## Organisms make ecosystems function

*Identifying functional indicators of anthropogenic stress in aquatic ecosystems*

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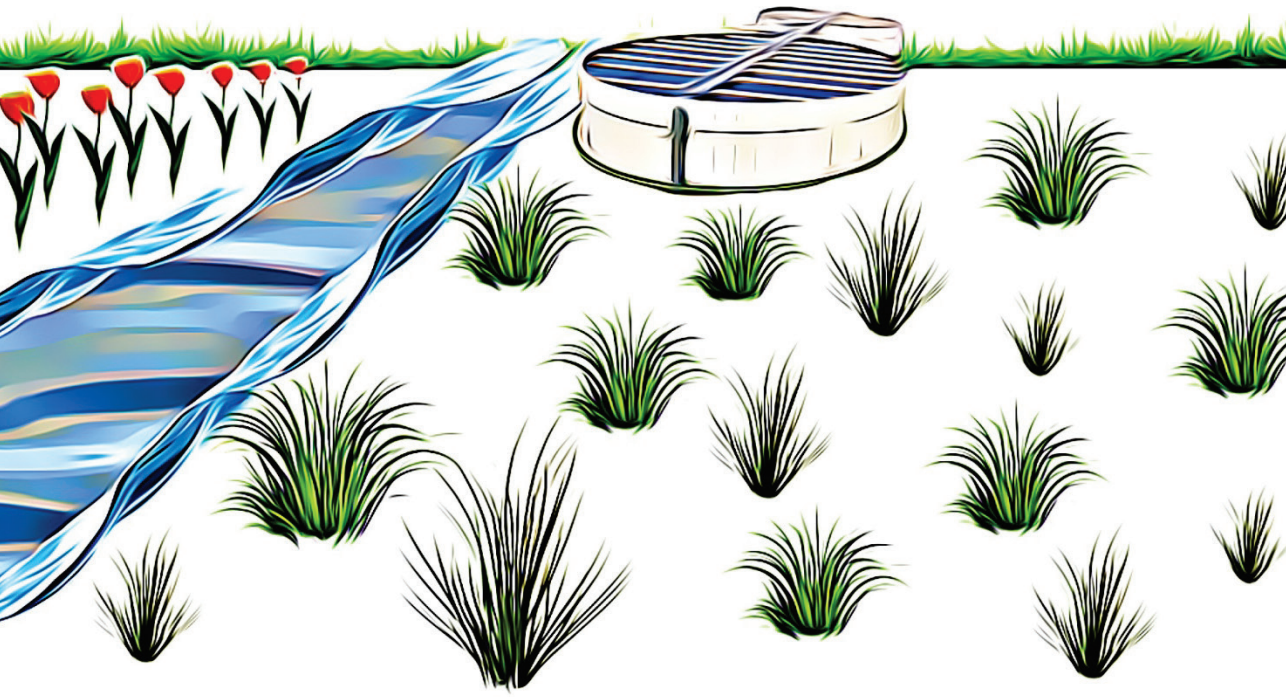
# CHAPTER 6

# STRUCTURAL AND FUNCTIONAL ASSESSMENT OF MULTI-STRESSED LOWLAND WATERS



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## ABSTRACT

Water bodies in densely populated lowland areas are commonly impacted by pollution from agricultural activities and wastewater treatment plant (WWTP) discharges. Yet, the selection of appropriate water quality assessment methods for these water bodies is under debate. Therefore, we aimed to compare the use of structural and functional metrics in their ability to 1) detect adverse effects from anthropogenic stress on aquatic ecosystems, and 2) diagnose the potential causes of the observed adverse effects. For this purpose, we compared the responses of several structural (both taxonomic and trait-based) and functional (i.e. ecological process) metrics to a series of stressors in 20 lowland water bodies impacted to a varying degree by agricultural activities and WWTP discharges. The measured stressors included nutrients, dissolved oxygen saturation, water temperature and proxies for pesticides, pharmaceuticals and personal care products. The results showed a significant negative relation between the combined proxy for organic toxicants and the taxonomic-based evenness metric, as well as the trait-based $SPEAR_{organic}$ and $SPEAR_{pesticides}$ metrics. The microbial decomposition and invertebrate consumption showed an opposite and more complex relation to the stressors. These findings suggest that process-based metrics may be able to detect patterns of anthropogenic stress that were not evident from the structural metrics alone, and thereby provide complementary information to aid water quality assessment. In terms of diagnostic value, the results indicated that both SPEAR metrics may be used to diagnose the combined presence of organic toxicants from agricultural activities and WWTPs discharges. It is concluded that taxonomic, trait-based and functional metrics provide complementary information that, when integrated, allow for more thorough water quality assessment strategies.

## INTRODUCTION

Freshwater ecosystems in densely populated lowland areas are generally impacted by a combination of hydro-morphological degradation and pollution, resulting in poor water quality (Paul & Meyer, 2001; Riis & Sand-Jensen, 2001; Needelman et al., 2007; Schinegger et al., 2012). The mixtures of pollutants entering these water bodies can largely be attributed to agricultural and urban land use (Allan, 2004; Burdon et al., 2019). Pesticides and excess nutrients may enter the water via run-off and spray drift from agricultural activities (Schulz, 2004; De Zwart, 2005; Bracewell et al., 2019). Urbanization can lead to an increase in almost all types of pollutants originating from non-point source run-off and municipal wastewater treatment plant (WWTP) discharges (Paul & Meyer, 2001). Depending on the particular processes used to treat influent wastewater, WWTPs may represent a major source of input of nutrients, pharmaceuticals and personal care products (PPCPs), pesticides and a suite of generally unknown contaminants (Carey & Migliaccio, 2009; Rosi-Marshall & Royer, 2012; Petrie et al., 2015; Munz et al., 2017). The input of excessive nutrients by agricultural land use and WWTPs can result in eutrophication, thereby indirectly altering dissolved oxygen regimes (Paul & Meyer, 2001; Van der Lee et al., 2018). Moreover, water temperatures tend to be higher due to increased solar radiation resulting from riparian vegetation removal and the warming influence of wastewater inputs into receiving water bodies (Allan, 2004; Kinouchi et al., 2007). Although several studies have demonstrated the negative effects of agricultural activities and WWTP discharges on the water quality, the selection of the appropriate assessment methods to aid effective management in multi-stressed water bodies is still under debate (Pascoal et al., 2003; Bonada et al., 2006; Hagen et al., 2006; Schäfer et al., 2007; Friberg et al., 2011; Peters et al., 2013).



