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Prioritization of contaminated watercourses using an integrated biomarker approach in caged carp

Sébastjen Schoenaers¹,⁵, Lucia Vergauwen², An Hagenaars², Lynn Vanhaecke³, Hamada AbdElgawad⁵,⁶, Han Asard⁵, Adrian Covaci⁴, Lieven Bervoets¹, Dries Knapen²,¹

¹ Systemic Physiological and Ecotoxicological Research (SPHERE), Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerpen, Belgium
² Zebrafishlab, Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium.
³ Research group of Veterinary Public Health and Zoonoses, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
⁴ Toxicological Centre, Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium
⁵ Integrated Molecular Plant Physiology Research (IMPRES), Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerpen, Belgium. sebastjen.schoenaers@uantwerpen.be.
⁶ Department of Botany, Faculty of Science, University of Beni-Suef, Beni-Suef 62511, Egypt.

Corresponding author:

Sébastjen Schoenaers
INTEGRATED MOLECULAR PLANT PHYSIOLOGY RESEARCH (IMPES)
Dept. Biology
Groenenborgerlaan 171
2020 Antwerpen
sebastjen.schoenaers@uantwerpen.be
tel +32 3 265 34 16
fax +32 3 265 34 17
www.uantwerpen.be/sebastjen-schoenaers
Abstract

Because of the ever increasing complexity of environmental contamination profiles, there are limitations to the use of analytical pollutant measurements for monitoring and prioritization of watercourses. The potential of biomarkers has been debated for many years, especially in laboratory settings, but there is a need for studies evaluating these approaches in the field. We evaluated the usefulness of a selection of biomarkers, mostly indicators of general physiological status and common stress responses such as oxidative stress, to discriminate among environmental pollution profiles, with the aim of prioritizing contaminated watercourses for targeted remediation efforts. To this end, juvenile common carp (Cyprinus carpio Lin.) were exposed in cages in the field to Flemish watercourses with varying pollution profiles. After six weeks of exposure, the bioaccumulation of key pollutants was measured, and a set of organismal, biochemical and transcriptional endpoints was determined in several tissue types. After data integration a discrete set of 14 parameters was identified, that could successfully distinguish all watercourses from each other. We show that an integrated biomarker approach, mainly targeting common stress responses, can offer the resolving power to discriminate among environmentally relevant exposure scenarios, and a means to prioritize watercourses for targeted remediation.

Keywords: prioritization, biomarkers, environmental pollution, field exposure, common carp
1. Introduction

The number of chemicals produced on a commercial scale has been rising for decades (American Chemical Society, 2015). A significant subset of these chemicals ends up in aquatic ecosystems, threatening ecological integrity. In the 21st century, surface waters are often characterized by the presence of complex mixtures of chemicals of which the potentially hazardous effects are often hardly understood. In an effort to improve surface and ground water quality, The European Water Framework Directive (WFD, EU Directive 2000/60/EC) was introduced to achieve a good ecological and chemical water quality by 2015 by all European member states. An additional directive (EU Directive 2008/105/EC) lists a set of 48 priority (PS) and priority hazardous substances (PHS). However, with respect to Environmental Risk Assessment (ERA), the concentrations in surface waters are measured systematically for only a few of these priority substances, often depending on regional policy. Prioritization for subsequent remediation in Europe is mainly based on chemical monitoring (of the water column and to a lesser extent of the sediment), on the composition of the local community structure of aquatic organisms and on standardized in vivo toxicity tests. Although indispensable, such an approach has many drawbacks with regard to cost effectiveness, biological relevance, interpretability and early detection.

Compared to chemical monitoring, biomonitoring often provides a more sensitive assessment of the pollution status, since it integrates the bioavailability and intrinsic toxicity of the pollutants present. Exposure-induced perturbations at different levels of biological organization can reflect the response to pollutants present in the environment (Hook et al., 2006). Scientific research has been moving towards screening for such sets of mode of action (MOA)- or toxicant-specific biomarkers (Bossus et al., 2014; Duman and Kar, 2013; Escher et al., 2005). Such biomarkers could be used to characterize the effects of exposure to surface water samples. However, as these studies are generally performed under controlled laboratory conditions, their usefulness needs to be evaluated in the field.

To be able to verify laboratory based tests, it needs to be established whether a biomarker approach is sensitive enough to discriminate among field situations, and if such an approach can result in biologically relevant information that can be used for prioritization in ERA. In the context of ‘ease of
interpretability’, future tools should be able to identify a predominant MOA resulting from exposure to a complex mixture of compounds, as is found in nature.

Many studies have focused on the use of biochemical or transcriptional data to discriminate among model toxicants and even toxicants of the same functional class, both in field and laboratory conditions (Ankley et al., 2009; Kausch et al., 2008; Novais et al., 2012). Biomarkers have also been used to distinguish among different field sites (Falciani et al., 2008; Tiili et al., 2010). However, using the response at a single level of biological organization often does not provide the biological insight needed for prioritization or the resolution needed to distinguish among predominant MOAs (Ricciardi et al., 2010). Thus, in an effort to simultaneously increase biological insight and resolving power, we opt for using an integrated biomarker approach.

