



## Disentangling multiple chemical and non-chemical stressors in a lotic ecosystem using a longitudinal approach



Markus Weitere<sup>a,\*</sup>, Rolf Altenburger<sup>b,h</sup>, Christine Anlanger<sup>a</sup>, Martina Baborowski<sup>a</sup>, Ilona Bärlund<sup>c</sup>, Liza-Marie Beckers<sup>d,3</sup>, Dietrich Borchardt<sup>c</sup>, Werner Brack<sup>d,h,n</sup>, Lisa Brase<sup>e,2</sup>, Wibke Busch<sup>b</sup>, Antonis Chatzinotas<sup>f,g</sup>, Björn Deutschmann<sup>h</sup>, Jens Eligehausen<sup>c,i</sup>, Karin Frank<sup>j</sup>, Daniel Graeber<sup>c</sup>, Christian Griebler<sup>k,l</sup>, Jeske Hagemann<sup>c</sup>, Peter Herzsprung<sup>m</sup>, Henner Hollert<sup>h,n</sup>, Pedro A. Inostroza<sup>d,1</sup>, Christoph G. Jäger<sup>c,o</sup>, René Kallies<sup>f</sup>, Norbert Kamjunke<sup>a</sup>, Bernhard Karrasch<sup>a</sup>, Sigrid Kaschuba<sup>k</sup>, Andrew Kaus<sup>c</sup>, Bernd Klauer<sup>p</sup>, Kay Knöller<sup>q</sup>, Matthias Koschorreck<sup>m</sup>, Martin Krauss<sup>d</sup>, Julia V. Kunz<sup>c</sup>, Marie J. Kurz<sup>r,4</sup>, Matthias Liess<sup>s,t</sup>, Margarete Mages<sup>a</sup>, Christin Müller<sup>q</sup>, Matthias Muschket<sup>d</sup>, Andreas Musolff<sup>r</sup>, Helge Norf<sup>a,c</sup>, Florian Pöhlein<sup>m</sup>, Lena Reiber<sup>s,t</sup>, Ute Risse-Buhl<sup>a</sup>, Karl-Werner Schramm<sup>u,v</sup>, Mechthild Schmitt-Jansen<sup>b</sup>, Markus Schmitz<sup>h,n</sup>, Ulrike Strachauer<sup>c</sup>, Wolf von Tümpling<sup>a</sup>, Nina Weber<sup>k</sup>, Romy Wild<sup>a</sup>, Christine Wolf<sup>p</sup>, Mario Brauns<sup>a</sup>

<sup>a</sup> Helmholtz Centre for Environmental Research – UFZ, Department River Ecology, Brückstr. 3a, 39114 Magdeburg, Germany

<sup>b</sup> Helmholtz Centre for Environmental Research – UFZ, Department of Bioanalytical Ecotoxicology, Permoserstr. 15, 04318 Leipzig, Germany

<sup>c</sup> Helmholtz Centre for Environmental Research – UFZ, Department of Aquatic Ecosystems Analysis, Brückstr. 3a, 39114 Magdeburg, Germany

<sup>d</sup> Helmholtz Centre for Environmental Research – UFZ, Department of Effect-Directed Analysis, Permoserstr. 15, 04318 Leipzig, Germany, PAI

<sup>e</sup> Helmholtz Centre Geesthacht – HZG, Department of Aquatic Nutrient Cycles, Max-Planck-Straße 1, 21502 Geesthacht, Germany

<sup>f</sup> Helmholtz Centre for Environmental Research – UFZ, Department of Environmental Microbiology, Permoserstr. 15, 04318 Leipzig, Germany

<sup>g</sup> Leipzig University, Institute of Biology, Talstrasse 33, 04103 Leipzig, Germany

<sup>h</sup> RWTH Aachen University, Institute for Environmental Research, Worringer Weg 1, 52074 Aachen, Germany

<sup>i</sup> University of Kassel, Department of Landscape Ecology, Gottschalkstr. 26A, 34127 Kassel, Germany

<sup>j</sup> Helmholtz Centre for Environmental Research – UFZ, Department of Ecological Modelling, Permoserstr. 15, 04318 Leipzig, Germany

<sup>k</sup> Helmholtz Zentrum München – German Research Center for Environmental Health (GmbH), Institute of Groundwater Ecology, Ingolstädter Landstrasse 1, 85764 Neuherberg, Germany

