecological factors usually drive a number of variables simultaneously. As a result, there is a great deal of redundancy in the distribution of ecological data. Ordination techniques, i.e. Principal Component Analysis (PCA), use this redundancy to extract and describe the major independent gradients in multivariate data set. So, the principal components accounting for most of the data variability were subsequently used as environmental gradients (Gotelli and Ellison 2004).

So, the objectives of this study are (1) to explore the interaction among different kinds of leaves and environmental gradients and (2) to individuate the role of environmental gradients in the distribution patterns of functional feeding (FFG) and habit (FHG) groups of macroinvertebrates colonizing artificial leaf packs in sites showing different degree of impairment.

## 2.2 Materials and methods

### *2.2.1 Study area*

We analyzed six sites (hereafter called S1 – S6) in Seveso, Lura and Bozzente streams, all belonging to the Olona-Lambro basin in the piedmont area of Lombardy region in Italy (Figure 1). The lengths of the streams range from 37 km (Bozzente) to 52 km (Seveso) and their catchment areas from 130 $km^2$ (Lura) to 228 $km^2$ (Seveso). The area is heavily exploited by human presence and activities (over 500,000 equivalent inhabitants throghout the three basins). Seveso, Bozzente and Lura receive the effluents from large WWTPs, fed on industrial and domestic wastewater, responsible for most of their stream base flow. In some areas both untreated sewage and sewerage overflows are also discharged.

The sites are similar in order and slope, but different in water quality, catchment land use and hydromorphological features. We evaluated several environmental variables to ensure that the sites were distributed along environmental gradients. In the six sampling sites chemical and hydromorphological parameters were measured during low flow periods, while land cover characteristics (expressed as a percentage of the total basin area) were analyzed with GIS software QuantumGis, (Freeware version 1.8) (Table 1A-1B). Water samples were collected two times in the six sites: at the beginning and at the end of the experiment. Water quality analyses included temperature, electric conductivity, dissolved oxygen (DO), pH, chemical oxygen demand (COD), total phosphorus (TP), total and ammonia nitrogen (TN and $NH_4$–N), and *Escherichia coli*.

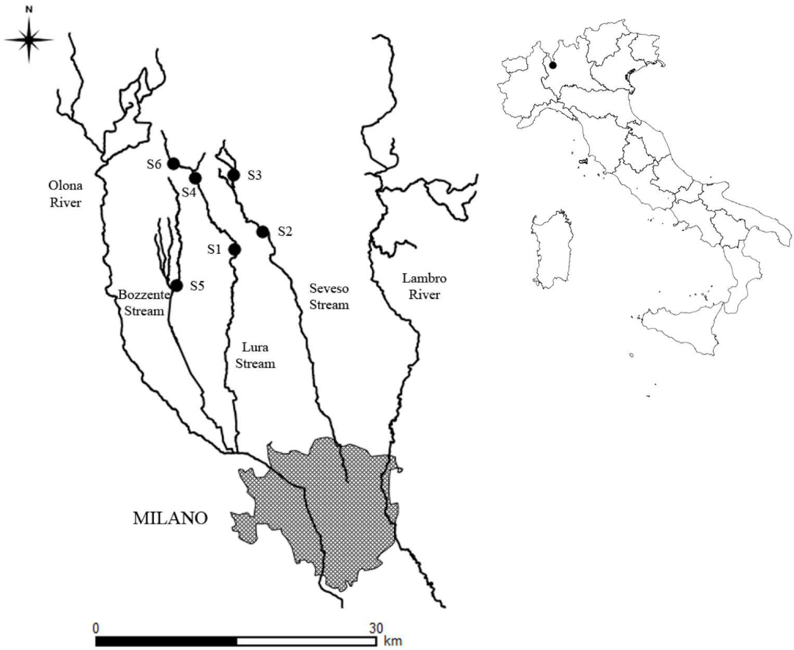


**Figure 1** Location of the six study sites (S1 – S6) in Northern Italy.

The three stream environments have similar vegetation assemblages. Alder (*Alnus glutinosa*), willow (*Salix alba*) and poplar (*Populus nigra*) are the typical riparian species and are present in all sampling sites. Alder is the most abundant, whereas willow and poplar are less copious. In the outermost zone the vegetation is chiefly made of oak (*Quercus robur*) and white hornbeam (*Carpinus betulus*). In the same area, locust-trees (*Robinia pseudoacacia*) have been introduced from centuries and are now widely spread across the basin. We examined natural leaf packs that could be found in streams and found them mainly composed by alder, oak, white hornbeam and locust-tree. In the urban portion of the basin laurel is being used for hedgerows, and we found packs of the pruned laurel (*Prunus laurocerasus*) leaves downstream of towns and cities.

### <u>2.2.2 Experimental design and data analysis</u>

On the basis of the environmental variables collected in the sampling sites (see above), a PCA was performed to evaluate the interactions among the measured variables and to determine the dominant gradients of variation (Johnson et al. 2006). Artificial leaf packs were placed in the study sites. We prepared three different kinds of leaf packs representative of native, naturalized and alien vegetation. The fallen leaves and twigs needed for leaf pack preparation were collected in May 2010. Leaves and twigs of alder, oak and white hornbean (native mix leaves) and locust-tree (naturalized leaves), common in riparian vegetation, were collected from a forest in the Lura stream basin. Leaves and twigs of laurel (alien "urban" leaves) were collected from urban parks in the same basin. Only intact leaves were used for the experiment. Leaves and twigs were brought to the laboratory shortly after collection and dried for 24 hours at 105°C to obtain standardized moisture contents (Spanhoff et al. 2007).

Leaves and twigs were placed in commercial net bags 15 x 40 cm (10 mm mesh size). Each net bag was filled with ca. 12 g of leaf litter and ca. 3 g of twigs and identified by an assigned number. We placed in the riverbeds a total of 54 leaf packs (9 packs for each site, 3 packs per leaf type); all packs were tied with polyester threads to metal rods that were knocked vertically into the sediment.

After 21 days leaf packs were removed from all sites and immediately transferred to the laboratory. Leaves were washed over 500 $\mu$m sieves and dried for 24 hours at 105° C in order to determine the remaining mass (Spanhoff et al. 2007). Mass loss ratio was calculated between initial and final dry mass of leaves. All macroinvertebrates found in leaf packs were stored in 90% ethanol and identified at genus level, except for Diptera order and Oligochaeta subclass that were identified at family level, using an Optika stereomicroscope (180x) and taxonomic keys (Campaioli et al. 1999; Sansoni 1992). Macroinvertebrates were assigned to the FFGs and FHGs, according to literature (Merritt and Cummins 1996; Tachet et al. 2000, Canobbio et al. 2010).
We used an ANCOVA model to evaluate the variation in the leaf breakdown rates and macroinvertebrate assemblages of leaf packs among leaf types and along impairment gradients. Within ANCOVA we set leaf types as treatment and 1st PC score (water quality gradient) and 2nd PC score (hydromorphological gradient) of PCA as the covariates (see Results section). Mass loss, number of individuals, taxa richness, Shannon Diversity Index (H'), FFGs and FHGs were used as dependent variables. For all tests, we set the threshold of significance $\alpha = 0.05$. All statistical analyses were performed using XLSTAT (Addinsoft, version 7) and R software (version 2.12).

**Table 1A** Variables and stream characteristics (means ± SD) among the six study sites, measured during low flow periods.

| **Site** | S1 | S2 | S3 |
|---|---|---|---|
| Stream | Lura | Seveso | Seveso |
| Slope (%) | 1.0 | 0.5 | 1.0 |
| Order | 2 | 2 | 2 |
| **Water quality** | | | |
| Temperature (°C) | 15.8 ± 6.9 | 20.1 ± 0.4 | 17.4 ± 4.2 |
| Conductivity (µS / cm) | 1372 ± 622 | 1101 ± 313 | 417 ± 66 |
| pH | 7.78 ± 0.43 | 7.63 ± 0.56 | 7.56 ± 0.58 |
| DO (%) | 94.48 ± 4.59 | 92.77 ± 10.85 | 98.40 ± 4.95 |
| TP (mg / L) | 2.10 ± 0.91 | 0.25 ± 0.07 | 0.13 ± 0.03 |
| TN (mg / L) | 18.81 ± 4.27 | 14.02 ± 5.88 | 3.43 ± 1.23 |
| $NH_4$-N (mg / L) | 0.06 ± 0.04 | 0.67 ± 0.55 | 0.07 ± 0.03 |
| COD (mg / L) | 53 ± 19 | 30 ± 14 | 17 ± 2 |
| *E. coli* (CFU / 100 ml) | 285 ± 21 | 543 ± 396 | 63 ± 35 |
| **Catchment and hydromorphological features** | | | |
| Catchment Area ($Km^2$) | 50.79 | 47.76 | 11.30 |
| Agrarian Area (%) | 44 | 40 | 40 |
| Woodland Area (%) | 27 | 32 | 36 |
| Urban Area (%) | 29 | 28 | 24 |
| Flow ($m^3$ / s) | 0.280 ± 0.007 | 0.449 ± 0.014 | 0.032 ± 0.005 |
| Stream width (m) | 5.8 | 8.4 | 3.8 |
| Current velocity (m / s) | 0.20 ± 0.17 | 0.30 ± 0.37 | 0.05 ± 0.07 |
| Water depth (m) | 0.17 ± 0.12 | 0.15 ± 0.09 | 0.20 ± 0.10 |

**Table 1B** Variables and stream characteristics (means ± SD) among the six study sites, measured during low flow periods.

| **Site** | S4 | S5 | S6 |
|---|---|---|---|
| Stream | Lura | Bozzente | Lura |
| Slope (%) | 0.6 | 0.5 | 1.0 |
| Order | 2 | 2 | 2 |
| **Water quality** | | | |
| Temperature (°C) | 10.5 ± 5.2 | 19.5 ± 4.7 | 14.6 ± 3.5 |
| Conductivity (µS / cm) | 403 ± 74 | 741 ± 24 | 463 ± 93 |
| pH | 7.97 ± 0.34 | 7.54 ± 0.35 | 7.99 ± 0.26 |
| DO (%) | 94.07 ± 33.06 | 67.35 ± 13.26 | 102.75 ± 12.34 |
| TP (mg / L) | 0.11 ± 0.13 | 4.96 ± 1.26 | 0.17 ± 0.10 |
| TN (mg / L) | 4.04 ± 1.29 | 14.70 ± 0.93 | 5.16 ± 1.19 |
| $NH_4$-N (mg / L) | 0.21 ± 0.29 | 2.44 ± 1.21 | 0.23 ± 0.34 |
| COD (mg / L) | 13 ± 15 | 27 ± 18 | 12 ± 4 |
| *E. coli* (CFU / 100 ml) | 2597 ± 525 | 2050 ± 1344 | 170 ± 44 |
| **Catchment and hydromorphological features** | | | |
| Catchment Area ($Km^2$) | 14.91 | 20.19 | 6.98 |
| Agrarian Area (%) | 47 | 44 | 45 |
| Woodland Area (%) | 38 | 36 | 43 |
| Urban Area (%) | 15 | 20 | 12 |
| Flow ($m^3$ / s) | 0.058 ± 0.001 | 0.058 ± 0.003 | 0.018 + 0.001 |
| Stream width (m) | 6.4 | 7.0 | 3.5 |
| Current velocity (m / s) | 0.09 ± 0.04 | 0.09 ± 0.06 | 0.04 ± 0.05 |
| Water depth (m) | 0.11 ± 0.08 | 0.16 ± 0.10 | 0.18 ± 0.10 |

## 2.3 Results

### *2.3.1 Environmental gradients*

Geographical, land use, water quality and hydromorphological variables were quantified for each sampling site (Table 1A-1B). They were used to investigate variable relations using PCA.
PCA output (Figure 2) showed few patterns that represent most of the dataset variability, because many variables were redundant.
Particularly, there were two clusters of variables that were significantly related (two-tails T-tests, $\alpha = 0.05$). The first cluster was composed by the water quality parameters. For example, DO was related with pH ($r = 0.914$), TP ($r = -0.945$), $NH_4$-N ($r = -0.904$) and COD ($r = -0.893$). The second cluster was constituted by most of the geographical, land use and hydromorphological data. For example, catchment area showed relations with woodland land use ($r = -0.896$), urban land use ($r = 0.844$), flow ($r = 0.926$) and current velocity ($r = 0.919$).
The linear combination of the variables resulted in two significant principal components, which accounted for 76.10% of the total variability of the dataset. The 1st PC axis (F1), explaining 42.84% of the variation, represented the water quality gradient and was principally outspread by pH, DO, TP, $NH_4$-N and COD. The 2nd PC axis (F2), explaining 33.26% of the variation, represented instead the hydromorphological gradient and was mainly explained by the woodland and urban land cover, catchment area, flow and current velocity (Table 2). The two significant principal components were used as environmental gradients (1st PC axis = water quality gradient; 2nd PC axis = hydromorphological gradient) in subsequent analyses.

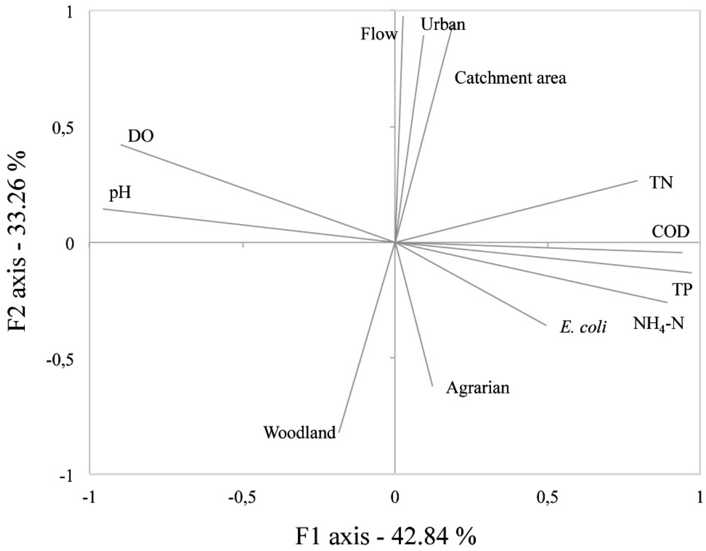


**Figure 2** PCA plot graph indicating relationships between environmental variables. Temperature, conductivity, stream width, water depth and current velocity variables have been graphically eliminated for clarity.

**Table 2** Eigenvectors of water quality, land use and hydromorphological variables. Loadings > 0.30 are shown in bold.

| | PC1 | PC2 |
|---|---|---|
| T | **0.340** | 0.060 |
| Conductivity | 0.262 | 0.214 |
| pH | **-0.344** | 0.060 |
| DO | **-0.323** | 0.172 |
| TP | **0.350** | -0.053 |
| TN | 0.286 | 0.108 |
| $NH_4$-N | **0.322** | -0.106 |
| COD | **0.338** | -0.019 |
| *E. coli* | 0.178 | -0.146 |
| Catchment area | 0.068 | **0.381** |
| Agrarian | 0.045 | -0.253 |
| Woodland | -0.066 | **-0.335** |
| Urban | 0.034 | **0.365** |
| Flow | 0.010 | **0.397** |
| Stream width | 0.188 | 0.251 |
| Current velocity | 0.043 | **0.387** |
| Water depth | -0.033 | -0.011 |

### 2.3.2 Leaf packs

Breakdown rates of leaf packs differed among sites and leaf types as reported in Table 3. Considering the whole of the sites, the greatest mass loss in leaf packs was observed in S2 (mean mass loss 76%), while the smallest in S3 (mean mass loss 40%). The three kinds of leaves were degraded differently: after 21 days of exposure, locust tree and native mix leaves were fragmented in pieces of different sizes, while laurel leaves showed delamination. Considering all sites, the mean mass loss of leaf packs was 60% for laurel, 50% for locust tree and 45% for native mix.

We collected aquatic macroinvertebrates belonging to 14 orders. A total of 45 taxa (genus and family) were identified. The taxonomic composition of the assemblages differed much among sites and this resulted in different values for H' and taxa richness (Table 3). Higher values of H' and taxa richness were found in S2, S3 and S6, while the number of individuals was higher in S2, S3 and S5. Large numbers of specimens belonging to taxa tolerant to pollution, such as Chironomidae, Oligochaeta and Trichoptera Hydropsychidae were found in all sites, whereas Bivalva, Hemiptera, Odonata and Plecoptera were completely absent in S1 and S5.

S3, S4 and S6 were dominated in numbers by Diptera (mostly Chironomidae), which accounted for 68%, 79% and 88% of the total individuals, respectively. Oligochaeta (principally Tubificidae and Naididae) were the most abundant individuals in S1 and S5 (83% and 75% respectively). S2 was dominated by both Diptera (44%) and Trichoptera (36%).

Other taxa, including Plecoptera and Ephemeroptera, were in general less abundant in the sampling sites. Plecoptera were found only in S3; Ephemeroptera were more abundant in S3 (4% of total specimens), while in the other sites their abundance was scarce (less than 1%). With the exclusion of S2, Trichoptera were scarce (always < 2%) in all the sites.

Crustacea (Gammaridae and Asellidae) were present in site S2 (10%), S3 (5%), S4 (< 1%) and S6 (4%). The remaining individuals belonged to Eteroptera, Coleoptera, Neuroptera, Odonata, Pulmonata, Veneroida and Arhyncobdellida orders and were always < 1% in every site.

Gathering-collectors (G-collectors) and burrowers were the dominant functional traits in all the sites and for all the leaf substrata (always > 50% of total specimen). Filtering-collectors (F-collectors) were more abundant in S2 for all leaf substrata (laurel: 37%; locust tree: 25%; native mix: 24%); in addition, S2 showed the highest concentration of sprawlers – in native mix leaves (18%). The other functional traits, such as predators, detritus-shredders (D-shredders), scrapers, clingers, climbers and swimmers, were less abundant and their presence was never over 15% of the total number of individuals in all sites and for all leaf types. Despite their limited presence, predators and D-shredders included large sized macroinvertebrates and so represented a high amount of the total mass (Figure 3).

**Table 3** Mass loss ratio of the three leaf types and macroinvertebrate metrics (mean ± SD) at the six study sites.

| Sites | Mass loss ratio | | |
|---|---|---|---|
| | Laurel | Locust tree | Native mix |
| S1 | 0.61 ± 0.14 | 0.58 ± 0.32 | 0.46 ± 0.13 |
| S2 | 0.84 ± 0.09 | 0.85 ± 0.13 | 0.59 ± 0.10 |
| S3 | 0.43 ± 0.07 | 0.34 ± 0.07 | 0.45 ± 0.11 |
| S4 | 0.55 ± 0.05 | 0.51 ± 0.07 | 0.36 ± 0.07 |
| S5 | 0.71 ± 0.09 | 0.34 ± 0.11 | 0.40 ± 0.08 |
| S6 | 0.48 ± 0.12 | 0.38 ± 0.14 | 0.42 ± 0.01 |
| Sites | Biological metrics | | |
| | Number of individuals per leaf pack | Taxa richness per leaf pack | H’ |
| S1 | 64 ± 35 | 5.3 ± 2.0 | 1.75 ± 0.66 |
| S2 | 178 ± 161 | 8.4 ± 1.5 | 1.73 ± 0.30 |
| S3 | 118 ± 51 | 8.8 ± 2.3 | 1.58 ± 0.45 |
| S4 | 63± 46 | 6.3 ± 2.5 | 1.14 ± 0.44 |
| S5 | 846 ± 685 | 4.3 ± 1.4 | 0.39 ± 0.34 |
| S6 | 82 ± 69 | 7.6 ± 3.3 | 1.20 ± 0.81 |

### *2.3.3 Mass loss and biological metrics response to environmental gradients*

According to ANCOVA analysis performed using the hydromorphological gradient (2nd PC axis) as covariate, mass loss variable showed a significant response ($p < 0.0001$) to the model. The variable value increased with the increasing of the hydromorphological gradient, as shown by the positive slope of the regressions. Treatment was significant ($p = 0.002$) and the three leaf substrata had different and significant degradation rate (Table 4B). Mass loss did not show a significant response to the ANCOVA model using 1st PC axis as covariate.

ANCOVA tests performed using the water quality gradient (1st PC axis) as covariate showed that number of individuals ($p = 0.006$), taxa richness ($p < 0.0001$) and H' ($p < 0.001$) had a significant response to the model. The covariate was highly significant, too ($p < 0.0001$ – see Table 4A).
The number of individuals increased with the increasing of the stream impairment (positive slope for all leaf substrata). The treatment (three leaf types) did not induce any significant difference. On the contrary, taxa richness and H' decreased with the increasing of the 1st PC values, as demonstrated by the negative slope for all leaf types. The response of taxa richness to water quality gradient was particularly strong for native leaves (slope = – 0.995 and $R^2 = 0.537$) and the differences among leaf packs were significant (treatment: $p = 0.013$).
G-Collectors (model: $p = 0.003$) and burrowers (model: $p < 0.01$) were significantly related to water quality gradient and were the only organisms that responded positively to increased impairment (positive slope of regressions). The other organisms, instead, were negatively influenced by the water quality gradient (negative slope of regressions).
Number of individuals, taxa richness and H' did not show a significant response to the hydromorphological gradient, even if taxa richness exhibited significant trends depending on leaf substratum (treatment: $p = 0.013$).
D-shredders, F-collectors, predators, and sprawlers responded significantly to the hydromorphological gradient (model: $p = 0.008$; $p = 0.034$; $p = 0.041$; $p = 0.005$, respectively). The other functional traits did not seem to have significant trends related to such gradient. FFG and FHG always showed no significant response to the treatment (no differences among the various kinds of leaf packs).