The goal of this study was to assess whether, in a field scenario, biomarkers assessing stress responses at multiple levels of biological organization can provide the resolving power and increase the biological relevance (as opposed to relying mainly on analytical measurements) needed for prioritization in ERA. Although such an approach would ideally involve species from different trophic levels, and even consider inter-species variability within trophic levels, this is not feasible within the current study. We selected carp as a representative from a high trophic level. By exposing juvenile carp in cages in the field, the potential of organismal, biochemical and transcriptional biomarkers to individually and jointly distinguish among locations with different patterns of environmental pollution was evaluated. Since an approach solely based on highly specific biomarkers would inherently lead to similar limitations as those encountered in analytical monitoring (specific biomarkers for all known MOAs would have to be included), we opted for a set of biomarkers probing for general health (e.g. condition factor, energy metabolism) and indicative of common MOAs (e.g. oxidative stress, disrupted ion-osmoregulation).
2. Materials and methods

2.1 Exposure scenario

2.1.1 Study area

Seven sites belonging to five watercourses in the drainage basin of the river Scheldt were selected (Fig. 1), based on chemical monitoring data from the Flemish Environmental Agency (‘Vlaamse Milieumaatschappij’ VMM) and previous research. Several sites were expected to have relevant concentrations of a (class of) pollutant(s) with distinctly different modes of action. In addition, three reference sites were selected with no indication of pollution. Physico-chemistry data of the water column was available for all sites except SN1 and CDS (Table 1).

The Scheppelijke Nete (SN) contains high Cd concentrations originating from groundwater historically polluted by metal industry (www.vmm.be). Three sites were selected; SN1 is situated upstream of the pollution source and was therefore an expected reference site within this watercourse. At sites SN2 and SN3 the Belgian and international water quality objectives (Cd: 0.25-1.5 µg L\(^{-1}\), Zn: 120 µg L\(^{-1}\)) were by far exceeded for both metals (European Commission, 2008; Reynders et al., 2008; US Environmental Protection Agency, 2009), although concentrations decreased downstream of the pollution source. The Desselse Nete is located in the same basin and was selected as an expected reference site for the basin of the Nete. Including an internal reference (SN1) and external reference belonging to the same basin (DN) aimed to test the discriminatory power of the acquired data, since fish were exposed to streams with similar physico-chemistry, only differing in the presence of certain key chemicals. During 2011, dimethoate (DM) concentrations in the Vliet (V) exceeded the water quality standards proposed by the WFD twice (www.vmm.be/geoview; European Commission, 2012). Other pesticides designated as priority substances by the WFD were detected though not exceeding the quality standards. These pesticides likely originate from upstream agricultural runoff. The canal Dessel-Schoten (CDS) was expected to contain high concentrations of PCB-153 and BDE-47, presumably from upstream industrial activities (Belpaire et al., 2011; Roosens et al., 2010). The Zwalmbeek (Z) was chosen as an external reference site, situated in the Scheldt basin but not in proximity of the other watercourses.
2.1.2 Test species, exposure and follow-up

Common carp (*Cyprinus carpio*) can be found in most surface water across Europe and, given its availability as legislated test-species, its resilience and ability to cope with prolonged exposure in cages (Reynders et al. 2008; Bervoets et al. 2009; Snyder et al., 2004), is a useful test species in ecotoxicological studies involving molecular biomarker assessment (e.g. De Smet et al., 2001; Tlili et al., 2010). Moreover, common carp was found in electric fishing campaigns (2003-2010) throughout the Nete basin, in CDS and Z and its tributaries (www.inbo.be).

This study was approved by the Institutional Ethical Committee of the University of Antwerp (project number 2011-05) and experiments were executed in strict accordance with the European Council Directive (2010/63/EC). Juvenile common carp (6.97±0.96 cm, 5.31±2.59 g; University of Wageningen) were temperature acclimated (18°C) during one month prior to exposure in a 500 L flow-through aquarium with softened, copper and chlorine free water. Oxygen (>60% saturation), ammonia (≤0.25 mg L⁻¹) and nitrite (<0.3 mg L⁻¹) concentrations were kept within acceptable boundaries. 15 fish that were acclimated in the laboratory were not exposed in the field and were included in the analysis as a zero-state reference (LAB). Immediately after acclimation, multiple fish were exposed to surface water for 6 weeks, using a set-up similar to that of Bervoets et al. (2009). Increasing exposure periods increase the extent to which caged fish reflect the actual pollution status of a watercourse. At the same time we chose to avoid excessive seasonal variation. Therefore we opted for a 6 week exposure which corresponds to half a season. A 10 day pilot experiment showed that the set-up was strong enough to withstand differences in flow and sediment load, and allowed normal feeding. As such, we observed no mortality or differences in the relative condition factor (RCF) when comparing the RCF of the same fish before and after exposure. Subsequently, at each location 4 cages (two plastic pond baskets tied together, 60x40x40 cm with mesh size 2x4 mm), each containing 7 fish, were exposed for six weeks (SN1, SN2, SN3, DN, V, Z, placed on September 13 and 14th 2012, CDS placed October 9th 2012). Weekly, the cages were lifted out of the water for a few seconds and checked for the amount of accumulated sediment and cage integrity. When there was mortality, the cages were opened partly in the water, the dead fish were removed and the closed cages were put back. During exposure, water temperature, pH, dissolved oxygen concentration (DOC) and conductivity were measured weekly (Hach HQ30D).
2.1.3 Biological sampling

After 6 weeks, fish were dissected in the field immediately after removal from the water. Prior to dissection, length and whole body mass were determined. Fish were sacrificed by decapitation. The liver was isolated first, weighed and snap frozen. The kidney, whole brains, laterodorsal muscle tissue (1x0.5 cm) and gills without gill arches were isolated, snap frozen, transported in liquid nitrogen and stored at -80°C. Remaining muscle tissue was also isolated, kept on ice and stored at -20°C. In order to collect enough tissue for all the assays, including pollutant and biomarker assessment, the samples of each tissue type were pooled per cage by joined homogenization in liquid nitrogen, resulting in minimum 3 but mostly 4 pools per site. Each pool contained organs from minimum 4 but mostly 7 fish. Table 1 provides an overview of the number of samples per location.

