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PII: S1382-6689(19)30175-9

DOI: https://doi.org/10.1016/j.etap.2019.103301

Reference: ENVTOX 103301

To appear in: *Environmental Toxicology and Pharmacology*

Received Date: 14 June 2019

Revised Date: 12 November 2019

Accepted Date: 14 November 2019

Please cite this article as: van der Oost R, McKenzie DJ, Verweij F, Satumalay C, van der Molen N, Winter MJ, Chipman JK, IDENTIFYING ADVERSE OUTCOME PATHWAYS (AOP) FOR AMSTERDAM CITY FISH BY INTEGRATED FIELD MONITORING, *Environmental Toxicology and Pharmacology* (2019), doi: https://doi.org/10.1016/j.etap.2019.103301

IDENTIFYING ADVERSE OUTCOME PATHWAYS (AOP) FOR AMSTERDAM CITY FISH BY INTEGRATED FIELD MONITORING

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HIGHLIGHTS

- Integrated environmental field survey on biomarkers, fish physiology & populations
- Identification of adverse outcome pathways of complex chemical mixtures
- Biomarker inductions at most polluted sites, associated with swimming behavior
- Biomarker and bioassay assessments were good indicators for adverse effects on fish
- No complete AOP could be determined due to missing data

Summary

The European City Fish project aimed to develop a generic methodology for ecological risk assessment for urban rivers. Since traditional methods only consider a small fraction of substances present in the water cycle, biological effect monitoring is required for a more reliable assessment of the pollution status. A major challenge for environmental risk assessment (ERA) is the application of adverse outcome pathways (AOP), i.e. the linking of pollutant exposure via early molecular and biochemical changes to physiological effects and, ultimately, effects on populations and ecosystems.

We investigated the linkage between responses at these different levels. Many AOP aspects were investigated, from external and internal exposure to different classes of micropollutants, via molecular key events (MKE) the impacts on organs and organisms (fish physiology), to changes in the population dynamics of fish. Risk assessment procedures were evaluated by comparing environmental quality standards, bioassay responses, biomarkers in caged and feral fish, and the impact on fish populations. Although no complete AOP was observed, indirect relationships linking pollutant exposure via MKE to impaired locomotion were demonstrated at the most polluted site near a landfill for chemical waste. The pathway indicated that several upstream key events requiring energy for stress responses and toxic defence are likely to converge at a single common MKE: increased metabolic demands. Both fish biomarkers and the bioanalytical SIMONI strategy are valuable indicators for micropollutant risks to fish communities.

Key words: adverse outcome pathways, micropollutants risk assessment, biochemical & physiological biomarkers, ecological studies

1 Introduction

The environment is continuously exposed to xenobiotic chemicals released by urban communities, agriculture and industries. Since the ultimate sink for many of these contaminants is the aquatic environment, the aquatic fate and effects of chemical substances have been extensively studied (Stegeman and Hahn, 1994). It is virtually impossible to make a reliable assessment of environmental quality using only chemical monitoring methods, because only a limited number of target substances are analyzed. The risk of non-target and unknown substances in the aquatic environment is therefore unclear (Escher and Leusch, 2012). Moreover, environmental chemicals do not occur as single substances, but in complex mixtures with potential synergism or antagonism. While concentrations of individual chemicals can be below lowest observed adverse effect concentrations (LOAEC) or detection limits, the entire mixture may still cause adverse effects (Silva et al., 2002). In addition, transformation products of micropollutants may be more toxic and persistent than the parent compounds (Escher and Fenner, 2011). Therefore, it is generally

acknowledged that, in addition to chemical analyses, biological effect monitoring is required for a more reliable assessment of the pollution status of aquatic ecosystems (e.g., Van der Oost et al., 2003). One of the main challenges for environmental risk assessment (ERA) is the application of adverse outcome pathways (AOP), i.e. the linking of pollutant exposure *via* early molecular and biochemical changes to physiological effects and, ultimately, effects on populations and ecosystems (Ankley et al., 2010). Until now, the emphasis on AOP development has mainly been in relation to identifying pathways responsible for toxicity in individual organisms and for individual chemicals. Apart from a limited number of papers, such as the whole-lake exposure study with ethinylestradiol by Kidd et al. (2007) and the impact of pulp mill effluents by Miller et al. (2015), the AOP concept has not been widely applied to field-based datasets, particularly in relation to the environmental impact of complex mixtures. The effects of “real-life” chemical mixtures are a challenge for risk assessors. In this context, AOP networks could prove to be a vital tool to explore mixture effects in order to focus attention on chemicals and AOPs that really matter and thus to prioritise remediation efforts (Villeneuve et al., 2014).

In environmental toxicology, the term ‘biomarker’ is generally used broadly to include almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological (WHO, 1993). In the context of ecotoxicology, a biomarker can be defined as any biotic feature of an organism, ranging from the molecular level through cellular and physiological processes to behavioural features, that is responsive to an exposure or toxic effect of xenobiotic chemicals (Peakall, 1994). Specific biochemical responses are generally the most sensitive biomarkers to be used as an early warning for deviations from the normal status, although their ecotoxicological significance is often unclear (Adams et al., 1989). By use of biomarkers that can monitor the activation of specific cellular signalling pathways, potential risks of many compounds can be screened simultaneously. Physiological adaptation to an altered environment is considered to influence the success with which an animal performs in a particular ecological niche (Prosser, 1950). Physiological responses, therefore, generally have a higher ecotoxicological relevance. Traits of physiological adaptation provide valid information about the

functional integrity of fish exposed to environmental contaminants, especially those traits that are a product of processes at many levels of organismal organisation (Schreck, 1990).

Numerous biochemical and molecular 'biomarkers' have proven to be specific sub-lethal indicators of the exposure of fish to particular classes of pollutants in the environment (reviewed by Van der Oost et al., 2003). Indeed, studies in both the laboratory and the field have demonstrated that biochemical markers can be used to accurately assess organismal exposure to specific groups of environmental pollutants as an early warning for irreversible adverse effects. An evaluation according to six fish biomarker criteria revealed that hepatic phase I biotransformation enzymes and biliary biotransformation products are considered the most valuable molecular biomarkers for ERA procedures (Van der Oost et al., 2003). Although not as sensitive, specific and diagnostic as phase I enzymes, phase II biotransformation enzymes and antioxidant enzymes can be considered as additional biomarkers for exposure to and effects of various classes of micropollutants (Van der Oost et al., 2003). In addition to biochemical changes, the levels of biliary xenobiotics metabolites are extremely sensitive biomarkers to assess recent exposure to easily biodegradable substances, such as polycyclic aromatic hydrocarbons (PAHs) (Collier and Varanasi, 1991). However, the exact significance of all molecular and biochemical responses for the functional integrity of the organism has still to be established. It is interesting, therefore, to investigate whether the expression of biomarkers can be linked to physiological evidence of functional impairments as well as the impact on fish populations, to improve the predictive value of biomarker analyses.

Metabolic rate, the energetic cost of occupying a given habitat, is a unifying currency of physiological adaptation to the environment (Wikelski and Ricklefs, 2001), with ecological ramifications that can include impacts on growth, behaviour, and life-history strategy (Metcalfe and Monaghan, 2001, Chabot et al., 2016a). In fish, standard metabolic rate (SMR), the minimum energetic cost for organismal maintenance at the prevailing temperature (Chabot et al., 2016b), increases following exposure to sub-lethal concentrations of pollutants such as heavy metals, chlorobenzenes and ammonia (e.g. Wilson et al., 1994; Yang and Randall, 1997; Shingles et al., 2001). Swimming (exercise) performance is a particularly valid measure of functional integrity because it requires the integrated activity of systems at various levels of organisation (Randall,

1982; Moyes and West, 1995), and it is widely accepted that swimming ability should influence the ability of a fish to thrive in its environment (Brett, 1958; Fry, 1971). Performance traits such as maximum sustainable aerobic swimming speed (U_{crit}, Brett, 1964) have been used widely to reveal the sub-lethal impact of stressors, and U_{crit} has proven to be sensitive to pollutants such as low pH, heavy metals and ammonia (e.g. Butler et al., 1992; Wilson et al., 1994; Beaumont et al., 1995; Shingles et al., 2001). Most research on the effects of pollutants on metabolic rate and exercise performance is laboratory-based, and mainly comprises the exposure of salmonid species to single toxicants (McKenzie et al., 2007). In nature, fish can be exposed to complex mixtures of pollutants but the potential effects of this on their metabolic rate and performance remain to be explored. The use of classical techniques of swimming respirometry (Brett, 1964) permits simultaneous analysis of metabolic rate (as oxygen consumption) and exercise performance (Brett, 1964; Fry, 1971; Randall and Brauner, 1991).

A cost-effective alternative for *in vivo* biomarkers is the application of *in vitro* bioassays. The two-tiered Smart Integrated Monitoring (SIMONI) strategy (Van der Oost et al., 2017a), for instance, assesses the environmental risks due to organic micropollutants. The first tier of the strategy is hazard identification of organic micropollutants, based upon the combination of field-exposed passive samplers (PS), one in situ bioassay and fourteen laboratory bioassays (apical endpoints and specific modes of action) exposed to the PS extracts. The first tier SIMONI is being applied in the Netherlands to identify the 'hot spots' of chemical water pollution by evaluating all bioassay responses, using effect-based trigger values (EBT) as criteria for potential ecological risks. A model has been designed to calculate an overall SIMONI Risk Indication (SRI) to quantify potential ecological risks. The second tier is a customized risk assessment, based upon the results of tier 1 and additional information on various aspects of the water system (influences of other ecological key factors). Only a limited number of sites, where bio-effects indicate ecological risks, should be examined by this more expensive tier 2 for the actual risk assessment. In this way, more advanced and expensive chemical analyses, effect-directed analysis (EDA) and bioanalytical methods (e.g., *in vivo* fish biomarkers) are only carried out at sites where they are most relevant. The SIMONI model is comparable to the more comprehensive strategy described by Schroeder et al. (2016),

which uses qualitative and semi-quantitative predictions of bio-effects based upon chemical analyses, as well as a high-throughput surveillance of more than 80 bioanalytical endpoints. Both strategies use the AOP knowledge base to identify the most relevant AOPs at a site in order to predict hazards and select the most relevant endpoints for future monitoring.

The ecological relevance of chemical environmental quality standards (EQS), responses of *in vitro* bioassays, and molecular or physiological biomarkers in fish can be established by comparing these responses with higher tier consequences in exposed fish populations. The EU City Fish project aimed to contribute in developing a generic methodology for ecological risk assessment and the development of water quality criteria for urban river basin management plans. Traditional risk assessment methods involving animal testing at high doses of individual chemicals and extrapolation to environmentally relevant levels are vastly overwhelmed in capacity by the innumerable chemicals and transformation products potentially present in the water cycle (Leusch and Snyder, 2015). Therefore, the City Fish project tried to develop environmental quality criteria with reference to fish, the primary health indicators of river ecosystems, and to determine sub-lethal factors that limit the restoration of sustainable fish populations in polluted urban rivers. The project introduced the concept that sensitive criteria, needed to monitor the recovery of urban river systems, can best be developed by measuring pollutant effects on endemic species of coarse fish at several biological levels enabling linkage between environmental, molecular, physiological and ecological responses. Several potential adverse outcome pathway events were followed, from external and internal exposure to different classes of micropollutants, via molecular key events (MKE) and their apparent impact on organs and organisms (fish physiology), to changes in the dynamics of fish populations. The present study was focussed on evaluating different risk assessment procedures at five selected sites in and around the city of Amsterdam, The Netherlands, by establishing adverse outcome relationships between biochemical and physiological responses of caged mirror carp *(Cyprinus carpio)* and feral bream *(Abramis bramis)* and the impact on fish populations. Relatively unpolluted sites were compared to sites, polluted with either a domestic waste mixture (containing effluent of a sewage treatment plant and polycyclic aromatic hydrocarbons [PAHs]) or a complex industrial waste mixture (containing

polychlorinated biphenyls [PCBs], polychlorinated dibenzodioxins [PCDDs], chlorobenzenes and organochlorine pesticides). In addition, the interpretation of the data was compared to the results of a tier 1 bioanalytical SIMONI strategy (Van der Oost et al., 2017a).

