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# Phenotypic variation as an indicator of pesticide stress in gudgeon: Accounting for confounding factors in the wild

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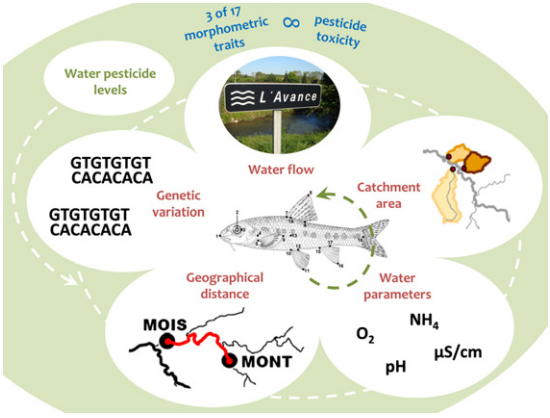
## HIGHLIGHTS

- We tested the link between pesticide levels in rivers and morphology of fish.
- Our model also considered covariables: genetics, geographical distances, etc.
- 1/6 of morphological traits were significantly correlated with pesticide toxicity.
- It is important to consider the many sources of inter-organism phenotypic variability.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The response of organisms to environmental stress is currently used in the assessment of ecosystem health. Morphological changes integrate the multiple effects of one or several stress factors upon the development of the exposed organisms. In a natural environment, many factors determine the patterns of morphological differentiation between individuals. However, few studies have sought to distinguish and measure the independent effect of these factors (genetic diversity and structure, spatial structuring of populations, physical–chemical conditions, etc.).
Here we investigated the relationship between pesticide levels measured at 11 sites sampled in rivers of the Garonne river basin (SW France) and morphological changes of a freshwater fish species, the gudgeon (*Gobio gobio*). Each individual sampled was genotyped using 8 microsatellite markers and their phenotype characterized via 17 morphological traits. Our analysis detected a link between population genetic structure (revealed by a Bayesian method) and morphometry (linear discriminant analysis) of the studied populations. We then

*Abbreviations:* $Q_{ST}$ or $P_{ST}$, genetic differentiation measured with quantitative traits (e.g. body size, growth rate); $F_{ST}$, genetic differentiation measured with neutral traits, generally measured using neutral loci such as microsatellites; msPAF (multi-substance Predicted Affected Fraction), quantifies the toxic pressure put on an ecosystem due to the presence of a mixture of chemicals, indicating the fraction of all species that is predicted to be exposed above an effect-related benchmark, such as the EC50 (median effect concentration) or the NOEC (no observed effect concentration) for standard toxicity test species; TU (Toxic Units), a risk quotient to reveal whether the measured bioavailable concentrations are higher than the known L(E)C50 (Lethal/Effect) for a certain species.

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Feral fish
Morphometry
Indicator
Confounding effects

developed an original method based on general linear models using distance matrices, an extension of the partial Mantel test beyond 3 matrices. This method was used to test the relationship between contamination (toxicity index) and morphometry ($P_{ST}$ of morphometric traits), taking into account (1) genetic differentiation between populations ($F_{ST}$), (2) geographical distances between sites, (3) site catchment area, and (4) various physical–chemical parameters for each sampling site.
Upon removal of confounding effects, 3 of the 17 morphological traits studied were significantly correlated with pesticide toxicity, suggesting a response of these traits to the anthropogenic stress. These results underline the importance of taking into account the different sources of phenotypic variability between organisms when identifying the stress factors involved. The separation and quantification of the independent effect of such factors provides an interesting outlook regarding the use of these evaluation metrics as indicators of ecosystem health.


## 1. Introduction

At the individual level, phenotype integrates the multiple effects of one or several stress factors upon the development of exposed organisms (Franssen, 2011), and determines part of the fitness of organisms (Orr, 2009). Therefore, via studying phenotypic variation between conspecifics exposed to different environments, we may gain insight from variation in fitness linked to environmental conditions. Phenotypic changes have thus been used as bio-indicators of chronic exposure of various organisms to pollutants as well as when facing habitat degradation (e.g. Klisarić et al., 2014; Monna et al., 2011; Sánchez-Chardi et al., 2013; Smakulska and Górniak, 2004). The developmental stability – reproducible development of a genotype under given environmental conditions – of the individuals of a population can be assessed by studying the degree of morphological variation (direct measurements between body landmarks). While some studies have shown that developmental instability increases with increase of environmental stress, others failed to establish clear relationships.

Because human pressure generally modifies more than one environmental factor at a time, and pressures from several sources often coincide, a multiple-stressor approach is the most adequate when conducting studies in the natural environment (Franssen et al., 2013; Ormerod et al., 2010). In environmental risk assessment, the determination of causality of toxic effects requires specificity of association, i.e., differentiation between stressor independent effects and environmental or genetic variability (Theodorakis, 2003). Indeed, phenotypic variation observed in wild populations can be affected by the presence of contaminants but also by a range of other factors that co-vary spatially with contaminants. Inferring the independent effect of collinear variables is possible through the use of adequate statistical tools (for a technical review see Murray and Conner, 2009). For quantitative traits, the genetic pool of populations can also be a key factor affecting between-population variation, while gene flow among populations can dampen the effect of contaminants on phenotypic variation expected between populations. Although genetic diversity and structure have been regarded as being potentially affected by contaminants (Bourret et al., 2008), they have rarely been considered as potential confounding effects in phenotypically-based ecotoxicological studies. The effect of these potentially confounding factors can be teased apart by comparing, between populations, the genetic differentiation measured with quantitative traits (e.g. body size, growth rate; $Q_{ST}$) to the genetic differentiation measured with neutral traits (i.e. generally measured using neutral loci such as microsatellites; $F_{ST}$). Such an approach (the $Q_{ST}/F_{ST}$ approach, or $P_{ST}/F_{ST}$ approach in wild populations) has proven powerful to differentiate the role of natural selection vs. genetic drift on natural populations (Merilä and Crnokrak, 2001; Raeymaekers et al., 2007), but has rarely been applied within an ecotoxicological context.

In this study, the main objective was to develop a method aiming at testing, in the wild, the independent effects of contaminants on the phenotype of individuals, while accounting for confounding environmental and genetic factors. Such a method was developed on wild populations of a Cyprinid fish (the European gudgeon, *Gobio gobio*) occurring along a river basin gradient (the Garonne watershed, south-western France) and exposed to different levels of water pollution. The method we developed is based on General Linear Models and is rooted on the partial Mantel test framework (Manly, 1991; Legendre, 2000). This method is specifically designed to test the effect of pesticide contamination on the morphometry of gudgeon populations, while simultaneously taking into account the influence of various physical–chemical parameters, the geographic isolation of populations, the genetic structure of these populations, and genetic drift. The applicability of this approach for ecotoxicological assessment of ecosystem health is discussed.