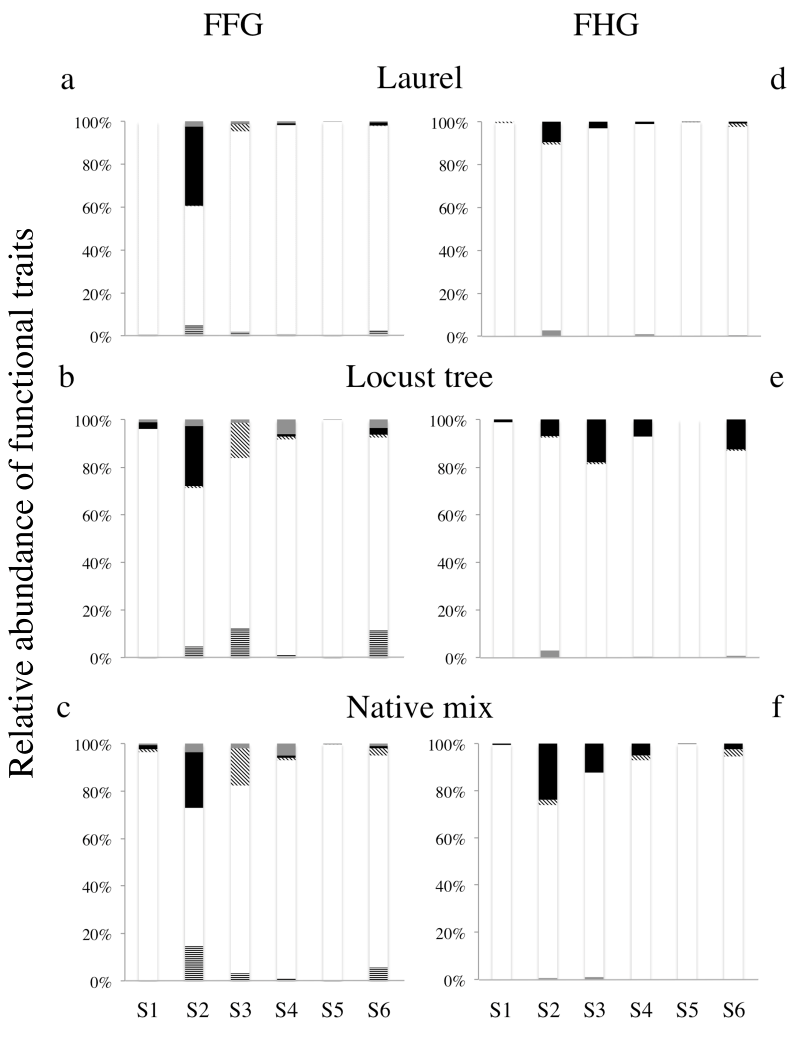


**Figure 3** FFG (a, b, c): G-collectors (white bars), F-collectors (black bars), predators (grey bars), scrapers (slanted lined bars), D-shredders (horizontal lined bars). FHG (d, e, f): burrowers (white bars), sprawlers (black bars), climbers (grey bars), swimmers (slanted lined bars); clinger (not visible; less than 3% in all the sites and for all leaf types).

**Table 4A** Summary statistics for ANCOVA model (N = 54; model df = 5; residual df = 48). Values shown in bold are significant ($p < 0.05$).

| | F-ratio | Model *P-value* | Covariate *P-value* | Treatment *P-value* | Laurel | | Locust Tree | | Native Mix | | Interaction *P-value* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Slope | $R^2$ | Slope | $R^2$ | Slope | $R^2$ | |
| *PC1 gradient* | | | | | | | | | | | |
| Mass loss | 2.124 | 0.079 | 0.889 | **0.029** | **0.031** | **0.116** | **-0.016** | **0.017** | **-0.020** | **0.123** | 0.234 |
| N° of individuals | **3.714** | **0.006** | **< 0.0001** | 0.970 | 89.986 | 0.221 | 158.316 | 0.341 | 92.741 | 0.216 | 0.520 |
| Taxa richness | **6.670** | **< 0.0001** | **< 0.0001** | **0.013** | **-0.474** | **0.123** | **-0.903** | **0.332** | **-0.995** | **0.537** | 0.410 |
| H’ | **5.772** | **< 0.001** | **< 0.0001** | 0.115 | -0.150 | 0.174 | -0.263 | 0.408 | -0.234 | 0.402 | 0.569 |
| D-Shredders | 1.721 | 0.148 | **0.020** | 0.372 | -0.881 | 0.109 | -1.844 | 0.176 | -2.332 | 0.102 | 0.691 |
| G-Collectors | **4.251** | **0.003** | **< 0.0001** | 0.950 | 95.722 | 0.261 | 163.122 | 0.356 | 100.886 | 0.252 | 0.528 |
| Scrapers | **2.723** | **0.03** | **0.006** | 0.313 | -0.447 | 0.117 | -1.795 | 0.109 | -3.041 | 0.260 | 0.235 |
| F-Collectors | 0.306 | 0.907 | 0.390 | 0.738 | -4.223 | 0.018 | -1.226 | 0.025 | -2.390 | 0.020 | 0.920 |
| Predators | 0.599 | 0.701 | 0.418 | 0.464 | -0.185 | 0.019 | 0.060 | 0.003 | -0.383 | 0.042 | 0.684 |
| Clingers | 0.535 | 0.749 | 0.147 | 0.824 | -4.669 | 0.022 | -3.083 | 0.114 | -5.511 | 0.103 | 0.945 |
| Climbers | 0.433 | 0.823 | 0.243 | 0.739 | -0.144 | 0.012 | -0.090 | 0.024 | -0.213 | 0.153 | 0.924 |
| Burrowers | **4.307** | **< 0.01** | **< 0.0001** | 0.948 | 95.820 | 0.262 | 163.605 | 0.358 | 102.286 | 0.258 | 0.529 |
| Swimmers | 0.923 | 0.475 | 0.210 | 0.277 | -0.112 | 0.019 | -0.118 | 0.152 | -0.290 | 0.041 | 0.834 |
| Sprawlers | **2.493** | **0.044** | **0.011** | 0.161 | -0.910 | 0.095 | -1.977 | 0.171 | -3.531 | 0.154 | 0.423 |

**Table 4B** Summary statistics for ANCOVA model (N = 54; model df = 5; residual df = 48). Values shown in bold are significant (p < 0.05).

| | F-ratio | Model *P-value* | Covariate *P-value* | Treatment *P-value* | Laurel | | Locust Tree | | Native Mix | | Interaction *P-value* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Slope | $R^2$ | Slope | $R^2$ | Slope | $R^2$ | |
| *PC2 gradient* | | | | | | | | | | | |
| Mass loss | **11.739** | **< 0.0001** | **< 0.0001** | **0.002** | **0.079** | **0.513** | **0.110** | **0.507** | **0.038** | **0.293** | 0.063 |
| N° of individuals | 0.033 | 0.999 | 0.916 | 0.978 | 10.503 | 0.002 | -20.664 | 0.004 | -2.283 | 0.000 | 0.947 |
| Taxa richness | 1.714 | 0.150 | 0.970 | **0.045** | **0.470** | **0.083** | **-0.251** | **0.018** | **-0.247** | **0.023** | 0.389 |
| H’ | 2.039 | 0.19 | **0.019** | 0.192 | 0.231 | 0.283 | 0.098 | 0.039 | 0.124 | 0.077 | 0.652 |
| D-Shredders | **3.553** | **0.008** | **0.017** | 0.318 | 1.960 | 0.370 | -0.999 | 0.036 | 4.833 | 0.301 | **0.014** |
| G-Collectors | 0.052 | 0.998 | 0.687 | 0.965 | -8.910 | 0.002 | -23.166 | 0.005 | -15.183 | 0.004 | 0.989 |
| Scrapers | 1.281 | 0.288 | 0.091 | 0.357 | -0.273 | 0.030 | -1.301 | 0.039 | -2.497 | 0.121 | 0.518 |
| F-Collectors | **2.642** | **0.034** | **0.003** | 0.688 | 16.853 | 0.198 | 4.808 | 0.261 | 9.593 | 0.221 | 0.326 |
| Predators | **2.530** | **0.041** | **0.010** | 0.402 | 0.872 | 0.294 | -0.006 | 0.000 | 0.970 | 0.184 | 0.171 |
| Clingers | 2.045 | 0.089 | **0.011** | 0.800 | 16.580 | 0.193 | 3.433 | 0.097 | 6.916 | 0.112 | 0.267 |
| Climbers | 1.964 | 0.101 | **0.015** | 0.706 | 0.653 | 0.170 | 0.337 | 0.234 | 0.082 | 0.016 | 0.265 |
| Burrowers | 0.052 | 0.998 | 0.686 | 0.963 | -9.230 | 0.002 | -23.039 | 0.005 | -15.175 | 0.004 | 0.989 |
| Swimmers | 0.718 | 0.613 | 0.467 | 0.284 | 0.177 | 0.033 | -0.037 | 0.010 | 0.225 | 0.017 | 0.791 |
| Sprawlers | **3.874** | **0.005** | **0.021** | 0.132 | 2.322 | 0.425 | -1.313 | 0.052 | 5.669 | 0.273 | 0.014 |

## 2.4 Discussion

We analysed the combined effects of different kinds of leaves (native and alien) and other environmental variables on the composition and abundance of macroinvertebrate assemblages colonizing leaf packs in impaired streams. We used environmental gradients to represent the various conditions that could be found in a system of streams, where reference sites were lacking. The evaluation of ecosystem dynamics in different leaf pack types could involve important management applications in river restoration. For example, finding that native leaves are responsible for increased invertebrate biodiversity or functional diversity could be a useful starting point for planning riparian vegetation restoration, a source of CPOM in streams. However, it is necessary to understand if other conditions, such as gradients of impairment or changes in hydromorphology, could influence the ecological patterns in impaired streams.
PCA was used to identify environmental gradients and to have a closer representation of those patterns. From PCA we obtained two gradients. The 1st PC axis represented the water quality gradient, principally due to the input of untreated or inadequately treated wastewater. The second gradient obtained from PCA (2nd PC axis) was interpreted as a hydromorphological gradient, chiefly related to the longitudinal variation of streams. This variation was due to habitat changes, induced by both the natural increase of flow and different land uses.
Statistical analysis did not show a significant relation between the mass loss and the water quality gradient, while it showed a highly significant ($p < 0.0001$) relationship with the hydromorphological gradient. We hypothesized that the main driving factor in this gradient influencing mass loss could be flow. Thus, increasing flow positively influenced the loss rate. The treatment resulted significant for both gradients ($p = 0.029$ and $p = 0.002$, respectively). Laurel, locust tree and native mix packs showed differences in the loss rate as well, probably due to their different structural properties.
The analysis of macroinvertebrate assemblages showed that the overall taxonomic and functional diversity was low, because all sites were located in an impaired basin. However, differences were seen especially

along the water quality gradient, where changes in abundance and distribution of macroinvertebrates could be observed. ANCOVA model showed that taxa richness decreased ($p < 0.0001$) and the number of individuals increased ($p = 0.006$) with increasing impairment because of the drop of sensitive taxa and of the proliferation of tolerant taxonomic groups, such as Oligochaeta and Chironomidae. The different kinds of leaf packs caused significant changes ($p = 0.013$) only for taxa richness, probably due to the different value of leaf types as refuges (Davies & Boulton, 2009). On the other hand, ANCOVA model principally showed that the different leaf types did not cause significant variations in the distribution of all macroinvertebrate functional traits. ANCOVA model for D-shredders, the most active functional feeding group involved in leaf decomposition, was not significant with the 1st PC gradient, but the covariate was significant ($p = 0.020$) and the slopes of the three leaf substrata (laurel slope = − 0.881; locust tree slope = − 1.844; native mix slope = − 2.332) pointed out that the number of D-shredders was inversely related to the 1st PC gradient, underlining that the use of leaves as food by macroinvertebrates is influenced more by water quality than leaf type in impaired sites. The response of G-collectors and burrowers, the most abundant FFG and FHG, to water quality was similar (significant relation with 1st PC gradient; model: $p = 0.003$; $p < 0.01$), showing no significance with treatment (colonization was similar in all leaf types).
Our data seemed to be in contrast to those found in literature. Many authors (i.a. Graça 2001; Ormerod et al. 1993) demonstrate that different kinds of leaf packs could modify macroinvertebrate distribution in pristine freshwater ecosystems, although Lacan et al. (2010) reported that alien leaves did not influence taxonomic composition of macrobenthos. We found that only taxa richness was significantly influenced by different kinds of leaf packs. Our results for all the other biological metrics were influenced by water quality impairment. The water quality along all the impairment gradient, even in sites showing a lower level of pollution, acted as a limiting factor in determining ecological patterns, such as composition of macroinvertebrate assemblages in leaf packs. It is well known that the major stressors affecting the integrity of streams, and thus the

distribution of the macroinvertebrate assemblages, are urbanization (Walsh et al. 2005), organic pollution, nutrient enrichment (Coimbra et al. 1996; Spänhoff et al. 2007) and alterations of hydromorphology (Nelson and Lieberman 2002; Wills et al. 2006). These conditions are all met in Seveso, Lura and Bozzente streams and influenced leaf packs colonization by macrobenthos.
ANCOVA model showed that the mass loss increased with the 2nd PC axis ($p < 0.0001$, positive slope for all leaf substrata), probably due to the greater flow in sites with larger catchment areas. In fact, local hydrology and hydraulic gradient can influence many stream patterns and processes (Vervier and Naiman 1992; Wagner et al. 1993; Marmonier et al. 1995), as well as natural leaf pack degradation (i.a. Tillman 2003). Thus, it was probable that, in relation to 2nd PC gradient, the main cause of leaf degradation was the mechanical action of water. More copious flows resulted in more pronounced leaf degradation.
Number of individuals, taxa richness and H' did not show a significant response to the ANCOVA model using hydromorphological gradient as the covariate. However, taxa richness in the three kinds of leaf packs laid out different trends: in laurel leaves, the number of taxa increased with the increase of 2nd PC gradient score, while in locust tree and native leaves the opposite situation occurred. The different structure of laurel leaves probably offered refuge to a higher number of taxa.
Shifts in macrobenthos assemblages may be due to the changes of stream flow (Mérigoux and Dolédec 2004; Dolédec et al. 2007). Our results show that the response of D-shredders (model: $p = 0.008$), F-collectors (model: $p = 0.034$), predators (model: $p = 0.041$) and sprawlers (model: $p = 0.005$) was significantly related to the 2nd PC gradient. In general, the slopes for each leaf substrata are positive, meaning that a better diversification of functional groups occurs with the hydromorphological diversification following natural longitudinal gradient, even in streams of the same orders.

D-shredders presented a good correlation with mass loss of leaf packs, in particular for laurel and native mix leaves (linear regression: p =

0.022; $p < 0.001$ respectively). This correlation would confirm the importance of macroinvertebrates in the leaf decomposition.
Of course, a comprehensive balance of the leaf mass loss should include microbial activity. Fungi, bacteria and actynomicetes are known to play an important role in the process (Gulis and Suberkropp 2003) and their colonization seems to affect positively the subsequent attack by macroinvertebrates (Wohl and McArthur 2001). Despite worse quality waters are richer in nutrients, organic matter and bacteria, in our case, no significant correlation has been found between water quality gradient and mass loss.
G-collectors and Burrowers responded strongly to the 1st PC gradient, but every relation was absent against the 2nd PC axis. While the presence of these groups was often correlated with the nutrient enrichment and the deposition of organic matter, as discussed above, no relation with the size of the catchment and therefore with the increase of flow was observed.

## 2.5 Conclusions

The adopted approach permitted to quantify the variation of data due to different sources (different leaf types and environmental gradients) and to evaluate their mutual influence. The results showed that leaf type influenced only taxa richness, while environmental gradients related to water quality and hydromorphology influenced most of the measured macroinvertebrate metrics.
From this point of view, it is possible to use this approach to determine the priority of river restoration interventions. The restoration of native riparian vegetation and, in general, interventions focused on the improvement of habitat quality are important and coherent to the objectives of the European Water Framework Directive. However, our results show that water quality is the main driving factor causing changes in the macroinvertebrate assemblages of impaired streams. Thus, our research demonstrates that, in the examined basin, a better treatment of wastewater should be the priority in river restoration programs in order to obtain the enhancement of macroinvertebrate functional diversity.

## 2.6 Acknowledgements

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# 3 INFLUENCE OF LEAF TYPES, EXPOSURE TIME AND WATER POLLUTION ON LEAF PACK BREAKDOWN AND MACROINVERTEBRATE COLONIZATION

**Abstract**

Invasion by exotic plant species is known to affect terrestrial systems and macroinvertebrate communities of neighbouring streams. Macroinvertebrate prefer some types of detritus over others, because of chemical composition, physical structure and levels of degradation and microbial conditioning of leaf substrata. Detritus characteristics can vary with exposure time in freshwater and so the attractiveness of detritus can also change with time.

The objectives of our work are (1) to analyse the effect of different leaf types, exposure time and environmental variables on the breakdown of leaf pack breakdown and macroinvertebrate colonization and (2) to understand is native riparian vegetation restoration is a useful tool for habitat quality improvement in *high modified water bodies* (HMWBs).

Our results pointed out that, in the studied HMWB, leaf breakdown varied significantly in relation to exposure time, while macroinvertebrate colonization of leaf packs was influenced simultaneously by time and by water quality, significantly. Substrate type affected only taxa richness. Native and locust tree leaf packs showed the maximum values of taxa richness and probably provided both shelter and food for macroinvertebrate assemblage.

Despite positive result of native and locust tree leaves in improving biodiversity, water quality influences more than leaf types the macroinvertebrate assemblages colonizing leaf packs. In the examined HMWB, a better treatment of wastewater should be the priority in river restoration programs in order to obtain the enhancement of macroinvertebrate functional diversity and should be preliminary to restoration of native riparian vegetation.

**Keywords**

Macroinvertebrates, functional traits, leaf packs, high modified water bodies

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Influence of leaf types, exposure time and water pollution on leaf pack breakdown and macroinvertebrate colonization

## 3.1 Introduction

Species diversity and factors responsible for its maintenance or decline are key issues in ecology. Streams receive substantial amounts of carbon inputs in the form of detritus from adjacent terrestrial habitats (Cummins et al., 1989; Benfield, 1997). Detritus inputs affect ecosystem function within aquatic systems (Wallace et al., 1997). Additionally, a change in the species of leaves entering a stream is known to alter the structure of macroinvertebrate communities and decomposition (Smock & MacGregor, 1988, Swan &Palmer, 2004). Invasion by exotic plant species is known to affect terrestrial systems (Levine et al., 2003), and additional studies indicate that replacement of native riparian tree species with exotics is likely to affect the ecosystem function and macroinvertebrate communities of neighbouring streams (Swan et al., 2008).

Such changes can modify detritus processing through changes in microbial communities or macrodetritivorous colonization (Barlocher, 2005; Reinhart &VandeVoort, 2006). All these changes finally alter nutrient cycling and community structure of the aquatic ecosystems.

Macrodetritivorous invertebrates play a key role in the breakdown process of the allochthonous material in aquatic ecosystems because they fragment coarse particulate organic matter (CPOM, diameter > 1 mm) into fine particulate organic matter (FPOM, diameter between 1 and 0.0005 mm) (Cummins et al., 1973) accessible to microorganisms, therefore contributing to recycling of nutrients.

Some types of detritus types are more attractive to invertebrates than others, as a function of their chemical composition, physical structure, and levels of degradation and microbial conditioning. Since the characteristics of detritus vary with the time of exposure in the water, the attractiveness of detritus can also change with time (Abelho, 2001; Graca et al., 2001). Several studies in temperate regions have demonstrated the importance of invertebrates, especially shredders, in the decomposition of leaf detritus (Webster & Benfield, 1986; Haapala et al., 2001, Graca, 2001).

The input of organic matter derived from deciduous leaves is an important point also in basins affected by anthropogenic activities.

These basins were defined according to the European Water Framework Directive "highly modified water bodies" (HMWBs). They distinguished by strongly chemical and hydromorphological alterations (Taylor, 2002); the presence of high concentrations of pollutants and habitat loss (e.g. artificial channels, sparsely vegetated river banks) contributed to the biodiversity loss and to a decrease in ecosystem functionalities (Boyle et al., 2003; Canobbio et al., 2008).
To have a better comprehension about the dynamics of macroinvertebrate colonization of retention structures (such as leaf packs) in HMWBs we analysed the effect of different leaf types, exposure time and environmental variables on the breakdown of leaf pack breakdown and macroinvertebrate colonization.

## 3.2 Materials and methods

### *3.2.1 Study area*

Lura stream is 45 km long and passes through 17 municipalities in Lombardy (Italy), north of Milan, as shown in Fig. 1. Its catchment (about 130 km2) is long and narrow, as is the typical case of lowland streams in this area. Lura receives water from superficial groundwater, wet meadows and small creeks, both on right and left side, and merges into the Olona river at Rho, close to Milan. Impairment is due to both the widespread urbanization, generating high polluting loads and catchment imperviousness, and to the presence of several industrial settlements.
WWTP discharges constitute most, and sometimes the only, stream flow. One of the existing WWTPs, Alto Lura, about 150,000 Equivalent Inhabitants (EI), discharge directly into Lura, the another one (Livescia, about 40,000 EI) into a small tributary, called Livescia.
We analysed two sites (hereafter called S1 and S2) in Lura stream (Figure 1). The sites are similar in order and slope, but different for water quality and catchment land use.
In the sampling sites chemical and hydromorphological parameters were measured during low flow periods, while land cover characteristics (expressed as a percentage of the total basin area) were analysed with GIS software QGis, version 1.8 (Table 1). Water samples

were collected in the two sites: water quality analyses included temperature, electric conductivity, dissolved oxygen (DO), pH, COD, total phosphorus (TP), total and ammonia nitrogen (TN and $NH_4$–N), and *Escherichia coli*.
Alder (*Alnus glutinosa*), willow (*Salix alba*) and poplar (*Populus nigra*) are the typical riparian species and are present the two sampling sites. Alder is the most abundant, whereas willow and poplar are less copious. In the outermost zone the vegetation is chiefly made of oak (*Quercus robur*) and white hornbeam (*Carpinus betulus*). In the same area, locust-trees (*Robinia pseudoacacia*) have been introduced from centuries and are now widely spread across the basin. We examined natural leaf packs that could be found in streams and found them mainly composed by alder, oak, white hornbeam and locust-tree. In the urban portion of the basin laurel is being used for hedgerows, and we found packs of the trimmed laurel (*Prunus laurocerasus*) leaves downstream towns and cities.

### *3.2.2 Experimental design and data analysis*

Artificial leaf packs were placed in the study sites. We prepared three different kinds of leaf packs representative of native, naturalized and alien vegetation. The fallen leaves and twigs needed for leaf pack preparation were collected in April 2011. Leaves and twigs of alder, oak and white hornbean (native mix leaves) and locust-tree (naturalized leaves), common in riparian vegetation, were collected from a forest in the Lura stream basin. Leaves and twigs of laurel (alien “urban” leaves) were collected from urban parks in the same basin. Only intact leaves were used for the experiment. Leaves and twigs were brought to the laboratory shortly after collection and dried for 24 hours at 105°C to obtain standardized moisture contents (Spanhoff *et al*., 2007).
Leaves and twigs were placed in commercial net bags 15 x 40 cm (10 mm mesh size). Each net bag was filled with ca. 12 g of leaf litter and ca. 3 g of twigs and identified by an assigned number. We placed in the riverbeds a total of 108 leaf packs (54 packs for each site, 18 packs per leaf type); all packs were tied with polyester threads to metal rods that were knocked vertically into the sediment.