Traditionally, most biomonitoring schemes have relied on structural metrics based on taxonomic inventories of various organism groups, with invertebrates being the most widely used (Boulton, 1999; Bonada et al., 2006; Resh, 2008). Species diversity, i.e. species richness, evenness and composition, is commonly used in water quality assessment, as the detrimental effects of human disturbances on species diversity are generally well established (Metcalfe, 1989; Norris & Thoms, 1999; Cao & Hawkins, 2019). However, most of the metrics calculated directly from taxonomic data respond to general degradation, and thus have limited value in identifying the impact of specific stressors on the ecological status of freshwater ecosystems (Clews & Ormerod, 2009; Friberg et al., 2011; Gieswein et al., 2017; Lemm et al. 2019). Metrics based on species traits may provide the specificity that is lacking in taxonomic metrics (Statzner & Bêche, 2010). A promising trait-based method is the SPEcies At Risk (SPEAR) approach, designed to distinguish between the impacts of different types of stressors on freshwater invertebrates (Liess & Von der Ohe, 2005). The $SPEAR_{organic}$ is based on taxon-specific sensitivities to organic contaminants (i.e. carbon-

based synthetic chemicals) gleaned from databases of ecotoxicity studies providing lethal effect concentrations ($LC_{50}$) (Liess & Von der Ohe, 2005; Beketov & Liess, 2008). The $SPEAR_{pesticides}$ combines the $SPEAR_{organic}$ with life history traits that differentiate a taxon's ability to recover from pulses of contamination typical of pesticide exposure, such as generation time, migration ability, and timing and duration of life stages (Liess & Von der Ohe, 2005; Knillmann et al., 2018). Several studies reported significant correlations between the $SPEAR_{pesticides}$ and the presence of elevated pesticide concentrations in streams (Liess & Von der Ohe, 2005; Schäfer et al., 2007; Beketov et al., 2009; Schäfer et al., 2012), and between $SPEAR_{organic}$ and concentrations of petrochemicals and surfactants (Beketov & Liess, 2008; Kuzmanović et al., 2016). An increased understanding of the diagnostic value of this approach could thus aid water quality assessment in multi-stressed lowland water bodies.

In addition to improving diagnostic value of water quality assessment approaches, there is also a recognized need to adopt indicators of ecosystem function, as anthropogenic stress may impact ecosystem structure and function differently (Bunn, 1995; Gessner & Chauvet, 2002; Young et al., 2008). Specifically, species composition may change without affecting ecological processes, i.e. the loss of a species is compensated for by a species with a functionally similar role within the ecosystem (Jackson et al., 2016). Alternatively, ecological processes may change in absence of a change in species composition, e.g. an increase in growth rate may enhance productivity, but does not change species composition (Bunn, 1995; Sandin & Solimini, 2009). To include functional indicators in assessment approaches, several direct measures of ecosystem processes have been suggested (e.g. metabolism and nutrient cycling), and particularly organic matter breakdown has been advocated as a sensitive parameter to assess the impact of human-induced stress (Gessner & Chauvet, 2002; Young et al., 2008). For example, organic matter breakdown may be enhanced by nutrient enrichment (Ferreira et al., 2015) and higher water temperatures (Ferreira & Chauvet, 2006), but could be inhibited by toxic contaminant concentrations (Schäfer et al., 2007; Young et al., 2008). Despite the importance of including both structural and functional metrics in water quality assessments, few studies have actually done so and usually only a single stressor is considered (but see Schäfer et al., 2011; Matthaei et al., 2010, Piggott et al. 2012).

Because the goal of many water-quality assessments is to detect whether multiple anthropogenic stressors have changed ecosystem structure and functioning, there is a legitimate question as to the sufficiency of the currently used metrics. Hence, our objective was to determine whether functional metrics provide added value in the detection of anthropogenic stress. We evaluated this by comparing responses of several structural (both taxonomic and trait-based) and functional (i.e. based on ecological process) metrics to a series of stressors. We hypothesized that functional metrics can reveal patterns that would

not be evident from structural metrics alone. As the goal of water-quality assessments is also to diagnose the potential causes of the observed adverse effects, we aimed to determine whether the trait-based SPEAR-approach offers value in distinguishing toxic pollution from agricultural activities and WWTPs discharges from other stressors in multi-stressed lowland water bodies.

## MATERIALS AND METHODS

### Study sites

The present field study was performed in 20 permanent lowland water bodies throughout the Netherlands. The selected water bodies were small (mean ± SD = 4.5 ± 1.8 m) and shallow (0.8 ± 0.2 m) with a minimal flow (5.3 ± 6.8 cm/s) and often with a channelized geomorphology (i.e. including drainage ditches). To comprise a wide range of stress from nutrients, low dissolved oxygen concentrations, water temperature, pesticides and PPCPs, sites were selected which were impacted to varying degrees by agricultural activities and WWTP effluent (Table 1; details in Supplementary material 1). Four sites were chosen that were primarily surrounded by nature in the riparian zone. In addition, 12 sites were selected that were surrounded to varying degree by agricultural land use in the riparian zone of which five sites were surrounded by intensive horticulture. Four other sites were chosen that directly received WWTP effluent. Since water bodies in the Netherlands are generally strongly connected, we used a measure of the anthropogenic sources of gadolinium (used in medical procedures) as an index of the degree to which each study site was influenced by effluent input from WWTPs (details in Supplementary material 1). A natural gadolinium anomaly (Gd*) without any WWTP impact is around 1.3 (Rabiet et al., 2005; Petelet-Giraud et al., 2009). Hence, the Gd* values reported in Table 1 indicate that WWTP effluent also reached several sites that did not directly receive WWTP discharge. Sampling was conducted from the 20$^{th}$ of August (week 34) until the 23$^{rd}$ of October (week 43) 2018 (Table 2).



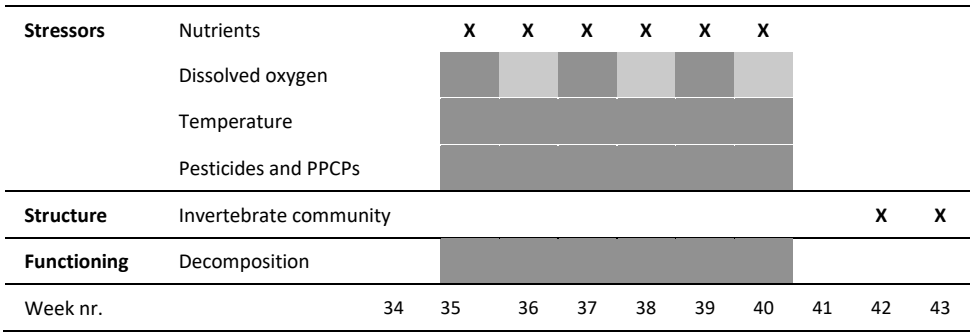
### Stressors

Nutrients – One surface water grab sample was collected weekly at each site for six weeks, filtered over a 1.2 µm filter and analyzed for total dissolved nitrogen (TDN) and orthophosphate ($PO_4$-P) on a continuous flow analyzer (SAN++ system, Skalar Analytical B.V., Breda, The Netherlands). The mean nutrient concentrations over the six weeks were calculated for further analysis.