## 2. Methods

### 2.1. Site selection and physical–chemical characterization

The 11 selected sampling sites were geographically dispersed throughout different streams of the Adour-Garonne river basin, SW France (Fig. 1), covering a range of varying pesticide levels classified according to a 5-level environmental quality index developed by the Adour-Garonne water agency (MEDD and Agences de l'Eau, 2003). Given the main objective of the study in including a range of different environmental (and genetic) factors in the final phenotype-pesticide contamination assessment, we gathered as much physical and chemical data possible for the selected sampling sites. The following paragraphs explain the structure and specific treatments of each dataset.

In order to characterize each sampling site, the Adour-Garonne water agency pesticide concentration databases between 2006 and 2008 (time period corresponded to the immediate three years prior to field sampling of fish) were used to calculate two toxicity indices: the msPAF (multi-substance Predicted Affected Fraction; Posthuma and De Zwart, 2006) and TU (Toxic Units; Von der Ohe et al., 2008). Each index calculation used the concentrations of 74 pesticides (of 23 different toxic modes of action) measured by the Adour-Garonne water agency in grab-samples taken at each sampling site 3 to 5 times per year.

The msPAF quantifies the toxic pressure put on an ecosystem due to the presence of a mixture of chemicals, indicating the fraction of all species that is predicted to be exposed above an effect-related benchmark, such as the EC50 (median effect concentration) or the NOEC (no observed effect concentration) for standard toxicity test species. As pesticide concentrations varied within and among years, an average msPAF value for each sampling site was calculated according to Posthuma and De Zwart (2006) for each year (2006, 2007 and 2008), and the maximum value out of the three years was retained in our analysis. The calculation of PAF levels is based on chemical-specific species sensitivity distributions (SSDs) that describe the variation in sensitivities for a set of species under acute or chronic exposure to a certain compound. The single substance PAF (ssPAF) can be used as an approximation of the ecological risk of a single substance to the

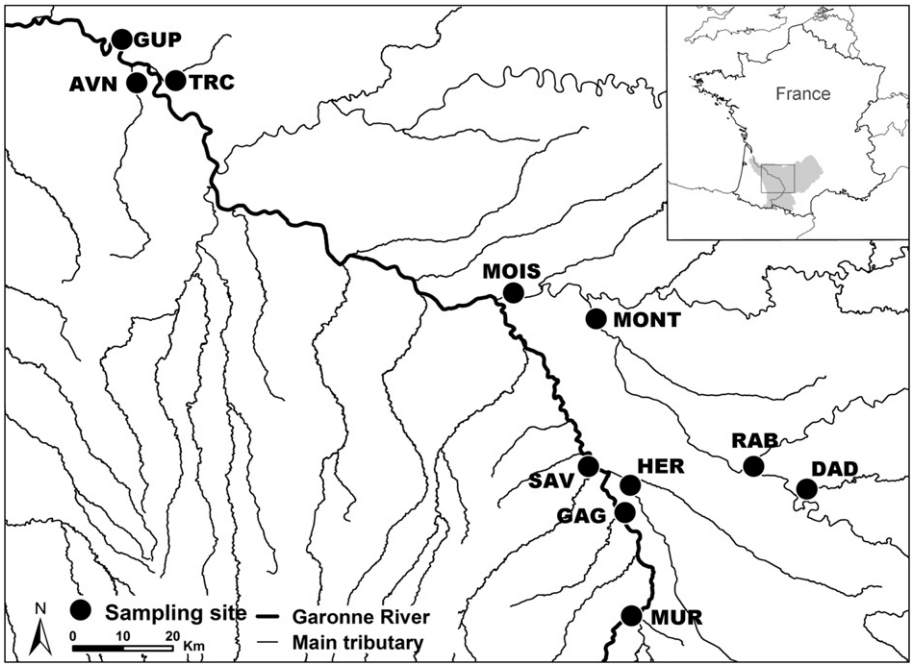


**Fig. 1.** Selected sampling sites throughout the Garonne river basin (grey area in insert map). Sampling site abbreviations as explained in Table 2. Main tributaries flow towards/into the Garonne River, which flows from South to Northwest.

ecosystem at measured or predicted ambient concentration and is calculated by

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

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

$$PAF_{TMoA} = \left(1 + e^{-\left(\log\left(\sum HU_{TMoA^{TMoA}}\right)/\beta_{TMoA}\right)}\right)^{-1} \quad (2)$$

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

$$msPAF = 1 - \prod_{TMoA} (1 - PAF_{TMoA}). \quad (3)$$

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

$$TU = C_i/LC50_i. \quad (4)$$

A principal component analysis (PCA) was then used to eliminate the colinearity between these two toxicity indices. The first axis of the PCA, accounting for 98.9% of the total variation, was kept as a synthetic index of toxicity. Pairwise differences between the toxicity of all pairs of sites were calculated using Euclidian distances along the first axis of the PCA (hereafter referred to as the "TOX" matrix).

For the same time period, data of 16 physical–chemical parameters ($NH_4$, calcium, $Cl^-$, conductivity, biological organic demand, chemical oxygen demand, hardness, $Mg^{2+}$, solid matter, $NO_3^-$, $NO_2^-$, $HPO_4^{-2}$, dissolved oxygen, pH, $SO_4^{-2}$, temperature) were averaged for each sampling site. Two integrative environmental variables (Env1 and Env2) were derived from the first two axes of a PCA (axes 1 and 2 accounted for 46.2 and 23.5% of the total variation respectively) on the 16 environmental parameters (see Appendix A for details). Pairwise differences were calculated between Env1 and Env2 values respectively, for all pairs of sites (matrices "ENV1" and "ENV2").

Table 1 summarizes a selection of environmental variables of each sampling site. Overall, sites with higher conductivity levels (HER, SAV, TRC, AVN, GUP) were also those with a log TU above the −4 toxicity limit beyond which effects on the fish community are expected, and two of which also had an msPAF above the 3% risk limit. The overall Water Quality Index (WQI) was in general considerably lower in those same sites than all the others.