After 1, 2 and 3 weeks 36 leaf packs (18 packs for each site, 6 packs per leaf type) were removed from all sites and immediately transferred to the laboratory. Leaves were washed over 500 $\mu$m sieves and dried for 24 hours at 105° C in order to determine the remaining mass (Spanhoff *et al*., 2007). Mass loss ratio was calculated between initial and final dry mass of leaves. All macroinvertebrates found in leaf packs were stored in 90% ethanol and identified at genus level, except for Diptera order and Oligochaeta subclass that were identified at family level, using an Optika stereomicroscope (180x) and taxonomic keys (Campaioli *et al*., 1999; Sansoni, 1992). Macroinvertebrates were assigned to the function feeding groups (FFGs), according to literature (Merritt & Cummins, 1996; Tachet *et al*., 2000, Canobbio et *al*., 2010): shredders, scrapers, gatherer-collectors (G-collectors), filterer-collectors (F-collectors) and predators groups were assigned.
We used a three-way ANOVA model to evaluate the variation in the leaf breakdown rates and macroinvertebrate assemblages of leaf packs among leaf types, sites and time. Within ANOVA we set leaf types, sites and time as factor. Remained mass, taxa richness and FFGs were tested. In all ANOVA tests, where significant differences were detected, Tukey's post-hoc tests were used to determine which groups were significantly different. For all tests, we set the threshold of significance $\alpha = 0.05$. All statistical analyses were performed using XLSTAT (Addinsoft, version 7) and R software (free version 2.12).

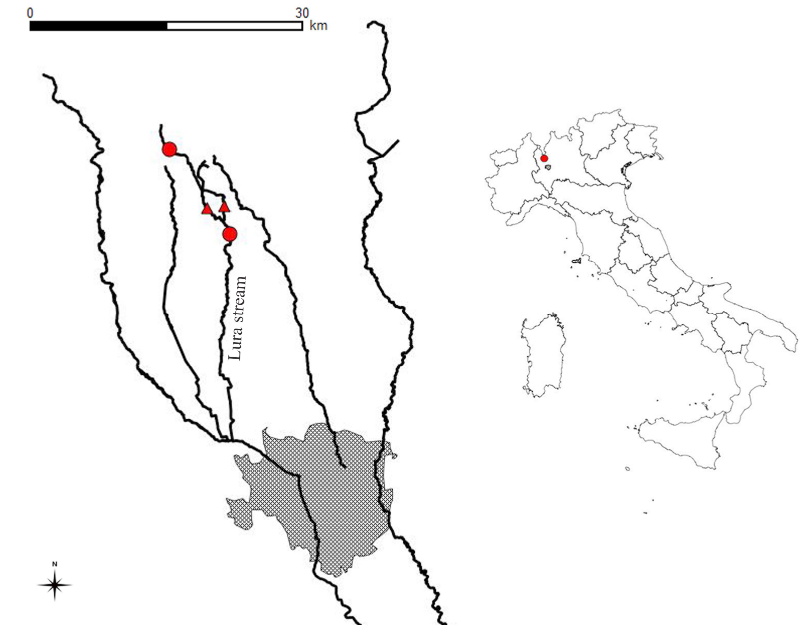


**Figure 1** Study sites (dots) and WWTPs (triangles) localization.

**Table 1** Physico-chemical and hydromorphological parameters at two sampling sites.

| **Water quality** | | |
|---|---|---|
| **Parameters** | **S1** | **S2** |
| T (°C) | 16.0 ± 3.8 | 17.4 ± 5.9 |
| Conductivity (ms/cm) | 437 ± 77 | 1057 ± 529 |
| pH | 7.8 ± 0.5 | 7.7 ± 0.4 |
| OD% | 100.33 ± 8.64 | 80.91 ± 17.16 |
| OD (mg/l) | 11.02 ± 1.72 | 8.12 ± 2.54 |
| P tot (mg/l) | 0.15 ± 0.07 | 3.53 ± 1.84 |
| N tot (mg/l) | 4.20 ± 1.45 | 16.75 ± 3.60 |
| $NH_4$-N (mg/l) | 0.14 ± 0.22 | 1.65 ± 1.55 |
| COD (mg/l) | 7 ± 4 | 40 ± 22 |
| *E. coli* (UCF/100 ml) | 117 ± 76 | 1168 ± 124 |
| **Catchment and hydromorphological features** | | |
| **Parameters** | **S1** | **S2** |
| Catchment Area ($Km^2$) | 6.98 | 50.79 |
| Agrarian Area (%) | 45 | 44 |
| Woodland Area (%) | 43 | 27 |
| Urban Area (%) | 12 | 29 |
| Flow ($m^3$ / s) | 0.018 + 0.001 | 0.280 ± 0.007 |
| Stream width (m) | 3.5 | 5.8 |
| Current velocity (m / s) | 0.04 ± 0.05 | 0.20 ± 0.17 |
| Water depth (m) | 0.18 ± 0.10 | 0.17 ± 0.12 |

## 3.3 Results

After the treatment period, we were forced to discard one laurel packs because after 9 weeks we found it in a dry riffle.
Remaining mass of leaf packs was significantly related to exposure time (Table 2A). After a phase of slow loss, the decrease was very rapid between 6 and 9 treatment weeks (ANOVA: $p < 0.0001$), with no differences among leaf types within the same time (Fig. 2). No differences were found between sites.
Entirely, in the leaf packs we collected 18,442 macroinvertebrates belonging to 65 taxa. The maximum values of taxa richness were recorded in the native mix and locust tree leaves with values that reach 20 taxa per pack in S1 after 3 treatment weeks. Taxa richness was significantly related to site, exposure time and substrate (Table 2A). Taxa richness strongly decreased among exposure time and between sites. In particular, Tukey's test showed that taxa richness after 3 weeks was significantly different between S1 and S2 ($p < 0.01$); this situation occurs also after 6 weeks ($p < 0.05$). After 9 weeks no differences between sites are shown. Independently, native mix and locust tree was significantly different among exposure times in S1, while laurel did not show this trend. In S2 no differences were found among leaf types and among exposure times (Fig. 3).
As shown by ANOVA results, sites and exposure time significantly influenced shredder abundance. While in S1 no differences between 6 and 9 treatment weeks were shown (Fig. 4), after 9 weeks in S1 (S1: *6 weeks vs 9 weeks*, Tukey's test: $p < 0.01$) and at all exposure time in S2 there was a reduction of the shredder abundance. We not found significant differences among leaf types. Nevertheless, the locust tree and native mix leaves clearly attracted a greater number of shredders with a maximum of 23 shredder specimen per pack in S1.
G-collectors were more abundant in S2 at 3 and 6 treatment weeks (Fig. 5). The greater concentration of G-collectors was recorded in laurel and locust tree leaves in S2 with a maximum of 2000 G-collector specimen per pack after 6 treatment weeks. After that, there was a strong decrease of G-collectors after 9 weeks in S2 (S2: *6 weeks vs 9 weeks*, Tukey's

test: $p < 0.01$). In S1 few individuals were present in leaf packs. Sites and exposure time significantly influenced G-collector abundance.
About F-collectors, no significant differences were found between sites, among exposure times and leaf types. We found a maximum of 5 filterers per packs (Fig. 6).
Scrapers were significantly influenced by sites and exposure times, as shown by ANOVA results (Table 2B). Despite no significant differences were found among leaf types, we observed a preference of scrapers respect native mix leaves with packs that recorded up to 38 scraper individuals in S1 after 6 treatment weeks (Fig. 7).
Sites, exposure times and substrate significantly influenced predator abundance in leaf packs. In S1 after 3 treatment weeks, predator abundance in native mix differed from abundance in laurel (Tukey's test: $p < 0.01$) and in locust tree (Tukey's test: $p < 0.05$).
Total individuals reflected the G-collector trends. The entire amount of G-collector was equal to 17,728 individuals, 96% of total collected macroinvertebrates. Sites and exposure time significantly influenced total number of individuals in leaf packs (respectively, $p < 0.01$ and $p < 0.01$), despite low significance of general model ($p = 0.052$). The maximum number of individuals was registered in locust tree after 6 treatment weeks in S2.

**Table 2A** 3-way ANOVA results. Site, Time, Substrate and their interaction are tested. Only significant tests are shown.

| Biological metrics | | DF | Sum of squares | Mean of squares | F value | P value |
|---|---|---|---|---|---|---|
| Remained mass | Model | 13 | 6.544 | 0.503 | 17.043 | **< 0.0001** |
| n = 107 | Residuals | 93 | 2.747 | 0.030 | - | - |
| | Total | 106 | 9.291 | - | - | - |
| | Time | 2 | 5.902 | 2.951 | 99.912 | **< 0.0001** |
| | Time*Substrate | 4 | 0.425 | 0.106 | 3.593 | **0.009** |
| Taxa richness | Model | 13 | 903.682 | 69.514 | 8.541 | **< 0.0001** |
| n = 107 | Residuals | 93 | 756.935 | 8.139 | - | - |
| | Total | 106 | 1660.617 | - | - | - |
| | Site | 1 | 152.507 | 152.507 | 18.738 | **< 0.0001** |
| | Time | 2 | 557.101 | 278.551 | 34.224 | **< 0.0001** |
| | Substrate | 2 | 61.511 | 30.756 | 3.779 | **0.026** |
| | Site*Time | 2 | 100.302 | 50.151 | 6.162 | **0.003** |
| Shredder abundance | Model | 13 | 759.329 | 58.410 | 3.315 | **0.0004** |
| n = 107 | Residuals | 93 | 1638.634 | 17.620 | - | - |
| | Total | 106 | 2397.963 | - | - | - |
| | Site | 1 | 306.525 | 306.525 | 17.397 | **< 0.0001** |
| | Time | 2 | 141.056 | 70.528 | 4.003 | **0.021** |
| | Site*Time | 2 | 117.681 | 58.841 | 3.339 | **0.040** |

**Table 2B** 3-way ANOVA results. Site, Time, Substrate and their interaction are tested. Only significant tests are shown.

| | | | | | | |
|---|---|---|---|---|---|---|
| G-collector abundance | Model | 13 | 2921211.082 | 224708.545 | 2.357 | **0.009** |
| n = 107 | Residuals | 93 | 8866522.114 | 95338.947 | - | - |
| | Total | 106 | 11787733.196 | - | - | - |
| | Site | 1 | 1212224.631 | 1212224.631 | 12.715 | **0.001** |
| | Time | 2 | 1025358.105 | 512679.052 | 5.377 | **0.006** |
| Scraper abundance | Modello | 17 | 1715.263 | 100.898 | 2.307 | **0.006** |
| n = 107 | Residui | 89 | 3893.167 | 43.743 | - | - |
| | Totale | 106 | 5608.430 | - | - | - |
| | Site | 1 | 437.829 | 437.829 | 10.009 | **0.002** |
| | Time | 2 | 401.386 | 200.693 | 4.588 | **0.013** |
| Predator abundance | Modello | 17 | 107.304 | 6.312 | 4.478 | **< 0.0001** |
| n = 107 | Residui | 89 | 125.462 | 1.410 | | |
| | Totale | 106 | 232.766 | | | |
| | Site | 1 | 21.309 | 21.309 | 15.116 | **0.000** |
| | Time | 2 | 42.599 | 21.299 | 15.109 | **< 0.0001** |
| | Substrate | 2 | 18.746 | 9.373 | 6.649 | **0.002** |
| | Site*Time | 2 | 9.485 | 4.742 | 3.364 | **0.039** |
| Total ind. | Modello | 17 | 2913852.824 | 171403.107 | 1.725 | 0.052 |
| n = 107 | Residui | 89 | 8842551.681 | 99354.513 | - | - |
| | Totale | 106 | 11756404.505 | - | - | - |
| | Site | 1 | 1175027.003 | 1175027.003 | 11.827 | **< 0.001** |
| | Time | 2 | 1046967.526 | 523483.763 | 5.269 | **< 0.01** |

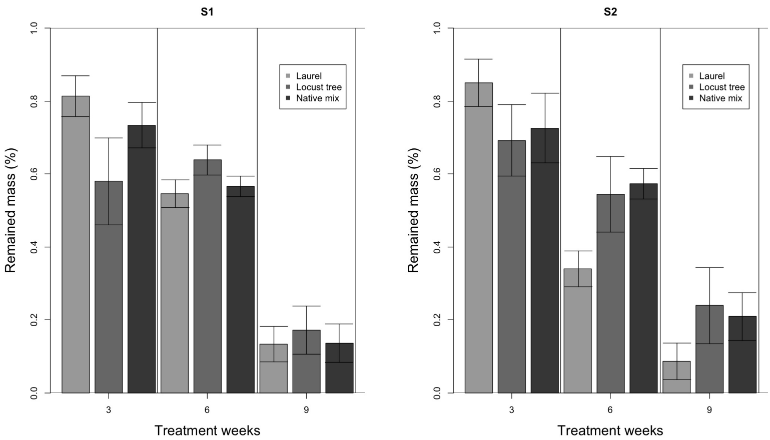


**Figure 2** Mean (±SE) percentage of remained mass in dry weight of laurel, locust tree and native mix leaf packs after 3, 6 and 9 treatment weeks.

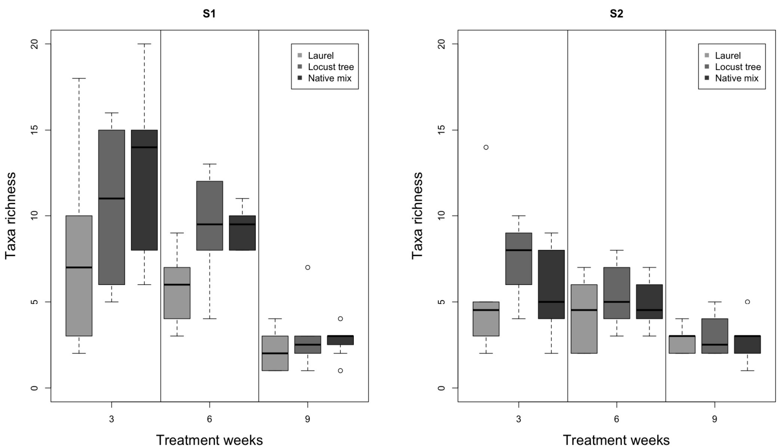


**Figure 3** Boxplot of taxa richness per packs of laurel, locust tree and native mix leaf packs after 3, 6 and 9 treatment weeks. Median (black horizontal line), first and third quartile (box extremes), confidence interval endpoints (whiskers) and outliers (dots) are indicated.

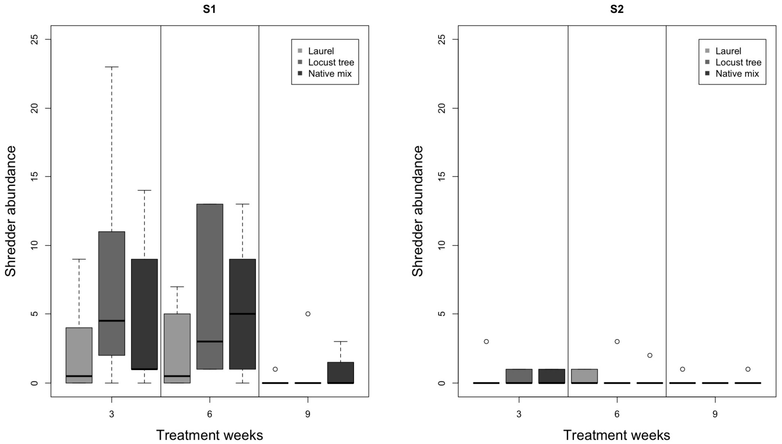


**Figure 4** Boxplot of shredder abundance per packs of laurel, locust tree and native mix leaf packs after 3, 6 and 9 treatment weeks. Median (black horizontal line), first and third quartile (box extremes), confidence interval endpoints (whiskers) and outliers (dots) are indicated.

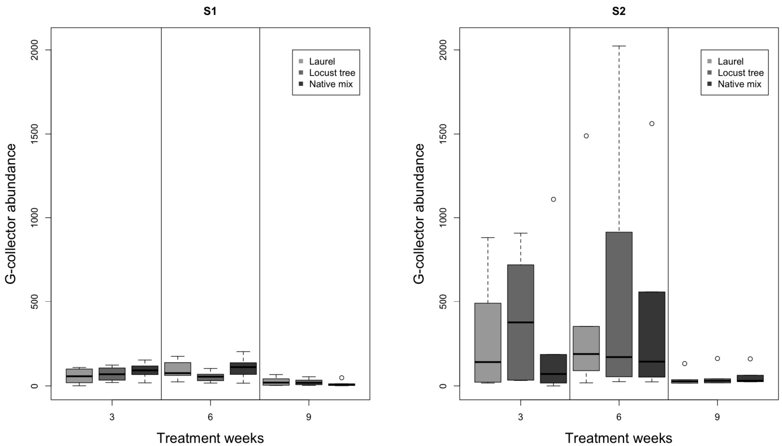


**Figure 5** Boxplot of G-collector abundance per packs of laurel, locust tree and native mix leaf packs after 3, 6 and 9 treatment weeks. Median (black horizontal line), first and third quartile (box extremes), confidence interval endpoints (whiskers) and outliers (dots) are indicated.

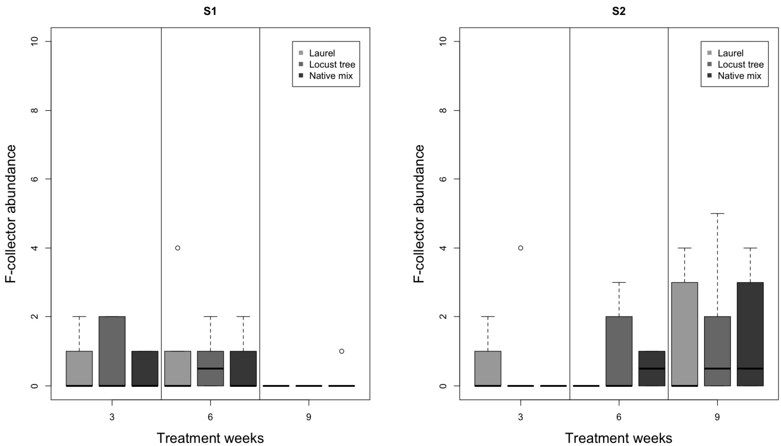


**Figure 6** Boxplot of F-collector abundance per packs of laurel, locust tree and native mix leaf packs after 3, 6 and 9 treatment weeks. Median (black horizontal line), first and third quartile (box extremes), confidence interval endpoints (whiskers) and outliers (dots) are indicated.

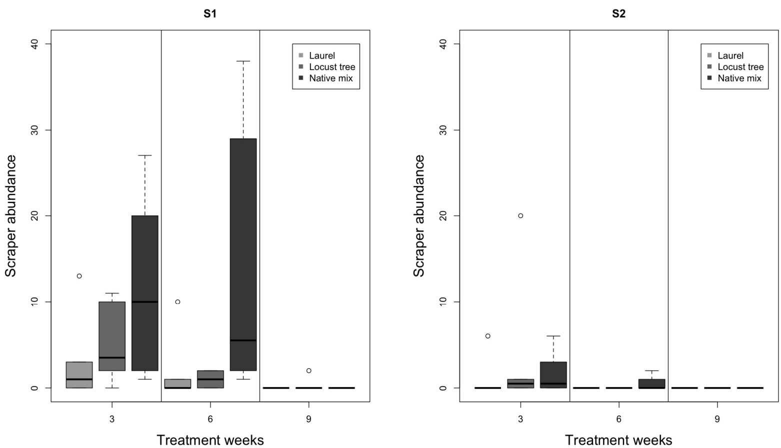


**Figure 7** Boxplot of scraper abundance per packs of laurel, locust tree and native mix leaf packs after 3, 6 and 9 treatment weeks. Median (black horizontal line), first and third quartile (box extremes), confidence interval endpoints (whiskers) and outliers (dots) are indicated.

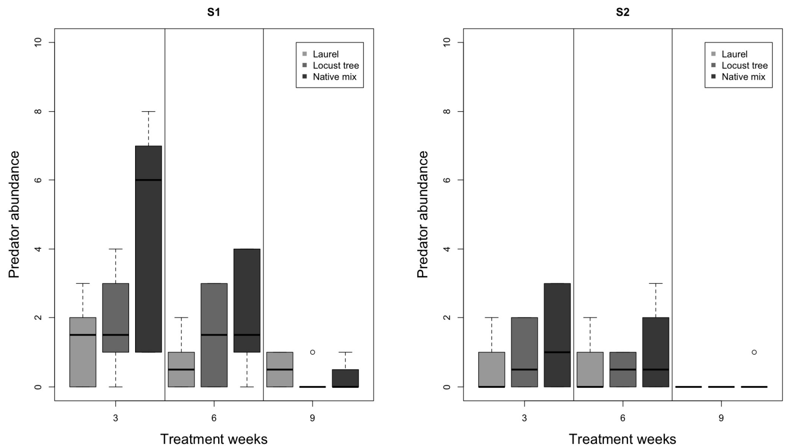


**Figure 8** Boxplot of predator abundance per packs of laurel, locust tree and native mix leaf packs after 3, 6 and 9 treatment weeks. Median (black horizontal line), first and third quartile (box extremes), confidence interval endpoints (whiskers) and outliers (dots) are indicated.

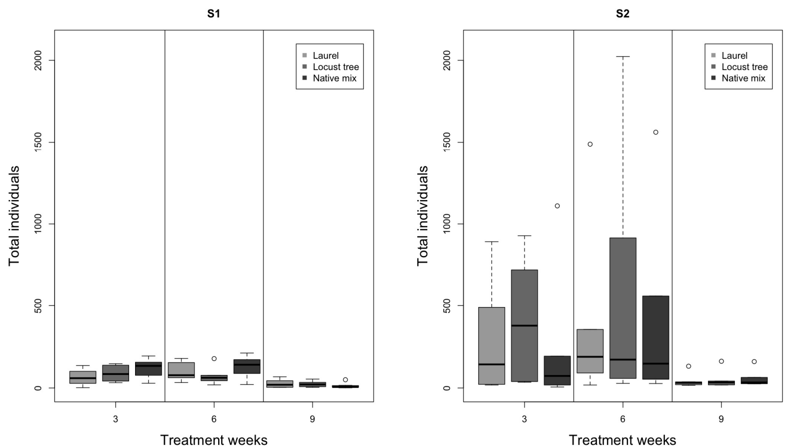


**Figure 9** Boxplot of total individuals per packs of laurel, locust tree and native mix leaf packs after 3, 6 and 9 treatment weeks. Median (black horizontal line), first and third quartile (box extremes), confidence interval endpoints (whiskers) and outliers (dots) are indicated.

## 3.4 Discussion

We analysed the combined effects of different kinds of leaves (native and alien), exposure time and environmental variables on the composition and abundance of macroinvertebrate assemblages colonizing leaf packs in impaired streams. The analysed stream is part of an HMWB. HMWBs have unique water quality characteristics that, in most cases, are comparatively different from normal stream conditions upstream of the discharge or at regional reference sites (Taylor, 2002; Brooks et al., 2004). The increase of urban development often results in the absence of reference sites in HMWBs (Chessman and Royal, 2004) and this leads in difficulties to define a target condition for restoring urban stream sites (Meyer et al., 2005).

In this situation, the evaluation of ecosystem dynamics in different leaf pack types could involve important management applications in river restoration. For example, to know how different leaf types influence macroinvertebrate assemblages could be a useful starting point for planning riparian vegetation restoration, a source of CPOM in streams. This is inevitably accompanied by the study of the interactions between macroinvertebrate communities and other environmental conditions, first of all, the water chemistry.