2 Materials and methods

2.1 Experimental

The experiments described in the present paper were carried out at five freshwater sites near the city of Amsterdam (Figure 1).

Figure 1

Caging experiments were carried out with genetically identical male carp (*Cyprinus carpio*) of a cultured F1 hybrid fish line, which was developed at Wageningen University and Research. Eight carp were exposed for 4 weeks in 0.6 m^3 wired cages at selected field sites, together with semipermeable membrane devices (SPMDs). The fish swimming experiments were performed on site, after 3 weeks of exposure. After measuring these traits the fish were allowed to recover for a week in the cages, prior to transport to the laboratory where muscle tissues, liver, bile and blood were isolated for the biomarker studies. Feral bream (*Abramis brama*) were caught at the three Amstel river sites (A1-3) for bioaccumulation and biomarker assessments. Fishing methods are described in section 2.8 (Ecological quality assessment).

During the caging experiment, sediment and water samples were taken at each site. A minimum of 10 grabs per sample of top-layer sediment (total weight approximately 3-5 kg) were collected using an Eckmann grab sampler. Homogenized sediment samples were stored at 4°C in clean glass jars until analysis. Water samples were stored at 4°C in brown glass bottles and analysed for standard water quality parameters.

The three Amstel river sites (A1-3), which were the main focus of the EU City Fish project in The Netherlands, were studied most extensively. Feral bream only were collected at these three sites. Since all carp caged at the A2 site in 2002 died during exposure, probably due to reduced oxygen

levels through sewage water overflows, no swimming and metabolism studies could be performed at this site. Therefore, fish swimming and metabolism studies were performed in fish at the A1, A3 and VM sites. Biomarker levels in caged carp, however, were measured at all five sites across three sampling programs from 2000-2002.

2.3 Chemical analyses in sediment, fish and SPMD

Fish muscle tissue and SPMDs were stored at -20°C until extraction. Biofouling was removed from SPMDs directly after sampling by wiping the SPMDs with paper tissues that were wetted with water from the sites. Methods for the chemical analyses of organic micro pollutants are described in detail by Verweij et al. (2004). Briefly, fish muscle tissue was dissected, homogenized, dried and extracted with hexane/acetone (1:1) in a Tecator® Soxtec apparatus. Lipid weight was determined gravimetrically. The extract was washed with purified water, dried with sodium sulphate and eluted with hexane through a 15 g alumina (6% H_20) column to remove lipids. Each extract was separated on silica gel SPE columns (Baker) in two fractions with different polarity. Sediment was shaken with 10 ml of acetone and, after equilibration, hexane was added, and the mixture was shaken again. The extracts were washed with saturated NaCl to remove the acetone. Concentrated extracts were eluted with hexane through a 1 g alumina SPE column (Baker) and separated by silica gel SPE. For PAH analyses, sediment was mixed with acetone and subsequently with 10 mL of hexane. Acetone-hexane extracts were centrifuged for 3 minutes at 500 rpm, and, after addition of sodium sulphate, shaken and centrifuged again. After addition of 3.5 mL of a 2% ethanediol solution in methanol, the dried extract was concentrated and stored at 4 °C until HPLC analysis. The organic matter of the sediments was determined by measuring the weight loss-on-ignition (LOI) between 500 and 900°C. SPMDs (30 cm long, 2.54 cm wide, containing 0.27 g triolein, 95%, Sigma) were prepared according to Booij et al. (1998). The triolein filling was spiked with performance reference compounds (PRCs), PCB 4, PCB 29, PCB 155 and PCB 204. SPMDs were extracted twice with 100 mL hexane for 24h. The extracts were combined, concentrated, and divided in two equal parts, for PAH and PCB/OCP analyses. The PAH part was

concentrated after addition of 4 ml of ethanol. The fraction for PCB/OCP analyses was eluted through alumina and separated in two fractions using silica gel SPE columns, as described for fish. All samples were analysed under quality assurance (QA) protocols, as described by Verweij et al. (2004). PCBs and OCPs were analysed with a gas chromatograph (GC) with electron capture detection (ECD). Quantified chemicals were divided into groups of related compounds (Sum PCB: PCBs 28, 52, 101, 118, 138, 153 and 180 ; Sum drins: dieldrin, endrin, aldrin, telodrin, isodrin; Sum DDTs: p,p-DDT, o,p-DDT, p,p-DDE, o,p-DDE, p,p-DDD, o,p-DDD; Sum HCHs: α-HCH, β-HCH, γ-HCH; Sum Chlorobenzenes: pentachlorobenzene, hexachlorobenzene). PAHs were analysed with high performance liquid chromatography (HPLC) with UV detection. Sum PAHs: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene. SPMD-based water concentrations were calculated according to Verweij et al. (2004), using the dissipation rate constants of PRCs and the absorbed amounts of the individual compounds.

2.2 Physico-chemical water and sediment conditions

Physico-chemical conditions, such as water temperature, dissolved oxygen levels, or extremes of pH, can have a profound influence on metabolic rate and exercise performance in fish (Brett 1964; Ye and Randall, 1991), so these parameters were monitored with a multiparametric probe (Hydrolab) immersed in the surface water throughout the assessment periods at each site. Standard water quality parameters were determined at the sites where swimming experiments were performed: ammonium, chloride, nitrate, nitrite, total nitrogen, total phosphate, particulate matter, sulphate and biological oxygen demand (BOD).

2.4 Biomarker analyses

A suite of molecular markers was analysed in caged carp (average of summer sampling programs in 2000, 2001 and 2002) and feral bream (average of autumn 2000, spring 2001 and autumn 2001

sampling programs). Most biomarker procedures are previously described by Van der Oost et al., 1996a.

Sample preparation for fish biomarkers. Fish were anaesthetized with a solution of tricaine methyl sulphate (0.2 mg/l) and sodium bicarbonate (0.4 mg/l). Blood was collected from the caudal vein with a heparinized needle and syringe. The blood was centrifuged for 5 minutes at 10,000 rpm and the supernatant (plasma) was stored at –80°C. Directly after killing the fish, the liver and the gall-bladder were dissected. Bile fluid was collected from the gall-bladder and directly stored in Eppendorf tubes at –80 °C. Liver homogenates were centrifuged for 25 minutes at 12,000 g in a 50 mM phosphate buffer (pH 7.4) containing 155 mM NaCl. Supernatants were then centrifuged for 60 minutes at 100,000 g. Resulting 100,000 g supernatants (cytosol) were stored at -80°C until analysis. Microsomal pellets were washed in the same buffer and centrifuged again for 60 minutes at 100,000 g. The pellets were suspended in a 100 mM phosphate buffer (pH 7.4) containing 0.1 % EDTA and 25 % glycerol and stored at –80°C until analysis. Hepatic biomarker results were expressed per mg protein. Protein levels were determined with a Pierce BCA protein kit, according to procedure TPRO-562 (Sigma-Aldrich) with bovine serum albumin provided as a standard. Interaction between BCA (Bicinchoninic Acid) and proteins gives a purple product with a specific absorption at 562 nm.

Ethoxyresorufin-O-deethylase (EROD). EROD activity was measured with a microplate reader, using a modified version of the original method of Burke and Mayer (1974). Each well of a 96-well microplate contained 25 µl of microsomal suspension (30-100 µg protein), and 2 µM of 7-ethoxyresorufin in a total volume of 310 µl phosphate buffer, pH 8.0. The reaction was started by adding 30 µl of a 2.9 mM NADPH solution (final concentration 0.26 mM NADPH). The assay was carried out at 30°C, using an external resorufin standard to quantify the slope of resorufin (pmole/min) formed during the assay. Detection limit: 0.05 pmole/min/mg protein.

Cytochrome P4501A (CYP1A). CYP1A microsomal protein levels from liver were determined using a semi-quantitative ELISA (enzyme-linked immunosorbent assay), as developed by Goksøyr (1991). 96-Well plates with samples and standards were incubated for one hour at 37°C (primary antibody) and room temperature (secondary antibody). Rabbit anti-cod P450 IgG (Biosense) was used as a primary antibody, and goat anti-mouse IgG Horseradish Peroxidase Conjugate (Biorad) was used as a secondary antibody. Development for 15 minutes with 0.04 % o-phenylenediamine dihydrochloride (Sigma) containing 0.012 % H_2O_2. (stopped with 4N H_2SO_4). Detection limit: 0.1 OD (492 nm) units/mg protein.

Uridinediphosphate glucuronyltransferase (UDPGT). UDPGT activity in liver microsomes was measured using a modified version of the incubation method of Förlin et al. (1986), with *p*-nitrophenol (pNP) as a substrate. Incubation mixtures contained 0.5-1 mg microsomal protein (pretreated for 5 minutes with 0.25% Triton X-100 on ice), 100 mM Tris/HCl buffer (pH 7.0), 5 mM $MgCl_2$, 1 mM p-nitrophenol and 10 mM UDPGA in a total volume of 240 µl. The reaction was initiated at 26°C by adding the UDPGA and terminated after 20 minutes by adding 1 ml of cold 3% trichloroacetic acid (TCA). After centrifugation, 1 ml of the supernatant was treated with 5 M KOH (50 µl) and the amount of p-nitrophenol remaining was measured spectroscopically at 405 nm after dilution. Detection limit: 1 nmole/min/mg protein.

Glutathione-S-transferase (GST). GST activity in cytosol was determined by monitoring the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) spectrophotometrically at 340nm according to the method of Habig et al. (1974) with slight modifications as described by Wilbrink et al. (1991) and converted to 96-well microplate format. Detection limit: 0.1 pmole/min/mg protein.

Superoxide dismutase (SOD). SOD in liver cytosol was determined according to the method of Sazuka (1989). In this assay, superoxide anion radicals are formed using xanthine-xanthine oxidase. The superoxide anion radicals reduce nitroblue tetrazolium (NBT), which can be measured spectrophotometrically at 560 nm. SOD activity was quantified by measuring the

decrease in NBT reduction caused by the addition of the sample. One unit of activity (U) is defined as 50% inhibition of the reaction. Detection limit: 10 U/mg protein.

Aspartate Transaminase (AST). AST in blood plasma was determined using INFINITY™ AST reagent (Sigma Diagnostics). Each well contained 20 µl of non-haemolysed plasma and 200 µl of AST reagent. Measurements were carried out in 96 wells microplates at 30°C, using a TECAN spectrafluor plus microplate reader. In order to determine the AST activity, the decrease in NADH concentration was followed spectrophotometrically for five minutes at 340nm. One unit of activity (U) was defined as one µmole of substrate transformed per minute. Detection limit: 3 U/L plasma.

2.5 Biliary PAH metabolites

Biliary (1-hydroxypyrene-like) PAH metabolites were analyzed using a semi-quantitative fixed wavelength fluorescence method, as described by Aas et al. (2000), using excitation/emission wavelengths of 340 and 380 nm (pyrene-like PAH metabolites) or 380 nm and 410 nm (BaP-like PAH metabolites). For fluorescence measurement bile samples were diluted 1:2000 in 48% methanol. In order to compensate for differences in bile density due to feeding status, all fluorescence values were corrected for the optical density specific for the biliary pigment biliverdin. Biliverdin concentrations were estimated by the absorbance at 380 nm of 100-fold diluted bile (Aas et al., 2000).

2.6 Gross Indices

Morphological parameters that are often determined in field research are the liver somatic index (LSI), to identify possible liver injury, and the condition factor (CF), to assess the general condition of fish. These gross indices were calculated for all investigated fish. The condition factor was determined as (body weight [g] * 100) / (length [cm])^3 (Bagenal and Tesch, 1978). Directly after killing the fish, livers were removed and weighed. The liver somatic index (LSI) was determined as (liver weight [g] * 100) / whole body weight [g]) (Slooff et al., 1983).