2.2 Pollutant bioaccumulation

The bioaccumulation of a series of metals (liver, kidney), persistent organic pollutants (POPs; muscle) and pesticides (muscle) was quantified. Tissue samples were dried (60°C, 48h) and digested using HNO₃ (overnight) and subsequent heating (3 x 110°C for 30 min). The concentrations of Ag, As, Cd, Cr, Cu, Ni, Pb and Zn were determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x ICP-MS, Santa Clara, USA) following the method of Reynders et al. (2008). The concentrations of 28 PCBs, 7 PBDEs, HCB, DDT and metabolites, 5 chlordanes and 3 lindane isomers were determined according to the method of Erdogrul et al. (2005). POPs were extracted in 3:1 hexane:acetone. Lipids were removed by acid-silica Solid Phase Extraction. The samples were concentrated and dissolved in iso-octane. PCBs were measured by gas chromatography coupled to an electron capture detector (HP 6890 GC-µECD with HT-8 capillary column). PBDEs were measured by Gas Chromatography coupled to Mass Spectrometry (GC-MS, HP 6890GC-5973MS, HT-8 column). The concentrations were calculated based on an internal standard. Analytic accuracy was determined using reference tissue (whale blubber with known POP concentrations; SRM 1945; Erdogrul et al. 2005). Pesticides were extracted using pressurized liquid extraction (1:1 acetonitrile:methanol) and Solid Phase Extraction (Isolute ENV+ 200 mg columns, Biotage, Uppsala, Sweden, acetonitrile/methanol as eluent). The concentrations of 14 pesticides were determined using Ultra-high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (U-HPLC-QqQ-MS/MS, Nucleodur C18
pyramid U-HPLC column, TSQ vantage triple stage quadrupole MS) and electron spray ionization, in accordance with the method of Wille et al. (2011). Concentrations were derived from standard curves based on a dilution series using reference carp liver (containing known concentrations of the measured pesticides), and normalized to an internal standard.

2.3 General fish condition parameters

2.3.1 Relative condition factor

The RCF was calculated according to formula 1 (Bagenal and Tesh, 1978). With \( W = \text{weight (g)}, L = \text{length of fish (mm)}, a = \text{intercept}, b = \text{regression coefficient.} \)

All fish were weighed and measured before exposure. The regression coefficient and intercept were derived from the mass ~ length relationship \( W = aL^b \) from these zero-state values \((a=1.06\times10^{-5}; b=3.076; R^2=0.98)\)

\[
RCF = \frac{W}{aL^b} \quad (1)
\]

2.3.2 Hepatosomatic index

The HSI was calculated as the ratio between wet weight of the liver and the whole fish’s fresh weight (FW), after exposure (Hagenaars et al., 2008).

2.4 Biochemical parameters

A broad set of biochemical parameters was selected with the purpose of acquiring an overall reflection of the physiological status of the exposed fish (Table 2).

2.4.1 Energy reserves and electron transport system activity

Muscle protein, carbohydrate and lipid concentrations were determined colorimetrically in 50 mg of lateral muscle tissue. Carbohydrate content was measured based on the Anthron-assay (Trevelyan et al., 1952), using a glycogen dilution series. Protein content was determined according to the method of Bradford (1976). The lipid-content was measured in accordance with the method of Bligh and Dyer (1959), based on a tripalmitin standard. The ETS activity was determined in 15 mg of lateral muscle tissue following the method of Packard (1971).
2.4.2 Oxidative stress parameters

A liver extract was prepared in 80% EtOH from 100 mg liver homogenate. The supernatant (after 13,300 g, 10 min, 4 °C) was assayed for FRAP, total polyphenol and MDA content. 150 mg of the same liver homogenate was suspended in potassium phosphate buffer (0.05 M PVP, 0.4 mM EDTA, 0.2 mM PMSF, 1 mM ascorbic acid, pH 7.0). The supernatant (after 14,000 g, 15 min, 4°C) was assayed for enzyme activities, \( \text{H}_2\text{O}_2 \) content and protein carbonylation.

The \( \text{H}_2\text{O}_2 \) content was measured in accordance with the method of Bellincampi et al. (2000), and normalized to tissue fresh weight (FW). The total antioxidative capacity was quantified based on the ‘ferric ion reducing antioxidant power (FRAP)’ assay (Benzie and Strain, 1996), and expressed as Trolox equivalents per gram FW. MDA level and superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), xanthine oxidase (XOX) and peroxidase activity (POX) were determined according to the method of El-Soud et al. (2013). The total polyphenol content was based on the Folin-Ciocalteau method (Zhang et al., 2006), and expressed as mg gallic acid-equivalents per gram FW. The activities of glutathione-S-transferase (GST), glutathione reductase (GR), monodehydroascorbic acid reductase (MDHAR), dehydroascorbic acid reductase (DHAR), ascorbic acid peroxidase (APX) were determined spectrophotometrically based on the workflow of Murshed et al. (2008).