**Table 1**: Overview of study sites: coordinates, water body dimensions, land use of the riparian zone (150m on both sides along 600m), and a proxy for the degree of WWTP effluent influence indicated by the gadolinium anomaly. Natural Gd* values in water bodies without any WWTP impact are around 1.3 (Rabiet et al. 2005, Petelet-Giraud et al. 2009). Details of methods in supplementary material 1.

| Coordinates (WGS 84) | | Water body dimensions | | | Land use riparian zone | | | Effluent influence |
|---|---|---|---|---|---|---|---|---|
| Latitude | Longitude | Depth (m) | Width (m) | Flow velocity (cm/s) | Agri-culture (%) | Nature (%) | Urban (%) | Gadolinium anomaly (Gd*) |
| 52°49'22.7"N | 5°54'26.5"E | 1.0 | 6.0 | 0.9 | 2 | 98 | 0 | 4.3 |
| 53°00'22.3"N | 5°48'43.4"E | 0.7 | 2.5 | 2.0 | 4 | 96 | 0 | 5.3 |
| 52°53'29.0"N | 4°49'34.8"E | 0.6 | 4.5 | 2.2 | 93 | 0 | 7 | 6.8 |
| 52°45'51.4"N | 4°40'52.0"E | 1.2 | 6.0 | 11.6 | 88 | 0 | 12 | 4.5 |
| 52°40'33.0"N | 4°50'02.3"E | 0.8 | 6.0 | 2.6 | 0 | 0 | 100 | 4.2 |
| 52°17'07.2"N | 4°32'34.6"E | 0.8 | 8.0 | 2.3 | 100 | 0 | 0 | 13.8 |
| 52°17'23.2"N | 4°30'37.7"E | 0.6 | 4.0 | 1.3 | 89 | 0 | 11 | 4.4 |
| 52°17'05.3"N | 4°29'54.7"E | 1.2 | 5.5 | 1.0 | 99 | 0 | 1 | 5.6 |
| 52°08'08.2"N | 4°48'37.6"E | 1.0 | 2.8 | 1.4 | 26 | 73 | 1 | 1.5 |
| 52°12'43.4"N | 4°53'10.6"E | 0.8 | 1.5 | 1.5 | 16 | 0 | 84 | 26.7 |
| 52°15'20.5"N | 5°05'15.2"E | 1.0 | 5.0 | 1.3 | 78 | 0 | 22 | 1.2 |
| 51°25'40.9"N | 4°46'46.8"E | 1.0 | 3.0 | 1.3 | 92 | 0 | 8 | 1.6 |
| 51°30'46.1"N | 4°50'57.2"E | 0.4 | 1.5 | 7.3 | 71 | 0 | 29 | 30.3 |
| 51°33'54.2"N | 4°59'13.1"E | 0.6 | 4.5 | 3.4 | 78 | 21 | 1 | 4.8 |
| 51°36'08.3"N | 5°04'32.9"E | 0.4 | 5.0 | 25.5 | 43 | 17 | 41 | 50.3 |
| 51°30'15.0"N | 5°10'19.9"E | 0.4 | 4.0 | 14.7 | 64 | 0 | 36 | 25.6 |
| 51°24'27.2"N | 5°41'37.0"E | 0.8 | 4.0 | 4.7 | 79 | 15 | 6 | 1.1 |
| 51°17'52.0"N | 5°36'18.8"E | 0.8 | 5.5 | 1.4 | 84 | 16 | 0 | 1.1 |
| 51°13'50.3"N | 5°37'31.4"E | 0.6 | 3.0 | 2.2 | 11 | 82 | 7 | 2.5 |
| 51°18'09.7"N | 5°29'09.6"E | 0.6 | 8.0 | 17.7 | 81 | 18 | 1 | 9.2 |

**Table 2**: Sampling scheme per week in 2018. A cross indicates a grab sample and a filled bar indicates a time-integrated sample. The dissolved oxygen measurements and invertebrates sampling was split between different weeks for the different sites, indicated by dark and light grey bars and crosses.



Dissolved oxygen and temperature – Dissolved oxygen (DO) concentrations (mg/L) were measured with optical HOBO® Dissolved Oxygen loggers U26-001, protected by the antifouling protective guard U26-GUARD-2 (Onset Computer Corporation, Bourne, MA, USA). Water temperature (°C) was measured with HOBO® Temperature/Light loggers UA-002-64 (Onset Computer Corporation, Bourne, MA, USA). Both Dissolved Oxygen and Temperature/Light loggers were placed mid-channel, 15 cm under the water surface (Van der Lee et al., 2018). Measurements were taken every ten minutes. Temperature/Light loggers were placed at each site for six weeks continuously, while the Dissolved Oxygen loggers were placed three times during the six week period for six consecutive days, rotating weekly between the sites (Table 2). Percent DO saturation was calculated from the DO concentrations and temperature, assuming 0 ‰ salinity and 1 atm barometric pressure, using DOTABLES developed by the U.S. Geological Survey (2011). Thereafter, the percent time that the dissolved oxygen levels were below 10 % saturation (DO < 10) was calculated, as many invertebrate taxa do not tolerate these low oxygen levels (Connolly et al., 2004).

Pesticides and PPCPs – Polar organic chemical integrative samplers (POCIS) containing 200 mg of Oasis hydrophilic-lipophilic balance sorbent (Waters, MA, USA) were applied for the sampling of polar compounds from the surface water (Alvarez et al., 2004; details in Supplementary material 2). At each site, four POCIS retained in stainless steel cages were deployed for six weeks in the middle of the water column. After field exposure, the POCIS were cleaned in the field with local water and a scrubbing sponge to remove any biofouling that had accumulated on the polyether sulfone membranes and stored at -20 °C. Frozen POCIS were freeze-dried overnight at −53 °C in a Scanvac CoolSafe freeze-dryer. Dry sorbent of the four POCIS per site was pooled and eluted three times with 3 mL LC grade acetonitrile under vacuum in a glass solid phase extraction column. Finally, the extracts were topped up to 10 mL with acetonitrile by weight and stored at -20°C until analysis.



POCIS acetonitrile extracts were subjected to three *in vitro* chemical activated luciferase gene expression (CALUX®) bioassays at the BioDetection Systems laboratories (Amsterdam, The Netherlands). Extracts were converted to dimethylsulphoxide before exposure in the bioassays. Estrogen receptor (ERα), androgen receptor antagonism (anti-AR) and progesterone receptor antagonism (anti-PR) CALUX assays were performed according to previously described protocols (Hamers et al., 2006; Sonneveld et al., 2004; Van der Linden et al., 2008). The activities of the extracts were expressed as bioanalytical equivalents of the corresponding reference compounds. Subsequently, the bioanalytical activities were divided by the effect-based trigger (EBT) value of each assay to obtain a measure of the ecotoxicological risk caused by the bioactive compounds present at the study sites (Brion et al., 2019; Escher et al. 2018; supplementary material 2 Table S1). ERα risk was considered to be a proxy for the presence of pharmaceuticals and personal care products (PPCPs) (Välitalo et al., 2016) and the mean of anti-AR and anti-PR risks was

considered to be a proxy for the presence of pesticides in the surface waters (Pieterse et al., 2015). In addition, we calculated a combined proxy for presence of organic toxicants using the mean of the proxy for PPCPs and pesticides. Although these proxies do not provide information on the concentrations of individual chemical compounds, they do provide a promising tool to interpret the harmful effects of groups of often (un)known, unregulated and unmonitored compounds present in surface waters (De Baat et al., 2019).