2.7 Swimming experiments and metabolic rate

The metabolic rate and exercise performance of the carp were measured at three sites in the prevailing surface water, using portable swimming respirometers designed to exercise individual fish in a non-turbulent water flow with a uniform velocity profile (Steffensen et al. 1984). All methods are described in detail by McKenzie et al., 2007. Briefly, carp were transferred to the respirometer and permitted 4h recovery from the handling stress while swimming at low speed of 20 cm s^{-1}, then exposed to progressive increments in swimming speed of 10 cm s^{-1} every 20 min until fatigue. Fatigue was unambiguous in the carp, which swam vigorously until they collapsed against the back screen. The carp were then allowed 40 min recovery from the first swim test (T1) after which they were exposed to exactly the same protocol a second time (T2). Maximum sustainable aerobic swimming speed (U_{crit}) was calculated in body lengths/s. The repeat performance ratio was calculated as T2/T1.

Instantaneous O_2 uptake (M_{O2}) was measured by intermittent stopped flow respirometry, over a 10 min cycle that alternated a six min closed recirculation 'measurement' period with a four min period where the respirometer chamber was flushed with aerated water (Steffensen 1989). During the measurement period, O_2 partial pressure (pO_2) declines over time due to O_2 consumption by the fish, which was recorded by an O_2 electrode. The flush period then replaced the O_2 consumed. For both T1 and T2, M_{O2} at each swimming speed was calculated as the average of two respirometry cycles. Extrapolation back to the y-intercept, a notional swimming speed of zero, provided a valid estimate of the standard metabolic rate (SMR) (Chabot et al., 2016b). For both T1 and T2, active (maximum) metabolic rate (AMR) was estimated as the M_{O2} measured at highest swimming speed immediately prior to fatigue (Norin & Clark, 2016). For T1, net aerobic scope was estimated as AMR minus SMR (Brett, 1964).

2.8 Ecological quality assessment

The ecological status at the three Amstel river sites (A1-3) was assessed by determination of macro-invertebrates and fish populations. Macro-invertebrates and fish were investigated in four sampling programs (spring and autumn 2000 and 2001). Macro-invertebrates were collected by

different sampling methods for different matrices, such as deep bottom sediment by Eckman grab samplers, near-shore sediments, wooden shore sheeting and water plants by macro-invertebrate landing nets, stones and wood by scraping off the animals. All habitats (water, sediment, stones, wooden sheeting, vegetation) were sampled proportionally at each site. Because no fish population studies are allowed in the Netherlands from April to November, all sampling programs were carried out in the late autumn and early spring. In cooperation with the Amsterdam Angling Association (AHV), seine net (40 mm mesh) and electro-aggregate fishing were applied to collect fish at the open water and less accessible spots close to the waterfront, respectively. Length and weight of the fish was determined in the field. Apart from some selected bream with comparable length that were collected for biomarker and bioaccumulation studies, all fish were returned to the water at the sites where they were caught. Macro-invertebrate and fish data were used to determine biodiversity (Shannon & Weaver, 1949) indices. Procedures for the classification of the ecological status of macroinvertebrates and fish (2006-2012), according to the Water Framework Directive (WFD), are described in the updated “Guideline WFD monitoring of surface water and protocol for assessment and classification” of the Dutch water authorities (Ohm et al., 2014, in Dutch).

2.9 SIMONI bioanalytical risk assessment

The recently developed Smart Integrated Monitoring (SIMONI) strategy is a cost-effective way to analyse the risks of organic micropollutants to aquatic ecosystems. This strategy is described in detail by Van der Oost et al. (2017a,b). In short, water concentrates were obtained with passive samplers (PS) for polar and non-polar compounds: Polar Organic Chemical Integrative Samplers (POCIS) and silicon rubbers, respectively. An *in-situ Daphnia magna* bioassay (survival after one week of surface water exposure) was performed during PS deployment, while the extracts of passive samplers were tested in a battery of 14 bioassays (Table S1, supplementary information). Bioassay results were compared to effect-based trigger values (EBT) that indicate potential ecological risks. The overall environmental risk of micropollutants was assessed by combining relative bioassay responses (i.e., response divided by EBT) of all bioassays by calculating the

SIMONI Risk Indication (SRI), also known as SIMONI-score (Van der Oost at al., 2017a). SIMONI sampling programs were performed from 2012 to 2016, at the three Amstel River sites (A1-3) only.

2.10 Statistical analyses

Statistical tests were carried out with SPSS 10.0 for windows (Statistical Product Service Solutions, Chicago, Il, USA). Biomarker data were analysed for statistical significance using the non-parametric Mann-Whitney rank sum test. Significant differences were determined at a 0.05 level of probability, using a 2-tailed test. The difference between swimming performance of the 3 exposed groups was tested with one-way analysis of variance (ANOVA). Prior to ANOVA analysis, homogeneity of variance was tested with Levene test. If ANOVA showed difference between groups, the Bonferroni multiple comparison was used to determine the difference between means. Paired T-tests were performed to compare differences within the same group between swim tests 1 and 2. Again, significant differences were determined at a 0.05 level of probability (p).

3 Results & discussion

3.1 External exposure: quality of water and sediments

The results of the standard water quality parameters and EQS are shown in Table 1. European MAC-EQS (WFD) are applied when available, otherwise Dutch quality standards are used. These data show that some EQS exceedances were observed at all sites, except for the GP reference. The Amstel river sites were most eutrophic, as indicated by the elevated phosphate and nitrate levels. Elevated ammonium and chloride levels were observed at the heavily polluted VM site. Water quality at the GP site was therefore classified as good, and for all other sites as moderate. Metal levels were generally not high at the investigated sites, with only slight EQS exceedances for mercury (A2) and lead (VM). Quality of these two sites is classified as moderate, while other sites showed good quality based on metal levels.

Table 1

The amounts of organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) that accumulated in the SPMDs were used to calculate water concentrations. Summed concentrations and EQS comparisons are listed in Table 2, while individual substances are presented in the supplemental information (Table S2). At the most polluted VM and A2 sites, high exceedances were observed for the ESQ criteria for DDDs and PAHs (fluoranthene), respectively, and their quality was therefore classified as poor.

Table 2

Sediment chemistry (Table S3, supplementary information), which in the present study was only determined at the three Amstel river sites, generally reflected the elevated water pollutant levels of PAH and mercury at the A2 site. Sediment PAH levels were extremely high at the A2 site, which is most likely a result of the fire at the Cindu chemical plant (Uithoorn) in 1992. In addition, elevated levels of copper, nickel and mineral oil were found in the sediments at this site. Elevated levels of mercury, copper and PAHs were found in sediments of the A3 site. Significant levels of OCPs were detected at all sites, but levels were below Dutch threshold values. According to the classification system (RIVM, 2008) the sediments were classified as heavily polluted (A2), moderately polluted (A3) and slightly polluted (A1). Sediment quality of the GP reference site (slightly polluted) and the VM site (heavily polluted) were determined in a previous study (Van der Oost et al., 1996b)

3.2 Internal exposure: fish bioaccumulation & metabolites

The internal exposure of micropollutants was measured by analysing levels of persistent compounds (PCBs and OCPs) in fish muscle, and levels of metabolites from biodegradable compounds (PAHs) in fish bile. Average lipid-based tissue levels of three sampling programs in caged carp (five sites) and feral bream (three Amstel river sites) are presented in Figure 2. PCB and OCP muscle tissue levels in feral bream from the Amstel river were much higher than those found in carp that was caged for four weeks at the same sites. Clearly elevated OCP tissue levels were found in caged carp at the heavily polluted Volgermeerpolder site (VM). The high average OCP concentrations were mainly due to increased chlorobenzene levels, followed by DDTs and

HCHs (Table S4, supplemental information). Based on these data the VM site was classified as poor quality (more than 5 times the reference), Amstel river sites and the GP site as good quality (no significant difference with the reference site).

Figure 2

Levels of biliary metabolites are considered sensitive biomarkers for recent exposure to polycyclic aromatic hydrocarbons (Collier and Varanasi, 1991). Generally, 1-hydroxypyrene (OH pyrene) accounts for a large percentage of all PAH metabolites in the bile of PAH-exposed fish (Krahn, 1987), and may be used as an indicator for total PAH exposure. It was demonstrated that the biliary PAH metabolite concentrations as well as the bile volumes were highly influenced by the feeding status of the fish (Collier and Varanasi, 1991; Brumley et al., 1998). In order to reduce these variations in PAH bile metabolite levels due to feeding status, Collier and Varanasi proposed a procedure in which metabolite concentrations are related to biliary pigment contents. Very high levels of both pyrene-like and benzo[a]pyrene (BaP)-like PAH metabolites in carp bile were found at the A2 site of the Amstel river (Figure 3). Pyrene-like metabolites were significantly increased at four sites compared to both carp from the culture (CC) and carp at the clean Lake Gaasperplas (GP) site, while BaP-like metabolites were only significantly higher at the A2, A3 and VM sites. BaP-like metabolite levels at the GP and A1 sites did not differ significantly, but were both higher than those in cultured carp. Similar patterns (A2>A3>A1) were observed in feral bream caught at the Amstel river (Table S5, supplemental information).

All patterns of PCB and OCP bioaccumulation and PAH metabolites in fish bile correlated with the PCB, OCP and PAH concentrations in SPMDs that were exposed simultaneously at the same sites (Figure S1: supplemental information). Although no guidelines are known for internal exposure, the results of the present study indicate that organic micropollutant levels at the A2 (PAHs) and VM (OCPs) sites posed the highest risks for fish populations. Based upon the internal exposure the quality of these two sites was classified as poor (significant difference and more than 5 times higher than reference site), quality of the A1 and A3 sites as moderate (significant difference with reference site), and GP quality as good (not significantly different from reference site).

Figure 3

3.3 Molecular key events: fish biomarkers

Several biochemical reactions in fish can be used as potential indicators of pollutant exposure and/or effects (Van der Oost et al., 2003). Phase I biotransformation, catalysed by the cytochrome P450 system, transforms lipophilic xenobiotics to more water-soluble compounds by oxidation, reduction or hydrolysis. Most oxidative phase I biotransformations in fish are catalysed by the cytochrome P450-dependent monooxygenases (MOs), predominantly located in the endoplasmic reticulum of the liver (Stegeman et al., 1993). A pronounced Ah-receptor-mediated induction of the cytochrome P4501A subfamily (CYP1A) has been observed in fish after exposure to a variety of non-polar xenobiotics, including PAHs and polyhalogenated aromatic hydrocarbons such as PCBs, polychlorinated dibenzodioxins and dibenzofurans (Stegeman and Hahn, 1994; Bucheli and Fent, 1995). It has been demonstrated that ethoxyresorufin-O-deethylase (EROD), the most sensitive indicator of CYP1A catalytic activity (Goksøyr and Förlin, 1992), as well as the CYP1A protein levels themselves are highly sensitive indicators of contaminant exposure in fish (Whyte et al., 2000; Van der Oost et al., 1996a). Both CYP1A and EROD assays are well established *in vivo* fish biomarkers of exposure to such xenobiotic contaminants.

EROD activities were significantly elevated in the livers of carp exposed at the A2, A3 and VM sites as compared to those found in the hatch control (CC, cultured carp) and the unpolluted GP site (Figure 4A). Extremely high EROD activities were found in the livers of the carp from the VM site, which were in line with previous results in eel (Van der Oost et al., 1996a) and reflect the highly polluted status of this site. A similar pattern was observed for the CYP1A protein levels (Figure 4B): the highest levels were found in carp exposed at the VM site, followed by A2 and A3. The CYP1A difference between VM and the Amstel river sites, however, is less pronounced than was seen for EROD activity. Both EROD and CYP1A in carp that were caged at the GP and A1 sites were close to those in unexposed carp from the culture (CC). Similar trends were found for EROD activities and CYP1A levels in feral bream (Table S6, supplemental info). A provisional quality

classification based upon phase I biomarkers is: VM poor (more than five times higher than reference site), A1-3 moderate (significant difference with reference site) and GP good.