In this study, we show that leaf breakdown in HMWBs varied significantly in relation to exposure time, while macroinvertebrate colonization of leaf packs was influenced simultaneously by time and by water quality.

Exposure time has been shown to be a crucial factor in the breakdown and macroinvertebrate colonization of leaf packs by several works (i.a. Fenoglio et al., 2006; Sanpera-Calbet et al. 2009; Ligeiro et al., 2010). In our results, exposure time significantly influenced remained mass of leaf packs (for all substrate), taxa richness and FFGs, with the exception of F-collectors. The small number of filterers found probably did not make statistical tests significant. Anyway, this functional trait did not show a distinct trend, both over time and between the two considered sites (Fig. 6).

The main difference between the study sites is the water chemistry, in particular the values of OD, total P, total N, ammonia nitrogen and

COD (Table 1). Despite the different sites seemed not to influence breakdown of leaf packs (Remaining mass: *S1 vs S2*, ANOVA: p = ns), taxa richness and FFG abundance were significantly affected by site differences. Taxa richness decrease in S2 was probably ascribable to poor water quality. S2 was deeply impacted by WWTP effluents as describe previously. Other studies have shown the water quality affect differently the leaf pack breakdown and macroinvertrate colonization (i.a. Spanhoff et al., 2007).
The substrate type seemed not to affect weight loss. There were no significant differences between values of remained mass of different substrates at the same site and after the same exposure time. Taxa richness was affected by the substrate type. Different leaves have led to different significantly different trends of taxa richness, as shown in Figure 3 (ANOVA: substrate, p = 0.026). Native mix and locust tree showed the maximum values of taxa richness. These kind of leaves probably provided both shelter and food as reported by many works (e.g. Richardson, 1992; Reinhart & VandeVoort, 2006; Davies & Boulton, 2009), promoting an increase in taxa richness values both in S1 and in S2 where the water chemistry negatively acts on biological communities.

## 3.5 Conclusions

An understanding of the ecological dynamics that regulate the degradation and colonization of leaf packs in a HMWB are useful for planning riverbanks restoration programs. Our work indicates that different leaf types do not show different trend of colonization. In any case, the reintroduction of native species on the riverbanks is preferred as native leaves tend to increase biodiversity values.
The improvement of habitat quality with the restoration of native riparian vegetation is important and coherent to the objectives of the European Water Framework Directive. However, our results show that water quality is the main driving factor causing changes in the macroinvertebrate assemblages of impaired streams. Thus, our research demonstrates that, in the examined HMWB, a better treatment of

wastewater should be the priority in river restoration programs in order to obtain the enhancement of macroinvertebrate functional diversity.

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# 4 SHORT-TERM EFFECTS OF AN OIL AND SEWAGE SPILL ON THE MACROINVERTEBRATE ASSEMBLAGES OF AN URBAN RIVER


**Abstract**

In 2010 thousand tons of fuel oil were poured into the sewage system north of the city of Milan (Italy), causing the breakdown of the local treatment plant. 1000 tons of oil were spilled into the Lambro River, and wastewater was discharged therein for a month. The short-term effects on the benthic invertebrate communities were analyzed in the following weeks, comparing data collected before/after, and upstream/downstream the spill.

The shifts of several metrics in macroinvertebrate assemblages were analyzed at site and microhabitat level. Three kinds of response were identified. A group of metrics, keyed to taxa richness and other specialized functional groups such as filterers, was affected both by pre-spill conditions and by the event, and failed a short-term recovery due to the sewage leakage. A second group, keyed to the abundance of tolerant organisms, was mainly affected by the oil spill and its recovery is likely to require longer time. A third group, keyed to specialized functional groups such as shredders and scapers, was mainly affected by pre-spill conditions and could not be used for data interpretation. These findings show that the assessment of the effects of a spill in an already impaired river is difficult and requires the evaluation of different metrics compared with standard monitoring. Metrics pertaining to tolerant taxa are more successful descriptors than the others.



**Keywords**

Oil spill; wastewater leakage; freshwater; macroinvertebrates; Lambro River


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Short-term effects of an oil and sewage spill on the macroinvertebrate assemblages of an urban river

## 4.1 Introduction

On February 23rd, 2010, unknown subjects spilled into the local sewage system the content of several diesel and oil fuel tanks at Villasanta, north of Milan (Lombardy, Italy). The oily mass, estimated over 2.5 million liters (more than 15700 barrels), entered the Wastewater Treatment Plant (WWTP) of Monza (680000 EI), inactivating all the treatment processes. Most of the spilled oil (estimated amount 1000 tons) then reached the Lambro River, where the effluent of the WWTP in usually discharged.

This event caused considerable harm to the river environment, already affected by the urban stream syndrome (Walsh et al, 2005), not only for the oil spill effects, but also for the damages caused to the WWTP: untreated sewage was discharged for almost one month into the Lambro River before the reactivation.

The literature on the impacts of diesel, oil and crude oil spills on freshwater ecosystems is growing (i.a. Coghlan and Lund, 2005; Lytle and Peckarsky, 2001; Poulton et al, 1997; Smith et al, 2009), but is still less exhaustive than the literature concerning marine environments. Currently, it is difficult to compare spills affecting water bodies of different kind and size, while also the quantity of oil spilled can vary significantly (Smith et al, 2009). Each hydrocarbon can have specific direct or indirect toxic effects (Bhattacharyya et al, 2003) which can involve various biological and ecological functions, and can be harmful in several ways. McKee's report (1956), revisited by Bury (1972), is still relevant: water soluble fractions can have a direct toxic action; free oil can affect the epithelial surfaces of fish and coat both plankton and algae; oil settling to the bottom can coat benthic communities; organic compounds in general may deoxygenate the water due to both oxygen consumption for degradation and to decreased oxygen recharge at the water/air interface; heavy coatings on the water surface may hamper not only reaeration but also photosynthesis.

Oil spills can affect already impaired ecosystems, especially in urban and industrial areas, where multiple stressors and confounding factors are present (Marshall et al, 2010; Nedeau et al, 2003). In such situations the biological responses are extremely variable and reference sites may be absent. Couceiro et al (2006) studied the combined effects of sewage and oil spills on the invertebrate communities of a stream and of the riparian ecosystem, but further analyses of the joint action of these two stressors are currently lacking. For all these reasons, studies evaluating the effects of oil spills on macroinvertebrate assemblages report responses varying within a broad range of magnitude, spatial scale and recovery periods.

Normally, most studies focus on the performance of various macroinvertebrate metrics, such as biotic, diversity and community comparison indexes (Pontasch and Brusven, 1988a); taxa richness, ephemeroptera/plecoptera/trichoptera (EPT) richness, density of individuals (Crunkilton and Duchrow, 1990); taxonomical ratios and Functional Feeding Group (FFG) abundance (Poulton et al, 1997); density of dominant taxa (Lytle and Peckarsky, 2001). Given the variability of responses, however, further knowledge is needed about the impact mechanisms of oil at different spatial scales. Above all, knowledge of meso- and microhabitat level ecological dynamics following this kind of events could help to identify general responses of macroinvertebrate assemblages. Poulton et al (1997) reported different impacts and, thus, diversified responses from the invertebrate communities in riffle and backwater habitats after a 3.3 million liter oil spill in the Gasconade River (Missouri, USA). This can have great implications in managing the emergency and the restoration efforts following a spill. However, no other studies are known to analyze the communities along a microhabitat gradient in such situations. The analysis along such gradients can be particularly useful when analyzing the effects of accidental or deliberate spills in rivers that are already heavily impaired, such as those in urban areas. In these cases it can be very difficult to discriminate among the effects induced by pre-existent impairment and the various impacts consequent to the event at a higher scale.

We have studied the short-term effects on macroinvertebrate communities in the weeks following the oil and sewage spill in the Lambro River. The aim of the work was to define the magnitude of the impact and the difference of the responses among several macroinvertebrate metrics in habitats with varying physical features.

Little is known about the damage caused by oil and sewage spills on invertebrate communities in already impaired streams and rivers, and understanding some of the processes occurring in such complex circumstances can be useful. Based on evidence and the literature, thus, an attempt was made to assess which alterations could be caused by the oil spill and which ones by the sewage leakage, or were due to pre-existing conditions.

## 4.2 Methods

### *4.2.1 Study site*

The Lambro River is a 127 km long left tributary of the Po, the main Italian river. The whole course of the Lambro is in Lombardy, the most densely urbanized and heavily industrialized region of the country, and its watershed includes a significant portion of the metropolitan area of Milan, with a population exceeding 3000000 inhabitants (Fig. 1). The anthropogenic pressure on the river is very high, and the ecological quality of most of its course has been classified for decades as “very bad” with various biotic and physico-chemical indices, defined by the regional environmental agency (ARPA) according to law. Several published studies assess the often critical condition of the river (i.a. Bettinetti et al, 2003; Pettine et al, 1996; Viganò et al, 2008; Zullini, 1989). The oil spill occurred in the mid-course of the river, upstream of the city of Milan, in an already heavily impaired sector of the river. After the spill several interventions were carried out to manage the emergency. Most of the oil (an estimated 1550 tons) was stopped in the WWTP, inactivating it. Fixed and floating booms were positioned, and most of the remaining 1,000 tons of oil were recovered using the existing basins for hydroelectrical power production in the downstream section of the Lambro River and in the Po River. 400 tons of oil are still missing and were probably dispersed into the environment. After the spill, Monza WWTP discharged untreated sewage for about a month.

### *4.2.2 Sampling procedures*

A first assessment, based on the same protocol adopted by ARPA, was performed in three sites upstream and downstream of the spill: Lesmo (a few kilometers upstream of the spill), Cologno (less than 1 km downstream of the spill) and S. Zenone (more than 15 km downstream of the spill – see Figure 1). The sampling was carried out in order to compare the results with pre-spill data in the same sites, monitored by ARPA. Macroinvertebrates have been monitored for years preceding the spill with qualitative sampling (obtained kicking the substrate with a net along transects), and only few biological metrics were available: Extended Biotic Index (EBI, an Italian index derived from Woodiwiss, 1978), taxa richness and EPT richness. A qualitative estimate of the abundance of macroinvertebrate individuals was also accessible.

BACI (Before-After, Control-Impact) experimental design, considering data both before- after and upstream-downstream the oil spill, is to be preferred when assessing the extent of damage and the recovery of the affected ecosystem in this kind of occurrence (Stewart-Oaten & Bence, 2001). The lack of data collected before the spill, apart from the metrics available from ARPA, made it necessary to also define an experimental design based only on samples collected upstream and downstream of the oil spill after the event, in order to quantify new and more useful biological metrics, and to analyze the effects of the event along the microhabitat gradient.

We used pre-spill data to select upstream and downstream sectors of the river showing the most similar impairment before the event. Within these sectors we identified sampling sites showing comparable hydromorphological features, thus trying to minimize the confounding factors given by already present stressors and longitudinal variation of morphology and ecology. Thus, a site near Lesmo (upstream of the spill) and a site near S. Zenone (downstream of the spill) were selected. These sites were the only two showing similar hydromorphological conditions. In fact, after the discharge from Monza WWTP, the Lambro River enters the urban area of Milan, where it becomes narrower, deeper and channelized; only downstream of the city, and only for a few kilometers upstream of S. Zenone, it recovers morphological

features more similar to those observed at Lesmo (presence of riffles and backwaters; riparian vegetation; a limited meandering).
In the selected sites we collected quantitative data (for a total of 60 samples covering 0.1 m2 each) using a surber net. The sampling procedure was based on Barbour et al (1999), and focused on a multi-habitat scheme (as shown in Furse et al, 2006) designed to sample all the available microhabitats (mainly riffles, canals, pools, backwaters). Environmental variables used to describe the microhabitats, such as water velocity (measured with a electromagnetic velocity flow meter), depth and substratum size, were also determined.
Each sample of macroinvertebrates was kept in 4% formaldehyde and transported to the laboratory. All macroinvertebrates were then sorted, identified and stored in 90% ethanol. All specimens have been identified at the genus level.

### *4.2.3 Data Analysis*

Various macroinvertebrate metrics have been determined: density of individuals, taxa richness, EPT richness, Shannon Diversity Index (all calculated on a family or genus level basis, in order to compare them with available pre-spill data based on standard biomonitoring protocols); richness, density and ratio of the various Functional Feeding Groups (FFG) such as shredders (Shred), scrapers (Scrap), filtering collectors (F-coll), gathering collectors (G-coll) and predators (Pred); richness and ratio of the various Functional Habit Groups (FHG) such as swimmers (Swim), clingers (Cling), climbers (Climb), sprawlers (Spraw) and burrowers (Burr); top-down control (ratio of predators/preys). FFGs and FHGs were assigned on the basis of the available literature (Merritt & Cummins, 1996; Tachet et al, 2000), as shown in Canobbio et al, 2010.
Statistical tests (t-student and Hotelling's T2), Principal Component Analysis (PCA) and Analysis of Covariance (ANCOVA) have been performed with XLSTAT 7 and R 2.12 software.
PCA was used to individuate a microhabitat gradient generated by hydromorphological variables (water velocity, depth, substrate size, Froude number). ANCOVA has been performed to quantify the

microhabitat gradient as a source of variation in the model and to obtain a stronger test for the spill effects on macroinvertebrate assemblages. We considered the hypothesis that the variation in the response of the biological metrics was driven by the different impairment rating in the upstream and downstream sites (given by the pre-spill conditions, the oil and the untreated sewage spill), as well as by a covariate representing hydromorphological (i.e. microhabitat) features, from riffle to backwater. We tested the upstream and downstream site division as the main categorical variable (treatments) and we chose PCA axis 2 as the continuous variable (covariate) representative of the microhabitat gradient (see results). The significance of the whole model, treatment, covariate and interaction between treatment and covariate for every biological metric as response variable has been tested.

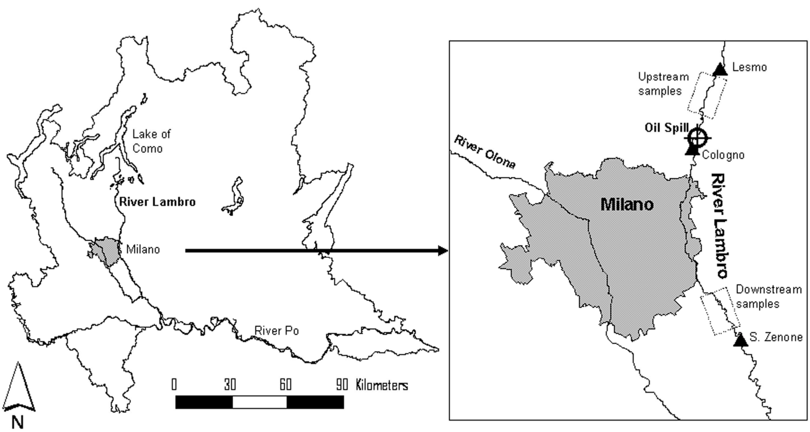


**Figure 1** The study site in the Lombardy region area. On the right, the position of the city of Milan, the oil spill (crossed circle), the sampling sites used for the before/after oil spill comparison (triangles) and the areas used for the upstream/downstream quantitative sampling (rectangles).

## 4.3 Results

### *4.3.1 Before and after the oil spill*

The data collected from 2005 to 2009 by ARPA (samples: n = 15 for Lesmo site; n = 12 each for Cologno and S. Zenone sites) were analyzed to assess the conditions of the different sites in pre-spill conditions. The situation already showed differences. Upstream of Monza WWTP the taxa richness at Lesmo site (calculated by ARPA mainly at the genus level) for the whole period showed 19 taxa as maximum and 11 (mean = 14.0, st.dev. = 2.2) as minimum, while EPT richness showed a mean value of 3.9 (st.dev. = 0.5; range from 3 to 5). This brought to a mean EBI value of 6.9 (st.dev. = 0.5; range from 6 to 8), which is representative of an already impaired situation, due to the urban land use and to the input of effluents from other WWTPs upstream – see Figure 2. The number of individuals ranged from "common" (at least one taxon with 102 individuals) to "abundant" (one dominant taxon with 103 individuals).
Downstream of the WWTP, the values generally dropped. At the Cologno site, taxa richness showed an average score of 5.7 (with wide fluctuations leading to a st.dev. of 2.2, minimum value = 3, maximum value = 9), while in S. Zenone, 15 km after Monza WWTP, the values recovered to a mean value of 9.2 (st.dev. = 1.9; range 6-12). EPT richness was generally extremely low for both sites in the whole 2005-2009 period (mean = 1.0, st.dev. = 1.0, range 0-3 in Cologno; mean = 1.4, st.dev. = 0.9, range 1-3 in S. Zenone). Consequently, EBI values were generally low, too. In Cologno, the mean value was 3.8 (st.dev. = 1.5, range 2-6), while in S. Zenone the mean value was 5.0 (st.dev. = 1.0, range 4-6). The number of individuals was described in Cologno as "common" in 4 sampling campaigns, "abundant" in 6 , and "very abundant" (at least two taxa with 103 individuals) in 2. In S. Zenone, the number of individuals ranged from "common" (n = 6) to "abundant" (n = 3) and "very abundant" (n =3).
Pre-spill data were tested (paired t-tests) in order to find if significant differences existed between sites. The values of all the three metrics were significantly lower ($p < 0.0001$) in Cologno and S. Zenone than in Lesmo, showing that differences in the impairment level were already

present before the spill. Metrics in S. Zenone showed a significant recovery from Cologno ($p < 0.0001$ for taxa richness; $p = 0.035$ for EBI value), except for EPT richness. The recovery, however, was never enough to raise the values back to Lesmo levels.
Data collected 4 weeks after the oil spill in 2010 with the same sampling protocol while the Monza WWTP was still inactivated showed a value of taxa richness equal to 15 in Lesmo, 6 in Cologno and 7 in Lesmo. The EPT richess was equal to 5, 0 and 0 respectively. This kind of assemblage led to an EBI value of 7 in Lesmo, and of 3 both in Cologno and in S. Zenone – see Figure 2 for comparison. The number of individuals was "abundant" in Lesmo and "rare" (no taxa with at least 102 individuals) in Cologno and S. Zenone. The sites downstream of the spill appeared as heavily affected from both the oil and sewage spill. The river water contained a huge amount of untreated wastewater, presenting low Dissolved Oxygen (DO) levels, grey color, turbidity and sewage smell. The oil was still present in the finer sediments of backwater and riparian areas: kicking the sediments, a thick layer of oil could be observed on the water surface. In riffle areas, on the contrary, the coarser substrate showed no oil presence. In these habitats, few periphytic algae were observed downstream, while they were abundant upstream.

### *4.3.2 Upstream and downstream of the oil spill*

Two river sectors upstream and downstream of the oil spill were selected for quantitative analysis. To confirm the hydromorphological similarity of the sites for every sample we collected some environmental variables: water velocity, depth, substrate size and Froude number. The results are reported in Table 1 with DO and conductivity values. The environmental variables showed significant collinearity (substrate size and Froude number with water velocity, depth and substrate size with Froude number; all $p < 0.01$, $r > 0.40$) in both sites. The range of the sampled habitats did not show differences between upstream and downstream of the sites. For example, water velocity had a mean value of 0.574 m/s upstream of the spill and 0.589 m/s downstream. The range of hydromorphological conditions in the two sites was tested using a Hotelling's T2 test applied to all the four

variables. The test showed no significant difference between sites (T2 4, 55 = 1.6962; p = n.s.) confirming that the range of available habitats was similar in the two sites.
For further analyses we determined a gradient representing the availability of different microhabitats: to detect it environmental variables were further analyzed by a PCA multivariate analysis. The main relationships among the variables is shown in Figure 3, where the first two components of the PCA are displayed (explaining about 77% of the total data variation). The first axis shows the variation keyed to the upstream-downstream gradient, while the second axis shows the variation keyed to the microhabitat gradient (given by the hydromorphological variables: positive values of Axis 2 represent riffle habitats, while negative values represent pool and backwater habitats). Samples from upstream and downstream were divided in two distinct clusters on axis 1. We used axis 2, the habitat gradient, as the covariate in the ANCOVA analysis (see below).

**Table 1** Environmental variables determined during macroinvertebrate sampling

| | Upstream | Downstream |
|---|---|---|
| Water velocity (m/s) | 0.574 ± 0.335 | 0.589 ± 0.320 |
| Depth (m) | 0.34 ± 0.18 | 0.32 ± 0.15 |
| Froude number | 0.364 ± 0.248 | 0.356 ± 0.197 |
| Substrate (Φ, $\log_2$(Ø; mm)) | 6.3 ± 1.2 | 5.8 ± 1.1 |
| DO (% saturation) | 111.0 ± 3.3 | 71.5 ± 23.3 |
| Conductivity ($\mu$S/cm) | 528 ± 31 | 795 ± 82 |

A total number of about 66000 macroinvertebrate specimens belonging to 35 taxa were collected and identified in the samples. The average values of the densities in different kinds of microhabitats are shown in Table 2, while the average values of the obtained biological metrics for upstream and downstream samples are reported in Table 3A-3B. The density of total individuals was far higher upstream (with a mean value of about 20000 invertebrates/m2) than downstream (less than 2000 invertebrates/m2). Generally, the dominant taxa in every site were those tolerant to impairment. In the samples collected upstream of the spill, the most abundant specimen belonged to Chironomidae (especially Chironomini) and most of all oligochaetes (especially Naididae and Tubificidae, up to 110000 specimens/m2). Downstream of the spill, the dominant taxa were Chironomidae (only Chironomus spp., up to 1500 specimens/m2) and Tubificidae (up to 10000 specimens/m2). No plecoptera were found. Ephemeroptera (Baetis and Caenis spp.) and trichoptera (belonging to families Hydropsychidae, Hydroptilidae, Polycentropodidae, Psychomidae, Rhyacophilidae) were found in substantial quantities (mean number of ephemeroptera/m2 = 200; trichoptera/m2 = 126) upstream, and only sporadically downstream (only one Baetis and one Hydropsyche specimen in two samples). Thus, the difference in the EPT richness was highly significant (upstream mean value = 2.5; downstream mean value = 0.1). Generally, the number of collected taxa was higher in the upstream samples (up to 15 taxa in a single sample, mean = 8.5), while it was very low downstream (with a maximum number of taxa in a sample equal to 11, but a mean value of 4.7). The different distribution of many taxa in the various microhabitats was evident upstream, but not so much downstream. The values of the Shannon Index, influenced more by the densities of dominant taxa (evenness) than by taxa richness, showed an odd result, being higher downstream (mean value 1.23) than upstream (mean value 0.95).

Density of individuals, taxa richness and EPT richness were positively related with Axis 1 of the PCA (figure 3) and, thus, with upstream sites ($r = 0.262$, $0.327$, $0.384$ respectively, $p < 0.05$). Taxa richness was positively related also with the microhabitat gradient represented by Axis 2 ($r = 0.378$, $p < 0.05$). Density of individuals showed no relationship with Axis 2. Shannon Index showed negative relationship with Axis 1 (and, thus, it was positively related to downstream sites, as already noted before), but positive relationship with Axis 2 (riffle microhabitats). Both relationships, however, resulted not significant.