The concentrations of oxidized and reduced ascorbic acid and glutathione were determined in 100 mg whole brain tissue by RP-HPLC (C-18, LiChroSpher, Alltech, Deerfield, IL; LC-10ADVP, Shimadzu, Colombia, MD) in accordance with the method of AbdElgawad et al. (2016).

Oxidative damage to proteins was estimated by the concentration of carbonylated proteins, following the method of Levine et al. (1994). Calculations were based on the molar extinction coefficient for aliphatic hydrazones (22000 M\(^{-1}\) cm\(^{-1}\)) and normalized to protein content (Bradford, 1976).

2.4.3 Metallothionein

The total MT-content was measured in the liver and kidneys using the Cd-saturation method (Bartsch et al., 1990). \(^{109}\text{Cd}\) gamma-radiation was counted for 1 min (Perkin Elmer, Wizard 1480). MT concentrations were calculated assuming a molar ratio of Cd/MT of 7.
2.4.4 Acetylcholinesterase activity

AchE-activity was determined in 50 mg of whole brain tissue according to the method of Ellman et al. (1961). The brain tissue was homogenized in 0.1 M phosphate buffer (pH 7.4). Results were expressed as µmol thiocholine min⁻¹.

2.4.5 Na⁺/K⁺ and Mg²⁺+Ca²⁺-ATPase activity

The Na⁺/K⁺ and Mg²⁺+Ca²⁺-ATPase activities were determined in the gills according to the method of Zaugg (1982). Gill arches were removed and gills were homogenized in SEID-EGTA buffer (2.4 mM sodium deoxycholate in SEI-EGTA buffer; 250 mM sucrose, 10 mM EGTA, 50 mM imidazole in water, pH 7.3). The Na⁺/K⁺-ATPase activity was determined as the difference between the Ouabain insensitive and Ouabain sensitive portion. The Mg²⁺+Ca²⁺-ATPase activity was derived from the Ouabain insensitive fraction only.

2.5 Gene transcription analysis

2.5.1 Gene selection and primer design

Based on literature and prior results, 8 genes (CYP1A: cytochrome p450 1A1, MT1 and MT2: metallothionein 1 and 2, Mn-SOD: Mn-superoxide dismutase, CAT: catalase, Hbα2: hemoglobin subunit α2, 18sRNA: 18sRNA homologue, IGFBP1: insulin-like growth factor binding protein 1) were selected for qPCR (SI Table 1). β-actin, Elongation Factor 1α (EF1α) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as potential housekeeping genes. Primers (Eurogentec, Seraing, Belgium) were designed using LightCycler probe software (Roche Diagnostics, Vilvoorde, Belgium).

2.5.2 RNA extraction and qPCR

Total liver RNA was extracted using the Qiazol method (Qiagen, Belgium), followed by DNase treatment (Fermentas, St. Leon-Rot, Germany) and phenol:chloroform extraction. RNA quantity and quality were determined by spectrophotometry (NanoDrop Technologies, Wilmington, DE) and Qiaxcel gel-electrophoresis (Qiagen, Belgium) respectively. First strand cDNA was transcribed from 1 µg of RNA (RevertAid H Minus kit, Fermentas, St. Leon-Rot, Germany) with oligodT primers. Reactions were performed in a Stratagene mx3005p using Brilliant II SYBR Green Master Mix (Agilent
Technologies). Thermal cycling consisted of an initial denaturation period of 10 min at 95°C, followed by 45 cycles of 20 s at 95°C, 40 s at 55°C and 50 s at 72°C. Melting curves and primer efficiencies were determined.

2.5.3 Relative quantification
The fold change (FC) was calculated using the method of Pfaffl (2001) and Vandesompele et al. (2002). Quantities were calculated using the \( \Delta Ct \) method (Pfaffl, 2001). A normalization factor (NF) was defined for each sample, being the geometric mean of the quantities (Q) of the two housekeeping genes with the most stable transcription ratio (\( GAPDH \) and \( EF1\alpha \)). The FC was then defined as the NF-normalized quantities. Results were expressed as relative transcription, with respect to the mean FC of the laboratory reference.

2.6 Statistical analysis
Statistics were performed in R (R Core team, 2013). Significance was assessed by analysis of variance (ANOVA) or Kruskal-Wallis tests followed by a TukeyHSD or pairwise Wilcoxon rank sum test. Correlations were calculated using a spearman rank correlation test and visualized in a correlation matrix (SI Fig. 1).

For multivariate analysis, matrix normality was checked by multivariate Shapiro Wilkinson tests. Data were normalized using a Z-score, calculated separately for each parameter. Dimensionality reduction was performed using principal component analysis (PCA).