Sampling site catchment areas were obtained using the geographical information system in ESRI© ArcMap™ 9.2. Here we assumed that taking into account site catchment area adjusts for river size. Pairwise differences between catchment areas and between distances to the respective sources of all pairs of sites were calculated ("BAS" and "DIST" matrices, respectively). Geographical distances between all pairs of sampling sites ("GEO" matrix) were measured along the stream

**Table 1**
Sampling sites with respective multi-substance Potentially Affected Fraction of species (msPAF), fish Toxic Units (logTU), average temperature, dissolved oxygen, conductivity, and Water Quality Index (WQI) for the period 2006 to 2008. An msPAF of 3% and above is expected to place primary producers (Faggiano et al., 2010), invertebrates and fish at risk and a log TU of −4 and above is considered to be toxic to fish (von der Ohe et al., 2008); msPAF and TU values of risk are indicated with an asterisk. The WQI was calculated based on Pesce and Wunderlin (2000), integrating all 16 physical–chemical parameters used for the calculation of the ENV1 and ENV2 variables.

| Site | msPAF (%) | logTU | Temp. (°C) | Oxygen (mg/L) | Cond. (μS/cm) | WQI |
|---|---|---|---|---|---|---|
| MUR | 0.0000 | −5.22 | 15.20 | 10.84 | 265.00 | 66.85 |
| GAG | 0.0022 | −4.67 | 16.15 | 10.47 | 262.13 | 58.22 |
| HER | 0.0728 | −3.83* | 16.12 | 9.25 | 627.50 | 24.19 |
| SAV | 0.1023 | −3.29* | 14.71 | 9.69 | 462.76 | 35.19 |
| DAD | 0.2329 | −4.48 | 15.51 | 9.61 | 356.53 | 58.85 |
| RAB | 0.0238 | −4.54 | 15.77 | 10.57 | 309.39 | 60.07 |
| MONT | 0.0014 | −5.23 | 16.10 | 9.61 | 280.23 | 48.93 |
| MOIS | 0.0041 | −4.94 | 16.33 | 9.77 | 310.27 | 43.67 |
| TRC | 9.1237* | −2.17* | 15.18 | 10.12 | 776.23 | 28.70 |
| AVN | 3.9204* | −2.82* | 14.43 | 9.90 | 445.27 | 49.63 |
| GUP | 1.6850 | −2.80* | 14.23 | 9.56 | 826.68 | 31.85 |

networks using ArcMap. Pairwise differences of average water velocities and water height for 2006 between all pairs of sites (one measurement a day by automatic devices; data provided by the Adour-Garonne water agency) were used to construct the "VEL" and "HIGH" matrices, respectively.

### 2.2. Fish sampling and morphometric data

The gudgeon, *G. gobio* (L.) is a benthopelagic cyprinid fish common in both polluted and non-polluted areas in Western Europe (Knapen et al., 2009). We considered that the fish captured at a certain site have been exposed to the conditions measured at that site. Maximum dispersal distances for gudgeon were calculated using the R package "Fishmove" (Radinger and Wolter, 2013), resulting in a range of 52–126 m for stationary individuals (1.3–4.1 km for the mobile component of the population) of, respectively, 1 and 8 years (maximum reported lifespan for gudgeon; Maitland and Campbell, 1992). Riparian distance between pairs of sites ranged between 12.35 km (GUP-AVN) and 230.19 km (GUP-DAD), which makes it unlikely that important exchanges of individuals between sites occurred within a gudgeon lifespan.

Between August and November 2008, electrofishing was performed on foot or by boat, and was standardized over all sampling sites: an area of 500 $m^2$ (approximately 50 × 10 m) was sampled during one pass so as to ensure adequate sampling of the population. Up to 20 gudgeon individuals (Table 2) were captured, sacrificed on-site and transported on ice to the laboratory where they were kept, individually wrapped in aluminium foil, at −20 °C until further processing.

To obtain morphometric traits of gudgeons, after unfreezing, both sides of each gudgeon – placed beside a metric ruler for scaling – were photographed. Both pelvic fins of each fish were then removed and stored in 95% ethanol for DNA analysis. It is noteworthy that photos for morphometric measurements can be taken of anaesthetized specimens on-site, and a section of the pelvic fin (or a few scales) removed without jeopardising the fitness of the fish, after which the fish can be released back into the wild immediately after having recovered from anaesthesia. Here we sacrificed the fish because we performed, within the scope of another project, additional analyses requiring dead individuals.

Photographs were analysed using Visilog 6.4 Demo® to obtain X–Y coordinates of the landmarks intended for morphometric measurements (Fig. 2). A total of 17 euclidean distances between 18 landmarks were calculated for both sides of each fish. All subsequent analysis (except measurement error estimation) was performed using Aitchinson log-ratio transformed measurements to account for individual size-effects (Peres-Neto and Magnan, 2004). The transformation follows the equation

$$Y_{ij} = \log x_{ij} - 1/p \cdot \sum_{i}^{p} \log x_{ij} \quad (5)$$

in which $Y_{ij}$ is the transformed distance of the *j*th trait for the *i*th individual, $p$ is the number of morphological traits and $x_{ij}$ the original value for the *i*th individual and the *j*th trait.

Of the 17 morphometric traits, 9 are subject to left-right side asymmetry (traits I to IX; e.g. V, pectoral fin length) while the remaining 8 are not (traits X to XVII; e.g. XII, dorsal fin height). Based on the left-right differences of morphological traits not subject to left-right asymmetry, the dataset presented an average measurement error of 2.74% (minimum 0.70%, maximum 5.56%). Gudgeon measured in average 8.48 cm (standard deviation: ±1.81).

The among-population divergence between genes that code for quantitative traits (Merilä and Crnokrak, 2001), such as morphometric traits, is quantified by

$$F_{ST} = \alpha_b/(\alpha_b + \alpha_w) \quad (6)$$

(Wright, 1951), in which the total genetic variation in neutral marker loci is partitioned into within ($\alpha_w$) and between population ($\alpha_b$) components. Quantitative genetic differentiation for natural populations ($P_{ST}$) is based on phenotypic data derived from wild individuals (Raeymaekers et al., 2007). The $P_{ST}$ index is the phenotypic analog of the $F_{ST}$ index. If $P_{ST}$ were estimated from allele frequencies at the loci determining the quantitative trait, $P_{ST}$ would be expected to be equal

**Table 2**
Number of fish sampled (N fish), toxicity index (Tox), average genetic parameters according to eight microsatellite loci: unbiased expected heterozygosity ($H_E$), observed heterzygosity ($H_O$), inbreeding coefficient ($F_{IS}$; Weir and Cockerham estimates), number of alleles per locus (A), and allelic richness (AR) for 11 sampling sites MUR (Garonne at Muret), GAG (Garonne at Gagnac), HER (Hers mort at St. Sauveur), SAV (Save at Grenade), DAD (Dadou at Peyrières), RAB (Tarn at Rabastens), MONT (Tarn at Montauban), MOIS (Tarn at Moissac), TRC (Trec at Longueville), AVN (Avance at Pont des Sables), GUP (Gupie). Tox: the higher the value, the lower the toxicity; for the determination, see Section 2.1. $H_E$, $H_O$, $F_{IS}$, A, and AR: for determination see Section 2.4. Standard errors in brackets.