FFG and FHG values reflected the described situation. Being chironomids and oligochaetes the most common macroinvertebrates, the dominant FFG resulted G-Coll (with a mean ratio of 0.975 upstream and 0.924 downstream), while the dominant FHG was Burr (with a mean ratio of 0.956 upstream and 0.925 downstream). Predator/prey ratio (Top-Down Control) was higher downstream, basically for the smaller amount of available preys. The FHG Climb was never found upstream or downstream.

A summary of the results given by the application of the ANCOVA model is shown in Table 3A-3B. We detected three kinds of responses from macroinvertebrate metrics and grouped them consequently.

For the first group of metrics the model showed strong overall significance ($p < 0.0001$). The treatment and at least one between the covariate and the interaction (usually both) were significant, too. Taxa richness and EPT richness are the most representative metrics of this group, which identifies the behavior of the most sensitive taxa. The most specialized functional guilds (represented by F-coll, Cling and Swim richness) that can be found with a considerable number of individuals in the Lambro River are in this group, too. For all the metrics the significance of the covariate was given by a clear response to the habitat gradient upstream of the spill: the values of the response variable were positively related to PCA Axis 2, meaning a higher number of taxa and specialized individuals were present in riffle areas, where more DO and suspended organic matter are available even in impaired environments. The interaction resulted generally significant because the treatment acts not only against the metrics themselves (with lower richness and individuals downstream of the spill), but also against their relationship with the habitat gradient: in fact, no clear relationship is visible downstream.

The second group consists of those metrics related to the density and dominance of tolerant taxa. The metrics involved are the density of total individuals, Shannon Index (related to the greater evenness in the downstream samples), top-down control (related to the lower number of preys downstream), the number (but not the richness) of Swim, and non specialized guilds such as G-coll and Burr. The ANCOVA model for these metrics resulted significant as well as the treatment, meaning that there were considerable differences between upstream and downstream samples, usually referable to a drop in the number of tolerant individuals and taxa. In this second group, however, the covariate and the interaction always resulted not significant, meaning that the metrics did not follow a pattern along the microhabitat gradient. This is once more explainable considering the ubiquitous and tolerant nature of the invertebrates involved in such metrics. They generally can live in huge numbers both in the periphytic algae of riffle substrates and in the finer sediments of pools and backwater habitats.

For the third and last group of metrics the ANCOVA model resulted non significant. These metrics referred to the density and richness of specialized functional groups (such as Shred, Scrap, Pred and Sprawl) which were not found in sufficient number of individuals or richness to show some intelligible pattern, even upstream of the spill.

**Table 2** Mean distribution of the invertebrate families (n. of individuals / m$^2$) in the *riffle*, *canal* and *pool* microhabitats, upstream and downstream of the spill.

| Family | Upstream | | | Downstream | | |
|---|---|---|---|---|---|---|
| | Riffle | Canal | Pool and Backwater | Riffle | Canal | Pool and Backwater |
| Baetidae | 304 | 74 | 92 | 0 | 0 | 1 |
| Caenidae | 41 | 8 | 4 | 0 | 0 | 0 |
| Hydropsychidae | 130 | 104 | 12 | 0 | 0 | 1 |
| Hydroptilidae | 2 | 0 | 0 | 0 | 0 | 0 |
| Polycentropodidae | 12 | 18 | 4 | 0 | 0 | 0 |
| Psychomidae | 39 | 19 | 1 | 0 | 0 | 0 |
| Rhyacophilidae | 10 | 0 | 0 | 0 | 0 | 0 |
| Elmidae | 3 | 1 | 0 | 0 | 0 | 0 |
| Chironomidae | 3213 | 1490 | 966 | 356 | 689 | 218 |
| Ceratopogonidae | 0 | 3 | 0 | 0 | 0 | 0 |
| Chaoboridae | 0 | 4 | 0 | 0 | 0 | 0 |
| Limonidae | 1 | 0 | 0 | 0 | 0 | 0 |
| Muscidae | 0 | 0 | 0 | 0 | 0 | 1 |
| Psychodidae | 2 | 1 | 2 | 1 | 0 | 1 |
| Simuliidae | 215 | 55 | 27 | 3 | 0 | 0 |
| Tabanidae | 0 | 0 | 1 | 0 | 0 | 0 |
| Gomphidae | 2 | 0 | 0 | 0 | 0 | 0 |
| Asellidae | 2 | 1 | 2 | 0 | 0 | 0 |
| Gammaridae | 1 | 0 | 0 | 0 | 0 | 0 |
| Valvatidae | 0 | 1 | 1 | 1 | 0 | 0 |
| Physidae | 0 | 0 | 2 | 1 | 0 | 1 |
| Planorbidae | 2 | 0 | 0 | 0 | 0 | 0 |
| Bithyniidae | 2 | 0 | 0 | 0 | 0 | 0 |
| Erpobdellidae | 61 | 28 | 57 | 49 | 91 | 5 |
| Glossiphoniidae | 4 | 0 | 0 | 22 | 38 | 10 |
| Enchytraeidae | 9 | 20 | 13 | 453 | 16 | 26 |
| Lumbricidae | 195 | 248 | 9 | 23 | 0 | 0 |
| Lumbriculidae | 48 | 9 | 2 | 6 | 0 | 0 |
| Naididae | 19038 | 12326 | 10158 | 285 | 1 | 11 |
| Tubificidae | 342 | 135 | 9094 | 1568 | 369 | 579 |
| Mermithidae | 0 | 1 | 1 | 1 | 0 | 0 |

**Table 3A** Mean values (± st.dev.) of the macroinvertebrate metrics determined in the samples collected upstream and downstream of the spill. Densities are expressed as number of individuals/$m^2$. Significance (model, treatment, covariate and interaction) of the ANCOVA tests ($F_{3,56}$) are shown as well.

| | Upstream | Downstream | ANCOVA | model | treatment | covariate | interaction |
|---|---|---|---|---|---|---|---|
| | (mean ± st.dev.) | (mean ± st.dev.) | $F_{3,56}$ | *P* | *P* | *P* | *P* |
| TAXA Richness | 8.5 ± 3.1 | 4.7 ± 1.9 | **14.923** | **< 0.0001** | **< 0.0001** | **0.011** | n.s. |
| EPT Richness | 2.5 ± 1.4 | 0.1 ± 0.25 | **37.962** | **< 0.0001** | **< 0.0001** | **0.013** | **0.035** |
| G-COLL Richness | 5.2 ± 1.8 | 2.9 ± 0.9 | **15.618** | **< 0.0001** | **< 0.0001** | **0.046** | n.s. |
| F-COLL Richness | 1.6 ± 0.8 | 0.1 ± 0.3 | **42.793** | **< 0.0001** | **< 0.0001** | **< 0.001** | 0.051 |
| F-COLL density | 214 ± 253 | 0 ± 1 | **15.828** | **< 0.0001** | **< 0.0001** | **0.001** | **0.010** |
| SWIM Richness | 0.8 ± 0.5 | 0.0 ± 0.2 | **38.862** | **< 0.0001** | **< 0.0001** | **0.003** | **0.007** |
| CLING Richness | 2.3 ± 1.5 | 0.2 ± 0.6 | **22.795** | **< 0.0001** | **< 0.0001** | **0.005** | n.s. |
| CLING density | 247 ± 297 | 3 ± 8 | **15.281** | **< 0.0001** | **< 0.0001** | **0.001** | **0.011** |

**Table 3B** Mean values (± st.dev.) of the macroinvertebrate metrics determined in the samples collected upstream and downstream of the spill. Densities are expressed as number of individuals/m$^2$. Significance (model, treatment, covariate and interaction) of the ANCOVA tests ($F_{3,56}$) are shown as well.

| | Upstream (mean ± st.dev.) | Downstream (mean ± st.dev.) | ANCOVA $F_{3,56}$ | model $P$ | treatment $P$ | covariate $P$ | interaction $P$ |
|---|---|---|---|---|---|---|---|
| Shannon Index | 0.95 ± 0.63 | 1.23 ± 0.46 | **3.263** | **0.028** | **0.042** | n.s. | n.s. |
| Density | 20273 ± 24922 | 1734 ± 2050 | **5.949** | **0.001** | **< 0.001** | n.s. | n.s. |
| Top-Down Control | 0.009 ± 0.020 | 0.096 ± 0.181 | **2.470** | **0.071** | **0.012** | n.s. | n.s. |
| G-COLL density | 20016 ± 24872 | 1659 ± 2043 | **5.918** | **0.001** | **< 0.001** | n.s. | n.s. |
| SWIM density | 179 ± 222 | 0 ± 2 | **8.537** | **< 0.0001** | **< 0.0001** | n.s. | n.s. |
| BURR Richness | 3.9 ± 1.4 | 2.9 ± 1.0 | **3.947** | **0.013** | **0.002** | n.s. | n.s. |
| BURR density | 19770 ± 24726 | 1658 ± 2043 | **5.879** | **0.001** | **< 0.001** | n.s. | n.s. |
| SHRED Richness | 0.2 ± 0.5 | 0.0 ± 0.2 | 1.160 | n.s. | | | |
| SHRED density | 0 ± 1 | 0 ± 0.2 | 1.362 | n.s. | | | |
| SCRAP Richness | 0.4 ± 0.9 | 0.1 ± 0.4 | 1.231 | n.s. | | | |
| SCRAP density | 1 ± 2 | 0 ± 0.4 | 0.961 | n.s. | | | |
| PRED Richness | 1.1 ± 1.0 | 1.6 ± 1.3 | 1.108 | n.s. | | | |
| PRED density | 6 ± 11 | 8 ± 12 | 0.941 | n.s. | | | |
| SPRAWL Richness | 1.4 ± 1.0 | 1.6 ± 1.3 | 0.292 | n.s. | | | |
| SPRAWL density | 77 ± 116 | 76 ± 121 | 1.104 | n.s. | | | |

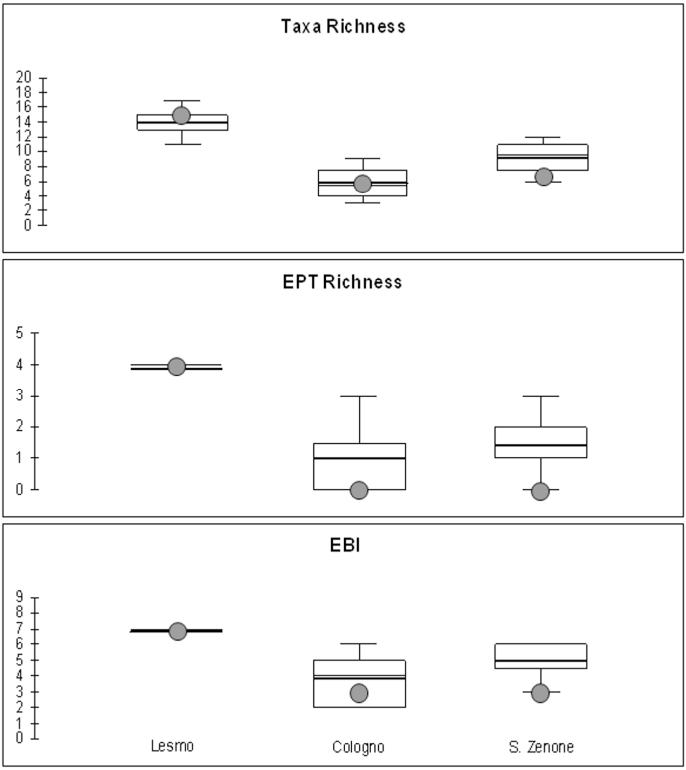


**Figure 2** Box plots representing the pre-spill situation upstream (Lesmo) and downstream (Cologno; S. Zenone) of Monza WWTP. The box plots represent the quartiles, while the bold line is the mean value. Grey dots are the values assessed 4 weeks after the oil spill.

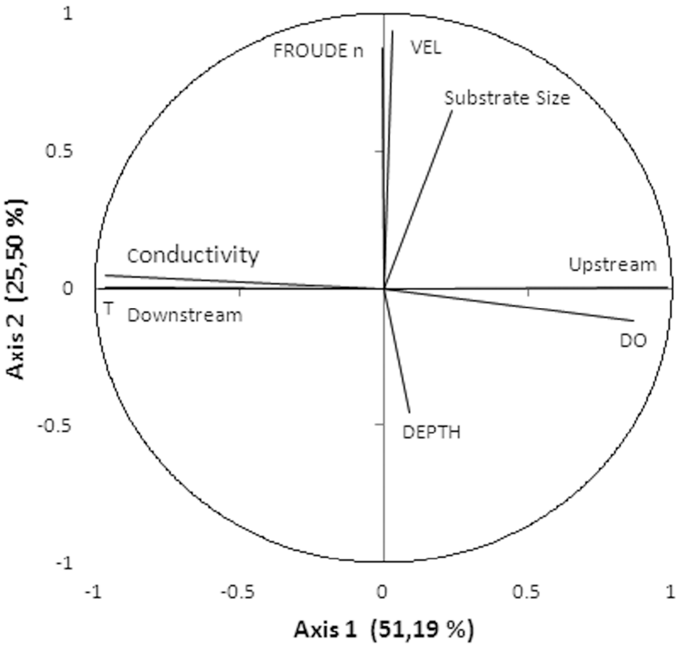


**Figure 3** PCA biplot graph showing relationships (first two axes, 76,79% of the total variance) among the environmental variables.

## 4.4 Discussion and conclusions

The analysis of macroinvertebrate metrics in the Lambro River (both before/after and upstream/downstream of the oil and sewage spill) allowed to indentify three kinds of responses to the pre-spill conditions and to the event.

Metrics classified in “group 3” within the ANCOVA analysis (related to specialized FFGs and FHGs – see Table 3A-3B) did not show any significant response to the spill or to the hydromorphological gradient. The influence of the pre-spill overall impairment (even in the upstream sector of the river) is so great that the richness and abundance of individuals of such guilds was too low to allow the detection of any difference among sites and microhabitats. This group, thus, is uniquely related to the pre-spill impairment and is not useful in helping to quantify the spill damage. It is composed of FFGs and FHGs that are more easily found in reference sites, and not in urban rivers.

Metrics classified in “group 1” are related to taxa richness, the presence of EPT and sensitive functional guilds such as filterers and clingers (which are functional traits mainly belonging to taxonomical groups such as trichoptera and some diptera). Metrics in this group showed a significant relationship with the habitat gradient upstream of the spill, with a noteworthy preference for riffle habitats, well known in the literature (i.a. Brown and Brussock, 1991; Merigoux and Doledec, 2004; Vinson and Hawkins, 1998 - and many more). The group also showed a significant response to the oil and sewage spill (the treatment in the ANCOVA model). However, in pre-spill conditions the upstream and downstream sectors of the river already showed significant differences at least in taxa and EPT richness values; these differences should be taken into account. The available metrics (taxa and EPT richness, EBI values) showed various levels of impairment in both the river sectors (upstream and downstream of the event yet to come), with low richness of ephemeroptera and trichoptera (up to a maximum of 5 taxa in the best situation) and dominance of tolerant taxa such as chironomids and oligochaetes. Moreover, the pre-spill impairment presented different magnitudes in the various monitored sites. The impairment greatly increased from Lesmo to Cologno; then, a recovery

could be observed from Cologno to S. Zenone, but the situation at S. Zenone was still not totally comparable to the Lesmo one. This must be taken into account not only in the "before/after the spill", but also in the "upstream/downstream of the spill" experimental design. The differing pre-spill situation in the various sites is likely to be a major confounding factor for the spill damage assessment. The observed trends in pre-spill conditions showed a drop in taxa and EPT richness from Lesmo to Cologno, where ephemeroptera and trichoptera approached complete disappearance, and a partial recovery thereafter. Another trend was the increase in the abundance of tolerant taxa, mainly oligochaetes, in the most impaired sites. This is in agreement with most of the literature (e.g. Alvarez-Cabria et al, 2011) and was already observed in other effluent-dominated streams of the Lambro basin (Canobbio et al, 2009).
Moreover, the time overlap in the oil and sewage spill makes it difficult to assess the specific action and the relative importance of the two events for biological metrics of "group 1".
Thus, is it not possible to understand the specific weight of the different alterations. The taxa and EPT richness in the sites (especially S. Zenone) downstream of the spill, however, are lower after the event if compared with the pre-spill dataset. It is probable that the spill had a further negative influence on metrics that were already compromised. Some authors (Pontasch and Brusven, 1988b; Poulton et al, 1998) report a short-term negative effect due to oil toxicity and coating on riffle assemblages, followed by a recovery. In the Lambro River it can be hypothesized that the initial oil and sewage spill worsened an already altered condition to the point that taxa belonging to EPT, clingers and filterers disappeared. The failed recovery after 4 weeks, instead, was probably caused by the continuous spill of sewage from the inactivated WWTP deoxygenating the river water, since no oil coating was visible anymore in riffle habitats during the sampling activities, and it did not prevent anymore fast flow habitats from recolonization.
The most noticeable metrics are those classified in "group 2" and mostly referring to tolerant taxa or guilds (swimmer abundance, but not swimmer richness, is in this group as well, since tolerant swimmers

such as some Baetis spp. can be abundant in impaired environments). Shannon Index is in this group too because of the increased evenness of downstream samples due to the drop of abundances of tolerant taxa. In group 2 the treatment (oil and sewage spill) affects the metrics causing a decrease of abundances (and a consequent increase of the Shannon Index), while the hydromorphology has no influence on such ubiquitous organisms, which can colonize both riffles (in the interstitial sediments and on the periphytic algae) and backwaters (in fine sediments and deposited organic matter). The metrics of this group show a response conflicting with the normal trends in impaired environments, especially those affected by the increase of organic pollution, and conflicting with the pre-spill situation in the Lambro River, as described above. According to the literature, abundances of total individuals as well as abundances of tolerant taxa and guilds should increase (i.a. see Alvarez-Cabria et al, 2010, and citations therein, although Grantham et al, 2012, report a decrease in abundance of tolerant specimens over high level thresholds of organic pollution), while the Shannon Index should decrease (i.a. Gray and Pearson, 1982). However, previous assessments of oil spill effects in other rivers (Lytle & Peckarsky, 2001; Pontasch and Brusven, 1988a) show that the trend associated with metrics in group 2 has been previously observed in similar situations, even if some contrasting observations by older studies (e.g. Harrel, 1985) report abundance increasing. Considering the performance of these metrics and their ecological meaning, it is probable that the metrics in group 2 have been mostly influenced by the oil spill, rather than by the sewage leakage. The direct effects of oil (toxicity and organism coating) were probably the cause of the drop in the abundances, while after 4 weeks the indirect effects (removal of periphyton in riffles and coating of fine sediment and organic matter in pools and backwaters) prevented a fast recolonization.

Hence, in the already impaired conditions affecting the Lambro River the most noticeable damages affecting macroinvertebrate assemblages that can be linked to the oil spill are those which occurred to the most tolerant and ubiquitous taxa. Other alterations, affecting sensitive taxa and specialized functional groups, are the effect of an overall impact caused by both the oil and the sewage spill, and by pre-spill conditions.

In riffle areas, the failed recovery of sensitive taxa to pre-spill conditions can be ascribed to the wastewater leakage that also continued after the end of the oil spill, while in backwaters the failed recovery of tolerant taxa can be related to the persistence of oil in the sediments and the removal of periphyton. As a consequence, and according to other experiences (Poulton et al, 1997; Ocon et al, 2008, in estuarine ecosystems), it is probable that invertebrate communities (and, thus, taxa and EPT richness) would recover faster to pre-spill conditions after the end of the sewage spill in fast flow (riffle) habitats. Backwater assemblages and, more generally, ubiquitous tolerant organisms are likely to require longer time for recovery.

Our findings suggest that it is possible to identify some useful invertebrate metrics for the evaluation of the effects of spills in already heavily impaired rivers, and that such metrics are those related to ubiquitous tolerant taxa and functional groups.

## 4.5 Acknowledgements

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# 5 A MULTIVARIATE APPROACH TO ASSESS HABITAT INTEGRITY IN URBAN STREAMS USING BENTHIC MACROINVERTEBRATE METRICS

**Abstract**

Benthic macroinvertebrates are widely used as indicators of the health of freshwater ecosystem, responding both to water quality and to the hydromorphological integrity. In urban streams, evaluations can be tricky for the synergistic effects of multiple stressors and confounding factors. In these situations, the most broadly used multimetric indices can be used to assess the overall damage to the invertebrate community and, thus, the overall anthropogenic pressure, but they do not allow to understand the specific causal effects. Particularly, habitat loss due to morphological alterations can be difficult to evaluate, especially due to the often concurrent disturbance caused by water pollution. We used a multivariate approach to focus on the characteristics of the streams and rivers in an urban district and to define which macroinvertebrate metrics should be used to assess the influence of the different kinds of alteration in a severely damaged environment. Some metrics enabling to assess habitat loss (ratio of oligochaeta, ratio of filterers) were identified. These metrics may help raising a better awareness in the evaluation of river restoration success and, thus, in supporting decision-making processes.

**Keywords**

Multivariate analysis; environmental gradients; macroinvertebrate assemblages; urban stream assessment.

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Canobbio S., Azzellino A., Cabrini R., V. Mezzanotte

A multivariate approach to assess habitat integrity in urban streams using benthic macroinvertebrate metrics

## 5.1 Introduction

In urban areas, streams and rivers may suffer from severe impairment, which has been described as the "urban stream syndrome" (Walsh *et al*., 2005). In catchments of this kind, the synergistic action of multiple stressors, such as - among the others – different types of pollution, increased flashiness of the hydrological cycle and habitat destruction (Canobbio *et al*., 2009) threaten the integrity of freshwater ecosystems. These stressors can also be confounding factors making difficult to accurately assess the cause of single alterations (Nedeau *et al*., 2003). Notwithstanding these difficulties, urban streams are often a priority within the goals of restoration programs (Bernhardt & Palmer 2007).
Countries and organizations worldwide, in the past decades, have been developing indicators to identify and quantify the stressor effects. Particularly, metrics associated with biological communities (i.a. fishes, macroinvertebrates, diatoms, macrophytes) have been widely used. The European Union Water Framework Directive (WFD) is an example of the attention that it is currently paid by law to the ecosystem health and ecosystem-related indicators. Among the others, macroinvertebrates are the most commonly used assemblages (Resh, 2008) because they integrate various desirable characteristics, such as ubiquity, different levels of tolerance to perturbations, and sampling cost-effectiveness (Rosenberg & Resh, 1993; Purcell *et al*., 2009). Multimetric indices and/or multivariate analyses are the most common approaches for the assessment of watercourse impairment based on macroinvertebrates, but usually they are calibrated over broad datasets considering both reference and impaired streams.
Indicators with a high degree of site-specificity are needed for urban streams in order to understand the different weight and the causal relationships of the various stressors acting locally and to optimize the efficiency of restoration efforts. Moreover, urban development can result in the absence of reference sites (Brooks *et al*., 2006) for this kind of streams. An issue is also the quantification of the damage deriving from habitat loss, because its effects are generally hidden by the effects of water pollution.