**Invertebrate community composition**

Three invertebrates samples were collected at each site on one occasion by sweeping a pond net (1mm mesh size, 25 cm width) three times over a length of 0.5 m of submerged vegetation (surface per sample = 0.125 $m^2$). The samples were stored overnight at 4°C with oxygen supply, washed over 1 mm and 250 µm sieves, sorted alive and preserved in 70% ethanol until identification. Invertebrates were identified to the genus level with a few exceptions, specifically Oligochaeta (order), Hydracarina (order) and Diptera (family). A total of 14968 individuals belonging to 94 invertebrate taxa were collected.

Numerous mathematical functions have been proposed for measuring diversity (Ludwig et al., 1988; Beisel et al., 2003), although few metrics have been specifically developed for small lowland water bodies with limited flow (Verdonschot et al., 2012). Here, we calculated richness in the simplest way as the total number of taxa in the sample (Ludwig et al., 1988). Evenness was estimated using the Smith & Wilson evenness (Evar) index, which describes the species abundance distributions using statistics to avoid dependence on species richness (Smith & Wilson, 1996; Heip et al., 1998). A commonly used water quality metric based on community composition in streams is the number or relative abundance of Ephemeroptera, Plecoptera and Trichoptera taxa, as these orders are considered sensitive to many pollutants in streams (Wallace et al., 1996; Norris & Thoms, 1999). However, these EPT metrics were not considered to be suitable for application in our drainage ditches, as Plecoptera are mostly absent from these types of water bodies and Ephemeroptera are represented by a few abundant insensitive species (Verdonschot et al., 2012). Hence, as alternative to the EPT-index for drainage ditches we used the number of Trichoptera families as taxonomic metric based on community composition, which was as recommended by Verdonschot et al. (2012).

As trait-based approach, we calculated the $SPEAR_{pesticides}$ index using the SPEAR calculator 2019.10 (Version 1.1.1) as implemented in www.systemecology.eu/indicate (Liess & Von der Ohe, 2005, recently revised by Knillmann et al., 2018). The $SPEAR_{organic}$ was calculated using the same calculator, but excluding the life history traits, i.e. manually setting all traits except for the taxon-specific sensitivities to organic contaminants to sensitive. The mean of each metric was calculated over the replicate samples per site for further analysis.

**Microbial decomposition and invertebrate consumption**

Decomposition was measured using standard substrates, the DEcomposition and COnsumption TABlets (DECOTABs) (Kampfraath et al., 2012). The DECOTABs were prepared by boiling 20 g/L of purified agar dissolved in deionized water for 3 minutes. The mixture was cooled down under continuous stirring to 60 °C at which point 60 g/L of powdered cellulose and 60 µmol/L ascorbic acid were added. The mixture was then poured into polycarbonate moulds (35 mm diameter, 6.7 $cm^3$ volume) and after cooling the DECOTABs were removed from the moulds and stored at 7 °C. The DECOTABs were deployed in cages (height 2 cm, diameter 10 cm) placed 15 cm under the water surface. To quantify decomposition by microbes the cages were closed off with fine mesh (width 51 µm) on both sides and to quantify the joint microbial decomposition and invertebrate consumption the cages were closed off at the top with coarse mesh (width 4 mm) and at the bottom with fine mesh to collect incompletely decayed DECOTAB fragments mobilized by decomposer activity (Brinson et al., 1981). At each site, five fine and five coarse mesh cages were deployed, each containing two DECOTABs. The DECOTAB cages were lost at one site. For the other sites, the cages were retrieved after 42 days of field exposure. In the laboratory, the DECOTABs were rinsed, dried in a stove (70 °C, 2 days) and weighed. Mass loss was calculated as mean initial DECOTAB weight (subset of 40 DECOTABs not deployed in the field) minus the individual DECOTAB weight after exposure in the field. Microbial decomposition was defined as the mass loss of the DECOTABs in the fine mesh cages. Invertebrate consumption was calculated by subtracting the mean mass loss of the DECOTABs in fine mesh cages from the mass loss of the DECOTABs in the coarse mesh cages. The mean mass loss was then calculated over the replicate DECOTAB cages per site for further analysis.



**Statistical analyses**

To test for collinearity between the measured stressors (i.e. co-occurrence of stressors at each sampling site) a Pearson correlation analysis was performed. To meet the assumption of normal distribution for this analysis, all stressor variables were $\log_{10}(x+1)$–transformed, except for the DO < 10 % which was logit transformed. Then, the relation between the different structural and functional end-points and stressors was analyzed using single- and two-variable regression analysis. For the single-variable models, we related each end-point and stressor using linear and unimodal (i.e. univariate quadratic function) relations. Unimodal relations were considered, as moderate amounts of stress may increase diversity and organic matter breakdown, while they may be suppressed under influence of high stress levels (Niyogi et al., 2002, Woodward et al., 2010). For the two-variable models, we related each end-point to each pair of stressors. The fits of the single (linear and unimodal) and two-variable models to the data were compared by using the small-sample version of

the Akaike Information Criterion (AICc). When the difference between the fits was relatively small (ΔAICc < 4) the simplest (i.e. linear) model was selected (Burnham & Anderson, 2004). For the two-variable models the variance inflation factor (VIF) was computed to estimate how much of the variance of a regression coefficient is inflated due to multicollinearity in the model, with VIF > 4 indicative of multicollinearity (Miles & Shevlin, 2001). The proxy for organic toxicants was only considered in the single-variable models, as it is a composite measure of the other measured toxic stressors. For the regression analysis the data were not transformed, as the residuals were approximately normally distributed (visual inspection of quantile-quantile plot). As we conducted analyses on seven independent variables, Bonferroni correction was applied to correct for multiple hypothesis testing (significance level of 0.05/7 = 0.007). All analysis were performed in R version 3.6.3 (R Core Team, 2019) using the 'codyn' package to calculate the Evar evenness index, the 'Hmisc' package to compute the Pearson correlation coefficients, the 'stats' package to fit the regression models, the 'AICcmodavg' package to compute the AICc, the 'cars' package to compute the VIFs.

## RESULTS

Several stressors were significantly positively correlated to each other (Table 3). The strongest correlation was between the proxy for pesticides and the $PO_4$-P concentrations ($r = 0.90$, $p = 0.001$). TDN concentrations were significantly correlated to all other stressors, except for the percent of time that the dissolved oxygen levels were below 10 % saturation. Moreover, water temperature was significantly correlated to the $PO_4$-P concentrations and the proxy for PPCPs.

For all metrics, the models fitted with a single stressor using the linear function performed better than the models using the unimodal function (Table 4) and the models including two stressors (Supplementary material 3 Table S1), except for the relation between microbial decomposition and the proxy for PPCPs, where the model using the unimodal function performed best (ΔAICc = 7.8). The structural metrics based on the taxa richness and the number of Trichoptera families did not relate to any of the stressors (Table 4, Figure 1). The evenness metric related significantly to the combined proxy for organic toxicants, i.e. the mean of the proxy for pesticides and the proxy for PPCPs. The variation in eveness values from the regression line reduced with higher values of organic toxicants. Both the $SPEAR_{pesticides}$ and $SPEAR_{organic}$ were also significantly related to the combined proxy for organic toxicants, although a higher proportion of variability was explained for the $SPEAR_{organic}$ ($R^2 = 0.57$, $p < 0.001$) than the $SPEAR_{pesticides}$ ($R^2 = 0.39$, $p = 0.002$). Moreover, the $SPEAR_{organic}$ also showed a negative relation to the proxy for PPCPs (Table 4, Figure 1).