| Site | N fish | Tox | $H_E$ | $H_O$ | $F_{IS}$ | A | AR |
|---|---|---|---|---|---|---|---|
| MUR | 13 | 1.12 | 0.809 (0.121) | 0.766 (0.188) | 0.062 (0.159) | 8.75 (1.19) | 7.79 (0.95) |
| GAG | 20 | 1.01 | 0.782 (0.130) | 0.747 (0.152) | 0.045 (0.124) | 9.38 (1.56) | 7.20 (1.03) |
| HER | 20 | 0.48 | 0.784 (0.103) | 0.649 (0.151) | 0.184 (0.140) | 9.00 (1.27) | 7.04 (0.88) |
| SAV | 20 | 0.12 | 0.807 (0.100) | 0.717 (0.124) | 0.113 (0.128) | 10.25 (1.46) | 7.46 (0.87) |
| DAD | 20 | 0.92 | 0.807 (0.067) | 0.720 (0.096) | 0.112 (0.078) | 8.13 (1.09) | 6.48 (0.67) |
| RAB | 20 | 1.12 | 0.775 (0.127) | 0.685 (0.194) | 0.131 (0.202) | 9.25 (1.37) | 6.95 (0.81) |
| MONT | 20 | 1.13 | 0.787 (0.097) | 0.717 (0.121) | 0.089 (0.121) | 8.88 (1.25) | 6.89 (0.78) |
| MOIS | 20 | 1.11 | 0.800 (0.095) | 0.648 (0.154) | 0.201 (0.153) | 8.75 (1.31) | 6.65 (0.76) |
| TRC | 20 | −3.62 | 0.754 (0.148) | 0.737 (0.197) | 0.031 (0.147) | 8.88 (1.26) | 6.83 (0.87) |
| AVN | 19 | −0.46 | 0.759 (0.154) | 0.631 (0.243) | 0.198 (0.251) | 8.50 (1.25) | 6.73 (0.96) |
| GUP | 19 | −2.93 | 0.835 (0.092) | 0.784 (0.139) | 0.065 (0.114) | 9.25 (1.19) | 7.48 (0.83) |

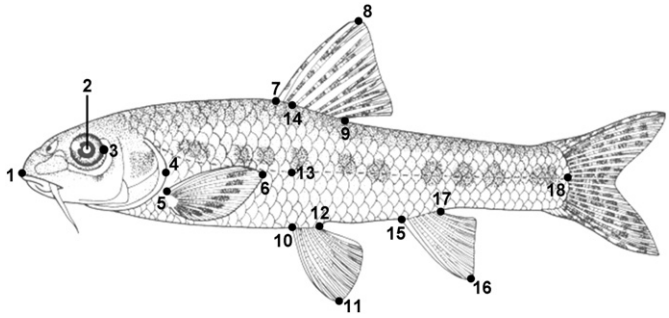


**Fig. 2.** Placement of landmarks on a gudgeon specimen sketch (Illustration by Susan Laurie Bourque, reproduced with permission from the Canadian Museum of Nature, Ottawa, Canada). Footnote: The following morphometric traits were measured: I (1–2), II (2–3), III (1–4), IV (4–5), V (5–6), VI (1–10), VII (10–11), VIII (10–12), IX (13–14), X (1–18), XI (1–7), XII (7–8), XIII (7–9), XIV (15–16), XV (15–17), XVI (1–15), XVII (10–14).

to $F_{ST}$ in the case where the trait carried an exclusively additive genetic basis (i.e., no gene interaction or epigenetic effects) and no linkage disequilibrium were to be present (Wright, 1951). The most common result is that $P_{ST} > F_{ST}$, meaning that directional/divergent natural selection has resulted in different phenotypes in different populations, as the level of quantitative trait differentiation exceeds that attained by genetic drift only (Merilä and Crnokrak, 2001; Raeymaekers et al., 2007). $P_{ST}$ values were estimated using the following equation

$$P_{ST} = \alpha_b^2/(\alpha_b^2 + 2\alpha_w^2) \quad (7)$$

(Merilä and Crnokrak, 2001), in which $\alpha_b^2$ is the between-population variance and $\alpha_w^2$ the within-population variance of the right-side measurement of each fish, per sampling site ($P_{ST}$-*I* to $P_{ST}$-*XVII*) obtained by analysis of variance on each trait. $P_{ST}$ values were computed in the same way for left-right differences of morphological traits I to IX and averaged over the 9 traits to obtain a general asymmetry $P_{ST}$ ($P_{ST}$-*ASY*).

### 2.3. Discriminant analysis of morphometric data

We used linear discriminant analysis (LDA) to illustrate the clustering of the sampling sites according to the main morphological differences among the 11 sampling sites, and to identify the traits that discriminate the sites. LDA was performed on all right-side morphometric traits (I to XVII, Fig. 2) using the R software (R Development Core Team, 2007; package ade4). The statistical significance of sites discrimination (ellipses in Fig. 3) was assessed using a Monte-Carlo permutation test (1000 permutations).

### 2.4. Microsatellite analysis, genetic variation and population structure

Microsatellite analysis was performed in order to obtain allelic data for further genetic variation and population structure analysis. Genomic DNA was extracted from the pelvic fins using a salt-extraction method (Aljanabi and Martinez, 1997). Eight microsatellites markers – those that did not present null alleles, and that displayed highly readable and repeatable profiles (Blanchet et al., 2010) – for gudgeon were used here: Ca01[a], Gob12[b], Gob15[b], Gob16[b], Gob22[b], Gob28[b], MFW1[c], Rhca20[d] (primer references: [a]Dimsoski et al., 2000; [b]Knapen et al., 2006; [c]Crooijmans et al., 1997; [d]Girard and Angers, 2006). The genetic analysis protocol followed Blanchet et al. (2009).