A multivariate approach can be adopted to understand the complex relationships among the environmental variables, representative of the various stressors in an urban stream, and between them and the biological metrics derived from the monitoring of invertebrate communities. Ordination techniques allow to detect patterns in the environment (i.e. gradients of various kinds of impairment) and, then, the response of biological communities to those patterns (McGarigal *et al.*, 2000). These techniques are specifically designed to individuate the major gradients that would explain most of the variability of the data set.

We applied Principal Component Analysis (PCA), a multivariate ordination technique, to a dataset of environmental variables collected in the stream system near the sprawling urban area of Milan, Northern Italy. Aim of the work was to identify independent environmental gradients accounting for water quality and hydromorphological conditions (i.e. habitat availability) and to analyze the response of macroinvertebrate communities to those gradients. The analysis was focused on invertebrate metrics that responded to the impact of specific anthropogenic pressures, and particularly to habitat loss, rather than to the overall impairment. We believe that the identification of these metrics is a first step for the unambiguous understanding of the weight and the effects of different pressures in multi-stressor environments, that could finally lead to the development of multimetric indices specifically designed for urban environments.

## 5.2 Materials and methods

### *5.2.1 Study site and data types*

Data have been collected from 20 sampling sites in the Lambro-Seveso-Olona system (Lombardy, Italy – Figure 1). It is the most urbanized watershed in Italy, with an average population density of 1600 inhabitants km$^{-2}$, hosting the sprawling conurbation of Milan, although in its southern part the prevailing land use is agricultural.

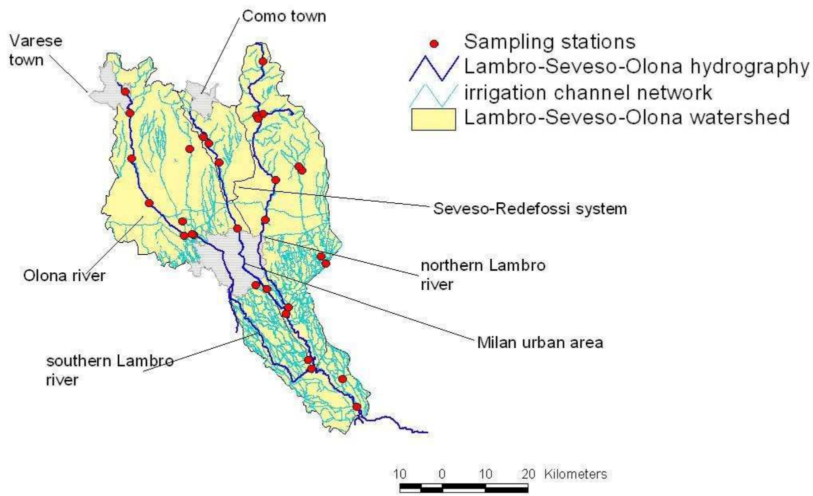


**Figure 1.** The Lambro-Seveso-Olona system.

Sampling has been distributed over 5 years (2005-2009) and a total of 225 samples were gathered by the local environment protection agency (ARPA Lombardia). For every sample, a wide range of environmental (abiotic) variables, including water chemistry and hydromorphological conditions of the site, were determined. Chemical analyses were performed according to standard methods (APHA, AWWA, WPCS, 1992). At the same time data about macroinvertebrate assemblages were collected seasonally by semi-quantitative samplings.

Water quality descriptors were obtained analyzing water samples, collected monthly, while hydraulic and morphological variables were defined on-site. Habitat integrity was quantified using the Italian adaptation of the RCE-2 protocol (Petersen, 1992; APAT, 2007) and expressed as the ratio between the actual condition and the best possible one. A theoretical optimal condition, described by the maximum values for RCE-2 protocol was assumed as a reference for hydromorphology.
On the whole, 54 macroinvertebrate metrics were defined, related to tolerance, abundance, richness, and diversity of both taxonomical units and functional feeding (FFGs) and habit (FHGs) groups. FFGs were classified as shredders, scrapers, filterers, collectors and predators. FHGs included clingers, climbers, sprawlers, burrowers and swimmers (Merritt & Cummins, 1996; Tachet *et al*., 2000). Most metrics were expressed as richness or abundance ratio of a given taxon or functional trait. Some aggregated ratios of functional groups were used to determine ecosystem attribute metrics, as shown in Canobbio *et al*., 2010. Two multimetric indices were also determined: Extended Biotic Index (EBI) (Woodiwiss, 1978) and STAR Intercalibration Common Metric Index (Star_ICMi, Erba *et al*., 2009), now broadly used in Europe to enforce the WFD.

### *5.2.2 Environmental gradient creation and biological metric screening*

A preliminary analysis of collected environmental variables showed that many water quality parameters and habitat descriptors resulted to be redundant. Only one metric from a group of redundant metrics with a Variance Inflation Factor (VIF) > 20 or $r > 0.7$ was considered. Relationships and patterns among the selected variables were analysed with a Principal Component Analysis (PCA).
Two screening criteria were used to screen each biological metric. These criteria were adapted from Purcell et al. (2009) that evaluated metric screening and techniques from several studies. This screening is important for the elimination of non-informative and redundant metrics. In the first step, the range of each metric (from minimum to maximum value) was examined to ensure that it was broad enough to discern differences in magnitude. The criteria provide that the range of

percentage metrics must be > 10 and that the range of richness metrics must be > 5 (e.g., Klemm et al., 2003).
In the second step, metrics were plotted to test for redundancy with Pearson correlation. If correlation of two metrics was greater than 0.7, only one metric from a group of redundant metrics was considered.
A multiple linear regression analysis was conducted between environmental gradients and the selected macroinvertebrate metrics to test gradient influence on biological communities.

## 5.3 Results

### *5.3.1 Environmental gradients*

The collected environmental variables reflected the heavy urbanization of the basin. Both water quality parameters and morphological descriptors showed a high degree of impairment (Table 1), with high concentrations of pollutants and low levels of habitat integrity. The collected variables showed also that in the whole basin no site had a quality comparable to the reference conditions.
Relationships and patterns among the collected environmental variables were analyzed by a PCA. Figure 2 shows the first two principal components and the factor loadings of the selected variables (Q, DO, COD, $N\text{-}NH_4$, $N\text{-}NO_3$, TP, RCE-2 ratio, leaf packs, habitat diversity and hydraulic integrity). The water quality parameters and the morphological indicators basically cluster in two different groups, identified by the first two components explaining about 55% of the total variance. Physico-chemical variables mostly correlate with axis 2, while morphological variables and Dissolved Oxygen (DO), as saturation percentage, correlate with axis 1. The factor scores of the first two principal components were subsequently used as new variables. The first one represents the gradient of hydromorphological conditions (*habitat gradient*), where higher values are associated with a greater morphological integrity. The second one represents the overall water quality gradient (*pollution gradient*), where the higher values are associated to higher pollution levels. Due to the mathematical properties of principal components, these are gradients that maximize variation and are independent from each other.

**Table 1** Values of the environmental variables collected in the Lambro-Seveso-Olona system during the survey. Habitat variables are expressed as the ratio between the actual condition and the best possible one.

| Variable | mean ± st.dev. | Variable | mean ± st.dev. |
|---|---|---|---|
| Flow - Q ($m^3/s$) | 1.833 ± 1.633 | Ecological flow | 0.85 ± 0.23 |
| Dissolved Oxygen - DO (%) | 74 ± 17 | Flooding area | 0.12 ± 0.11 |
| $BOD_5$ (mg/L) | 8 ± 6 | Leaf Packs | 0.27 ± 0.17 |
| COD (mg/L) | 31 ± 14 | Erosion | 0.18 ± 0.22 |
| *E. coli* (CFU/100ml) | $8.7*10^4 \pm 12.8*10^4$ | Section | 0.31 ± 0.24 |
| Total Nitrogen - TN (mg/L) | 11.118 ± 4.606 | Fish habitat | 0.88 ± 0.26 |
| $N\text{-}NH_4$ (mg/L) | 2.655 ± 2.302 | Riffle-Pool sequence | 0.47 ± 0.32 |
| $N\text{-}NO_3$ (mg/L) | 5.384 ± 2.446 | Macrophytes | 0.25 ± 0.12 |
| Total Phosphorus - TP (mg/L) | 1.043 ± 0.535 | Detritus | 0.36 ± 0.18 |
| $P\text{-}PO_4$ (mg/L) | 0.788 ± 0.445 | Hydraulic integrity | 0.44 ± 0.14 |
| Land Use | 0.18 ± 0.14 | Biota | 0.25 ± 0.08 |
| Riparian vegetation kind | 0.13 ± 0.11 | Riparian Vegetation | 0.20 ± 0.14 |
| Riparian veg. width | 0.25 ± 0.20 | Habitat diversity | 0.28 ± 0.14 |
| Riparian veg. continuity | 0.36 ± 0.22 | RCE-2 | 0.29 ± 0.10 |

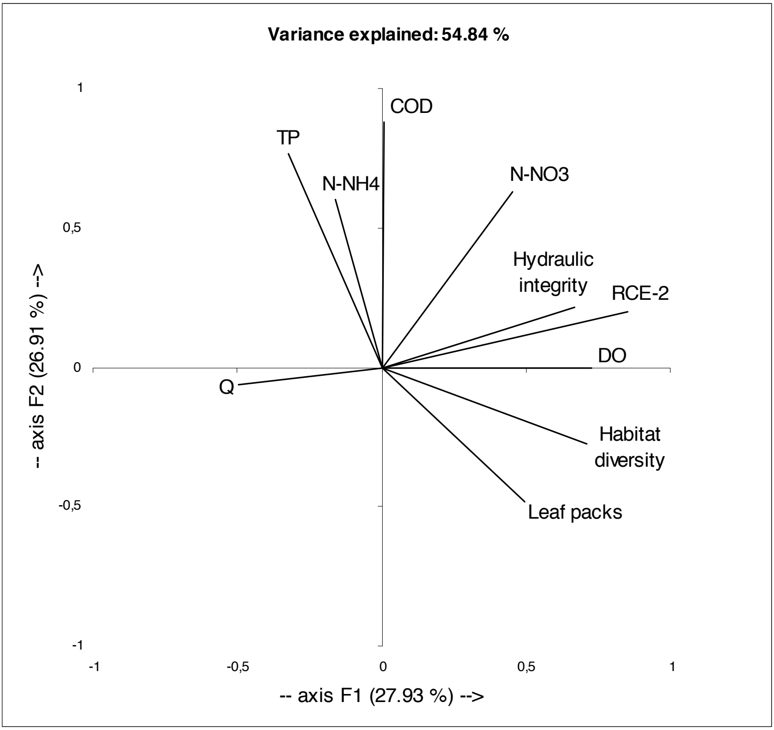


**Figure 2.** Factor Loading plot for the first two Principal Components, accounting for more than 50% of the total variance of the environmental variables.

### *5.3.2 Biological metric response to gradients*

The macroinvertebrate metric dataset was screened and reduced considering only those metrics, which showed a broad range (richness > 5 or abundance ratio interval > 0.1) and excluding redundant variables (Pearson correlation: $r > 0.7$). An exception was made with the six metrics composing the STAR_ICMi index. Although showing strong redundancy among them, they were maintained in the dataset because they are used by the Italian law for the stream classification, so they are often broadly available. Selected metrics are shown in Table 2, with the mean values of the dataset and their standard deviations.

**Table 2** Values of macroinvertebrate metrics used for the analysis (mean ± std. dev.).

| | |
|---|---|
| Density of total individuals (ind/m$^2$) | 1144 ± 1192 |
| *Ratios (individuals/total individuals)* | |
| Chironomidae Ratio | 0.34 ± 0.31 |
| *Baetis* Ratio | 0.08 ± 0.17 |
| Oligochaeta Ratio | 0.33 ± 0.30 |
| Filterers Ratio | 0.08 ± 0.13 |
| Predator Ratio | 0.06 ± 0.11 |
| Shredder Ratio | 0.07 ± 0.17 |
| *Richness* | |
| Predator Richness | 2.2 ± 1.5 |
| Scraper Richness | 0.8 ± 0.9 |
| Swimmer Richness | 1.2 ± 1.0 |
| G-collectors Richness | 4.3 ± 1.2 |
| *Ecosystem attributes* | |
| P/R ratio | 0.03 ± 0.28 |
| *Multimetric Indexes* | |
| EBI | 5.2 ± 1.5 |
| STAR_ICMi | 0.308 ± 0.102 |
| *STAR_ICMi metrics* | |
| ASPT | 2.94 ± 0.66 |
| Log(EPTD+1) | 0.012 ± 0.102 |
| 1-GOLD | 0.27 ± 0.29 |
| Family Richness | 9.3 ± 3.4 |
| EPT Family Richness | 1.7 ± 1.1 |
| Shannon Index | 1.566 ± 0.804 |

Based on PCA, a multiple linear regression analysis was conducted on the relationships between the two new environmental variables ("pollution gradient" and "habitat gradient") and the selected macroinvertebrate metrics. In Table 3 significant correlations (bilateral t-test, $\alpha = 0.05$) are highlighted by bold text. Most metrics respond to at least one of the identified gradients and, thus, are influenced by anthropogenic pressure.
The strongest correlations with the two gradients were, respectively, the EBI and the STAR_ICMi multimetric indices respectively with the habitat gradient ($r = 0.460$) and with the pollution gradient ($r = -0.517$). On the other hand, some of the metrics shown in Table 3 presented a significant relationship with only one of the two gradients.

**Table 3** Correlations ($r$) between macroinvertebrate metrics and environmental gradients. Significant relationships are in bold. Variables showing significant relationship with only one of the two gradients are marked with *.

| | Habitat | Pollution |
|---|---|---|
| Chironomidae Ratio* | -0.088 | **0.449** |
| Baetis Ratio | **0.176** | **-0.303** |
| Oligochaeta Ratio* | **-0.209** | 0.096 |
| Filterers Ratio* | **0.354** | -0.189 |
| Predator Ratio* | 0.002 | **-0.350** |
| Shredder Ratio* | 0.026 | **-0.235** |
| Predator Richness | **0.265** | **-0.283** |
| Scraper Richness | **0.237** | **-0.347** |
| Swimmer Richness* | 0.059 | **-0.444** |
| G-collectors Richness | **0.232** | **-0.217** |
| Density* | 0.003 | **-0.171** |
| P/R ratio | 0.083 | -0.102 |
| EBI | **0.460** | **-0.376** |
| STAR_ICMi | **0.372** | **-0.517** |
| ASPT | **0.251** | **-0.513** |
| LOG(EPTD+1) | 0.062 | -0.112 |
| 1-GOLD | **0.207** | **-0.496** |
| Family Richness | **0.378** | **-0.451** |
| EPT Family Richness | **0.315** | **-0.461** |
| Shannon Index | **0.278** | **-0.383** |

The ratio of oligochets to total individuals was found inversely correlated to the availability of habitats, while the ratio of filterers showed a positive correlation with the same variable. The ratio of chironomids (non-biting midges) to total individuals showed a positive correlation with the pollution gradient, while the fractions of predators and shredder and the richness of swimmer taxa were inversely correlated to it.

## 5.4 Discussion

Biological metric screening and environmental variable selection were helpful to select only the information variables to describe the data variability of the entire basin and to understand how biological metrics respond to environmental gradients.
The analysis of environmental parameters has led to individuate two independent gradients. The first one represents the habitat gradient, where higher values are associated with habitat integrity, habitat diversity, RCE-2 and DO. The second one represents the pollution gradient, supported by the higher values of COD, ammonia nitrogen and total phosphorous.
The positive correlation of DO with the habitat gradient can be explained by the fact that the overall DO levels in the basin are generally low (the mean DO value is 74%) and the maximum values are present only in sampling sites with high morphological diversity, where the higher water turbulence allows DO level to approach saturation.
Most of the considered biological metrics are influenced by human activity as suggested by the correlation among metrics and gradients. EBI and STAR_ICMi metrics showed positive correlation with habitat gradient and simultaneously negative correlation with pollution gradient. The significant relationships with both the gradients indicates that they are not suiTable  to differentiate the influence and weight of the various stressors acting simultaneously. This result was expected, since the multimetric indices commonly used for monitoring purposes are generally designed for the assessment of the overall impairment of a site with respect to a reference condition, and they are not intended to relate causes and effects.
On the contrary, some of the biological metrics can be used as indicators of habitat integrity independently from pollution effects, while other metrics respond to pollution independently from habitat conditions. For instance, the ratios of oligochets and chironomids to total individuals are two of the metrics responding to the pollution gradient. In the investigated basin both groups are, in fact, composed by tolerant taxa (most of the oligochets belong to the family Tubificidae,

while most of the chironomids belong to the genus *Chironomus*), which are the dominant and most abundant in the most impaired sites. The presence of these taxa normally contributes also to the determination of the most common multimetric indices (i.e. if they are dominant the index value drops). However, in the analyzed urban basin they showed a differential response to specific environmental gradients. This could be attributed to their ecological niche: in highly polluted conditions, strongly limiting for other invertebrates, oligochets proliferate in fine sediments and other undiversified habitats, while chironomids dominate in more diversified habitats and where coarser substrates are present. The analysis of the ratio of some functional groups, such as filterers, shredders or predators, to total individuals can also help to understand the efficacy of specific restoration actions in an overall impaired environment.

## 5.5 Conclusions

In this study, we wanted to identify a gradient of morphological conditions, or habitat, diversity in streams, aiming to understand if there was some variability component in the macroinvertebrate communities responding to this gradient and independent from the high level of water pollution. We successfully identified such gradient, and found some biological metrics that significantly correlate only with this gradient. The obtained results are site-specific, but we believe that the described methodology can be potentially used to identify any kind of gradient in any sort of impaired environment. The experimental design must allow the construction of a dataset wide enough to provide an exhaustive description of the different impairment levels within the studied environment and should incorporate replication and randomization to offset the problems introduced by confounding factors and non-independence. Gradients related to the overall urbanization (as in Bressler *et al*., 2009), gradients of different kind of pollution, or even gradients not related with anthropogenic stressors, such as the natural longitudinal modification of river ecosystems (e.g. due to the increasing size of the basin) can be identified and analyzed to understand in which way they modify the response of the biological communities.

Nevertheless, in wide dataset with high data variability and complex relationship among variables common statistical tools (such as those used in this paper) can be not appropriate. Usually, hypotheses about the central response of organisms to environmental gradients are tested, although the effects of other stressors may also influence such response and decrease the fit of the model, which may even become uninformative (Lancaster & Belyea, 2006). In this perspective, quantile regression, theorized by Koenker in 1978, allows the various stressors to be considered as “constraints” to the distribution of biological communities, without compromising the model causal relationship (Cade et al., 1999; Thomson et al., 1996).
The analysis of variables as limiting factors may be useful for assessing the potential of biological communities, particularly important in catchment strongly influenced by human activities. This kind of approach, not widely used in ecology, will offer a better awareness in the evaluation of river restoration success and, thus, in decision-making processes.

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# 6 LIMITING FACTORS IN HEAVILY MODIFIED WATER BODIES: THE QUANTILE REGRESSION APPROACH

**Abstract**

Biological indicators, particularly benthic macroinvertebrates, are widely used and effective measures of the impact of urbanization on stream ecosystems. In particular, in heavily modified water bodies (HMWBs) macroinvertebrate are useful to individuate the ecological potential, following European Water Framework Directive (WFD) dictates. In this work, we want to (1) develop gradients that accurately characterize water quality and habitat availability; (2) find the best model that describes data distributions of biological metrics against gradients using quantile regression to assess the role of environmental variables as constraints; (3) identify which metrics respond to gradients. Using PCA, we identified two gradients: the first one represents the gradient of hydromorphological conditions and the second one represents the overall water quality gradient. Various biological metrics, including taxonomic composition and richness, functional feeding and habit groups, were selected using several criteria. Quantile regression was used to select metrics that respond to gradients. Most of the analysed metrics have wedge-shaped relationship because of the limiting effects of water pollution and habitat loss on ecological status. In HMWBs water quality is the strongest driving force for the decrease of biodiversity and ecological status. However, some metrics have a preferential response to habitat gradient rather than to water quality. The response of such metrics help to quantify the effect of habitat loss on biological communities. The results underline the need to consider and address such large-scale pressures in river management and restoration because they potentially constrain the effects of local restoration measures (for example mesohabitat restoration). Furthermore, using the quantile regression approach it is also possible to assess how the considered gradient acts as limiting factor in order to define the ecological potential in HMWBs.

**Keywords**

Macroinvertebrate, heavily modified water bodies, ecological potential, environmental gradients, quantile regression

*Submitted manuscript*

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Limiting factors in heavily modified water bodies: the quantile regression approach

## 6.1 Introduction

Human activities have altered the environment for centuries leading to the loss of habitat and biodiversity. In particular, stream ecosystems are some of the most threatened in various parts of Europe. These alterations especially derive from urbanization and lead to stream degradation and aquatic communities decline (Paul and Meyer, 2001): natural hydrology of streams is dramatically affected by increased stormwater runoff from impervious surfaces in urban areas and this increases flood magnitude, duration, and frequency (Wissmar et al., 2004; Roy et al., 2005; Walsh et al., 2005b). Conversely, the increase of water consumption in urban areas can significantly reduce the stream flows and, so, the availability of habitats for biological communities, increase water temperature, and reduce dissolved oxygen concentration (Finkenbine et al., 2000; Groffman et al., 2003). Urban runoff can also carry nutrients from residential area and toxic chemicals from industrial areas into nearby streams (Neal and Robson, 2000).

Furthermore, the discharge of untreated or inadequately treated wastewater in streams may cause water pollution and the input of large amounts of FPOM (Chang, 2005). Thus, invertebrate communities downstream the discharge of wastewater are often impoverished and dominated by pollution-tolerant species (Canobbio et al., 2009), and show shifts in the composition of feeding groups (Rawer-Jost et al., 2000). In European Union Water Framework Directive (WFD: Directive, 2000/60/EC), developed in order to implement a more comprehensive approach to aquatic environments, the river basins with these characteristics are defined *heavily modified water bodies* (HMWBs).

HMWBs have unique water quality characteristics that, in most cases, are significantly different from normal stream conditions upstream of the discharge or at regional reference sites (Taylor, 2002; Brooks et al., 2004). Reference sites are commonly used in bioassessment studies to identify undisturbed or pristine conditions and hence to define management and recovery targets (Hughes, 1995; Prins and Smith, 2007). The increase of urban development in wide areas often results in the absence of reference sites for HMWBs (Chessman and Royal, 2004) and this makes difficult to define a target condition for restoring urban stream sites (Meyer et al., 2005).