Figure 4

In addition to the phase I CYP1A genes, the Ah gene battery also comprises phase II genes like NADPH menadione oxidoreductase, aldehyde dehydrogenase, UDPGT and GST (Celander,1993). The mechanism of induction for several phase II enzymes is, therefore, regulated via this receptor as well. Phase II metabolism involves a conjugation of the xenobiotic parent compound or its metabolites with an endogenous ligand (Van der Oost et al., 2003). Although the phase II system is less responsive to pollutants than phase I, several studies observed an induction of GST or UDPGT in fish from polluted sites (e.g., Gadagbui and Goksøyr, 1996). The less pronounced induction of phase II enzymes may be due to confounding natural variability factors such as sex, maturity, nutrition, season and temperature. Still, impairment of the phase II system may be harmful to an organism and induction shows that the organism is responding to a threat. The synthesis of glucuronides by microsomal UDP-glucuronyl transferases (UDPGTs) is a major pathway for the inactivation and subsequent excretion of both endogenous and xenobiotic organic compounds (Mulder et al., 1990; George, 1994). UDPGT activity appears to be the phase II parameter which is most responsive to pollutant exposure (Van der Oost et al., 2003). In caged carp, during the present study, UDPGT activities were not significantly affected after four weeks of exposure at the five freshwater sites (figure 4C). In feral bream, however, UDPGT was significantly increased at the most polluted A2 site, as compared to the other two Amstel river sites (Table S6, supplemental info).

The conjugation of electrophilic compounds with glutathione (GSH) is catalysed by the glutathione S-transferases (GSTs) that are mainly located in the cytosolic fraction of the liver. Apart from essential functions in intracellular transport and biosynthesis (e.g. prostaglandins), a critical role for the phase II system includes defence against oxidative damage of DNA and peroxidative products of lipids (George, 1994). The susceptibility of different fish species to chemical carcinogenesis may be modulated by the activity of GST (Varanasi et al., 1987). Here, cytosolic GST activity was

significantly induced in carp caged at the A2, A3 and VM sites, compared to both culture (CC) and GP reference site (Figure 4D). No significant GST differences were observed between feral bream from the three Amstel river sites (Table S6, supplemental info). These results clearly demonstrate species differences in sensitivity of phase II biomarker responses. If phase I bioactivation is highly increased while phase II detoxification is only slightly elevated, an increased biotransformation index (BTI) might increase the risk of genotoxic effects or cell damage (Varanasi et al., 1987; Van der Oost et al., 1998). This was supported by observations in a previous study of increased liver DNA adducts in eel from the VM site (Van der Oost et al., 1996a). A provisional quality classification based upon phase II biomarkers: VM, A2 and A3 moderate (significant difference with reference site), A1 and GP good.

Many micropollutants, such as aromatic diols, quinones or hydroxylamines, nitroaromatics, and bipyridyls are able to cause oxidative stress in fish (Winston and Di Giulio, 1991). Oxidative stress may cause oxidations of proteins, lipids and nucleic acids, as well as perturbed tissue redox status (Winston and Di Giulio, 1991). Defence systems that tend to inhibit oxyradical formation include the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPOX) and glutathione reductase (GRED). In many field studies a significant increase of hepatic SOD activity has been observed in various fish species (e.g., carp) at polluted sites (reviewed by Van der Oost et al, 2003). A significant induction of SOD activity, compared to both cultured carp (CC) and GP reference, was observed in carp caged at the VM site (Figure 4E). SOD in carp at the Amstel river sites was only induced in relation to the CC carp from the culture. SOD activity in feral bream from the A2 site was elevated as compared to the other two Amstel river sites, but this difference was not statistically significant (Table S6, supplemental information). No significant effects on GRED were found in either caged carp or feral bream (Table S6, supplemental information). A provisional quality classification based upon oxidative stress biomarkers is: VM moderate (significant difference with reference site), A1-3 and GP good.

3.4 Impact on individuals: gross indices and fish physiology

No significant differences were observed between the liver somatic indices (LSI) of caged carp and feral bream from different sites (Figure S2, supplemental info). Since no gross indications of liver injury could be demonstrated in the investigated fish, all sites were classified as good (no significant difference with reference site).

Typically, haematological parameters are non-specific in their responses towards chemical stressors, but may provide important information in effect assessment studies, for example by providing an indication as to the general physiology and health status of an organism including organ specificity of damage (Beyer, 1996). Increases of blood serum transaminases, such as alanine transaminase (ALT) or aspartate transaminase (AST), are sensitive indicators of cellular damage since the levels of these enzymes within the healthy cell exceed those in the extracellular fluids by more than three orders of magnitude (Moss et al., 1986). A significant AST decrease, however, was observed in carp caged at four sites, compared to the cultured carp (CC) and carp from unpolluted GP site (Figure 4F). As can be seen in the graph, inter-individual variances were very high for carp from CC and GP. The plasma AST activities of feral bream from the A2 Amstel river site were significantly higher than those at A3, but did not differ significantly from activities at the least polluted A1 site (Table S6, supplemental information). These results indicate that no clear evidence of tissue damage was demonstrated in the investigated fish of the present study. The lack of histological investigations, however, is a serious limitation in drawing firm conclusions on this outcome. Based upon the limited data on organ impact the quality of all sites can be classified as good (no significant elevation compared to the reference site).

No visible adverse health effects were observed in carp after four weeks of exposure in cages, or in feral bream, and the condition factors (CF) of both fish species were not significantly different between sites (Figure S2, supplemental info). Based upon these parameters, the quality of all sites was thus classified as good (no significant difference with the reference site).

Fish physiology was tested by measuring swimming speed and metabolic rate of carp caged at the A1, A3 and VM sites. Water temperature at all three sites was between 18°C and 19°C, and pH at all sites fell within the circum-neutral range (Table 1). Dissolved oxygen on the assessment days was, however, significantly different between sites, being extremely hyperoxic at the A1 site (150%

saturation) and moderately hypoxic at the A3 (50%) and VM (60%) sites. Figure 5 shows the relationship between swimming speed and oxygen uptake for the carp maintained at each site, for the repeated swim tests (T1 and T2). There is a clear exponential relationship with low variability between the animals, presumably because they are clones. Estimation of the SMR for T1 revealed that it was significantly higher at the VM site than at the A1 or A3 sites (Table 3). Since oxygen consumption, or metabolic rate, of a fish at rest indicates the amount of energy that fish requires to simply stay alive, the energetic costs of organismal maintenance were higher at the most polluted site. A metabolic load that may influence the ability to allocate oxygen for other metabolic activities, or ingested energy towards growth (Chabot et al., 2016a). Metabolic rate was also higher at the VM site when the fish were swimming at a constant aerobic speed of 20 cm s^{-1} prior to each swim test (Table 3), confirming that fish at the polluted site incurred a metabolic load. This was not linked to a significant difference in water temperature, and was not the result of hypoxia, as the A3 site was also hypoxic. This is instead consistent with exposure to the complex mixture of organic pollutants as well as biomarker responses of fish at the VM site. Based upon metabolic rate the VM quality was classified as moderate (significant difference with the reference), while A1 and A3 were good.

Figure 5

Table 3

The increased SMR of the carp at the VM site was not, however, associated with reduced aerobic scope or U_{crit} for T1 (Table 3). In this respect, the carp is different from the rainbow trout for which a toxicant-induced increase in SMR can act as a metabolic load that compromises the allocation of oxygen to exercising muscle and contributes to a decline in aerobic scope and U_{crit} (Wilson et al. 1994; Shingles et al. 2001). Indeed, it is interesting that the carp at the hypoxic A3 and VM sites had the same aerobic scope and were able to exercise to the same U_{crit} as the carp at the hyperoxic A1 site. Hypoxia typically causes a decline in U_{crit} in salmonids, by limiting aerobic scope (Jones 1978; Bushnell et al. 1984). The carp, however, is known to be exceptionally tolerant of

hypoxia (Rogers et al., 2016) and the degree of hypoxia at the A3 and VM sites was evidently not severe enough to compromise the delivery of enough O_2 to the working muscles.

Table 3 also shows the respirometry data obtained during T2. There was evidence of an increased metabolic rate as compared to T1, when swimming at 20 cm s^{-1} immediately prior to initiating T2. This was a post-exercise increase in M_{O2} derived from T1 (an "oxygen debt"), which was statistically significant in the carp at sites A3 and VM (Table 3). This indicates that the carp at these two sites had not recovered metabolic homeostasis prior to initiating T2. The impaired recovery was associated with a significantly lower U_{crit} for T2 at the A3 and VM sites relative to the A1 site (Table 3). However, although U_{crit} for T2 was lower at the A3 and VM sites, the repeat ratio was not significantly different from unity at any site (Table 3). A provisional quality classification based upon fish swimming was: VM and A3 moderate (significant differences with the reference site) and A1 good.

Other City Fish studies investigated metabolic rate and swimming performance of caged chub (*Leuciscus cephalus*) exposed to polluted rivers in Italy (River Lambro, Milan) and the UK (Blythe, Cole & Tame river system, Birmingham). In both these countries, exposure to polluted sites caused a profound, approximately 30%, reduction in the ratio of repeat U_{crit} performance, compared with chub exposed at relatively unpolluted sites (McKenzie et al., 2007). This pronounced effect may indicate a greater sensitivity to complex mixtures of pollutants in chub than in carp. Unlike the sites used in the present study, however, the UK and Italian rivers suffer from significant pollution by heavy metals and much lower levels of organic pollution (Winter et al., 2005). There is evidence in the literature that heavy metals can impair the capacity of fish for sustained exercise (Wilson et al. 1994; Beaumont et al. 1995), possibly due to disruptions to gill ion transport and an accumulation of endogenous ammonia (Beaumont et al. 1995; 2000; Shingles et al. 2001; McKenzie et al. 2003). On the other hand, organic pollutants such as PCBs do not appear to impair the capacity of fish for sustained exercise (Brauner et al. 1994; Yang and Randall, 1997).

3.6 Impact on populations: ecological quality

Since the ecological status of freshwater sites can be affected by many non-chemical stressors ('ecological key factors', such as eutrophication, light climate, habitat suitability etc.), it is likely that an impaired quality is only partly due to chemical water pollution (STOWA, 2017). It is therefore not possible to find a direct linkage between the effects of micropollutants and ecological quality, unless the potential impact of all non-chemical key factors is known.

Macro-invertebrates. A total of 99 macro-invertebrate species were found at the three Amstel river sites, during four sampling programs (Table 4). Most species (72) were found at the least polluted A1 site. The highest number of species was found for the group of sludge worms (oligochaetes), while in most Dutch waters highest numbers are observed for non-biting midge species. A relatively high number of bivalve species was found at all sites. Most species of oligochaetes, pea clams and chironomus species live in muddy sediment. Chironomids caught at the A3 site showed mentum deformations, which may be indicative of toxic stress (e.g. Hamilton and Saether, 1971). Although the same amount of sediment was investigated at all sites, virtually no bottom dwelling animals were observed at the A2 site, which might be explained by the high sediment pollution (Table S3, supplemental information). Some rare species of sludge worms were found at the A1 site. At each of the four sampling periods, highest numbers of macro-invertebrate individuals were found at the A3 site, which comprised more than 80% crustaceans (Table S7, supplemental info). This can be explained by the large quantity of moss-grown embankment walls, which appears to be a favourable habitat for these organisms. This indicates that non-chemical factors may have a significant impact on macrofauna populations. The lowest amounts of individuals as well the lowest numbers of species were found at the A2 site. Based upon the diversity index of Shannon & Weaver (1949), the A1 site was classified as good (S&W >3), while A2 and A3 were classified as moderate quality (S&W 2-3; Table 4). Although no research was performed for the City Fish project, previous reports on macroinvertebrate status demonstrated a poor quality at the VM site (Van Dijk et al, 1984). Both the number of taxa and individuals of various taxonomic groups (e.g. bivalves) were clearly reduced. The ecological quality of macroinvertebrates at four sites (GP and A1-A3) was also determined for the Water Framework Directive (WFD) monitoring from 2006-

2012. Macroinvertebrate classifications were similar as our S&W classification, i.e. good for A1 and GP (scores above the 'good ecological potential' of 0.5) and moderate for A2 and A3 (scores between poor and good [0.2-0.5]).

Table 4.

Fish. Four fish population studies have been carried out at the three Amstel river sites, in which a total of 15 species were caught (Table 5). Bream and roach appeared to be the most common species in the Amstel River. It was difficult to get a good representative overview of the existing fish population because the fishing method and location were more or less specific to particular fish species. Electro-fishing close to riverbanks, for instance, was most efficient for perch, eel, tench and juvenile fish, while dragnet fishing in the deeper parts of the river was most efficient for bream, pike perch and pike. Even if both methods were used with comparable intensity it was difficult to get an accurate overview of the total population at each site.