For each sampling site, observed and expected heterozygosity ($H_O$ and $H_E$) as well as inbreeding coefficient ($F_{IS}$) were estimated using GENETIX 4.05.2 (Belkhir et al., 2004). Number of alleles (A) and allelic richness (AR; based on minimum sample size) were calculated using the program FSTAT 2.9.3.2 (Goudet and Buchi, 2006). Departure from Hardy–Weinberg equilibrium and genotypic linkage disequilibrium between all pairs of loci for each population were checked using FSTAT,

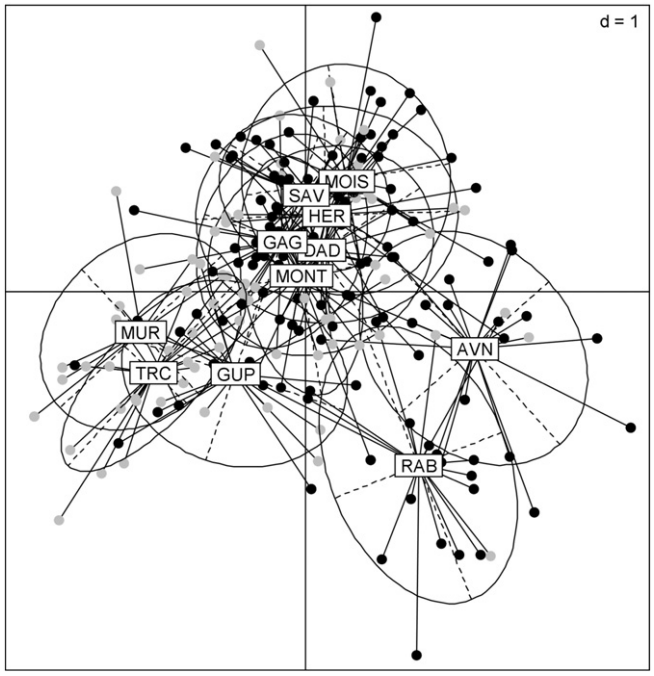


**Fig. 3.** Two dimensional distribution of the gudgeon individuals from the 11 sampling sites after Linear Disciminant Analysis (LDA) based on the right-side morphometric measurements (I to XVII, see Fig. 2); ellipses group individuals from each sampling site (Monte-Carlo test after 1000 permutations: $p = 0.001$). Point shading indicates the inferred genetic cluster to which individuals belong when $q$ (fractional membership) >70% (identified using STRUCTURE; K = 2; for details see Section 2.4). Sampling site abbreviations as explained in Table 2.

with significance levels Bonferroni-adjusted for multiple comparisons. Differences of $H_E$ and allelic richness between populations were tested using Kruskal–Wallis multiple comparisons test. $F_{IS}$ averaged over populations was tested regarding difference to zero via a Student's t-test. Allelic frequencies were estimated and differences among populations calculated by Fisher's exact test, both using GENEPOP 4.0 (Rousset, 2008).

The degree of genetic differentiation among populations was assessed using the standardized $F_{ST}$ approach. $F_{ST}$ were calculated using FSTAT for each pair of sampling sites, comparing within and among-population variance (Eq. (1) applied to allelic data). The statistical significance of $F_{ST}$ values was tested by 55,000 permutations and the significant level was adjusted by the Bonferroni procedure ($\alpha = 0.0009$). $F_{ST}$ ratios

$$F_{ST}/(1-F_{ST}) \quad (8)$$

were used to construct a $F_{ST}$-ratio distance matrix ("FST").

Population genetic structure was assessed via the Bayesian clustering method in STRUCTURE 2.3.3 (Pritchard et al., 2000a; Falush et al., 2003). Irrespective of sampling location, STRUCTURE allocates genotypes (individuals) to a number of genetic clusters ($K$), regrouping individuals according to their biological population, instead of predefined sampling sites. Ten replicates of each run from $K = 1$ to $K = 11$ were performed using the admixture model, $K$ being the number of genetic clusters. Each replicate was run for 20,000 Markov chain Monte Carlo (MCMC) generations (initial burn-in of 20,000 generations). Posterior probabilities $L(K)$ were estimated using the output of the runs, and $\Delta K$ calculated according to Evanno et al. (2005). As recommended by Evanno et al. (2005), the height of the modal value of the $\Delta K$ distribution was used here as the signal for the uppermost hierarchical level of genetic structure in our data set.

In addition, we considered the fractional membership ($q$) of each individual in each group (Pritchard et al., 2000). Two categories of

populations were differentiated according to their $q$ values when considering $K = 2$ (clusters C1 and C2): those with a $q$ higher than 70% for either of the clusters were considered to belong to that cluster; and populations that did not present any $q$ values above 70% were considered as sharing membership between clusters.

### 2.5. Relating toxicity and morphometry

Partial Mantel tests (Manly, 1991; Legendre, 2000) are commonly performed to check if two matrices are similarly correlated when controlling for a third matrix (Raeymaekers et al., 2007; Bourret et al., 2008). We extended the partial Mantel regression to more than three distance matrices by using general linear models (GLM). The advantage of this method is that GLM generalizes the multiple regression of matrices to non-Gaussian data (e.g. binomial). This analysis was performed manually in R (R Development Core Team, 2007). Posterior to this the "mRm" R package was developed with the ability to perform this same analysis. The analysis was thus run again with results from both approaches (manual and "mRm" package) in complete accordance.

The vectors of all distance matrices – FST, TOX, ENV2, GEO, BAS, VEL, $P_{ST}$ I to XVII, and $P_{ST}$-*ASY* (traits I to IX individually and average) – were extracted and the data scaled (transformed values are centred around zero and have a unit variance). For $P_{ST}$ I to XVII and for $P_{ST}$-*ASY*, GLMs was used to test, after 1000 permutations, the relationship between TOX and each of these traits, simultaneously taking into account FST, ENV1, ENV2, BAS, DIST, GEO, VEL, and HIGH. GLM output provided the significance of the correlation coefficients of simple (permuting one of FST, ENV1, ENV2, BAS, DIST, GEO, VEL, and HIGH, excluding TOX) and composed (permuting TOX, including all others) models (see Table 4). Significance levels were adjusted for multiple comparisons following the Bonferroni procedure. For all composed GLMs that were statistically significant, Pearson's correlation coefficient between average trait measurements and the toxicity index of each sampling site were calculated, thus obtaining the tendencies of those relationships.

## 3. Results

### 3.1. Morphological variation

The LDA revealed 3 clusters of sampling sites apparently separated along the first axis: MUR, TRC and GUP in the negative side of the horizontal axis, AVN and RAB in the positive side, and the remaining 6 sites in the centre (Fig. 3). Correlation values of each morphometric trait with the first two axes of the LDA are shown in Appendix B. Trait XVII had a strong (>0.5) correlation with the first axis, while traits III, IV, X, and XVI were strongly correlated to the second axis, contributing to the clear separation of the bigger cluster of 6 sites along the second axis (Fig. 3).

### 3.2. Genetic variation and population structure

The microsatellite allele dataset did not reveal departure from Hardy–Weinberg equilibrium nor present genotypic linkage disequilibrium for any pairs of loci. From 3 to 17 alleles per locus were detected, at an average of 19.4 over all loci. Among the 11 sampling sites and for 8 loci, 155 microsatellite alleles were detected. The minimum total number of alleles over all loci was observed for DAD (65 alleles) and the maximum for SAV (82). Allelic richness varied between 6.48 at DAD and 7.48 at MUR (Table 2). Differences between $H_E$ and AR were non significant ($p > 0.05$) for all population comparisons respectively.