The WFD requires that all waters achieve good ecological status and only slightly deviate from natural reference conditions. The ecological status is quantified in many European member states using multi-metric indices, and good ecological status corresponds to a specific score value. However, there is little information on the combined limiting effects of large-scale pressures on the biological metrics. In addition, WFD established exemptions for HMWBs as in these basins a good ecological status is unreachable. So, WFD accepts that different quality objectives are set on the basis of the so-called ecological potential attainable under current conditions of the basin. However, the European Directive does not state what are the criteria to establish ecological potential for HMWBs.

In this view, the individuation of which factors set limits to biological community development and of their respective values is of great interest for river managers and river restoration strategies. In urban streams it is usually hard to assess causal relationships among specific stressors and responses of biological communities using the most common statistical tools. Usually, hypotheses about the central response of organisms to environmental gradients are tested, although the effects of other stressors may also influence such response and decrease the fit of the model, which may even become uninformative (Lancaster & Belyea, 2006). In this perspective, quantile regression allows the various stressors to be considered as “constraints” to the distribution of biological communities, without compromising the model causal relationship (Cade et al., 1999; Thomson et al., 1996).

Quantile regression criteria characterize the spread and shape of the upper boundary of the data, when biological metrics are tested against environmental gradients (e.g., water quality, habitat availability). Quantile regression has been used instead of traditional central response model because it more effectively characterizes the upper boundary of the biological indices and gradient plots (Purcell et al., 2009). Figure 1 shows a comparison of traditional linear regression and quantile regression.

Many papers show the use of linear quantile regression models to describe biological metrics against gradients (i.a., Purcell et al., 2009; Kail et al., 2012). Often, biological metrics that describe macroinvertebrate assemblages do not vary linearly to an environmental gradient. Cade, Noon & Flather proposed a modified version of Akaine Information Criterior (AIC) corrected for small sample size (developed by Johnson & Omland, 2004) to select the best model for quantile regression (Appendix C in Cade et al., 2005).
Nevertheless, no papers in literature show how to choose the best quantile regression model (e.g., linear, logarithmic, exponential), which describes biological data against environmental gradients in HMWBs.
So, the objectives of this study are (1) to develop gradients that accurately characterize water quality and habitat availability; (2) to find the best model that describes data distributions of biological metrics against gradients using the quantile regression; (3) to identify which metrics respond to gradients.

## 6.2 Materials and methods

### *6.2.1 Study site*

All sites were sampled and their ecological status was assessed by regional authorities by the AQEM method (Assessment System for the Ecological Quality of Streams and Rivers throughout Europe using Benthic Macroinvertebrates, Hering et al., 2004).
We analyzed 19 sites (hereafter called S1 – S19) in Olona-Seveso-Lambro, the piedmont area of Lombardy region in Italy (Figure 1). The area is heavily exploited by human presence and activities (over 5,000,000 inhabitants throghout the whole basin and rhe presence of heavy industry).

### *6.2.2 Data types*

On the whole, 220 samples were collected and analyzed for the 19 study sites by different city agencies of ARPA (Agenzia Regionale per la Protezione dell'Ambiente, the Italian Regional Agency for environmental protection). Water quality, physical habitat and benthic macroinvertebrates data were collected during spring, summer and fall in the period 2005 - 2010. Water quality data included electric conductivity, dissolved oxygen (DO), pH, biological and chemical oxygen demand ($BOD_5$ and COD), total phosphorus (TP), total and ammonia nitrogen (TN and $NH_4$–N), and *Escherichia coli*.
Physical habitat data consisted of visual-based measures patterned following River Functionality Index (APAT 2007, adapted from RCE-2 protocol - Petersen, 1992). Hydromorphological parameters were measured during low flow periods, while land cover characteristics (woody vegetation, intensive agriculture and urban areas expressed as a percentage of the total basin area) were calculated based on CORINE 2000 data with GIS software QuantumGis, (Freeware version 1.8).
Biological data were collected semiquantitatively. All biological samples were stored in 90% ethanol and identified at genus level, except for Diptera order and Oligochaeta subclass that were identified at family level, using an Optika stereomicroscope (180x) and taxonomic keys (Campaioli et al. 1999; Sansoni 1992).

Macroinvertebrates were assigned to the FFGs and FHGs, according to literature (Merritt and Cummins 1996; Tachet et al. 2000). Based on macroinvertebrate functional traits, ecosystem attributes (Table 1) were calculated (Merritt et al., 2002; Canobbio et al. 2010).

### *6.2.3 Developing gradients*

71 environmental variables were used in developing gradients. A preliminary analysis of collected environmental variables showed that many water quality parameters and habitat descriptors resulted to be redundant. Only one metric from a group of redundant metrics with a Variance Inflation Factor (VIF) > 20 or r > 0.7 was considered. Relationships and patterns among the selected variables were analysed with a Principal Component Analysis (PCA) to create dominant gradients of variation.

### *6.2.4 Biological metric selection*

Four screening criteria, plus a fifth qualitative criterion, were used to screen 53 biological metrics (Table 2). These criteria were adapted from Purcell et al., 2009 that evaluated metric screening and techniques from several studies. This screening is important for the elimination of both non-informative metrics and of redundant ones.

**Table 1** Description of ecosystem attributes based on macroinvertebrate functional traits (Canobbio et al., 2010).

| Ecosystem attributes | Description | Traits (FFG and FHG) | General ecosystem attribute trend for a better functionality |
|---|---|---|---|
| P/R | Primary production as a proportion of community respiration | Scrapers as a proportion of shredders and total collectors | + (autotrophic system) |
| CPOM/FPOM | Storage CPOM as a proportion of FPOM in and on the sediments | Total shredders as a proportion of total collectors | + (normal shredder riparian system) |
| SPOM/BPOM | Suspended FPOM as a proportion of deposited FPOM | F-collectors as a proportion of G-collectors | + (enriched in SPOM) |
| Habitat FFG | Availability of sTable surfaces and non-shifting sediments | Scrapers + F-collectors as a proportion of shredders + G-collectors | + (sTable substrate) |
| Habitat FHG | Availability of sTable surfaces and non-shifting sediments | Clingers + Climbers as a proportion of burrowers + sprawlers + swimmers | + (sTable substrate) |

Notes: CPOM: coarse particulate organic matter; FPOM: fine particulate organic matter; SPOM: suspended fine particulate organic matter; BPOM: benthic fine particulate organic matter; G-collectors: gatherer-collectors; F-collectors: filterer-collectors.

**Table 2** Criteria for biological metric selection

| | |
|---|---|
| 1 | Range<br>Percent > 10%<br>Richness > 5 |
| 2 | Area-based effects examined (Vannote et al., 1980) determined using linear regression. No clear relationship between metrics and catchment area must be present. |
| 3 | Quantile regression<br>Metrics responding across the entire gradient |
| 4 | Redundancy - metrics considered redundant if r > 0.7 |
| (5) | Evaluation of eliminated metrics. Some metrics could be reconsidered. |

Note: Procedure adapted from Purcell et al., 2009.

First, the range of each metric (from minimum to maximum value) was examined to ensure that it was broad enough to discern differences in magnitude. The criterion provides that the range of percentage metrics must be > 10 and that the range of richness metrics must be > 5 (e.g., Klemm et al., 2003).

Second, the relationship of the metrics to the catchment area was examined using correlation analysis (as done in Klemm et al., 2003). This step is based on the natural faunal shifts occurring with increasing catchment size (River Continuum Concept, Vannote et al., 1980).

Third, the relationships of each biological metric to the two environmental gradients were examined using quantile regression criteria. Linear, exponential or logarithmic model (Table 3) was selected for each biological metric using the Akaine Information Criterion corrected for small sample size (AICc – Appendix C in Cade et al., 2005).

**Table 3** Equations used for quantile regression models

| Model | Equation |
|---|---|
| Linear | $y = a + b*x$ |
| Logaritmic | $y = a + b*\ln(x)$ |
| Exponential | $y = a + b*e^x$ |

For each relation between metrics and gradients, we chose the best model and the best quantile ($\tau$) to assess the limiting effect of the gradient on the metric. For this reason (the search of the limiting effect) we decided arbitrarily to consider only $\tau > 0.85$. The statistical analyses were conducted using both BLOSSOM software (Cade and Richards, 2005) and the quantreg package in R Project software (R Development Core Team 2012).

$\Delta AIC_c(\tau)$ was computed for each model by subtracting the $AIC_c(\tau)$ for the constant model from the $AIC_c(\tau)$ for more complex models.

The test is considered significant if two conditions occur simultaneously: we chose the $\tau$ at which there is the least $\Delta AIC_c(\tau)$ and to which the confidence interval for b parameter of the model is the narrowest (Kail et al., 2012; Fig. 1). If the difference between $AIC_c(\tau)$ for the second model and $AIC_c(\tau)$ for the best model was greater that 2, then the best model was determined to be significantly different from the second (Johnson & Omland, 2004). If no model had a significant difference with the null one or with the second one, then the biological metric was discarded, in order to eliminate metrics that have not a clear trend.

Fourth, metrics were plotted against each other to test for redundancy by Pearson correlation. If the correlation of two metrics was greater than 0.7, only one metric from a group of redundant metrics was considered, choosing the one that have the best quantile regression models.

Fifth, we conducted an evaluation of the eliminated metrics to reconsider, eventually, their ecological importance in this work.

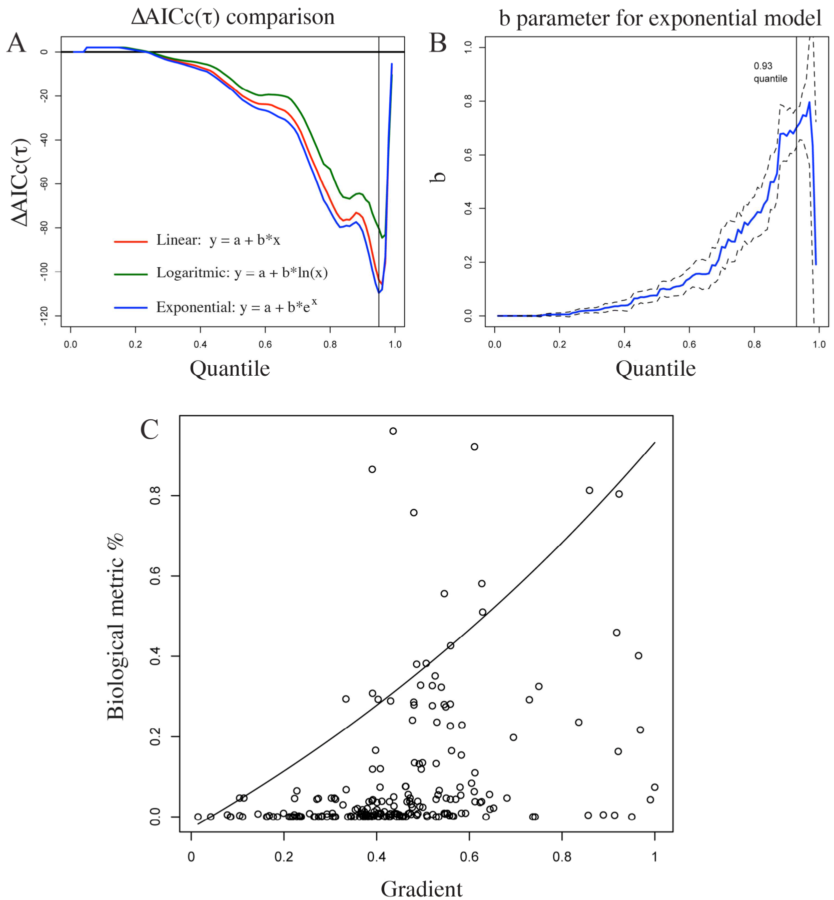


**Figure 1** In panel A $\Delta AIC_c(\tau)$ for each model for all $\tau$ are shown. In this case, the exponential model presents the minimum value of $\Delta AIC_c(\tau)$ at $\tau = 0.93$ (vertical black line). Simultaneously, at the same $\tau$, the confidence interval of b parameter for exponential model gets its minimum (panel B); for larger quantiles confidence intervals are wider and can included zero. In the panel C, the selected 0.93 exponential quantile regression (black line curve) was superimposed to a scatterplot between a biological metric and a gradient..

## 6.3 Results

### *6.3.1 Environmental gradients*

The collected environmental variables reflected the heavy urbanization of the basin. Both water quality parameters and morphological descriptors showed a high degree of impairment (Table 4), with high concentrations of pollutants and low levels of habitat integrity. The collected variables showed also that in the whole basin no site had a quality comparable to the reference conditions.
After the preliminary analysis of collected environmental variables, Q (flow), DO, COD, $N\text{-}NH_4$, $N\text{-}NO_3$, TP, RCE-2, presence of leaf pack retention structures in the riverbed, habitat diversity and hydraulic integrity were selected to develop gradients using PCA. Figure 2 shows the first two principal components of the PCA and the factor loadings of the selected variables. The water quality parameters and the morphological indicators basically cluster in two different groups, identified by the first two components explaining about 55% of the total variance. Physico-chemical variables mostly correlate with axis 2, while morphological variables and Dissolved Oxygen (DO), as saturation percentage, correlate with axis 1. The positive correlation of DO with the habitat gradient can be explained by the fact that overall DO levels in the basin are generally low (the mean DO value is 74%) and the maximum values are present only in sampling sites with high morphological diversity, where the higher water turbulence allows DO level to go closer to saturation. The factor scores of the first two principal components were subsequently used as new aggregated variables. The first one represents the gradient of hydromorphological conditions (*habitat gradient*), where higher values are associated with a greater morphological integrity. The second one represents the overall *water quality gradient*, where the lower values are associated to higher pollution levels. Due to the mathematical properties of principal components, these are gradients that maximize variation and are independent from each other.

### *6.3.2 Metrics response to gradients*

The metric screening procedure resulted in the selection of several metrics for use in describing limiting action of water quality and habitat gradients. The first step in the metric screening procedure eliminated ten metrics that did not have sufficient range of values (Diptera richness, Ephemeroptera richness, percentage of Plecoptera, Plecoptera richness, Trichoptera richness, F-collector richness, Shredder richness, percentage of Climbers, Climber richness – Table 5). The second step found that twenty metrics had a significant relationship (positive or negative) to the site catchment area; these metrics were eliminated. STAR_ICMi was one of the eliminated metrics in second step, but was retained for use in third step screening for its importance in the ecological status evaluation.

The third step was divided in two phases: in the first one, we analysed the relationship of each biological metric to the water quality gradient and in the second one the relationships to the habitat gradient, using quantile regression.

The first phase of the third step showed that most of the selected metrics were influenced by water quality. Quantile regression eliminated G-collector richness, percentage of Scrapers, Scraper richness and P/R. For all the metrics, b parameters of selected model (linear, logarithmic and exponential) included zero in its confidence interval (Table 6A).

The second phase of the third step showed that few metrics were affected by habitat gradient. Only percentage of Predators, Habitat FFG and STAR_ICMi met the two conditions to consider the test significant (Table 6B).

Selected metrics after quantile regression step were tested for redundancy (Pearson test). This step was divided in two phases. In the first one, six of the selected metrics after quantile regression against water quality gradient were found to be redundant with other ($r > 0.7$) and so were to be discarded (Table 7A). In the second phase the Pearson test (Table 7B) did not found redundant metrics after quantile regression selection (metrics against habitat gradient).

After four steps of screening, we selected seven metrics (percentage of Baetis, Oligochaeta and Predators, Clinger richness, Family richness, Shannon - Family level – and STAR_ICMi) to describe the limiting action of water quality (Fig. 3). Only three metrics were selected to describe the influence of habitat availability (Fig. 4).

**Table 4** Values of the environmental variables collected in the Lambro-Seveso-Olona system during the survey. Habitat variables are expressed as the ratio between the actual condition and the best possible one, using RCE-2 reference conditions.

| Variable | mean ± st.dev. |
|---|---|
| ***Water quality parameters*** | |
| Flow - Q ($m^3/s$) | 1.833 ± 1.633 |
| Dissolved Oxygen (%) | 74 ± 17 |
| $BOD_5$ (mg/L) | 8 ± 6 |
| COD (mg/L) | 31 ± 14 |
| *E. coli* (CFU/100ml) | $8.7*10^4 \pm 12.8*10^4$ |
| Total Nitrogen - TN (mg/L) | 11.118 ± 4.606 |
| $N\text{-}NH_4$ (mg/L) | 2.655 ± 2.302 |
| $N\text{-}NO_3$ (mg/L) | 5.384 ± 2.446 |
| Total Phosphorus - TP (mg/L) | 1.043 ± 0.535 |
| $P\text{-}PO_4$ (mg/L) | 0.788 ± 0.445 |
| ***Habitat variable (RCE-2 ratio)*** | |
| Land Use | 0.18 ± 0.14 |
| Riparian vegetation kind | 0.13 ± 0.11 |
| Riparian veg. Width | 0.25 ± 0.20 |
| Riparian veg. Continuity | 0.36 ± 0.22 |
| Ecological flow | 0.85 ± 0.23 |
| Flooding area | 0.12 ± 0.11 |
| Leaf Packs | 0.27 ± 0.17 |
| Erosion | 0.18 ± 0.22 |
| Section | 0.31 ± 0.24 |
| Fish habitat | 0.88 ± 0.26 |
| Riffle-Pool sequence | 0.47 ± 0.32 |
| Macrophytes | 0.25 ± 0.12 |
| Detritus | 0.36 ± 0.18 |
| Hydraulic integrity | 0.44 ± 0.14 |
| Biota | 0.25 ± 0.08 |
| Riparian Vegetation | 0.20 ± 0.14 |
| Habitat diversity | 0.28 ± 0.14 |
| RCE-2 | 0.29 ± 0.10 |

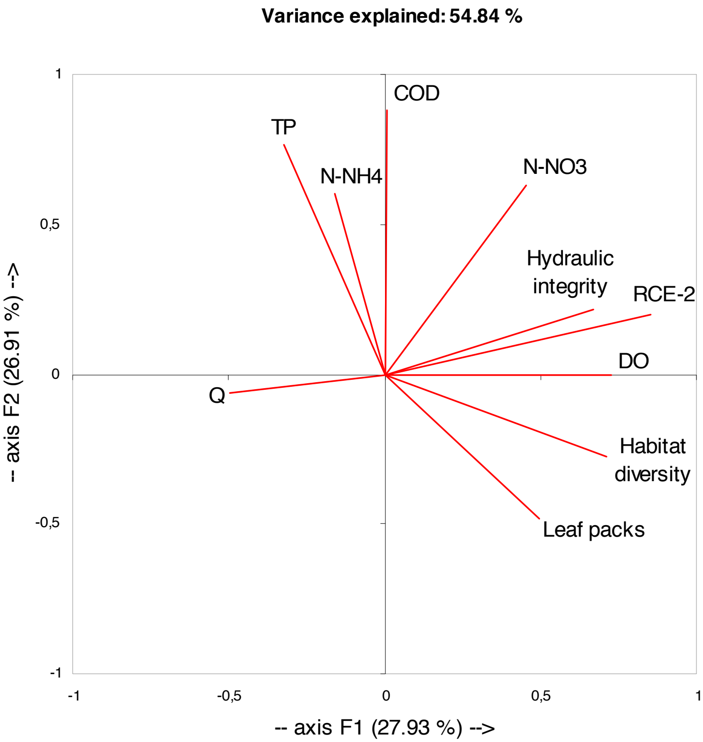


**Figure 2** Factor Loading plot for the first two Principal Components, accounting for more than 50% of the total variance of the environmental variables.

**Table 5A** Results of step 1 and step 2 screening for biological metrics.

| Metrics | Step 1 Range | Step 2 Area |
|---|---|---|
| Shannon diversity index* | √ | √ |
| Shannon (Family level)* | √ | √ |
| Simpson index* | √ | √ |
| Family richness* | √ | √ |
| EPT family richness* | √ | √ |
| EBI* | √ | √ |
| Chironomidae % | √ | X |
| Diptera % | √ | X |
| Diptera richness | X | – |
| Baetis %* | √ | √ |
| Ephemeroptera %* | √ | √ |
| Ephemeroptera richness | X | – |
| EPT %* | √ | √ |
| EPT Richness* | √ | √ |
| Oligochaeta %* | √ | √ |
| Oligochaeta richness | √ | X |
| Plecoptera % | X | – |
| Plecoptera richness | X | – |
| Trichoptera % | √ | X |
| Trichoptera richness | X | – |

Notes: “X” indicates that the metric was eliminated and a dash indicates that the metric was no longer considered in the selection process. A “√” indicates that the metric met the criteria of that step and was retained. Metrics with a “*” were retained for use in the step 3 screening. STAR_ICMi was retained for its importance for the research work.

**Table 5B** Results of step 1 and step 2 screening for biological metrics.

| Metrics | Step 1 Range | Step 2 Area |
|---|---|---|
| Dominant%* | √ | √ |
| Dominant richness | X | – |
| G-collector % | √ | X |
| G-collector richness* | √ | √ |
| F-collector % | √ | X |
| F-collector richness | X | – |
| Predator %* | √ | √ |
| Predator richness* | √ | √ |
| Scraper %* | √ | √ |
| Scraper richness* | √ | √ |
| Shredder % | √ | X |
| Shredder richness | X | – |
| Burrower % | √ | X |
| Burrower richness | √ | X |
| Climber % | X | – |
| Climber richness | X | – |
| Clinger % | √ | X |
| Clinger richness* | √ | √ |
| Sprawler % | √ | X |
| Sprawler richness | √ | X |
| Swimmer %* | √ | √ |
| Swimmer richness | √ | X |
| P/R* | √ | √ |
| CPOM/FPOM | √ | X |
| SPOM/BPOM* | √ | √ |
| Habitat FFG* | √ | √ |
| Habitat FHG | √ | X |
| ASPT | √ | X |
| BMWP | √ | X |
| LOG_EPTD | √ | X |
| 1-GOLD | √ | X |
| STAR_ICMi* | √ | X |

Notes: "X" indicates that the metric was eliminated and a dash indicates that the metric was no longer considered in the selection process. A "√" indicates that the metric met the criteria of that step and was retained. Metrics with a "*" were retained for use in the step 3 screening. STAR_ICMi was retained for its importance for the research work.