Cluster analysis was carried out in MultiExperiment Viewer (www.tm4.org/mev, version 4.8.1) with the normalized relative value compared to the laboratory reference. Hierarchical clustering (Euclidean distance) was combined with Multi-Class Significance Analysis of Microarrays (SAM) analysis (7 classes, 10 K-nearest neighbors, 100 permutations, \( \Delta=0.2, \text{FDR} < 1\% \)) to select the smallest number of best distinguishing parameters.
3. Results

3.1 Physico-chemical variation during the period of exposure

The water temperature fell gradually from 15°C to approx. 11°C at most locations. Z and V showed stronger fluctuations. pH ranged between 7.88 (CDS) and 6.44 (DN), and differed among streams, with lowest pH measured in locations within the Nete basin (7.09±0.15, n=7), followed by Z (7.44±0.07, n=7), V (7.65±0.11, n=7) and CDS (7.75±0.11, n=7). At the time of sampling, the pH was highest in CDS (7.73) and lowest in DN and SN3 (6.44). The DOC differed among streams and was lowest and most variable in Z (4.03 ± 1.87 mg L\(^{-1}\), n=7). Mean DOC was normal (>5.5 mg L\(^{-1}\), n=7) for other locations. The conductivity was highest for V (650±67 µS cm\(^{-1}\), n=7) and lowest for DN (329 ± 23 µS cm\(^{-1}\), n=7). Strongest fluctuations were observed in Z (514±143 µS cm\(^{-1}\), n=7). Locations at SN were similar (438±19 µS cm\(^{-1}\), n=7).

3.2 Fish survival

In Z, 11 fish died. The cause is unknown, however given the sudden occurrence of mortality but high RCF after exposure, this suggests a transient stress factor. In DN, 8 fish escaped following removal of water vegetation. The remaining fish from two cages were placed in one cage.

3.3 Pollutant bioaccumulation in liver, kidney and muscle

Figure 2 provides an overview of all detected pollutants by PCA (34.5% PC1, 15.3% PC2; full dataset: SI Table 2). SN3 replicates cluster in the upper right quadrant, due to high PCB accumulation (sum individual congeners = 142±62 ng g\(^{-1}\) FW, n=4) and Cd in liver and kidneys (0.15±0.01 and 0.89±0.11 µg g FW\(^{-1}\), n=4). SN2 replicates, located closer to the metal pollution source, cluster in the lower right quadrant, due to high concentrations of Cd in liver and kidneys (0.52±0.04 µg g FW\(^{-1}\) and 2.34±0.19 µg g FW\(^{-1}\), n=4). Z and V accumulated low concentrations of pesticides (terbuthylazine, isoproturon, β-HCH). Highest concentrations were found for p,p’-DDE and p,p’-DDD in muscle tissue (2.90±0.4 ng g FW\(^{-1}\) in Z, n=3, 2.86±0.4 ng g FW\(^{-1}\) in V, n=4). SN1, DN and CDS replicates cluster near the point of zero variation. One SN1 replicate had accumulated a high concentration of Cr (mean SN1: 2.85±2.11 µg g\(^{-1}\) FW, n=4). SN1\([\text{low}]\) fish accumulated low concentrations of oxime carbamates. All fish accumulated BDE-47 (0.79±0.23 ng g\(^{-1}\) muscle FW, n=26) and low concentrations of PCBs, Cu, Pb, As
and Zn. From here on, watercourses will be referred to as SN$_{[\text{low}]}$, DN$_{[\text{low}]}$, CDS$_{[\text{low}]}$ (low pollutant concentrations), SN$_{2[\text{Cd}]}$ (highest cadmium), SN$_{3[PCB,Cd\pm]}$ (PCB and cadmium), Z$_{[\text{pest.}]}$ and V$_{[\text{pest.}]}$ (low pesticide).

### 3.4 General condition and energy metabolism

The RCF differed among sites (Fig. 3). It was lowest for SN$_{1[\text{low}]}$ and SN$_{2[\text{Cd}]}$. The HSI differed among watercourses, with lowest values in SN, DN$_{[\text{low}]}$ and CDS$_{[\text{low}]}$, and intermediate and highest levels in V$_{[\text{pest.}]}$ and Z$_{[\text{pest.}]}$ respectively.

Muscle lipid concentrations were unaffected. Muscle carbohydrate and protein concentrations differed significantly ($p\leq0.001$). Carbohydrates were similar in all samples and correlated with the HSI ($\rho=0.73$; SI Fig. 1). Only in DN$_{[\text{low}]}$, muscle protein concentrations were significantly lower compared to other samples. The ETS activity did not differ among watercourses.