Total biomass of fish caught at the A1 site was much lower than at the more polluted A2 and A3 sites. Fish populations at A2 and A3 sites were strongly dominated by mature bream, while only a few breams were caught at the A1 site. Highest amounts of predator species, such as pike and pike perch, were caught at the least polluted A1 site. This might be due to the fact that the visibility at this site was much higher than at the two other Amstel River sites. The fish populations at the A2 and A3 site seemed to be comparable. The water near the outlets of wastewater treatment plants (SWTP), located at both sites, proved to be 'hot spots' for mature bream. Most of the fish from the A2 and A3 sites were caught within a distance of 100 meters of these SWTP outlets. Bream was the dominant species of fish at these outlet areas. High numbers of smaller bream (10-25 cm) were caught at the A2 site, most of them close to reed borders that were not found at the A1 and A3 sites. Condition factors of bream caught during the four population studies did not show significant differences between sites (Figure S2, supplemental information).

Table 5

The length classes of bream caught for the population studies at the three Amstel sites are presented in Figure 6. The exact length and weight of very large groups of breams caught at the A2 and A3 sites (more than 300 fish of 35-45 cm length) were not measured for these population studies. At the A1 site many juvenile fish (3-6 cm) were caught or spotted, especially during the 2001 sampling programs, while no bream smaller than 8 cm was caught at the A2 and A3 sites (Figure 6). According to Shannon-Weaver (1949), the biodiversity of the fish was highest at the least polluted A1 site (Table 5). Because of highest diversity and the availability of more juvenile and predator fish at the A1 site, the fish population quality was classified as good (S&W >3). The fish population qualities at the A2 and A3 sites were classified as moderate (S&W 1-2). The ecological quality of fish populations at four sites (GP and A1-A3) was determined for the Water Framework Directive (WFD) monitoring from 2006-2012. Fish classification was moderate for A3 (score below good ecological potential of 0.5), and good for A1, A2 and GP (scores above good ecological potential of 0.5). The good ecological status of fish populations at the polluted A2 site is notable, since it was classified as moderate in 2002 (although a different classification method was used). This could be due to a remediation of the A2 sediment in the summer of 2002. Fish classification of the A2 site will therefore be considered good/moderate.

Figure 6

3.7 SIMONI bioanalytical risk assessment

In recent years, several SIMONI sampling programs (Smart Integrated Monitoring strategy) were carried out at the three Amstel River sites (A1-3). This strategy combines passive sampling with effect-based analyses with a suite of bioassays. Like the SOLUTIONS model (Altenburger et al., 2015), this strategy is based upon the application of *in vitro* whole-organism and cell-based bioassays within an AOP framework for assessing the effects of complex mixtures of aquatic contaminants. Bioassay responses are compared to effect-based trigger values (EBT) and an overall hazard assessment is calculated by the SIMONI Risk Indication (SRI), formerly known as SIMONI-score (Van der Oost et al., 2017a). The SIMONI results are summarized in Figure 7.

According to the SIMONI data there is a low risk of adverse ecological effects due to micropollutants at the A1 site (SRI <0.5), despite slight EBT exceedances of PXR CALUX. The risks at both A2 and A3 sites were moderate, but acceptable (SRI 0.5-1.0). At the A2 site EBT exceedances of the *in-situ* Daphnia assay, the anti-AR and PAH CALUX assays were observed, while at the A3 site only the EBT of the anti-AR CALUX was exceeded. Although not all bioassays were applied in all sampling programs, SRI corrections were made to compensate for lacking bioassays (Van der Oost et al, 2017a). A striking result is that, despite the remediation in 2002, increased risks of PAH pollution were still detectable at the A2 site.

Figure 7

4 Water quality evaluation

An overview of the water quality classifications, based upon the parameters that were discussed in the former paragraphs, is presented in Figure 8. There was a lack of information on fish physiology (GP and A2), fish population studies (VM) and SIMONI Risk Indication (GP and VM).

Figure 8

Apart from the unknown parameters (fish physiology and SIMONI), the quality of the unpolluted Lake Gaasperplas site (GP) was considered good for all investigated exposure and key events. The ecological status of the GP site was considered good for macroinvertebrates and fish.
The least polluted Amstel river site (A1) showed a moderate quality for general water parameters, but the quality based upon external exposure of micropollutants was generally good. However, due to increased bioaccumulation of PCBs and OCPs and higher PAHs exposure in caged and feral fish, the internal exposure indicated a moderate quality. MKE and impact on organs and organisms all indicated a good water quality. Macroinvertebrate and fish populations indicated a good water quality based on biodiversity and (more recent) WFD ecological scores.
The most heavily polluted Amstel river site (A2) was classified as poor water quality due to external and internal exposure of PAHs. The induction of phase I and II biomarker responses indicated a moderate water quality with increased risk for the ecosystem, possibly due to PAH and PCB

exposure. No indications of oxidative stress, impact on organs and organisms were observed, but fish swimming and metabolic rate tests were not performed at this site. Most population studies indicated a moderate ecological quality at the A2 site, although more recent fish population studies with WFD ecological scores indicated a good ecological quality.

The Amstel river site close to the City of Amsterdam (A3), showed a good quality based on water levels of micropollutants, but quality based upon oxygen, phosphate and sediment pollution was moderate. Phase I and II biomarker responses indicated a moderate quality. No impact on organs and organisms was detected, apart from a slight reduction in swimming speed. Ecological surveys on populations (including more recent WFD ecological scores) indicated a moderate water quality. Fish population studies in the Amstel river indicated that fish tended to migrate to less polluted sites for spawning, since juvenile bream were only found at the least polluted A1 site. Moreover, the more polluted A2 and A3 sites had a lower fish biodiversity.

The water quality of the heavily polluted VM site was classified as poor, based upon external and internal exposure to pesticides and dioxin-like compounds (DLC). This was confirmed by molecular key events (CYP1A induction) leading to cellular responses, such as indications for the occurrence of oxidative stress by elevated activities of antioxidant enzymes. Fish physiology was impaired (increased metabolic rate and reduced swimming speed), indicating that energetic costs of organismal maintenance were higher, associated with pollutant exposure. No impact on growth (CF) and visible adverse effects were noted in fish that were caged at this site for four weeks. Since no population studies were performed at this site, the adverse outcomes due to the pollutant exposure on reproduction and mortality could not be confirmed.

5 Identifying potential adverse outcome pathways

Despite the extensive range of field data that were collected in the present study, many relevant variables are still lacking to verify all interactions among pollutant exposure, molecular initiating events (MIE), key events (KE) and adverse outcomes. First, complete datasets could only be obtained for two of the five sites (A1 and A3). Second, important molecular markers (endocrine disruption) and key events (histopathology and embryo toxicity) were not investigated. Establishing

the link between individual-based AOs and population-level responses is complicated by the fact that population outcomes are highly context-specific and depend on both internal (organism-specific) and external (site-specific) factors (Groh et al., 2015). Since is it very difficult to get reliable data on entire fish populations, most AOP-related field studies use population models (Kramer et al., 2011; Miller et al., 2015). The present study was not initially designed to study adverse outcome pathways, since most experiments were carried out when the AOP theory as such was not yet published (Ankley et al., 2010), but this dataset still contains interesting information on indirect relationships among molecular markers, key events and ecological data. In addition, the study is an example of how AOP could be applied in an environmental context and field studies like the present may point towards the potential to provide qualitative and quantitative predictive modelling.

Complex environmental mixtures are most probably able to cause an interplay between multiple pathways potentially contributing to an AO (Groh et al, 2015). In establishing the linkage between molecular events and those at the population level, a clear adverse outcome pathway could not be established with data from the present study. However, some indirect links between pollutant exposure and adverse effects on individuals could be demonstrated at the most polluted VM site (red arrows in Figure 9). Although a clear initiating event was not established, it is likely that the induction of CYP1A through aryl hydrocarbon receptor (AhR) activation is a useful surrogate for such, as it shows a clear molecular interaction. Activation of the AhR by DLC has been shown to cause a range of adverse effects in vertebrates, including hepatotoxicity, immune suppression, reproductive and endocrine impairment, teratogenicity, carcinogenicity, and loss of weight (Kawajiri and Fujii-Kuriyama, 2007). Two AOPs are being developed on AOP-Wiki that link AhR activation to embryo toxicity in fish (Figure S3, supplemental info), a pathway that was also demonstrated in the field (Cook et al, 2003).

Figure 9

The high CYP1A induction in fish caged at the VM site was associated with oxidative stress, as indicated by an increased SOD activity. Although no tissue damage was demonstrated by serum

transaminases and LSI in VM fish, physiology of caged carp was impaired, as indicated by altered metabolic rate and swimming behaviour. In the proposed AOP for cadmium-induced growth impairment, several upstream KEs that require energy to synthesize molecules involved in stress response and defence pathways all converge at a single common KE: increased metabolic demands (Groh et al., 2015). Apart from metals, many other compounds were shown to cause elevation of oxygen consumption, so energy reallocation can be viewed as a significant 'generic' KE occurring in response to many toxicants. No impacts on growth (CF) and visible adverse effects could be demonstrated in fish after four weeks of exposure at the VM site, but effects on survival and reproduction might occur after longer exposure times. It is obvious that certain adverse effects that were not measured, such as immune suppression, reproductive and endocrine impairment, teratogenicity, carcinogenicity, cannot be ruled out at this site. Unfortunately, many of these parameters such as abnormalities, embryo lethality and cancers (that can each lead to lack of survival) are difficult to measure in the field and no fish population studies were carried out at the VM site to test this.

Author contributions

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships

that could have appeared to influence the work reported in this paper.
The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

In summary, although no complete AOP could be observed in the dataset of the Amsterdam City Fish research, indirect relationships linking pollutant exposure via MKE to impaired locomotion were demonstrated at the most polluted site. When evaluating the entire dataset, both fish biomarkers and a bioanalytical monitoring strategy (SIMONI Risk Indication) seem to be valuable indicators of risks to macroinvertebrate and fish populations due to micropollutants.

6 Acknowledgements

This work is dedicated to the memory of our friend and colleague, the late Frank Verweij, who performed and coordinated the field work and laboratory biomarker analyses for this project. The research was funded by the City of Amsterdam, the European Union (CITYFISH project EVK1-CT-1999-00009) and the Waternet Institute for the Urban Water Cycle, Amsterdam. Laura Moria, Tim Pelsma, Gerard ter Heerdt (all Waternet) are acknowledged for critically reading the ecological parts of the manuscript and providing additional information on WFD ecological quality scores. Gert van Ee (HHNK water board), thanks for providing information on macroinvertebrate studies at the VM site. Tim Williams (University of Birmingham, UK), thanks a lot for critically reading and improving the initial manuscript. We thank Ted Taylor (University of Birmingham) for the smooth coordination of the City Fish project.