$F_{ST}$ ranged from 0.0006 to 0.1107, with an overall average of 0.0395 (Table 3). Significant differences were detected in 34/55 (63.6%) of the comparisons (Table 3). The $F_{ST}$-ratio was not correlated with geographical distances between sites ($r = 0.2009$, p-value = 0.1414). Out of the 17 morphological traits, 15 presented larger average $P_{ST}$ values than the average $F_{ST}$, 6 of which were significantly larger (95% confidence intervals beyond the upper $F_{ST}$ 95% CI level; Fig. 4). Thus for traits PST-III, IV, V, VIII, XVI and XVII, directional/divergent natural selection has resulted in different phenotypes among the different populations/sites, as the level of quantitative trait differentiation ($P_{ST}$) exceeded that attained by genetic drift only ($F_{ST}$).

FST were strongly related to 8 of the 17 morphological PST (GLMs permuting the FST component and without additional components; Table 4). Complete GLMs on PST*FST, permuting FST and accounting for all other variables gave the same significant results as simple PST*FST models, except for PST-II (data not shown). Of the 8 significant FST-PST relationships, 5 also presented significantly larger PST than FST (traits III, IV, V, XIV, and XVII; Fig. 4).

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

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

### 3.3. Relationship between toxicity and morphometry

Complete GLM permuting TOX and including all other components, revealed significant slopes for comparisons of toxicity and 3 out of 17 morphological trait $P_{ST}$ differences between sites (Table 4). With increasing toxicity differences, differences in fish eye position ($P_{ST}$-I), fish eye radius ($P_{ST}$-II), and body height ($P_{ST}$-XVII) increased significantly.

When testing the correlation between toxicity and the measurements of each significantly related morphological trait, eye radius significantly decreased ($r^2 = 0.2822$, $p < 0.001$) and body height significantly increased ($r^2 = 0.4105$, $p < 0.001$) with increasing toxicity. There was a tendency (although not quite significant; $r^2 = 0.1650$, $p = 0.0638$) for eye position to increase with increasing toxicity.

## 4. Discussion

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

### 4.1. Morphometry and genetics

First we studied the morphometric and genetic differentiation between populations separately. A general conformity is observed between the distribution of sampling sites according to morphology (LDA) and genetic data (STRUCTURE). For example, MUR, TRC and GUP were closely positioned within the LDA plot, while most individuals in those populations were allocated to the same cluster regarding membership values (obtained with genetic data). This type of pattern suggests that it is important to include genetic data in studies comparing phenotypic differentiation of populations. Without this

**Table 3**
Pairwise genetic ($F_{ST}$, index quantifying the among-population divergence between genes coding for quantitative traits; for the calculation procedure, see Section 2.4) and geographical distances between sampling sites. $F_{ST}$ values are below the diagonal (significance levels are indicated by asterisks: $^{*}p < 9 \cdot 10^{-4}$; $^{**}p < 1 \cdot 10^{-4}$; $^{***}p < 3 \cdot 10^{-5}$) and geographical distances in kilometres are above. MUR to GUP: sampling sites as explained in Table 2.

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

information we would not have been able to conclude that the significant relationships found between morphometry and toxicity were the result of the toxicity per se, and not that of genetic differences between populations.

The gudgeon populations studied are in general significantly differentiated genetically, although weakly. Indeed, most $F_{ST}$ values (70.9% of comparisons) can be considered as low genetic differentiation (i.e. $F_{ST}$ lower than 5%), whereas 29.1% of $F_{ST}$ values corresponded to moderate levels of population differentiation (Balloux and Lugon-Moulin, 2002). This is not surprising given that gudgeon are present in a large range of stream conditions typical of temperate rivers thus presenting a broad ecological niche (Knapen et al., 2009), with high prevalence in South-West France (Gevrey et al., 2009). A tendency for isolation-by-distance (IBD; Hutchison and Templeton, 1999) with a slight increase of genetic differentiation with distance was found for the ensemble of site comparisons. However, when testing for IBD within the clusters identified by Bayesian structuring analysis, the pattern showed no significant correlation between genetic differentiation and geographical distance, presenting high variance (results not shown here). According to Hutchison and Templeton (1999), such a pattern indicates a lack of regional equilibrium with genetic drift being more influential than gene flow within the identified genetic clusters. In addition to various types of more-or-less insurmountable physical barriers along rivers of the Garonne basin (Eau France, 2010), gudgeon present short dispersal distances (see Section 2.2.), which would explain reduced gene flow via migration.

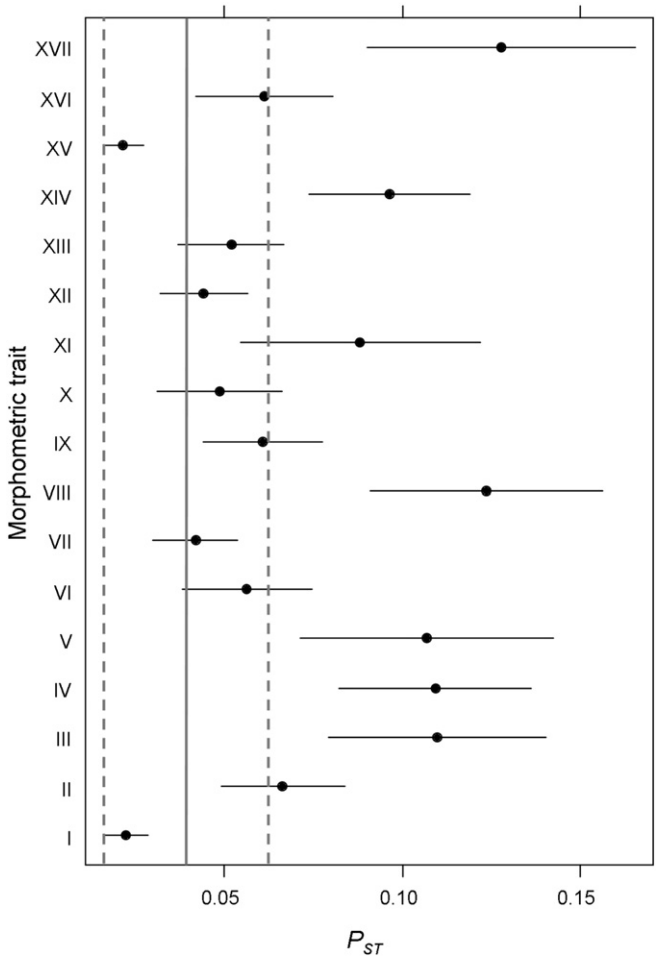


**Fig. 4.** Indices quantifying the genetic divergence of natural populations ($P_{ST}$) for the morphometric traits I to XVII (see Fig. 2) with the data of all 11 populations, and indices quantifying the among-population divergence between genes ($F_{ST}$) coding for quantitative traits. $P_{ST}$ = mean (black point) and 95% confidence interval (horizontal lines); $F_{ST}$ = mean of all population and loci (solid vertical line) and 95% confidence interval (dashed vertical lines).