The microbial decomposition related significantly to several stressors (Table 4, Figure 1), although the strongest fit was with the TDN concentrations using a positive linear

function ($R^2$ = 0.88, $p < 0.001$), followed by the more complex fit with the proxy for PPCPs using a unimodal function ($R^2$ = 0.69, $p < 0.001$). The invertebrate consumption showed a significant positive relation to the combined proxy for organic toxicants ($R^2$ = 0.60, $p < 0.001$) and the proxy for PPCPs ($R^2$ = 0.44, $p < 0.001$), as well as with TDN concentrations and water temperature when considering $p < 0.05$.

**Table 3**: Collinearity between stressors measured at 20 drainage ditches and lowland streams. Reported are the Pearson correlation coefficients with significant values in bold ($p < 0.05$). Stressors include total dissolved nitrogen (TDN) concentrations, orthophosphate ($PO_4$-P) concentrations, the percent time that the dissolved oxygen saturation is below 10 % (DO < 10), mean water temperature (temperature), the proxy for the presence of pesticides (pesticides) and the proxy the presence of pharmaceuticals and personal care products (PPCPs).

| | TDN | $PO_4$-P | DO < 10 | Temperature | PPCPs | Pesticides |
|---|---|---|---|---|---|---|
| TDN | 1 | **0.60** | 0.41 | **0.58** | **0.60** | **0.54** |
| $PO_4$-P | **0.60** | 1 | 0.37 | **0.45** | 0.13 | **0.90** |
| DO < 10 | 0.41 | 0.37 | 1 | 0.04 | 0.13 | 0.37 |
| Temperature | **0.58** | **0.45** | 0.04 | 1 | **0.56** | 0.35 |
| PPCPs | **0.60** | 0.13 | 0.13 | **0.56** | 1 | 0.09 |
| Pesticides | **0.54** | **0.90** | 0.37 | 0.35 | 0.09 | 1 |

## DISCUSSION



The present study compared the use of several structural (both taxonomic and trait-based) and functional metrics in water quality assessment of multi-stressed lowland water bodies. The sites were surrounded to a varying degree by agricultural land use and some sites directly received WWTP effluent. Yet, the gadolinium anomaly showed that several sites that did not directly receive WWTP discharge were also impacted by effluent to some extent through a WWTP higher upstream, sewage overflow, or the inlet of river water which is a common practice during dry summer months to retain a constant water level in The Netherlands (Verdonschot et al., 2012). These anthropogenic activities resulted in the combined presence of multiple stressors, including nutrient enrichment, increased water temperature and elevated proxies of pesticides and PPCPs. Due to the correlation and co-presence of these stressors, their individual contribution to changes in ecosystem structure and functioning could not be unraveled and neither could potential interactive effects between different stressors be determined (Ferreira et al., 2015; Piggott et al., 2015). For example, water temperature can affect the bioavailability of toxicants and thereby alter their subsequent effects on ecosystem structure and function (Peters et al., 2013).

**Table 4**: Regression statistics for single variable models using linear and univariate quadratic (Unim.) functions between each structural and functional metric and each stressor. When the difference between both fits was relatively small (ΔAICc < 4) the simplest (i.e. linear) model was selected (bold). Adjusted $R^2$ and *p*-value of selected best fitting models are presented (N = 20, except for microbial decomposition and invertebrate consumption N = 19). Significant regressions are presented in bold (Bonferroni corrected, p < 0.007). Stressors include total dissolved nitrogen (TDN) concentrations, orthophosphate (PO4-P) concentrations, the percent time that the dissolved oxygen saturation is below 10 % (DO < 10), mean water temperature (temperature), the proxy for the presence of pesticides (pesticides) and the proxy the presence of pharmaceuticals and personal care products (PPCPs), and proxy for combined presence of organic toxicants (Toxic).

| | **Taxonomic** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Richness (#)** | | | | | **Evenness (-)** | | | | | **Trichoptera fam. (#)** | | | | |
| | **AICc** | | | **Best model** | | **AICc** | | | **Best model** | | **AICc** | | | **Best model** | |
| | **Linear** | **Unim.** | **Δ** | **$R^2_{adj.}$** | ***p*** | **Linear** | **Unim.** | **Δ** | **$R^2_{adj.}$** | ***p*** | **Linear** | **Unim.** | **Δ** | **$R^2_{adj.}$** | ***p*** |
| TDN (mg/l) | **114.5** | 114.4 | 0.1 | 0.04 | 0.20 | **-22.8** | -20.3 | -2.5 | 0.11 | 0.08 | **47.6** | 50.6 | -3.0 | 0.07 | 0.13 |
| $PO_4$-P (mg/l) | **113.7** | 116.9 | -3.2 | 0.07 | 0.13 | **-20.0** | -17.7 | -2.3 | -0.02 | 0.47 | **49.8** | 50.6 | -0.8 | -0.04 | 0.59 |
| DO < 10 (%) | **116.3** | 117.8 | -1.5 | -0.05 | 0.80 | **-19.6** | -16.5 | -3.1 | -0.05 | 0.69 | **49.6** | 52.6 | -3.0 | -0.03 | 0.50 |
| Temperature (°C) | **115.7** | 114.1 | 1.6 | -0.02 | 0.43 | **-23.5** | -21.2 | -2.3 | 0.14 | 0.06 | **49.4** | 51.6 | -2.2 | -0.02 | 0.43 |
| PPCPs (%) | **114.5** | 115.8 | -1.3 | 0.04 | 0.21 | **-27.4** | -25.2 | -2.2 | 0.29 | 0.01 | **46.7** | 48.1 | -1.4 | 0.11 | 0.08 |
| Pesticides (%) | **113.3** | 115.5 | -2.3 | 0.10 | 0.10 | **-20.0** | -16.8 | -3.2 | -0.03 | 0.48 | **49.8** | 52.7 | -2.9 | -0.04 | 0.56 |
| Toxic (%) | **116.2** | 119.3 | -3.1 | -0.05 | 0.69 | **-29.8** | -27.0 | -2.8 | **0.37** | **0.003** | **46.6** | 48.3 | -2.7 | 0.16 | 0.05 |