### 3.5 Differential biochemical and transcriptional response

A list of all results is provided (SI, Table 3). Figure 4 provides a more comprehensive overview of the results, showing that the sites form clear clusters using the organismal and biochemical data alone (Fig. 4A; 42.8% PC1, 14.4% PC2) and upon including the transcriptional data (Fig. 4B; 31.8% PC1, 14.2% PC2). Using all data (Fig. 4B) results in distinct clusters for SN$_{1[\text{low}]}$, SN$_{2[\text{Cd}]}$, SN$_{3[PCB,Cd\pm]}$, DN$_{[\text{low}]}$ and Z$_{[\text{pest.}]}$. CDS$_{[\text{low}]}$ and V$_{[\text{pest.}]}$ group closely together. Z$_{[\text{pest.}]}$ samples are similar to the laboratory reference. All oxidative stress parameters were increased markedly in SN$_{2[\text{Cd}]}$ in the liver and brain. [H$_2$O$_2$] was highest in all SN and DN$_{[\text{low}]}$ replicates, as was lipid peroxidation ($p=1.10^{-6}$). The antioxidative response distinguishes SN$_{2[\text{Cd}]}$ ($p<0.005$), followed by SN$_{3[PCB,Cd\pm]}$ and SN$_{1[\text{low}]}$ which only showed high GR and SOD-activity respectively. The ASC and GSH status in the brain was affected in SN$_{1[\text{low}]}$, SN$_{3[PCB,Cd\pm]}$ and DN$_{[\text{low}]}$ exposed fish. [MT] in liver ($p=0.001$) and kidney ($p<0.001$) were highest in SN$_{2[\text{Cd}]}$ followed by SN$_{3[PCB,Cd\pm]}$. In the gills, Mg$^{2+}$+Ca$^{2+}$-ATPase activity ($p<0.001$) was the lowest in SN$_{2[\text{Cd}]}$ and the highest in CDS$_{[\text{low}]}$. The overall biochemical response in V$_{[\text{pest.}]}$ and CDS$_{[\text{low}]}$ was low compared to the laboratory reference. Na$^+$/K$^+$ and AchE activity were unaffected. We observed low muscle carbohydrate reserves at all sites, low protein content for DN$_{[\text{low}]}$ and the lowest RCF for SN$_{1[\text{low}]}$. 


and SN2$_{[Cd^{+}]}$ exposed fish.

No gene transcription data was included for two SN2$_{[Cd^{+}]}$ and SN1$_{[low]}$ replicates and one V$_{[pest.]}$ replicate, due to bad RNA quality. Transcription of MT2, MT1, Mn-SOD, CYP1A and IGFBP1 differed among locations (p≤0.01). SN1$_{[low]}$ MT2 transcription did not differ from the laboratory reference. MT1 transcription was the highest in SN2$_{[Cd^{+}]}$, SN3$_{[PCB,Cd^{±}]}$, V$_{[pest.]}$ and CDS$_{[low]}$.

MT protein levels in the liver correlated with MT2 (ρ =0.79) and MT1 (ρ =0.76) transcription. Mn-SOD transcription was downregulated in SN2$_{[Cd^{+}]}$, SN3$_{[PCB,Cd^{±}]}$ and DN$_{[low]}$, compared to the laboratory reference, and correlated negatively with SOD enzyme activity (ρ = -0.55). The HSI correlated with carbohydrate content (ρ =0.73). A correlation matrix (SI Fig. 1) shows strong correlation among oxidative stress parameters.

### 3.6 Cluster analysis and biomarker set selection

Figure 5 shows a HC using all data, and a HC and PCA after selection of the minimum number of 14 SAM-selected parameters (when the number of parameters was further decreased, good separation could not be obtained).

Based on all data, the replicates clustered together per location except for those of V$_{[pest.]}$ and CDS$_{[low]}$. However, based on the 14 best discriminating parameters, each location clustered separately. This subset included parameters related to oxidative stress in the brain (ascorbate and glutathione status) and liver (H$_2$O$_2$ content, MDA content, DHAR activity), osmoregulation in the gills (Mg$^{2+}$+Ca$^{2+}$-ATPase activity), metal and PCB detoxification in the liver (MT content, CYP1A$^1$ transcription) and kidneys (MT content), and general condition (HSI and RCF). IGFBP1α transcription was included, for which increased transcription could be indicative for DM exposure (unpublished results). SN1$_{[low]}$, SN2$_{[Cd^{+}]}$ and SN3$_{[PCB,Cd^{±}]}$ formed neighboring clusters. DN$_{[low]}$, while also part of the Nete basin, clustered separately. SN2$_{[Cd^{+}]}$ replicates formed the most distinct cluster. A PCA (PC1 39.6%, PC2 19.9%) based on this selected biomarker set resulted in full separation of exposure scenarios. SN2$_{[Cd^{+}]}$ replicates are most dissimilar compared to other sites. Other sites in the Nete basin cluster in the upper left quadrant,
with SN3_{PCB,Cd\eta} closest to SN2_{Cd\eta}. Sites with low bioaccumulation profiles cluster to the right, close to the laboratory reference. They form separate clusters, reflecting differences in biological response.

4. Discussion

The goal of this study was to establish if an integrated biomarker approach offers (1) the resolving power to discriminate among environmentally relevant exposure scenarios, and (2) a means to prioritize watercourses for remediation efforts. In order to obtain exposure to different pollution profiles, we exposed juvenile common carp for six weeks to watercourses that were expected to contain different pollutants (Table 1).

4.1 Pollutant bioaccumulation profiles differed among exposure sites

The accumulated pollutants reflected different pollution profiles (Fig. 2). With regard to metal accumulation, the findings for SN2_{Cd\eta}, SN3_{PCB,Cd\eta} were in agreement with available chemical monitoring data (VMM monitoring database; Reynders et al 2008). As expected, pesticides were detected in V_{pest.\eta}, and pollutant levels were low in SN1_{low} and DN_{low}. Against expectations, high levels of PCBs were found in SN3_{PCB,Cd\eta} whereas concentrations were low in CDS_{low}. Furthermore, Z_{pest.\eta} exposure led to unexpected accumulation of pesticides. Based on the observed differences in accumulation profiles, SN3_{PCB,Cd\eta} followed by SN2_{Cd\eta} had the most severe degree of pollution. Based on these bioaccumulation data, SN3_{PCB,Cd\eta} would be given the highest priority, followed by SN2_{Cd\eta}.