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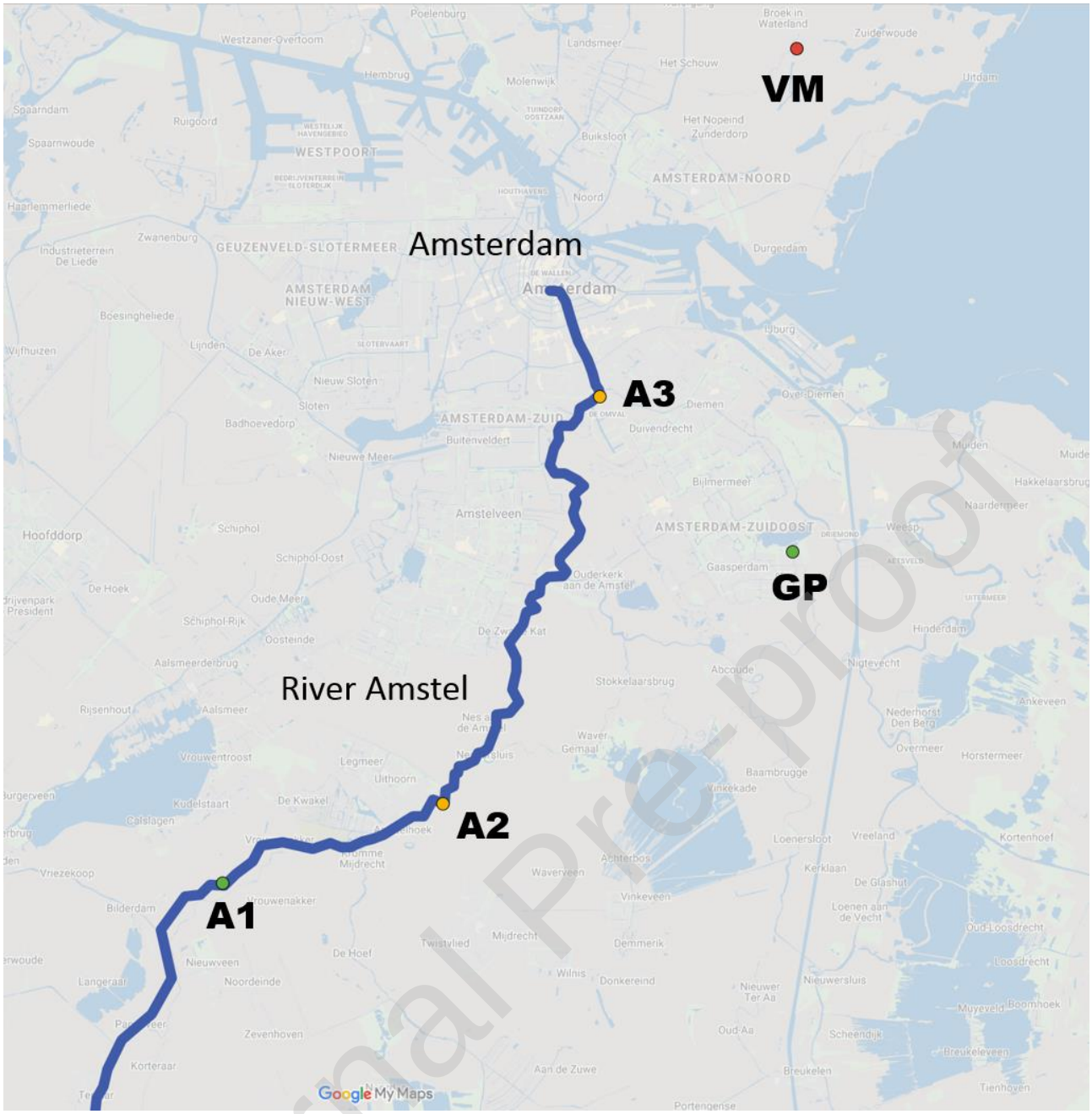


Fig. 1

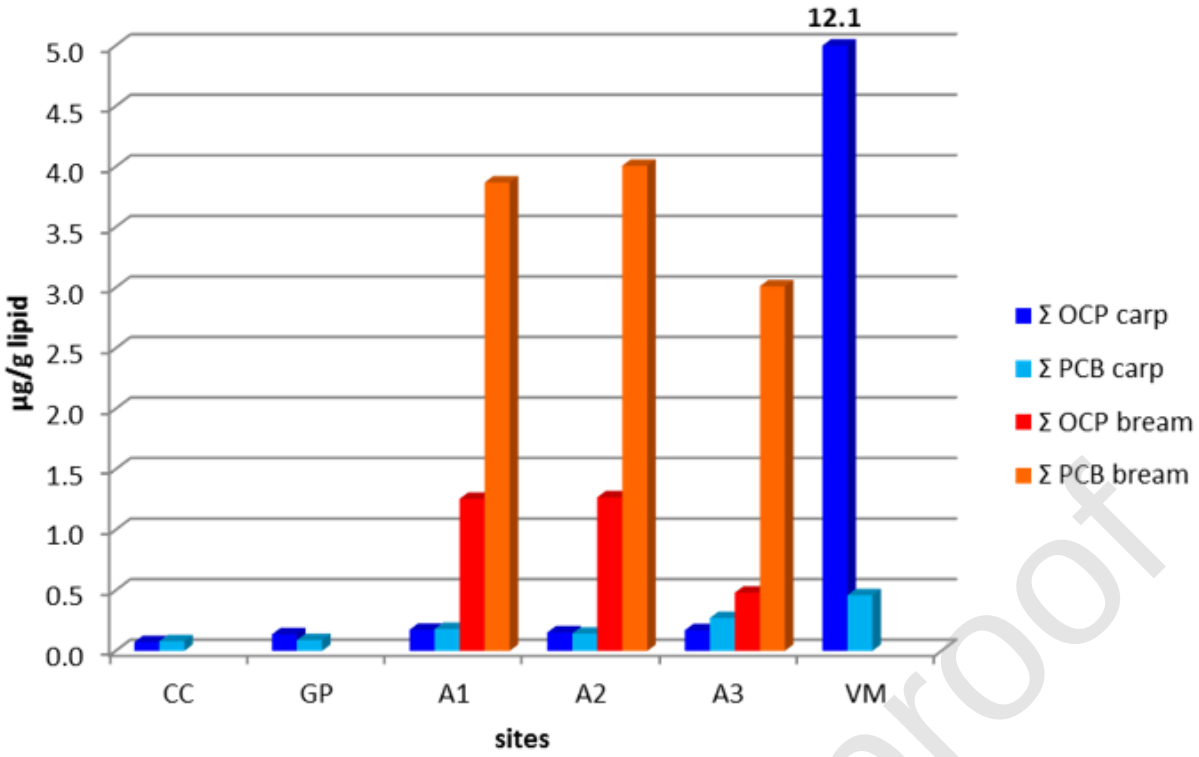


Fig.2

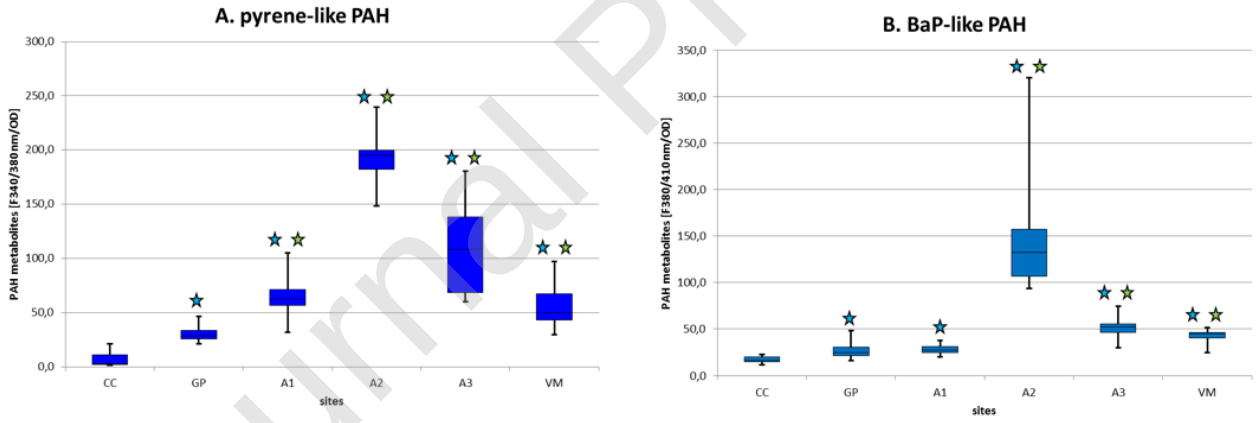


Fig.3

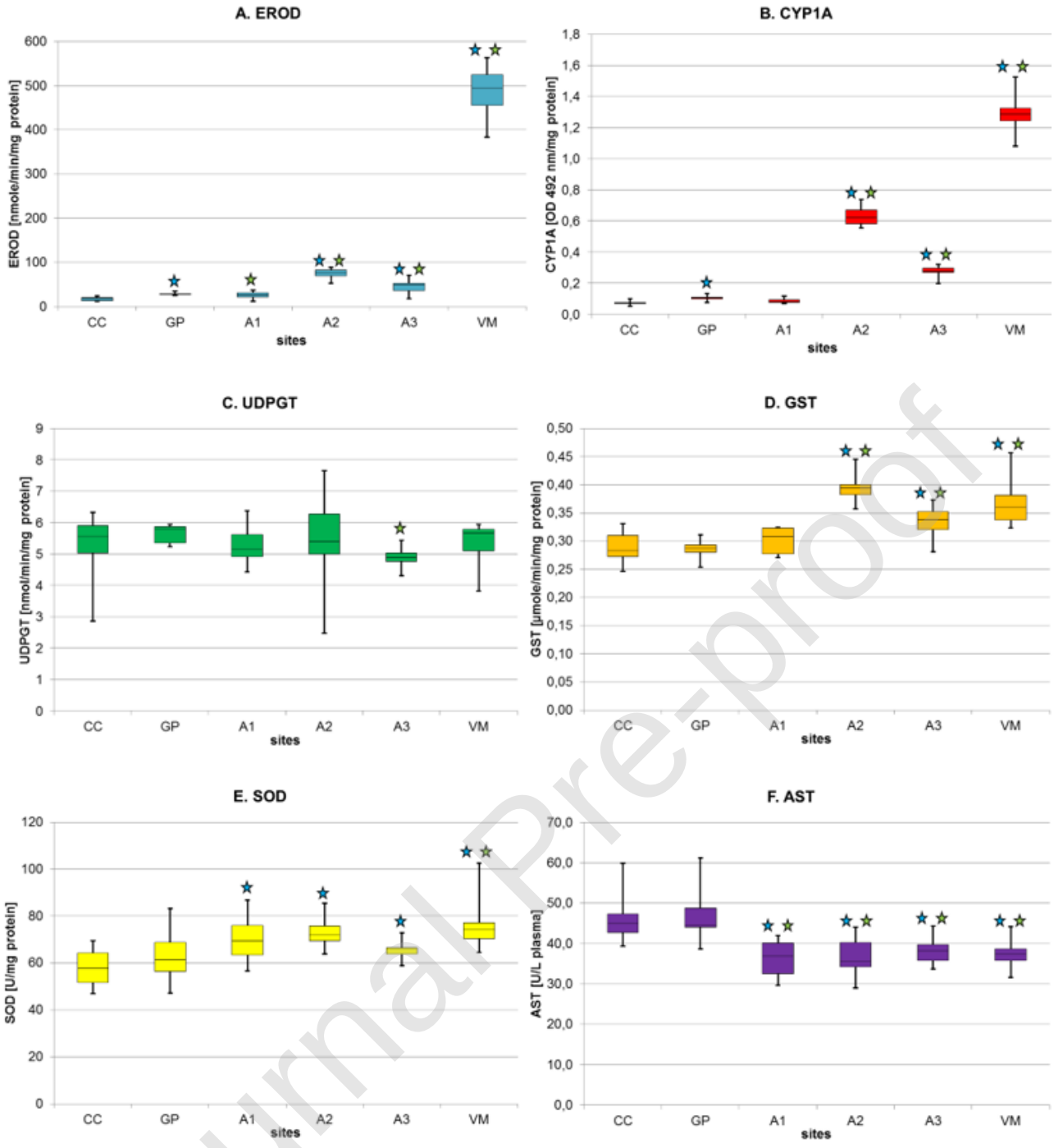
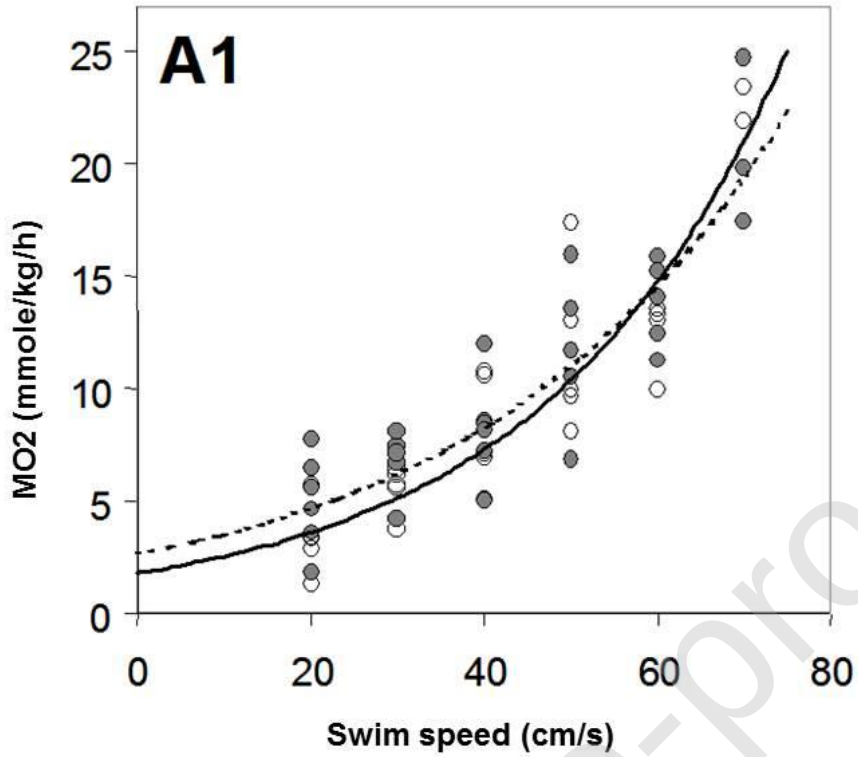
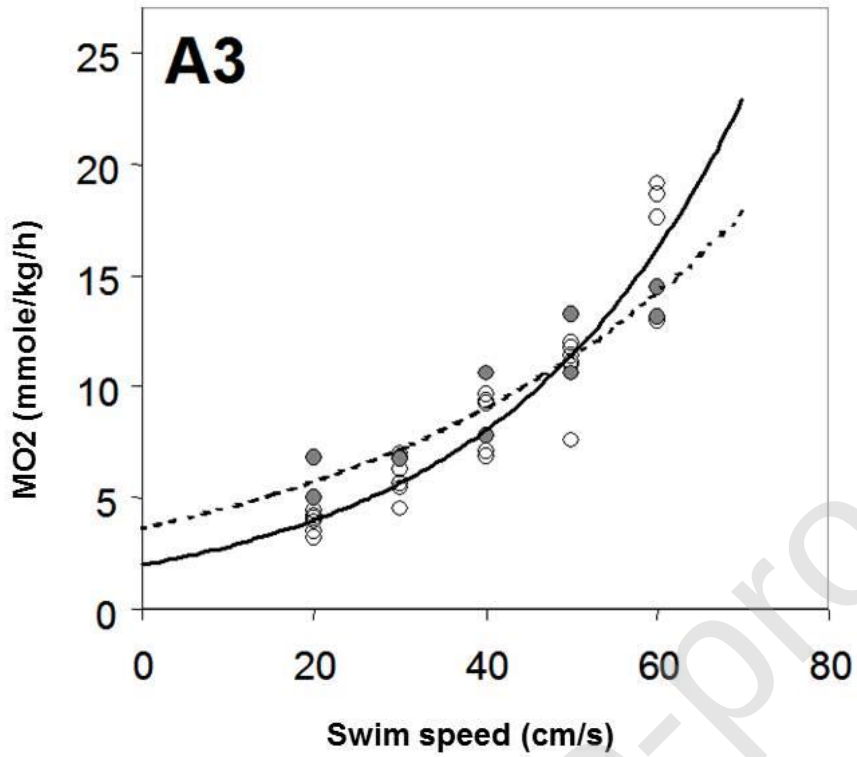