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

The genetic-morphometric conformity found with the LDA and STRUCTURE analysis is also apparent in the FST*PST GLM tests, for which 8 of the 17 morphological traits were significantly correlated to FST. Despite the fact that a quarter of $F_{ST}$ values showed moderate population differentiation, the modelling approach applied still revealed a significant relationship between morphometry and toxicity when accounting for all confounding variables (composed GLMs). The strong correlations between FST and some PST found here suggest that the extent of genetic differentiation in neutral marker loci can be considered fairly predictive of the extent of differentiation in loci coding for quantitative traits (Merilä and Crnokrak, 2001).

It would thus be interesting to continue this study using quantitative trait loci (QTL) analysis on markers (genes) that affect certain phenotypic traits (Raeymaekers et al., 2007). There is an interesting potential in using QTLs when a trait can be linked to one principal gene (i.e. non polygenic; also supported by Macnair, 1991) or a specific breeding design is experimentally implemented (Merilä and Crnokrak, 2001; Raeymaekers et al., 2007), although not easily applied to wild populations. QTL could be used as a tool to identify phenotypes that are variably sensitive to exposure to environmental contaminants. If

**Table 4**
Slopes of general linear models performed with all $P_{ST}$ (I to XVII and ASY) permuting different components of the model. $P_{ST}$ = quantitative genetic differentiation index based on morphometric traits I to XVII as shown in Fig. 2. ASY = general asymmetry *PST*. § indicates the complete model: permuting TOX and including all other variables. Significant tests after 1000 permutations are in bold and marked with an asterisk (significance level adjusted according to the Bonferroni procedure; p = 0.0029). See Fig. 2 for landmarks used for each $P_{ST}$. Variables: TOX, index of toxicity; FST, FST-ratio (degree of genetic differentiation among populations); ENV1 & ENV 2, integrative environmental variables of 16 physical-chemical parameters; BAS, catchment areas; DIST, distance of sampling points to the respective sources; GEO, geographical distances between sampling sites; VEL, average water velocity; HIGH, average water height.

| $P_{ST}$ | TOX § | TOX | FST | ENV1 | ENV2 | BAS | DIST | GEO | VEL | HIGH |
|---|---|---|---|---|---|---|---|---|---|---|
| I | **0.307*** | **0.397*** | **0.739*** | 0.191 | **0.344*** | −0.074 | 0.033 | 0.070 | 0.016 | −0.036 |
| II | **0.356*** | **0.310** | **0.401*** | **0.386*** | **0.684*** | −0.040 | 0.027 | 0.053 | 0.115 | −0.055 |
| III | −0.088 | 0.005 | **0.457*** | −0.041 | 0.116 | −0.164 | −0.112 | 0.124 | 0.004 | −0.143 |
| IV | −0.007 | 0.141 | **0.581*** | 0.100 | 0.304 | −0.145 | −0.108 | 0.099 | 0.062 | −0.170 |
| V | −0.132 | −0.023 | **0.296*** | −0.183 | −0.001 | −0.122 | −0.061 | 0.127 | 0.092 | −0.123 |
| VI | −0.003 | −0.097 | −0.025 | −0.158 | −0.034 | 0.008 | −0.064 | 0.028 | −0.152 | −0.016 |
| VII | −0.189 | −0.209 | −0.083 | 0.015 | 0.191 | −0.145 | −0.149 | −0.034 | −0.141 | −0.081 |
| VIII | 0.149 | 0.026 | −0.066 | 0.168 | 0.221 | 0.092 | −0.066 | −0.111 | −0.090 | −0.060 |
| IX | 0.271 | 0.230 | **0.262*** | 0.258 | 0.393 | **−0.285*** | −0.199 | 0.018 | −0.027 | −0.224 |
| X | 0.254 | 0.162 | 0.162 | 0.094 | 0.251 | −0.067 | 0.004 | 0.134 | −0.093 | 0.029 |
| XI | 0.102 | 0.033 | 0.062 | −0.109 | −0.006 | −0.143 | −0.069 | 0.044 | −0.150 | −0.038 |
| XII | 0.042 | −0.051 | −0.044 | 0.195 | 0.209 | **0.396*** | 0.092 | −0.110 | 0.106 | 0.115 |
| XIII | −0.155 | −0.191 | −0.135 | −0.135 | −0.040 | 0.122 | −0.012 | 0.139 | 0.011 | 0.019 |
| XIV | 0.070 | 0.221 | **0.732*** | −0.012 | 0.095 | −0.108 | −0.027 | 0.090 | −0.058 | −0.029 |
| XV | −0.102 | −0.114 | −0.046 | 0.043 | 0.084 | 0.002 | −0.042 | −0.054 | −0.092 | 0.020 |
| XVI | −0.016 | −0.062 | 0.096 | −0.069 | 0.099 | −0.139 | −0.146 | 0.109 | −0.157 | −0.084 |
| XVII | **0.647** | **0.587*** | **0.490*** | **0.493*** | **0.620*** | −0.247 | −0.038 | 0.148 | 0.123 | −0.124 |
| ASY | −0.240 | −0.195 | −0.100 | −0.002 | 0.154 | −0.139 | −0.086 | 0.112 | −0.075 | −0.049 |

functional characteristics or morphological traits that confer individuals an advantage in more stressful environments, can be linked to specific genes, ecological status (i.e. fitness) of wild populations can then be screened using either a quantitative or genomic approach.

### 4.2. Morphometry as a sign of pesticide stress

In our study we tested a modelling approach that is capable of taking into account a range of co-varying factors. Composed GLMs to assess the relationship between morphological traits ($P_{ST}$) and toxicity, while including all other variables in the model were performed. Upon removal of confounding effects, 3 of the 17 morphological traits studied were significantly correlated with pesticide toxicity, suggesting a response of these traits to the agricultural stress. Despite weak genetic differentiation between most sampling sites, the model enabled us to identify morphological traits that are related to sampling site toxicity levels after removal of genetic, environmental and geographical confounding effects.