| | **Trait-based** | | | | | | | | | | **Ecological process** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **$SPEAR_{pesticides}$ (-)** | | | | | **$SPEAR_{organic}$ (-)** | | | | | **Microbial decomposition (%)** | | | | | **Invertebrate consumption (%)** | | | | |
| | **AICc** | | | **Best model** | | **AICc** | | | **Best model** | | **AICc** | | | **Best model** | | **AICc** | | | **Best model** | |
| | **Linear** | **Unim.** | **Δ** | **$R^2_{adj.}$** | ***p*** | **Linear** | **Unim.** | **Δ** | **$R^2_{adj.}$** | ***p*** | **Linear** | **Unim.** | **Δ** | **$R^2_{adj.}$** | ***p*** | **Linear** | **Unim.** | **Δ** | **$R^2_{adj.}$** | ***p*** |
| TDN (mg/l) | **24.5** | 21.1 | 3.4 | -0.05 | 0.69 | **29.5** | 28.7 | 0.8 | -0.02 | 0.46 | **135.3** | 137.9 | -2.6 | **0.88** | **<0.001** | **160.7** | 162.2 | -1.5 | 0.17 | 0.04 |
| $PO_4$-P (mg/l) | **22.9** | 26.1 | -3.2 | 0.03 | 0.22 | **28.9** | 31.9 | -3.0 | 0.00 | 0.31 | **174.5** | 171.2 | 3.3 | 0.03 | 0.23 | **163.0** | 165.2 | -2.2 | 0.07 | 0.15 |
| DO < 10 (%) | **21.4** | 20.9 | 0.5 | 0.10 | 0.09 | **28.5** | 30.2 | -1.7 | 0.03 | 0.24 | **171.4** | 173.8 | -2.4 | 0.17 | 0.04 | **163.0** | 162.0 | 1.0 | 0.06 | 0.15 |
| Temperature (°C) | **24.6** | 26.9 | -2.3 | -0.05 | 0.79 | **27.6** | 29.5 | -2.1 | 0.07 | 0.14 | **168.7** | 171.9 | -3.2 | 0.28 | 0.01 | **160.4** | 160.6 | -0.2 | 0.18 | 0.04 |
| PPCPs (%) | **19.1** | 22.1 | -3.0 | 0.20 | 0.03 | **17.7** | 19.9 | -2.2 | **0.43** | **0.001** | 162.8 | **154.9** | 7.9 | **0.69** | **<0.001** | **153.4** | 156.4 | -3.0 | **0.44** | **<0.001** |
| Pesticides (%) | **22.1** | 24.7 | -2.6 | 0.07 | 0.13 | **29.0** | 31.0 | -2.0 | 0.00 | 0.32 | **175.9** | 178.4 | -2.5 | -0.04 | 0.62 | **164.0** | 167.1 | -3.1 | 0.01 | 0.28 |
| Toxic (%) | **13.7** | 16.7 | -3.0 | **0.39** | **0.002** | **12.0** | 12.2 | -0.2 | **0.57** | **<0.001** | **160.7** | 159.6 | 1.2 | **0.52** | **<0.001** | **146.9** | 149.9 | -3.0 | **0.60** | **<0.001** |

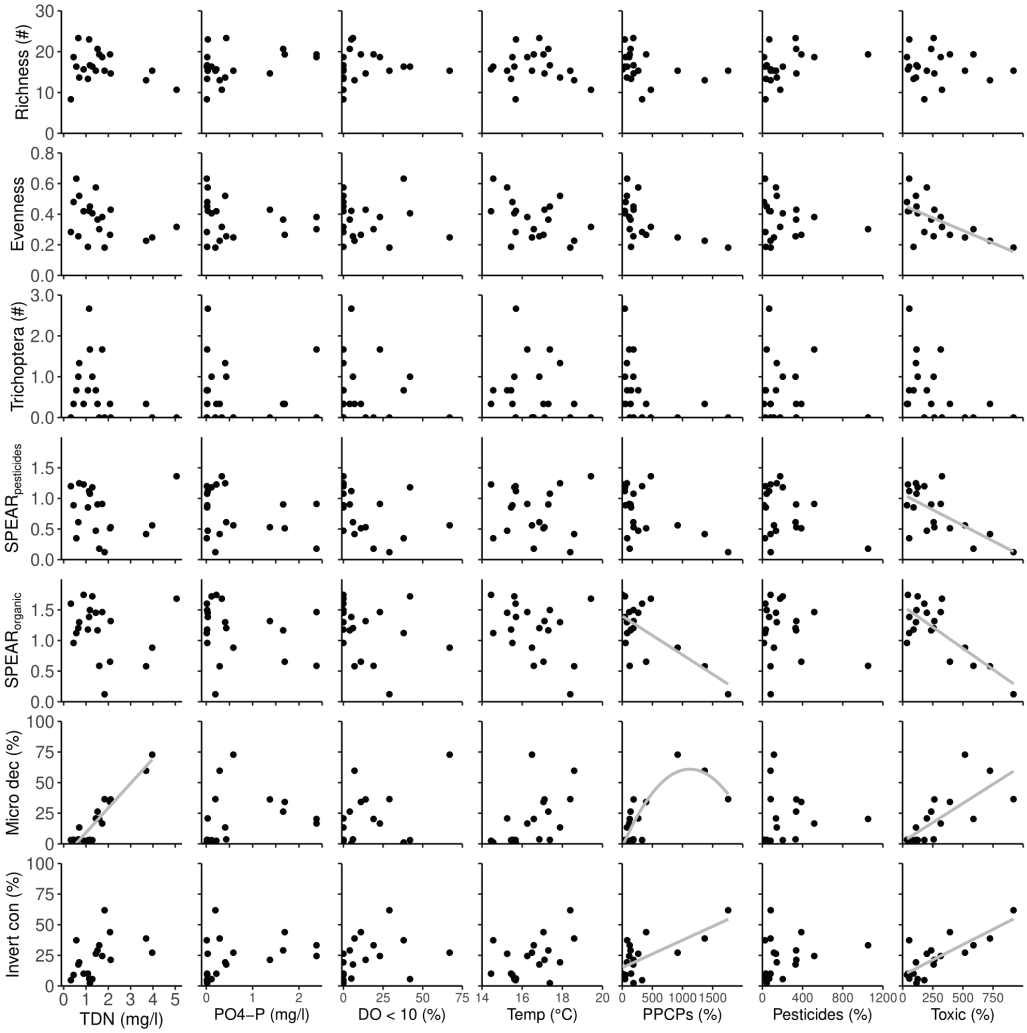



***Figure 1**: Relationships between structural and functional metrics and different stressors. Lines indicate significant regressions using best fitting linear or univariate quadratic function (Bonferroni corrected significance level of p < 0.007; N = 20, except for microbial decomposition and invertebrate consumption N = 19). See Table 4 for the details on regression statistics and abbreviations of labels*

To disentangle individual and combined effects of specific stressors on ecosystem structure and functioning other approaches may be needed, like mesocosm experiments (e.g. Townsend et al., 2008; Pigott et al., 2012). Since the combined presence of multiple stressors is common in water bodies in densely populated areas (Allan, 2004; Ormerod et al., 2010), the design of the present study did allow us to compare the ability of structural and functional metrics to 1) detect adverse effects from anthropogenic stress on aquatic ecosystems, and 2) diagnose the potential causes of the observed adverse effects in water quality assessment under realistic field conditions.