Fig.4





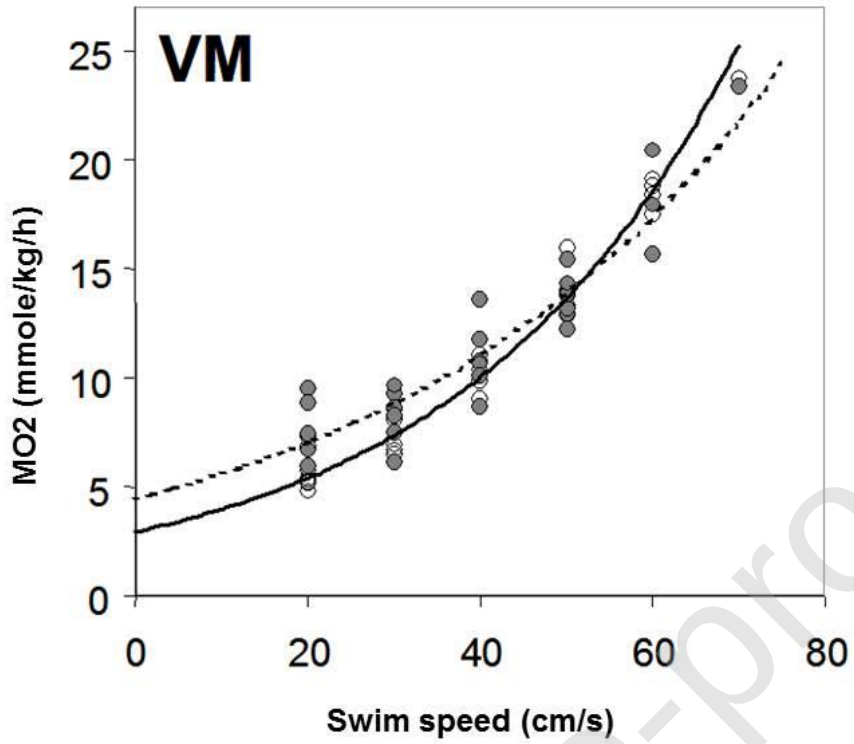


Fig.5

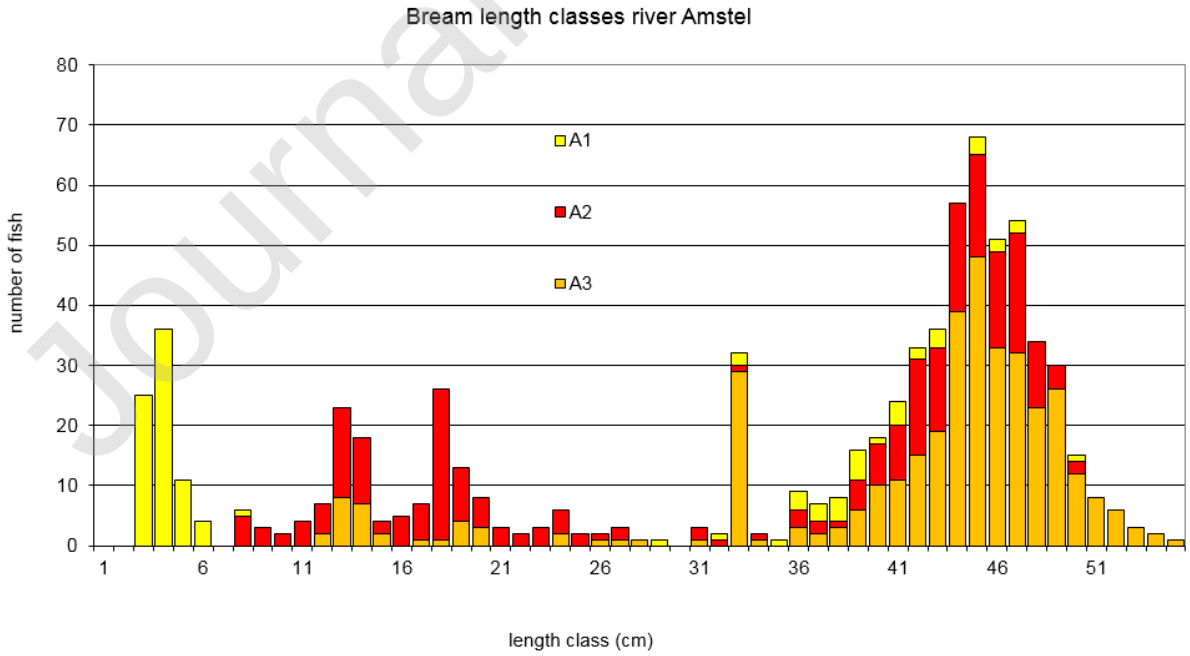


Fig.6

Site	year	Daphnia field	Microtox	Algaetox	Daphniatox	cytotox polar	cytotox non-polar	Era CALUX	anti-AR CALUX	GR CALUX	Antibiotics	PAH CALUX	PPARg CALUX	Nrf2 CALUX	PXR CALUX	p53- CALUX	p53+ CALUX	SRI
	EBT	20	0.05	0.05	0.05	0.05	0.05	0.5	25	100	50-500	150	10	10	3	0.005	0.005	
	unit	%M	TU	TU	TU	TU	TU	EEQ	FluEQ	DexEQ	AEQ	BaPEQ	RosEQ	CurEQ	NicEQ	TU	TU	
A1	2014	0.00	0.00	0.02	0.07		0.01	0.38	0.69	0.02	0.13		0.00	0.29		0.36		**0.22**
	2015	0.00	0.03	0.00	0.10		0.00	0.36	0.26	0.10	0.26		0.20	0.47	1.28	0.00		**0.36**
A2	2012	1.50	0.08	0.04	0.15			0.07	0.63	0.00	0.00	1.37	0.27			0.00		**0.75**
	2012	0.00	0.18	0.07	0.45			0.14	1.21	0.00	0.02	1.93	0.64			0.00		**0.61**
A3	2016	0.50	0.02	0.00	0.03	0.60	0.00	0.88	1.79	0.04	0.07	0.44	0.00	0.38	0.52	0.01	0.00	**0.61**

Fig.7

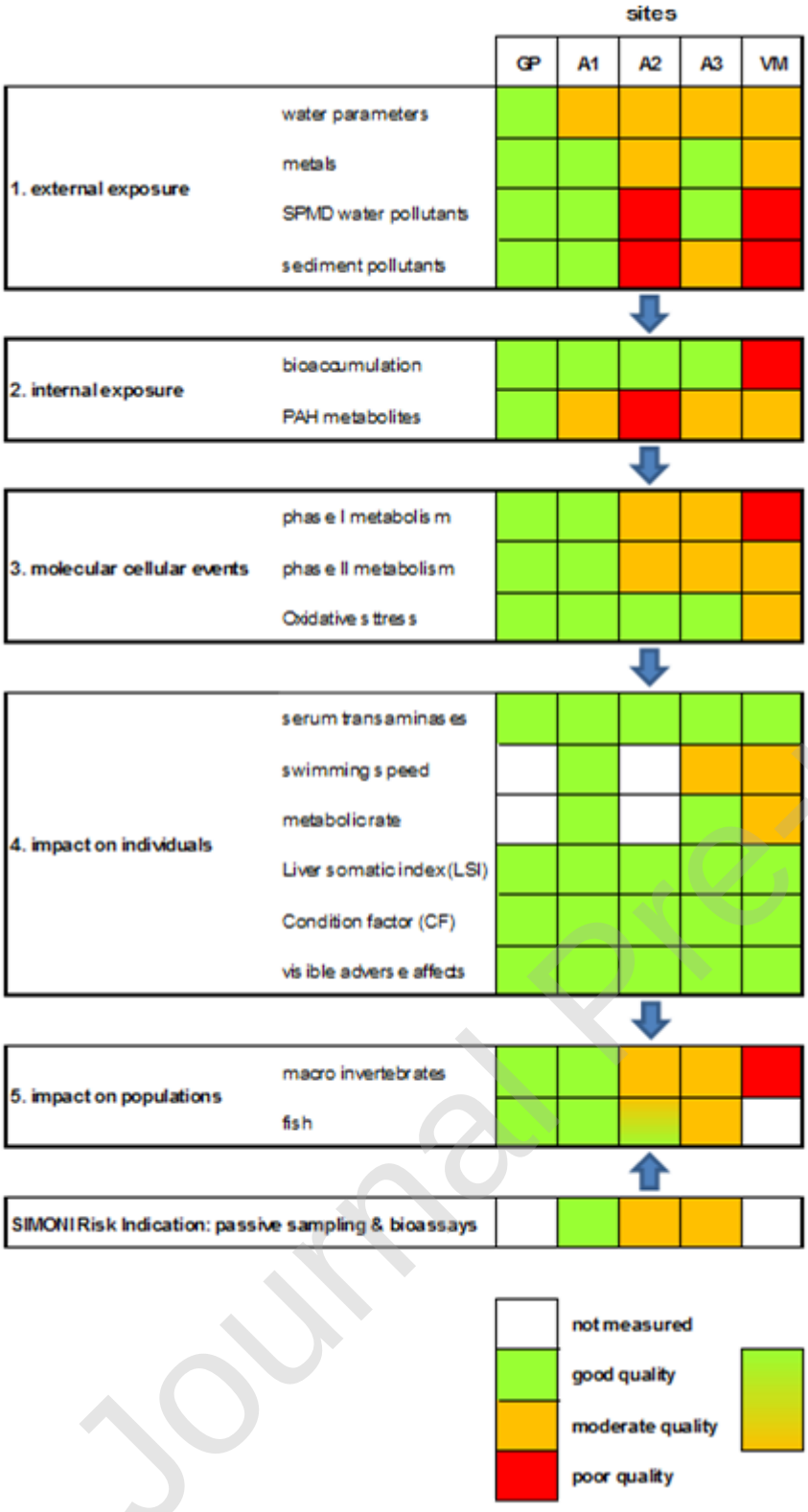

sites
GP
A1
A2
A3
VM
1. external exposure
water parameters
metals
SPMD water pollutants
sediment pollutants
2. internal exposure
bioaccumulation
PAH metabolites
3. molecular cellular events
phase I metabolism
phase II metabolism
Oxidative stress
4. impact on individuals
serum transaminases
swimming speed
metabolic rate
Liver somatic index (LSI)
Condition factor (CF)
visible adverse affects
5. impact on populations
macro invertebrates
fish
SIMONI Risk Indication: passive sampling & bioassays
not measured
good quality
moderate quality
poor quality