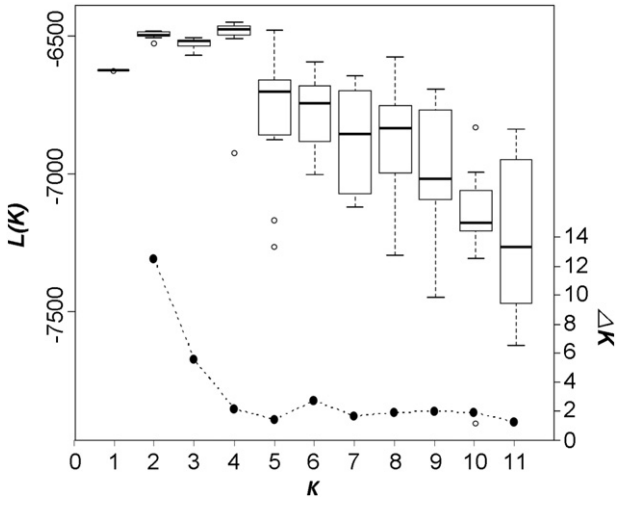


**Fig. 5.** Identification of population genetic structure based on Bayesian clustering. Given are posterior probabilities $L(K)$ (box plots) and second order rate of change in the likelihood of $K$ ($\Delta K$, full dots) according to the number of genetic clusters ($K$). See Section 2.4. for analysis details.

Gudgeon eye-diameter, eye position and body height were the three morphological traits that presented a response to pesticide toxicity levels: increasing differences (between sampling sites) in those traits corresponded to increasing differences in toxicity. Eye-diameter significantly decreased, and eyes tended to be positioned higher on the head with increasing toxicity levels. Previous studies have found that fish inhabiting more turbid areas than their conspecifics tend to present smaller eyes, compensated by the development of accessory sensory organs (Bruton, 1985; Evans, 2004). However, correlations between gudgeon eye-diameter and toxicity with suspended matter levels were both non-significant in our study. On the other hand, the initial stages of eye development in fish have been previously shown to be disturbed by the presence of environmental contaminants (Kruitwagen et al., 2006). This may explain why the two metrics related to eyes showed to be somewhat correlated to water toxicity in our composed GLMs. Thus our results are in line with previous findings although the mechanism behind the response cannot be determined here, nor which contaminants are responsible.

Gudgeon body height significantly increased with the level of toxicity. As previous studies have found negative relationships between water velocity and fish body height (Pakkasmaa and Piironen, 2000; but see also: Franssen et al., 2013; Peres-Neto and Magnan, 2004), we tested the relationship between water velocity and toxicity and between water velocity and fish body height. Average water velocities registered for year 2006 at the studied sampling sites did not exceed those suitable for gudgeon (Lamouroux and Capra, 2002). We found that sampling sites with higher toxicity levels also presented lower average water velocity but fish body height was not significantly correlated to water flow ($r^2 = 0.0932$, $p = 0.3613$). In any case, the GLM analysis we implemented here allowed eliminating water velocity as a determining factor of fish shape, thus we can hypothesize that toxicity does in fact affect development of exposed gudgeon, in this case body height.

In order to infer whether a given fish population (i.e. sampling site) is healthy or not, one can observe the sites for which the $P_{ST}$ values are the most extreme when plotted against the levels of toxicity. For all three significant GLM slopes (permuting TOX and including all other components) of $P_{ST}$-I, $P_{ST}$-II and $P_{ST}$-XVII, three sampling sites (TRC, GUP and MUR) were grouped at one end of the toxicity-morphological trait correlations, which highlights sites for which a specific management should be undertaken. Furthermore, all three sampling sites were grouped together in the LDA plot (Fig. 3). Interestingly, two of these

sites (TRC and GUP) are associated with the highest toxicity index, whereas the third one (MUR) is associated with the lowest toxicity index. This observation is intriguing and may reflect either a weakness of our approach (a site being detected as non-healthy whereas it is in fact healthy, e.g. MUR); or, on the contrary, a strength of our integrative approach which may detect non-healthy sites that are considered healthy via current toxicity index evaluation approaches (msPAF and TU).

In any case, this shows that our approach is highly complementary to approaches based on toxicity indices, ultimately leading to management of sites that otherwise would not be surveyed, but nevertheless supporting in the least a conservative methodology of ecological risk assessment.

### 4.3. Method advantages and applicability

Due to the flexible structure of the models (GLM) and testing framework (Mantel test) used in our study, the method developed here can be easily applied to other locations and species. In particular, we would like to highlight a few specific advantages and applications of the method:

1– Instead of focusing on a (fish) community, the tool developed here studies one species (of fish) in particular, and while this method doesn't take into account species interactions, it simplifies the approach regarding field sampling, so long as the one species can be found over all sampling sites intended to be studied.
2– Choosing any one species in particular to study over the whole sampling area has the advantage that that species can be selected according to its presence at all sampling points, overcoming restrictions related to the analysis of species-poor communities in certain climatological conditions (but see Hermoso et al., 2010) or in very altered habitats.
3– Our method accounts for abiotic differences between sites (inter-site variability), which often occurs over large water basins, in addition to the key problem in eco(toxico)logical studies of requiring reference points (CEC, 2000).

## 5. Conclusions

Morphology is one of many phenotypic traits that can be studied in order to quantify (or simply detect) organism responses to environmental stressors. Our results underline the importance of taking into account the different sources of phenotypic variability between organisms when identifying the stress factors involved. The separation and quantification of the independent effect of such factors provides an interesting outlook regarding the use of these evaluation metrics in the natural environment.

Although here we attempted to offer insight in the causality relationship between pesticide stress and morphological changes in exposed animals, the practical utility of the study mainly concerns the development of tools that can be easily implemented in environmental assessment programmes. The fact that such a tool has incorporated in its design a range of generally interfering factors confers robustness to the final conclusions and demonstrates that adequate statistical approaches can greatly simplify integration of multiple factors. One important requirement – among others (for an overview see Statzner and Bêche, 2010) – regarding the applicability of a biomonitoring tool is the stability of the trait(s) (in this case phenotypic) studied across large spatial scales. The inclusion of additional factors that quantify differences between geographically distanced sites can overcome this problem. We hope that more studies in environmental assessment of ecosystems will adopt similar approaches, as environmental risk analysis and subsequent management and protective measures can benefit from such improvements.

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## Appendix A. Correlation of each environmental variable with the first two axes of the principal component analysis. Absolute values greater than 0.5 are in bold

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

## Appendix B. Correlation of each morphometric trait with the first two axes of the linear discriminant analysis. Absolute values greater than 0.5 are in bold

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

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