All other water courses would likely be classified as ‘no priority’ since overall pollutant levels were found to be low. However, the ultimate objective of prioritization and subsequent water quality management is to ensure or improve local biological integrity by increasing the water quality. Importantly, biological integrity relies on the full complexity of the surrounding environment, rather than only its chemical composition. As such, while chemical monitoring data is valuable, it provides little insight in the effects of environmental exposure on biological integrity. Biomarker based data has been put forward in many instances as a means of tackling this problem (e.g. Budka et al., 2010; Devin et al., 2014). If a biomarker approach is to be applicable for prioritization in ERA, it has to provide (1) the resolving power to discriminate among environmentally relevant exposure scenarios, and (2)
interpretable, biologically relevant information, reflecting the exposure induced physiological status.

4.2 Environmental pollution profiles can be discriminated using an integrated biomarker approach

Due to differential pollutant accumulation among locations, we could test whether a broad set of biological parameters of different organizational levels could distinguish among environmentally relevant exposure scenarios. The parameters were chosen to provide a broad reflection of the physiological status of the exposed fish (Table 2).

Based on individual endpoints, the biological response of SN2\(_{[\text{Cd}^+]}\) and SN3\(_{[\text{PCB,Cd} \pm]}\) exposed fish was most prominent, possibly due to the high Cd and/or PCB levels that were detected. Cadmium can evoke a myriad of biochemical and transcriptional responses (Espinoza et al., 2012; Jia et al., 2011; Roméo et al., 2000). PCB126 accumulation was shown to affect biotransformation enzymes and the antioxidative response in lake trout at only 25 ng PCB126 g\(^{-1}\) liver (Palace et al., 1996). We observed a concentration dependent detoxification/sequestration response in the form of an increase in GST enzyme activity (SN2\(_{[\text{Cd}^+]}\)) and MT protein and gene transcription levels (SN2\(_{[\text{Cd}^+]}\), SN3\(_{[\text{PCB,Cd} \pm]}\)). In SN3\(_{[\text{PCB,Cd} \pm]}\) and SN2\(_{[\text{Cd}^+]}\) exposed fish oxidative stress exceeded the antioxidative capacity. In SN2\(_{[\text{Cd}^+]}\), severe oxidative damage to proteins was observed. Oxidative stress may have serious consequences on higher levels of biological organization as most oxidative stress parameters showed a strong negative correlation with the HSI and RCF (SI Fig. 1). Based on these individual biomarkers, one would reach a similar conclusion compared to the chemical analysis, namely that SN2\(_{[\text{Cd}^+]}\) and SN3\(_{[\text{PCB,Cd} \pm]}\) should be given highest priority for remediation. This shows that an unintegrated interpretation using single parameters can be useful for distinguishing heavily polluted watercourses from others, but does not provide the resolution and interpretability to further categorize the remaining watercourses.

Subsequent data integration greatly increased resolving power, and showed that the biological response indeed differed considerably among the remaining exposure sites (Fig. 4). Based on all biochemical and organismal data (Fig. 4A) the locations could be distinguished into clear groups related to (1) high oxidative stress and MT in liver, low general condition (SN2\(_{[\text{Cd}^+]}\); upper right quadrant), (2) good general
condition, low oxidative stress in liver (LAB, Z\text{pest.}; upper left quadrant), (3) oxidative stress in brain, low general condition (DN\text{low}, SN1\text{low}; lower quadrants), (4) low response (V\text{pest.}, CDS\text{low}; center), (5) moderate oxidative stress in liver, low general condition (SN3\text{PCB,Cd±}; lower right quadrant). Upon including the transcription data, the resolution further increased, separating DN\text{low}, SN1\text{low} and SN3\text{PCB,Cd±}, and LAB and Z\text{pest.}. The ‘biological response’ classes to which the different watercourses can now be assigned are of much greater value to prioritization efforts, due to higher discriminatory power and easier interpretation.

We further tested whether we could select a smaller set of biomarkers which is able to discriminate among the pollution profiles, since this would be more practical in future applications. Through SAM-analysis, we identified a highly diverse subset of 14 transcriptional, biochemical and organismal parameters that allowed us to distinguish among all exposure scenarios and obtain a similar grouping pattern compared to using all data (Fig. 5). This shows that a small but diverse subset of parameters, covering general energy status, oxidative stress, osmoregulation and metal/PCB detoxification suffices to provide (1) the resolution to discriminate among complex environmental exposure scenarios and (2) easy to interpret, biologically relevant information, reflecting the exposure induced perturbations.