In line with our hypothesis, the process-based metrics detected patterns of anthropogenic stress that were not evident from the structural metrics alone. Specifically, the microbial decomposition and invertebrate consumption showed an opposite and more complex relation to the stressors than the evenness metric, which was the only structural metric that showed a significant response in our study. Similar to previous studies, the microbial decomposition was stimulated by an increase in dissolved inorganic nutrient availability (see review by Ferreira et al., 2015). The DECOTABs used in this study, consisting only of cellulose, may be particularly sensitive to nutrient gradients in the water column, as microbes need to assimilate nutrients from the water column when decomposing low-nutrient substrates (Gulis et al., 2006; Schäfer et al., 2012). The microbial decomposition showed a unimodal relation to the proxy for PPCPs. The negative effects of toxicants on microbial decomposition may be limited by the replacement of sensitive species with tolerant ones, maintaining their function as decomposers (Blanck, 2002). Supporting this line of reasoning, no negative effects on microbial organic matter breakdown rates were observed in the laboratory when mixtures of pharmaceuticals (Hughes et al., 2016) and pesticides (Feckler et al., 2018) were added to microbial communities from disturbed sites. The invertebrate consumption was positively related to the proxy for organic toxicants and to a lesser extent TDN concentrations and water temperature. This finding is in contrast with other studies that have frequently observed impaired invertebrate consumption rates in relation to stress from agricultural activities (e.g. Lecerf et al., 2006; Schäfer et al., 2007; Piscart et al., 2009; Schäfer et al., 2012) and WWTP discharges (e.g. Englert et al., 2013, Münze et al., 2017). Possible explanations for the difference in functional response may relate to the characteristics of the receiving water bodies, variation in the composition of the stressors and the sensitivity of the dominant decomposer invertebrate species (Dangles & Malmqvist, 2004, Hagen et al. 2006, Solagaistua et al., 2018). The impact of anthropogenic stress on ecosystem processes thus appears to be context-dependent, meaning that the relation between taxonomic-based and functional metrics is not always straightforward. Therefore, functional metrics may provide additional information on degradation of the water bodies compared to only using structural metrics.

In terms of diagnostic value, the results of this study indicated that the $SPEAR_{pesticides}$ and $SPEAR_{organic}$ can both potentially be used to diagnose the combined presence of organic toxicants from agricultural activities and WWTPs discharges, however, they may have limited application in differentiating between both sources of pollution. Various studies have suggested that the $SPEAR_{pesticides}$ relates specifically to pesticide exposure, as the calculation includes life history traits that differentiate a taxon's ability to recover from pulses of contamination typical of pesticide exposure (e.g. Schäfer et al. 2007, Beketov et al., 2019, Knillmann et al. 2018). However, the results from the present study suggest that the $SPEAR_{pesticides}$ may also relate to other organic toxicants in WWTPs discharges (e.g. PPCPs), although we cannot exclude that the invertebrates at these sites did not integrate effects of pesticide peaks in WWTP discharges prior to the sampling period. Munz et al. (2017), for example, reported a high number of pesticide peaks in WWTP effluent in May and July, and considered these pesticide peaks the main explanatory factor for lowered $SPEAR_{pesticide}$ values. Various studies used the $SPEAR_{pesticides}$ to detect impact of WWTP discharges in flowing water, more or less depreciating the $SPEAR_{organic}$ (Burdon et al. 2016, Burdon et al. 2019). However, our results showed that a higher proportion of the proxy of organic toxicants was explained by the $SPEAR_{organic}$ than by the $SPEAR_{pesticides}$. One explanation could be that the reduced flow and hydro-morphological habitat degradation of lowland water bodies, including channelization, dredging and riparian vegetation removal and control, may have hampered the colonization of the sensitive $SPEAR_{pesticide}$ taxa, irrespective of the presence of organic toxicants (Rasmussen et al. 2012a). To distinguish between different sources of pollution, the SPEAR approach may need to be combined with other methods, like the quantification of sources of stress based on land use maps (De Vries et al. 2019) and using proxies for WWTP discharge such as the gadolinium anomaly used in this study. Moreover, the taxonomic-based evenness metric showed a wider variation in values at the sites with a low toxic pollution than at high toxic pollution, indicating that there may have been other (unmeasured) stressors that impacted the invertebrates. Therefore, it would be a valuable addition to future research efforts to also test the applicability of SPEAR metrics designed for other types of stressors in multi-stressed lowland water bodies, such as heavy metal pollution (Malaj et al. 2012) and salinity (Schäfer et al. 2011).

The selection of metrics should depend on the goals of the water quality assessment approach. If the goal is to detect adverse effects from anthropogenic stress on both ecosystem structure and functioning, we recommend to include process-based metrics besides taxonomic-based metrics, as their relation with anthropogenic stress may be opposite to the taxonomic-metrics and more complex. If the goal is to diagnose the potential causes of the observed adverse effects, the application of the $SPEAR_{organic}$ and $SPEAR_{pesticides}$ seems promising in distinguishing elevated levels of toxic pollution, although

this trait-based approach should still be verified for other types of stressors in multi-stressed lowland waters. If an additional goal is to identify sources of stress (i.e. determine whether the toxic pollution derived from agricultural land use or WWTP discharges), the SPEAR approach may need to be combined with other methods, extracting information from land use maps and proxies such as the gadolinium anomaly. In conlcusion, the taxonomic, trait-based and functional metrics may provide complementary information that, when integrated, allow for more thorough water quality assessment strategies.

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## SUPPLEMENTARY MATERIAL 1

**Water body dimensions**

Water body size (width and depth) and flow velocity were measured on a single occasion. Flow velocity was measured in the middle of the water body with an electromagnetic current meter (Valeport model 802, Devon, UK).

**Land use of the riparian zone**

The land use of the riparian zone (i.e. nature including forest, agriculture, or urban) was taken from the LGN5 map, providing land use types in 2003 and 2004 using the clipping function in ArcMap (Hazeu, 2005). The riparian zone was delineated as a zone of 150 m on both sides of the sampling site along a length of 600 m upstream in lotic waters and from the middle in lentic waters.

**Wastewater treatment plant effluent influence**

A proxy for the degree of direct and indirect effluent input from wastewater treatment plants (WWTPs) was quantified using the gadolinium anomaly (Gd*) in the water. Gadolinium (Gd) is a rare earth element (REE) that is used as a paramagnetic contrast agent in the form of stable Gd complexes in medical magnetic-resonance imaging (MRI). REEs have a specific geochemical behavior and occur in strict ratios with very little naturally occurring variation (Kulaksız & Bau, 2011). Gd complexes are excreted by people within hours after a MRI scan, and pass through the WWTP almost unchanged, thus providing a good tracer for WWTP effluent in surface waters (Rabiet et al., 2005; Petelet-Giraud et al., 2009).

One surface water grab sample was collected each week for six weeks, and 8 mL of each of the weekly samples were combined to obtain a composite sample per site. These composite samples were filtered over 0.45 µm filters, after which the filtrate was acidified with 0.334 mL 65% $HNO_3$ (Suprapur®) and stored at 4 °C until analysis on a Thermo Scientific i-Cap ICP-MS, with an APEX ESI (Omaha, US) sample introduction system. The geogenic background concentration of Gd ($Gd_{geo}$) was calculated for each sample individually, based on the presence of at least five other REEs in the sample which are naturally occurring and unaffected by anthropogenic activities. The gadolinium anomaly, i.e. Gd enrichment compared to $Gd_{geo}$, was calculated as follows: $Gd^* = Gd_{measured} / Gd_{geo}$ (Kulaksız & Bau, 2011). Natural Gd* values in water bodies without any WWTP impact are around 1.3 (Rabiet et al., 2005; Petelet-Giraud et al., 2009).