<sup>l</sup> University of Vienna, Department for Functional and Evolutionary Ecology, Althanstrasse 14, 1090 Wien, Austria

<sup>m</sup> Helmholtz Centre for Environmental Research – UFZ, Department Lake Research, Brückstr. 3a, 39114 Magdeburg, Germany

<sup>n</sup> Goethe University Frankfurt, Department of Evolutionary Ecology and Environmental Toxicology, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany

<sup>o</sup> Rosenheim Technical University of Applied Sciences, Centre for Research, Development and Technology Transfer, Hochschulstraße 1, 83024 Rosenheim, Germany

<sup>p</sup> Helmholtz Centre for Environmental Research – UFZ, Department of Economics, Permoserstraße 15, 04318 Leipzig, Germany

<sup>q</sup> Helmholtz Centre for Environmental Research – UFZ, Department Catchment Hydrology, Theodor-Lieser-Str. 4, 06120 Halle, Germany

<sup>r</sup> Helmholtz Centre for Environmental Research – UFZ, Department Hydrogeology, Permoserstraße 15, 04318 Leipzig, Germany

<sup>s</sup> Helmholtz Centre for Environmental Research – UFZ, Department of System-Ecotoxicology, Permoserstr. 15, 04318 Leipzig, Germany

<sup>t</sup> RWTH Aachen University, Institute for Environmental Research (Biology V), Aachen, Germany

<sup>u</sup> Helmholtz Zentrum München-German Research Center for Environmental Health (GmbH), Molecular EXposomics, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

<sup>v</sup> Technische Universität München, Department für Biowissenschaftliche Grundlagen, Weihenstephaner Steig 23, 85350 Freising, Germany

\* Corresponding author.

E-mail address: [markus.weitere@ufz.de](mailto:markus.weitere@ufz.de) (M. Weitere).

<sup>1</sup> Present address: Department of Biological and Environmental Sciences, University of Gothenburg, Box 461, SE-405 30 Gothenburg, Sweden.

<sup>2</sup> Present address: Universität Hamburg, Center for Sustainable Research Data Management, Monetastraße 4, 20146 Hamburg.

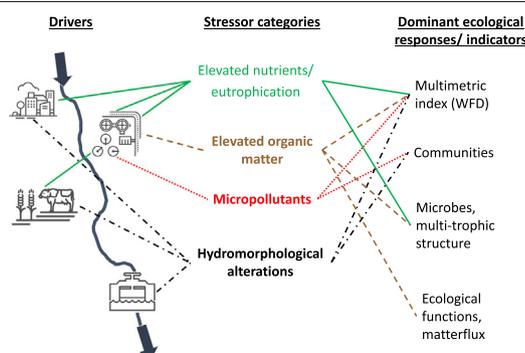
<sup>3</sup> Present address: Department of Aquatic Chemistry, Federal Institute of Hydrology (BfG), Am Mainzer Tor 1, 56068 Koblenz, Germany.

<sup>4</sup> Present address: The Academy of Natural Sciences of Drexel University, Patrick Center for Environmental Research, 1900 Benjamin Franklin Parkway, Philadelphia, PA, 19103, USA.

## HIGHLIGHTS

- Stressor (chemical/non-chemical) analyses in a stream under real-world conditions
- Effect and component-based chemical assessment combined with ecosystem analyses
- Wastewater treatment plant (WWTP) emitted micropollutants and affects communities
- Microbial density and function as important indicators for organic matter from WWTP
- Functional and multi-trophic assessment suitable to disentangle multiple stressors