Fig.8

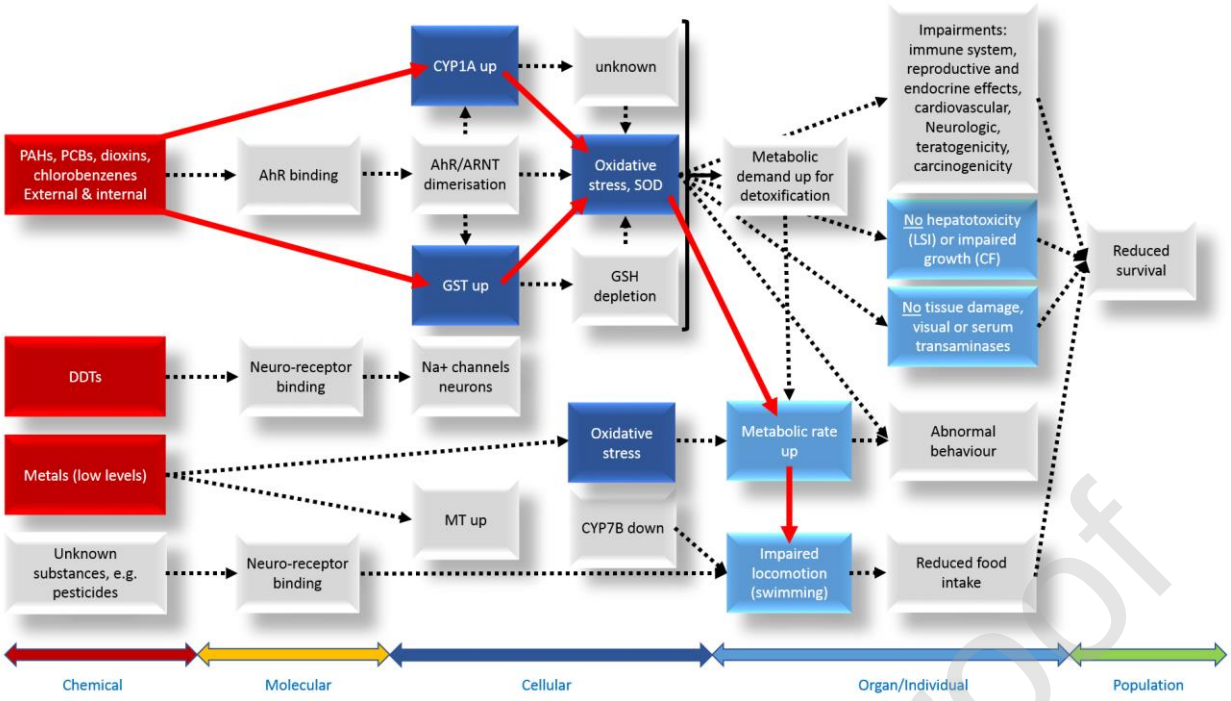


Fig.9

Table 1: standard water quality parameters

Parameters [mg/l]	unit	DQS	Sites			
			GP	A1	A2	A3	VM
ammonium		0.8		<0.1		1.3	0.3
chloride		200		55		400	330
nitrate				3.6		2.5	<0.05
nitrite		0.3		0.08		0.24	0.02
total nitrogen				5.6		6	1.8
total phosphate		0.2		0.94		0.68	0.12
particulate matter				21.6		14.3	4.6
sulphate		100		110		68	60
biological oxygen demand (BOD)		6		2.3		3	1.3

Table 1: Water quality parameters and environmental quality standards (EQS)

Parameters	unit	EQS	Sites				
			GP	A1	A2	A3	VM
temperature	°C	**<25**	16.3	14.2	13.7	15.7	15.6
oxygen	mg/l	**>5**	7.3	8.3	6.2	5	6.6
pH	pH	**6.5-9.0**	8.05	8.05	7.65	7.45	7.75
conductivity	mS/cm		0.71	0.95	1.31	1.72	1.19
salinity	ppt		0.15	0.3	0.4	0.7	0.4
ammonium	mg/l	**<0.8**	0.35	0.35	0.3	0.75	0.98
chloride	mg/l	**<200**	71	51	62	160	200
nitrate	mg/l		0.52	3.8	2.88	7.3	0.565
nitrite	mg/l	**<0.3**	0.035	0.09	0.075	0.195	0.055
total N	mg/l		1.8	6.9	5.6	9.9	3.3
total P	mg/l	**<0.15**	0.07	0.55	0.34	1.13	0.08
particulate matter	mg/l		4.75	12.35	17.05	3.5	2.1
sulphate	mg/l	**<100**	39.5	145	118	94	34.5
biological oxygen demand (BOD)	mg/l	**<6**	1.9	2.6	3	2.3	1.8
arsenic	µg/l	**<50**	<6	<6	<6	<6	<6
cadmium	µg/l	**<0.6**	<0.2	<0.2	<0.2	<0.2	<0.2
chromium	µg/l	**<50**	2.2	1.7	1.9	2.2	26.2
copper	µg/l	**<50**	<2	3.5	5	7.0	9.0
mercury	µg/l	**<0.07**	<0.02	0.03	0.07	0.05	0.03
lead	µg/l	**<14**	<3	3	5.5	5	18
nickel	µg/l	**<34**	<2	5	6	4	4
zinc	µg/l	**<200**	12	24	26	28	36

Table 2: Average levels of micropollutants in water determined with SPMD data

Substances	unit	EQS range	GP	A1	A2	A3	VM
Sum chlorobenzenes	ng/L	**50-300**	0.11	0.14	0.08	0.08	90.37
Sum DDTs	ng/L	**0.4-25**	0.01	0.09	0.12	0.08	2.88
Sum drins	ng/L	**0.9-12**	<0.02	<0.02	<0.02	<0.02	<0.02
Sum HCHs	ng/L	**800-3000**	0.25	0.21	0.16	0.58	4.8
Sum Heptachlors	ng/L	**0.3-0.5**	<0.02	<0.02	<0.02	<0.02	<0.02
Sum OCPs	ng/L	**0.3-3000**	**0.38**	**0.43**	**0.37**	**0.74**	**98.09**
Sum PCBs	ng/L	**no EQS**	**0.02**	**0.15**	**0.25**	**0.25**	**1.35**
Sum PAHs	ng/L	**8.2-130,000**	**94.22**	**57.25**	**827.24**	**71.60**	**115.61**

Table 3: Selected metabolic and performance variables in carp exposed to the three river sites, investigated with a repeated swimming respirometry protocol.

Swim Test 1	**A1**	**A3**	**VM**
SMR (mmole O_2/kg/h)	1.85 ± 0.39[a]	2.01 ± 0.21[a]	2.95 ± 0.15[b]
M_{O2} at 20 cm s^{-1} (mmole O_2/kg/h)	3.71 ± 0.69[a]	3.90 ± 0.19[a]	5.50 ± 0.29[b]
AMR (mmole O_2/kg/h)	16.5 ± 2.2[a]	15.2 ± 1.5[a]	17.7 ± 1.6[a]
Scope (AMR-SMR)	14.68 ± 2.28[a]	14.04 ± 1.65[a]	14.74 ± 1.83[a]
U_{crit} (body lengths s^{-1})	3.19 ± 0.17[a]	2.86 ± 0.10[a]	2.94 ± 0.15[a]
Swim Test 2			
M_{O2} at 20 cm s^{-1} (mmole O_2/kg/h)	4.98 ± 0.85[a]	5.98 ± 0.35[a,b]*	7.61 ± 0.54[b]*
AMR (mmole O_2/kg/h)	18.2 ± 1.5[a]	15.3 ± 1.4[a]	17.3 ± 1.7[a]
U_{crit} (body lengths s^{-1})	3.28 ± 0.14[a]	2.78 ± 0.08[b]	2.88 ± 0.14[b]
Repeat ratio (U_{crit} T1 / U_{crit} T2)	1.03 ± 0.03[a]	0.97 ± 0.03[a]	0.98 ± 0.01[a]

Values are the mean (± SD) of six samples in each case. The presence of a common superscript indicates no difference between sites for that variable. An * indicates a significant difference between swim tests 1 and 2 at a site.

Table 4: macro-invertebrate species diversity at three Amstel river sites; summary of four ecological surveys (spring/autumn 2000-2001).

	A1	A2	A3	Total
Sludge worms	15	6	16	**23**
Bristle worms			1	**1**
Leeches	9	6	2	**9**
Water mites	4			**4**
Crustaceans	4	3	4	**7**
Non-biting midges	14	11	15	**21**
Short-palped Crane Flies	1	1		**1**
Bivalves	8	8	8	**14**
Snails	11	4	1	**11**
Mayflies	1			**1**
Water beetles	2	1		**3**
Water bugs	1			**1**
Flatworms	1	1		**1**
Caddis flies	1			**1**
Sponge			1	**1**
Hydroids			1	**1**
Total number of species	**72**	**41**	**49**	**99**
Average number of organisms	**813**	**724**	**2519**	
Diversity index (Shannon & Weaver)	**3.47**	**2.82**	**2.24**	

Table 5: Total fish caught at three Amstel river sites in four fish population studies in 2000 and 2001

Species	A1 n	A1 length range [cm]	A2 n	A2 length range [cm]	A3 n	A3 Length range [cm]	Total
Bleak *(Alburnus alburnus)*	13	5-8					**13**
Perch *(Perca fluviatilis)*	49	7-12	67	7-19	59	2-29	**175**
Roach *(Rutilus rutilus)*	117	5-17	82	7-22	50	12-34	**249**
Bream *(Abramis brama)*	68	4-50	523	12-53	439	13-58	**1030**
Carp *(Cyprinus carpio)*			5	45-71			**5**
Pike *(Esox lucius)*	16	32-79	8	26-76			**24**
Pike perch *(Stizostedion lucioperca)*	15	32-75	7	31-82	19	8-69	**41**
Rudd *(Scardinius erythrophalmus)*	4	5-15	40	5-28	1	17	**45**
Asp *(Aspius aspius)*					2	43-46	**2**
Rufle *(Gymnocephalus cernua)*	4	6-10	1	7	1	11	**6**
Eel *(Anguilla anguilla)*	22	Total ±4700g	26	Total ±8200g	4	Total ±1100g	**52**
White Bream *(Blicca bjoerkna)*			4	12-14			**4**
Tench *(Tinca tinca)*	1	45	10	17-43	1	22	**12**
Gudgeon *(Gobio gobio)*	1	10	1	13			**2**
Stickelback *(Gasterosteus aculeatus)*					2	5-6	**2**
Total species	**11**		**12**		**10**		**15**
Diversity index (S&W)	**2.54**		**1.74**		**1.26**		