## SUPPLEMENTARY MATERIAL 2

POCIS were constructed using two stainless steel rings, with an inner diameter of 5.4 cm, to retain sorbent between two membranes, leaving approximately 46 $cm^2$ of surface area exposed to the surrounding water. Stainless steel rings (Exposmeter, Sweden), nuts and bolts, as well as all used tools were cleaned in acetone before assembly of the samplers. Polyether sulfone (PES) diffusion limiting membrane filters (Pall Corporation, NY, USA, 0.1 µm pore size, 90 mm diameter) were used to enclose the sorbent, and were cleaned before POCIS assembly in HPLC grade methanol:ultra-pure water (50:50, v:v) followed by rinsing in ultra-pure water. As a receiving phase, 200 mg of Oasis hydrophilic-lipophilic balance (HLB) sorbent (Waters, MA, USA) was enclosed between the PES membranes. The HLB was conditioned in its original column by sequentially eluting with 40 mL acetone, 40 mL dichloromethane and 40 mL methanol (Biosolve, The Netherlands, all chromatography grade) and dried under vacuum, followed by final assembly of the POCIS. POCIS were stored at 4 °C in food-grade Mylar zip lock bags until deployment.

At each site, four POCIS retained in stainless steel cages were deployed for six weeks in the middle of the water column. After field exposure, the POCIS were cleaned in the field with local water and a scrubbing sponge to remove any biofouling that had accumulated on the polyether sulfone membranes, and stored at -20 °C. Frozen POCIS were freeze-dried overnight at −53 °C in a Scanvac CoolSafe freeze-dryer. Dry sorbent of the four POCIS per site was pooled and eluted three times with 3 mL LC grade acetonitrile under vacuum in a glass solid phase extraction column. Finally, the extracts were topped up to 10 mL with acetonitrile by weight, and stored at -20°C until analyses.

POCIS acetonitrile extracts were subjected to three *in vitro* chemical activated luciferase gene expression (CALUX®) bioassays at the BioDetection Systems laboratories (Amsterdam, The Netherlands). Extracts were converted to dimethylsulphoxide before exposure in the bioassays. Estrogen receptor (ERα), androgen receptor antagonism (anti-AR) and progesterone receptor antagonism (anti-PR) CALUX assays were performed according to previously described protocols (Hamers et al., 2006; Sonneveld et al., 2004; Van der Linden et al., 2008). The activities of the extracts were expressed as bioanalytical equivalents of the corresponding reference compounds. Subsequently, the bioanalytical activities were divided by the effect-based trigger (EBT) value of each assay to obtain a measure of the ecotoxicological risk caused by bioactive compounds present at the study sites (Brion et al., 2019; Escher et al., 2018; Table S1).

ERα risk was considered a proxy for the presence of pharmaceuticals and personal care products (Välitalo et al., 2016), and the mean of anti-AR and anti-PR risks was considered a proxy for the presence of pesticides in the surface waters (Pieterse et al., 2015). In addition, we calculated a combined proxy for toxic pollution using the mean of the proxy for PPCPs and pesticides. Although these proxies do not provide information on the

concentrations of individual chemical compounds, they do provide a promising tool to interpret the harmful effects of groups of often (un)known, unregulated and unmonitored compounds present in surface waters (De Baat et al. 2019).

**Table S1**: Overview of CALUX assays performed on POCIS extracts. Effect-based trigger (EBT) values were previously defined by Brion et al. 2019 (ERα) and Escher et al. 2018 (anti-AR and anti-PR).

| CALUX assay | Endpoint | Reference compound | EBT | Unit |
|---|---|---|---|---|
| ERα | Estrogenic activity | 17β-estradiol | 0.28 | ng EEQ/L |
| anti-AR | Antiandrogenic activity | flutamide | 14.4 | µg FEQ/L |
| anti-PR | Antiprogestogenic activity | Ru486 | 0.013 | µg REQ/ml |

## SUPPLEMENTARY MATERIAL 3

**Table S1**: Regression statistics for two- variable models using linear functions between each structural and functional metric and each pair of stressors. A variance inflation factor (VIF) > 4 is indicative of multicollinearity. The ΔAICc was calculated against the best fitting single variable model (see Table 4, main text). Stressors include total dissolved nitrogen (TDN) concentrations, orthophosphate (PO4-P) concentrations, the percent time that the dissolved oxygen saturation is below 10 % (DO < 10), mean water temperature (temperature), the proxy for the presence of pesticides (pesticides) and the proxy the presence of pharmaceuticals and personal care products (PPCPs).

| | | VIF | ΔAICc | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Richness (#) | Evenness (-) | Trichoptera (#) | $SPEAR_{pesticides}$ (-) | $SPEAR_{organic}$ (-) | Microbial decomposition (%) | Invertebrate consumption (%) |
| TDN | PO4-P | 1.03 | -0.5 | -9.8 | -4.3 | -12.3 | -19.6 | -3.1 | -15.8 |
| TDN | DO < 10 | 1.08 | -3.9 | -10.1 | -4.3 | -10.9 | -19.4 | -3.3 | -16.7 |
| TDN | Temperature | 1.70 | -4.4 | -8.9 | -4.4 | -13.9 | -18.6 | -0.2 | -14.9 |
| TDN | PPCPs | 1.39 | -3.8 | -5.2 | -2.9 | -7.5 | -7.0 | 0.4 | -9.7 |
| TDN | Pesticides | 1.01 | -0.5 | -9.7 | -4.2 | -11.4 | -19.6 | -3.3 | -16.0 |
| PO4-P | DO < 10 | 1.02 | -3.6 | -12.8 | -6.3 | -9.5 | -18.7 | -38.0 | -17.3 |
| PO4-P | Temperature | 1.04 | -2.1 | -9.3 | -6.1 | -12.4 | -18.0 | -36.0 | -15.5 |
| PO4-P | PPCPs | 1.02 | -2.1 | -3.7 | -2.8 | -4.9 | -4.4 | -24.2 | -1.6 |
| PO4-P | Pesticides | 4.17 | -3.1 | -12.9 | -6.6 | -11.5 | -20.0 | -40.9 | -19.2 |
| DO < 10 | Temperature | 1.01 | -5.5 | -9.0 | -5.6 | -10.7 | -16.3 | -28.5 | -13.4 |
| DO < 10 | PPCPs | 1.10 | -3.9 | -5.4 | -3.5 | -6.8 | -8.7 | -28.0 | -9.2 |
| DO < 10 | Pesticides | 1.01 | -3.2 | -12.9 | -6.2 | -8.5 | -18.7 | -39.2 | -18.3 |
| Temperature | PPCPs | 1.46 | -4.4 | -5.1 | -3.5 | -6.6 | -8.8 | -29.6 | -9.6 |
| Temperature | Pesticides | 1.02 | -1.8 | -9.2 | -6.0 | -11.5 | -17.9 | -36.6 | -16.0 |
| PPCPs | Pesticides | 1.04 | -1.9 | -3.1 | -2.5 | -2.4 | -19.6 | -28.0 | -3.2 |