**Table 6**A: Results of step 3 screening for biological metrics against water quality gradient.

| Metrics | tau | CI of b parameter includes zero | ΔAICc (lin) | ΔAICc (log) | ΔAICc (exp) | ΔAICc (S-B) | Choosen model |
|---|---|---|---|---|---|---|---|
| Shannon diversity index* | 0,85 | no | -33,8 | -43,2118 | -27,37 | 9,41 | logaritmic |
| Shannon (Family level)* | 0,85 | no | -33,81 | -43,21 | -27,38 | 9,4 | logaritmic |
| Simpson index | 0,85 | no | -35,98 | -37,25 | -30,84 | 1,27 | logaritmic |
| Family richness** | 0,95 | no | -66,31 | -66,32 | -59,29 | 0,01 | logaritmic |
| EPT family richness | 0,92 | no | -104,11 | -101,19 | -102,68 | 1,43 | linear |
| EBI | 0,99 | no | -51,77 | -53,64 | -47,41 | 1,87 | logaritmic |
| Baetis %* | 0,95 | no | -103,85 | -80,27 | -109,61 | 5,76 | exponential |
| Ephemeroptera %* | 0,94 | no | -102,54 | -80,41 | -107,97 | 5,43 | exponential |
| EPT % | 0,88 | no | -76,6 | -67,55 | -76,09 | 0,51 | linear |
| EPT Richness* | 0,92 | no | -103,37 | -100,83 | -101,33 | 2,04 | linear |
| Oligochaeta %* | 0,88 | no | -9,59 | -6,31 | -12,72 | 3,13 | exponential |
| Dominant richness | 0,98 | no | -8,48 | -7,11 | -9,59 | 1,11 | exponential |
| G-collector richness | | yes | | | | | |
| Predator %* | 0,95 | no | -45,48 | -48,27 | -37,98 | 2,79 | logaritmic |
| Predator richness* | 0,97 | no | -41,2 | -48,75 | -34,85 | 7,55 | logaritmic |
| Scraper % | | yes | | | | | |
| Scraper richness | | yes | | | | | |
| Clinger richness* | 0,95 | no | -83,18 | -86,06 | -78,71 | 2,88 | logaritmic |
| Swimmer %* | 0,95 | no | -103,07 | -92 | -98,57 | 4,5 | linear |
| P/R | | yes | | | | | |
| SPOM/BPOM | 0,91 | no | -57,73 | -55,25 | -55,91 | 1,82 | linear |
| Habitat FFG | 0,92 | no | -51,03 | -46,76 | -52,05 | 1,02 | exponential |
| STAR_ICMi* | 0,89 | no | -53,08 | -67,98 | -43,06 | 14,9 | logaritmic |

**Table 6B**: Results of step 3 screening for biological metrics against habitat gradient.

| Metrics | tau | CI of b parameter includes zero | ΔAICc (lin) | ΔAICc (log) | ΔAICc (exp) | ΔAICc (S-B) | Choosen model |
|---|---|---|---|---|---|---|---|
| Shannon diversity index | | yes | | | | | |
| Shannon (Family level) | | yes | | | | | |
| Simpson index | | yes | | | | | |
| Family richness | | yes | | | | | |
| EPT family richness | | yes | | | | | |
| EBI | | yes | | | | | |
| Baetis % | | yes | | | | | |
| Ephemeroptera % | | yes | | | | | |
| EPT % | | yes | | | | | |
| EPT Richness | | yes | | | | | |
| Oligochaeta % | | yes | | | | | |
| Dominant richness | | yes | | | | | |
| G-collector richness | | yes | | | | | |
| Predator %* | 0.99 | no | -78.93 | -26.36 | -89.10 | 10.17 | exponential |
| Predator richness | | yes | | | | | |
| Scraper % | 0.97 | no | -43.81 | -5.58 | -45.02 | 1.21 | exponential |
| Scraper richness | | yes | | | | | |
| Clinger richness | | yes | | | | | |
| Swimmer % | | yes | | | | | |
| P/R | | yes | | | | | |
| SPOM/BPOM | | yes | | | | | |
| Habitat FFG* | 0.96 | no | -28.15 | -3.53 | -21.26 | 6.89 | linear |
| STAR_ICMi* | 0.93 | no | -33.29 | -22.73 | -30.02 | 3.27 | linear |

Notes: CI: Confidence interval. Metrics with a "*" were retained for use in the step 4 screening. If confidence interval of b parameter includes zero or ΔAICc(S-B) is < 2, metric is discarded. ΔAICc(S-B) indicates the difference between AICc for the second model and AICc for the best model. Metrics with a "**" were retained despite low significance in quantile regression analysis because of their ecological importance.

**Table 7** Results of step 4 screening for biological metrics.

| Step 4 | |
|---|---|
| **A – Water quality gradient** | |
| Metrics | Pearson < 0.7 |
| Shannon diversity index | X |
| Baetis % | √ |
| Ephemeroptera % | X |
| EPT Richness | X |
| Oligochaeta % | √ |
| Predator % | √ |
| Predator richness | X |
| Clinger richness | √ |
| Swimmer % | X |
| Family richness | √ |
| Shannon (Family level) | √ |
| STAR_ICMi | √ |
| **B – Habitat gradient** | |
| Metrics | Pearson < 0.7 |
| Predator % | √ |
| Habitat FFG | √ |
| STAR_ICMi | √ |

Notes: "X" indicates that the metric was eliminated. A "√" indicates that the metric met the criteria of that step and was retained.

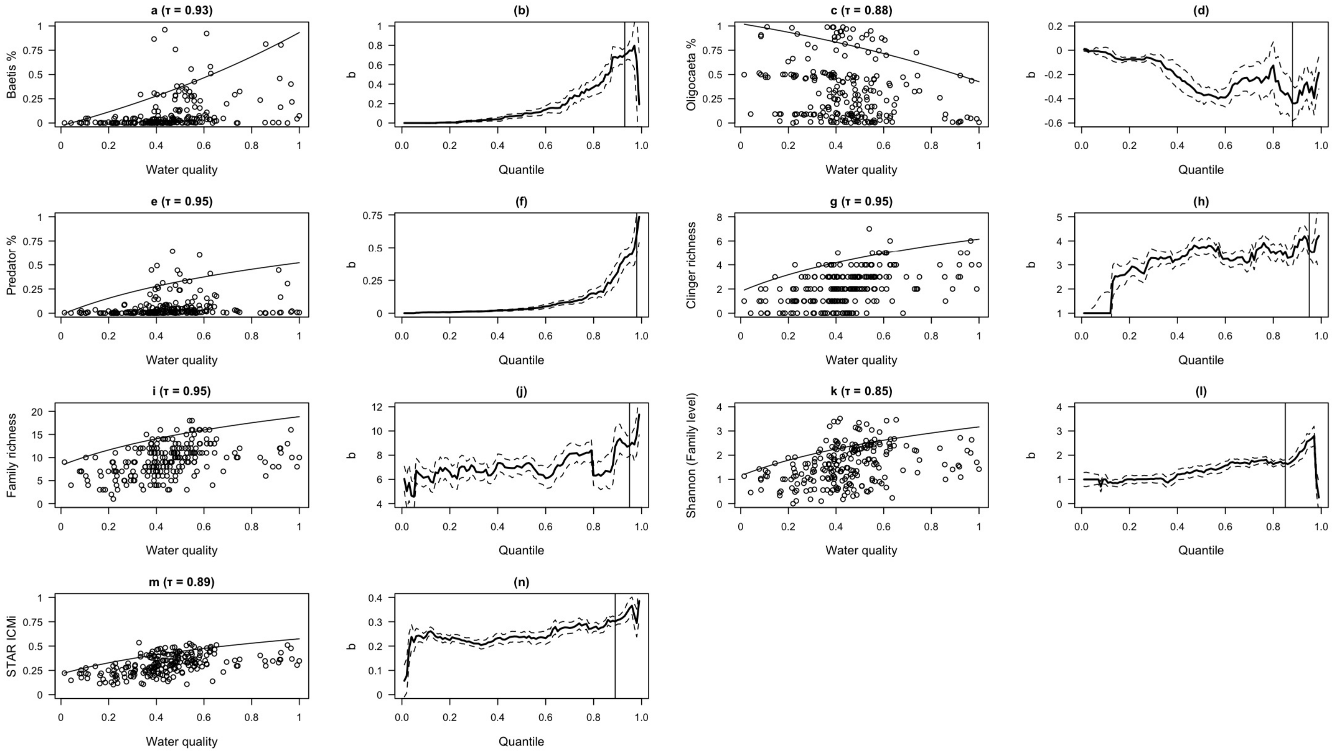


**Figure 3** Scatterplots of invertebrate metrics against water quality gradient (a, c, e, g, i, k, m) and their respective b parameter value for all τ (b, d, f, h, j, l, n – continuous lines) with 95% confidence intervals (dashed lines). The best quantile regression lines are given, based on results in Table 5A. Vertical black line indicates the selected τ.

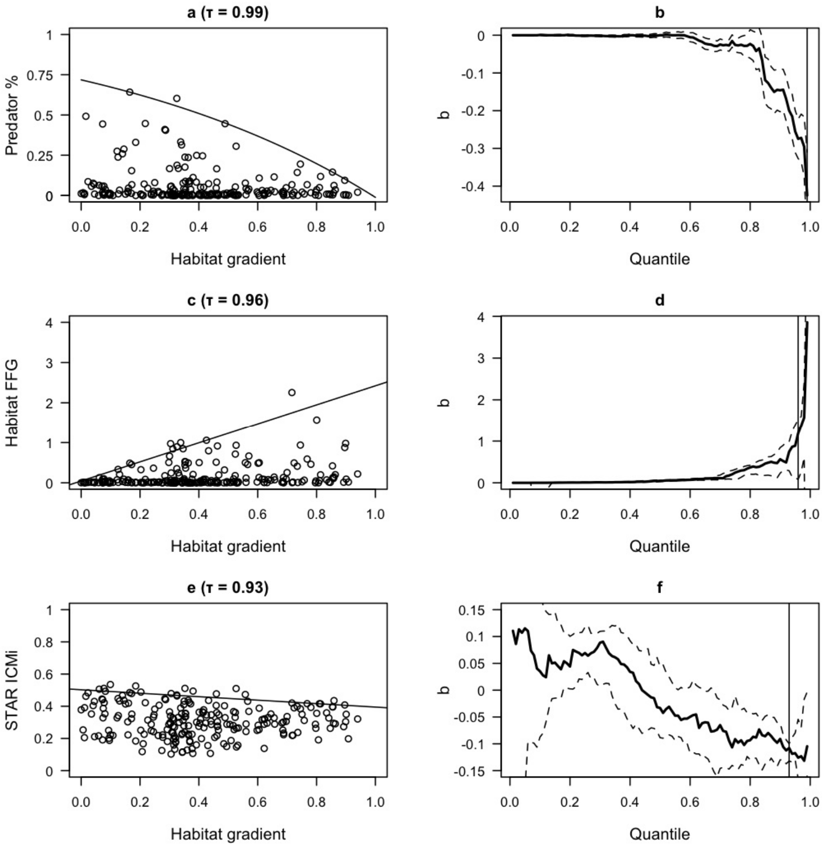


**Figure 4** Scatterplots of invertebrate metrics against habitat gradient (a, c, e) and their respective b parameter value for all τ (b, d, f – continuous lines) with 95% confidence intervals (dashed lines). The best quantile regression lines are given, based on results in Table 5B. Vertical black line indicates the selected τ.

## 6.4 Discussion

We analysed the combined effects of different stressors and other environmental variables on the composition and abundance of macroinvertebrate assemblages in Olona-Seveso-Lambro basin. We used environmental gradients to represent the various conditions that could be found in HMWBs, where reference sites were lacking.
From PCA we obtained two gradients. The axis 1 represents the habitat gradient, where higher values are associated with habitat integrity, habitat diversity, RCE-2 and DO, while the axis 2 represents the water quality gradient, negatively supported by the higher values of COD, ammonia nitrogen and total phosphorous.
The relationship between biological metrics and environmental variables revealed the presence of sensitive metrics for evaluating the complex effects of urbanization. In our study, biological metrics were eliminated in each step of the metric selection process. Several metrics were eliminated in step 1 (range criterion) and step 2 (area-based effect) because a lot of the considered metrics were not broad enough to discern differences in magnitude or had strong relation with the basin area. Other studies that have examined fish as biological indicators have found a stronger relationship with catchment area (e.g., Fausch et al., 1984). Others instead have found the absence of any relationship between catchment area and biological metrics (Purcell et al., 2009). Our study sites were located along the entire extension of the basin (from spring to plan zone) and so many metrics, such as the percentage of G-collectors, F-collectorsand burrowers, had a strong relationship with the basin area. The step 2 of the metric selection process ensured that only independent metrics from area were selected.
For different biological metrics (percentage of Baetis, Oligochaeta and Predators, Clinger richness, Family richness, Shannon - Family level – and STAR_ICMi) the water quality acts as a limiting factor (Fig. 3). Metric selection process allowed selecting only those metrics that can best describe the limiting action of water quality on biological communities. In six scatterplots (Fig. 3), there is a clear upper boundary indicating that the current maximum biological condition decreases as water quality decreases. Only percentage of Oligochaeta increased as

water quality decrease, indicating that in stream with good physico-chemical properties the less proliferation of tolerant taxa (in particular belonging to the Tubificidae family) allows the development of a more diversified biological community. Community richness and diversity increase were evidenced by the rise in Shannon Diversity Index value that is limited by water quality.

In our dataset, *Baetis* was the most sensitive taxon and the scatterplot in Figure 3 indicates that the percentage of *Baetis* increases as water quality increases. While metrics belonging to EPT (Ephemeroptera, Plecoptera, and Trichoptera) orders have strong negative responses to anthropogenic disturbances (Barbour et al., 1992; Carter and Fend, 2005), *Baetis* is usually a tolerant genus. In the sites of the studied basin the level of impairment, albeit different, is always high, and in such conditions *Baetis* represents the *borderlin*e taxon among different levels of pollution. Where it is dominant, the water quality increases enough to allow *Baetis* proliferation, while in the most impaired sites it tends to disappear letting Diptera and Oligochaeta to proliferate. Thus, *Beatis* can be considered a good basin-specific bioindicator, given the current conditions.

Clingers prefer sTable  and sediment-free substrate (Merritt et al., 2002), so a decreased substratum particle size in sites downstream of urbanized areas (enriched in FPOM) may not provide an adequate habitat for clingers. Quantile regression between clinger richness and water quality shows a decrease of clingers as water quality decreases; hence results confirm theory and makes clinger decrease a good proxy of urbanization.

Based on quantile regression results, predators increase with increasing water quality. The percentage of predators is low where the water quality is poor (low value of gradient) and tolerant taxa, such as oligochaetes and chironomids, proliferate. In situations of medium and low water quality water, the predators proliferate, probably because of the high availability of preys formed by tolerant collectors, but quickly decreases with improving water quality (high values of gradient).

In general, we found, as expected, that biodiversity strongly decreases with decreasing water quality. Family richness shows clear upper boundary indicating the limiting action of physico-chemical properties

on richness and biodiversity. Despite the low significance of Family richness metric in quantile regression analysis, we decided to retain this metrics for its ecological importance. Family richness may be an easy-to-use indicator of biodiversity that strongly respond to water quality gradient and it is widely used in the multimetric indices adopted across the EU state members. The low significance may depend on similar response of linear and logarithmic model to metric data distribution. Anyhow, ΔAICc(lin) and ΔAICc(log) values (respectively -66,31 and -66,32) are clearly distant from the AICc calculated by the null model and so we can be sure to select a metric whose distribution is significantly different from a constant.
STAR_ICMi, the actual index used for biomonitoring in surface water bodies in many parts of Europe, shows the same trends as the other metrics. Despite the narrow interval of STAR_ICMi values (from 0.1 to 0.6), we note a trend indicating the limiting effect of water quality also on this index. Such effect is shown in Figure 3 where, however, the slope of logarithmic quantile regression is slight.

After screening, only for three biological metrics (percentage of predators, Habitat FFG and STAR_ICMi) habitat gradient has been found to act as limiting factor (Fig. 4).
Predators significantly respond to the habitat gradient. Where simplified habitats are present (low values of the gradient), the percentage of predators increases. In this basin, the predator group is mostly composed of Hirudinea who prefer simple habitats (gravel or pebbles, uniform substrates - Merritt et al., 2002). The percentage of predators decreases with increasing habitat complexity (high values of the gradient) where taxa requiring complex habitats can proliferate and equilibrate the relative abundances of FFGs.
Habitat FFG strongly responds to habitat gradient. The use of filterers as a common functional feeding group has been widely cited as a metric that responds to disturbance (Kerans et al., 1992; Klemm et al., 2003; Ode et al., 2005). Our results show that filterer increase and G-collector decrease may promote “Habitat FFG” metric increasing.
STAR_ICMi behaves unclearly when tested against the habitat gradient. With increasing habitat complexity a slight decrement of

STAR index values results. On the other hand, the slope of the logarithmic regression representing the relationship between STAR index and water quality is also very slight. It is important to underline that values of STAR index never exceed the value of 0.6, corresponding to *sufficient* ecological status. STAR_ICMi has been created to assess more comprehensive datasets, that specifically need reference sites to be included. A better understanding of this metric response should probably need considering broader gradients, because it shows a low description capability among impaired sites

## 6.5 Conclusions

The results presented in this paper indicate that wedge-shaped relationships are common in large and complex datasets, which might be not considered with statistical methods that quantify changes in the central tendency along pressure gradients, such as least-square regression. Most of the analysed metrics have wedge-shaped relationship: this is an empirical evidence for the limiting effects of water pollution and habitat loss on the ecological status.

In the considered HMWBs, the rivers belonging to the Lambro-Olona basin, water quality is the strongest driving force for the decrease of biodiversity and ecological status, and *Baetis* relative abundance can be used as a biomonitoring tool in the current general conditions. However, some metrics have a preferential response to habitat gradient rather than to water quality. These metrics allow to disentangle the effect of habitat loss on biological communities in a context confounded by multiple stressors. This distinction becomes extremely important for the HMWB management. Knowing what are the metrics that answer to different gradient solicitations, decision-making processes can be helped to understand if a gradient-specific recovery strategy is successful, even if other stressors are still limiting the overall river ecosystem quality.

The results underline the need to consider and address such large-scale pressures in river management and restoration because they potentially constrain the effects of local restoration measures (for example habitat restoration at site or mesohabitat level).

Using the quantile regression approach it is also possible to assess how the considered gradient acts as limiting factor. This is a useful tool for the definition of the ecological potential in HMWBs, the definition of which is dictated by WFD. The upper boundary of a metric-gradient scatterplot allows to identify at each point of the gradient which is the ecological potential for each analysed metric. Since the gradient is considered as a set of coordinates that correspond to precise values of environmental variables, it can be possible to individuate, for each point of the gradient, the variable values that permit to obtain the ecological potential in a set of given circumstances. Further analyses will be carried out on this possible application.

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# 7 GENERAL CONCLUSIONS

## 7.1 Limiting effect of different simultaneous stressors

Population growth and the increase of anthropogenic impacts to freshwater ecosystems have led to the proliferation of research on heavily impacted rivers. The research of the last 10 years has allowed to better understand the effects of human impacts (i.a. pollutant discharges, habitat loss, hydromorphological alterations) on the aquatic biological communities and to identify the best way to maintain and improve the conditions of these ecosystems (Paul and Meyer, 2001; Rogers et al., 2002; Violin et al., 2011; Davidson et al., 2012).
The HMWBs, the study object of this thesis, are heavily impacted rivers and have a peculiar characteristic: they are freshwater ecosystem where impacts often occur simultaneously, causing uncertainty in the decision-making, management and monitoring of restoration efforts. In order to deal with this uncertainty, a better comprehension of the synergistic action of multiple stressors must be obtained at different spatial (and temporal) levels.

The first works presented in this thesis (Chapters 2 and 3) address the problem of habitat loss and alteration of physico-chemical water properties, respectively due to the oversimplification of the riverbeds and for discharges of WWTPs and untreated wastewater. This has been carried out at the microhabitat and site level. We evaluated the possible effects of native riparian vegetation restoration and retention structure increase to improve the input of organic matter in order to promote the development of more complex macroinvertebrate communities. It should be emphasized that the assessment of the combined effect of different leaf packs and environmental gradients has been scarcely used in literature, but we consider it important to indicate the best river restoration options in sites affected by the previously mentioned combined stressors.
The use of multivariate approach to create alteration gradients allowed to quantify the variation of data due to different sources (different leaf

types and environmental gradients) at microhabitat level. Both chemical and hydromorphological changes led to the decrease of the macroinvertebrate community quality and diversity. In this work it has been found that water quality is the main driving factor causing changes in the macroinvertebrate assemblages of HMWBs, even at microhabitat (leaf pack) level. Our research suggests that, in the examined basin, a better treatment of wastewater should be the priority in river restoration programs in order to obtain the enhancement of macroinvertebrate functional diversity.

Water quality was found to be primarily responsible for the biodiversity loss in the evaluation of sudden impacts (Chapter 4). The combined effect of an oil spill and the inactivation of all WWTP treatment processes have been evaluated at mesohabitat and site level,.

The discharge of untreated wastewater, and the deeply change of the physico-chemical conditions of the water downstream the studied spill, led to a strongly decrease of the few sensitive taxa colonizing in the river, with the proliferation of tolerant and ubiquitous taxa, as widely documented in literature (e.g.: Coimbra et al., 1996; Daniel et al., 2002; Zeilhofer et al., 2006; Canobbio et al., 2009). The direct effect of the oil spill resulted in the decrease of very tolerant taxa, mainly in the backwater microhabitat, where hydrocarbons sedimented. The two effects could be separated analysing the macroinvertebrate communities at the mesohabitat level. Thus, the work has allowed disentangling the combined action of two strong stressors acting on an already heavily impaired river ecosystem. These findings show that the assessment of the effects of a spill in an already impaired river is difficult and requires the evaluation of different metrics compared with standard monitoring. Metrics pertaining to tolerant taxa seem to be more successful descriptors than the others.

After specific works at microhabitat and site level, a whole basin analysis was conducted in chapter 5 and 6, to have a better comprehension of large-scale pressure effects on the biological metrics. We used a multivariate approach to focus on the characteristics of the streams and rivers in a urban district and to define which macroinvertebrate metrics should be used to assess the influence of the different kinds of alteration in severely damaged environments.

This work has allowed us to create environmental gradients with a multivariate approach. Thus, obtained gradients were used to describe how the various alterations (hydromorphological gradients and water chemistry gradient) affected macroinvertebrate assemblages.
The chapters from 2 to 4, presented at the level of micro- and mesohabitat, or site, and the 5th at the basin scale, present the same problem. Despite significant relationships were found between macroinvertebrate community health and environmental variables (both local and global), great variability in the data has been found, and this often makes poor fitness of the statistical tests. The strong variability observed is most likely due to the fact that the various stressors act simultaneously and therefore do not always succeed in discriminating the action of the single acting pressure.
In wide dataset with high data variability and complex relationship among variables, common statistical tools (such us those used in chapter 5) can be not appropriate. Usually, hypotheses about the central response of organisms to environmental gradients are tested, although the effects of other stressors may also influence such response and decrease the fit of the model, which may even become uninformative.
In this perspective, we decided to conduct analyses at the basin scale using quantile regression (chapter 6), which assessed the limiting action of the various stressors acting on biological communities.
We suggest that the use of quantile regression is an excellent tool for the analysis of pressures, especially at the large scale of site or basin, and it seems to be the only approach that can be considered highly informativein order to discriminate the limiting effect of different simultaneous stressors.

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