Ranking and prioritization of watercourses with regard to pollution severity and subsequent water quality management would greatly benefit from biological data which is cheap (opposed to relying on analytical methods) and easy to acquire, provides a broad and easily interpretable view of biological functioning, and the resolution to discriminate among complex environmental exposure scenarios. We show that these requirements can be met by using general biological parameters. For instance, based on the integrated dataset, it is clear that SN2\text{(Cd+)} exposure induced the strongest oxidative stress response and subsequent low RCF. This shows an important effect on fish wellbeing and therefore, based on these data, we would give SN2\text{(Cd+)} the highest priority for remediation. Moreover, SN2\text{(Cd+)} replicates cluster the furthest from locations with a response similar to the laboratory reference (CDS\text{low}, V\text{pest.}, Z\text{pest.}). The latter could then be considered as ‘no-priority’. SN3\text{PCB,Cd±} could be given second priority, with an intermediate oxidative stress response and low RCF, followed by SN1\text{low} and DN\text{low} where the GSH and ASC status in the brain is affected.
**4.3 Considerations for prioritization**

An important remark is that biological status can be affected by both chronic and transient (e.g. single pollution peak due to runoff) environmental perturbation. For instance, given the sudden occurrence of mortality in $Z_{pest.}$ and the yet high RCF after exposure, this suggests a transient stress factor. $Z_{pest.}$ physico-chemical data showed highly variable conditions throughout the period of exposure. This suggests that side by side evaluation of both data types (biological and physico-chemical) could be important for prioritization.

An important aspect of biological data acquisition for ERA is the ease of sample collection. Sampling from the environment or exposure in cages is laborious. A promising approach is to expose small laboratory animals (e.g. zebrafish embryos, daphnids) to water samples collected in the field (e.g. Bervoets et al., 1996; Fang et al., 2014).

Our results show that high discriminatory power can be achieved using a set of biomarkers probing for general health status (pollutant-aspecific markers). When solely using highly specific biomarkers, one could be at risk of neglecting the actual relevance to population survival. While we showed the value of our approach, we do feel that further addition of biomarkers targeting other physiological processes essential to population survival (e.g. immune function, reproductive health) would be useful. This could be done in future trials evaluating the approach on a larger scale (i.e. including more watercourses).

**5. Conclusions**

The potential of biomarkers to tackle the limitations of using analytical pollutant measurements for monitoring and targeted remediation efforts has been debated for many years. As a means of verifying such an approach, we evaluated the usefulness of a set of general biomarkers to discriminate among complex environmental contamination profiles. We show that an integrated biomarker approach, mainly targeting common stress responses, can offer (1) the resolving power to discriminate among environmentally relevant exposure scenarios, and (2) a means to prioritize watercourses for targeted remediation.
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water policy.


Figure 1: Locations of exposure sites. Map of Flanders showing the locations of exposure (green circles), situated in the Scheldt estuary. Z (Zwalmbeek; expected reference site), V (Vliet; expected dimethoate site), CDS (Channel Dessel-Schoten; expected PCB and BDE site), SN1 (Scheppelijke Nete site 1; expected reference site), SN2 (Scheppelijke Nete site 2; expected heavy cadmium pollution), SN3 (Scheppelijke Nete site 3; downstream of SN2; expected more moderate cadmium pollution), DN (Desselse Nete; expected reference site). Main canals and rivers (blue lines) and larger cities (black dots) are shown. The observed pollution profiles differed from those that were expected (clarified in section 3.3 and 4.1).

Figure 2: Principal component analysis of the accumulated pollutants. The PCA is based on Z-score normalized data, showing how the pattern of accumulation of different pollutants allows for the distinction of fish exposed at different locations. Accumulation of metals was measured in both liver (l) and kidneys (k).

Figure 3: The relative condition factor (RCF) and hepatosomatic index (HSI) after exposure. (A) ΔRCF (RCF after exposure – RCF before exposure) of fish that survived exposure. (B) the HSI. Error bars indicate the SD. Pairwise significance is shown as different letters.

Figure 4: Principal component analysis of the biological parameters. PCAs based on Z-score normalized data showing how the organismal, biochemical and transcriptional responses allow for the distinction of fish exposed at different locations. (A) PCA using organismal and biochemical data. (B) PCA using the organismal, biochemical and gene transcription data. No gene transcription data was obtained for one V and two SN1 and SN2 replicates. Mortality (MOR), Relative Condition Factor after exposure (RCFafter). Abbreviations refer to table 2.

Figure 5: Subset selection of best discriminating parameters. (left) heatmap and HC (Euclidean distance) of the complete, normalized dataset (field site vs laboratory reference). (right) HC (Euclidean distance) after SAM-selection of the smallest set of best discriminating parameters. Colors represent relative values compared to the laboratory reference. (bottom) PCA of SAM-selected parameters.

Table 1: Selected sites for exposure. Flemish Environmental Agency (VMM) accession numbers refer to www.vmm.be/geoview. SN1 is no VMM registered sampling point. Samples are presented as number of fish for which samples were collected, expressed as the sum of individual cages. *=fish taken from two cages. ND=No Data.

Table 2: List of biochemical parameters that were quantified in different organs. The same organs were pooled per cage.
Figure 1:
Figure 2:
Figure 4:
Table 1:

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<th>expected pollution profile</th>
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<td>DN</td>
<td>308000</td>
<td>51°14'53.63&quot;N 5°5'1.81&quot;E</td>
<td>(VMM 2009: 1 data point) pH: 7 [O₂]: 9.3 mg L⁻¹ hardness: 357 µS cm⁻¹</td>
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<td>7+7+6*</td>
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<td>Z</td>
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<td>4*+6+7</td>
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<tr>
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<td>(VMM 08/2011-08/2012) pH: 7,8±0,2 [O₂]: 6,7±2,2 mg L⁻¹ hardness: 809±152 µS cm⁻¹</td>
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