## GRAPHICAL ABSTRACT



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## ABSTRACT

Meeting ecological and water quality standards in lotic ecosystems is often failed due to multiple stressors. However, disentangling stressor effects and identifying relevant stressor-effect-relationships in complex environmental settings remain major challenges. By combining state-of-the-art methods from ecotoxicology and aquatic ecosystem analysis, we aimed here to disentangle the effects of multiple chemical and non-chemical stressors along a longitudinal land use gradient in a third-order river in Germany. We distinguished and evaluated four dominant stressor categories along this gradient: (1) Hydromorphological alterations: Flow diversity and substrate diversity correlated with the EU-Water Framework Directive based indicators for the quality element macroinvertebrates, which deteriorated at the transition from near-natural reference sites to urban sites. (2) Elevated nutrient levels and eutrophication: Low to moderate nutrient concentrations together with complete canopy cover at the reference sites correlated with low densities of benthic algae (biofilms). We found no more systematic relation of algal density with nutrient concentrations at the downstream sites, suggesting that limiting concentrations are exceeded already at moderate nutrient concentrations and reduced shading by riparian vegetation. (3) Elevated organic matter levels: Wastewater treatment plants (WWTP) and stormwater drainage systems were the primary sources of bioavailable dissolved organic carbon. Consequently, planktonic bacterial production and especially extracellular enzyme activity increased downstream of those effluents showing local peaks. (4) Micropollutants and toxicity-related stress: WWTPs were the predominant source of toxic stress, resulting in a rapid increase of the toxicity for invertebrates and algae with only one order of magnitude below the acute toxic levels. This toxicity correlates negatively with the contribution of invertebrate species being sensitive towards pesticides (SPEAR<sub>pesticides</sub> index), probably contributing to the loss of biodiversity recorded in response to WWTP effluents. Our longitudinal approach highlights the potential of coordinated community efforts in supplementing established monitoring methods to tackle the complex phenomenon of multiple stress.

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## 1. Introduction

Surface water ecosystems are often altered by multiple stressors among which hydrological and morphological alterations, high nutrient loading and corresponding eutrophication, invasive species, as well as chemical pollution from both urban and agricultural origin, are common (e.g., Nöges et al., 2016; Grizzetti et al., 2017). Large-scale studies revealed that 90% of European lowland rivers are multi-impacted and that patterns of impacts vary among ecoregions in Europe (Schinegger et al., 2012). Schäfer et al. (2016) made a first step towards the assessment of potential cumulative risks from habitat degradation, nutrients, invasive species and organic toxicants in running waters and revealed that more than 80% of sites are at risk from at least three stressors, confirming the findings of Schinegger et al. (2012). A recent study on the European scale demonstrated that rivers are more often affected by multiple stressor effects (additive and interactive effects) compared to lakes, in which nutrient enrichment is more often the overriding stressor (Birk et al., 2020). As a consequence of anthropogenic stressor impacts, many water bodies fail to reach ecological quality standards.

On the European scale, for example, more than 60% of water bodies do not reach the target “good ecological status” (EEA, 2018).

The central importance of multiple stressors in altering the ecological status of aquatic ecosystems is increasingly recognized and addressed in both individual and coordinated research projects (e.g., Townsend et al., 2008; Hering et al., 2015; Navarro-Ortega et al., 2015; Birk et al., 2020). Despite this substantial community effort, it is still a significant challenge for management-oriented research to identify relevant stressors (i.e., those which substantially affect ecological response variables) in complex environmental settings. Tackling multiple stressor effects requires a set of different approaches. Factorial experimental approaches have a strong potential to identify stressor effects and often highlight the role of stressor interactions (e.g., Townsend et al., 2008; Rotter et al., 2013; Schmitt-Jansen et al., 2016; Graeber et al., 2017; Beermann et al., 2018; Polst et al., 2018). Data-driven statistical analyses of large monitoring programs from catchment to national and continental scale (e.g., Leps et al., 2015; Feld et al., 2016; Birk et al., 2020) have significantly advanced our understanding of multiple stressor effects in real-world scenarios. However, such approaches

strongly depend on the available data and their outcome is at least partly dependent on the type and quality of input data, both with respect to stressor variables and to appropriate response variables describing the aquatic ecosystem. Given the complex nature of multiple stressor effects, it is important to apply a comprehensive characterization of stressor variables as well as ecosystem response variables jointly, using state-of-the-art approaches. This requires data-dense model field sites. Here we see critical shortcomings of established multiple stressor analyses based on regular monitoring programs concerning both stressor and response variables:

With respect to stressor variables, the effect of chemical stress is often underrepresented relative to non-chemical stress. Holistic approaches which aim to detect relevant stressors in multi-stressor contexts often consider chemical pollution by highly aggregated parameters such as “agriculture/ urban land use” or “physicochemical conditions” (e.g., Leps et al., 2015; Gieswein et al., 2017). This does not allow specific conclusions on chemical effects. Often only a selected number of chemicals (priority substances) is considered by specific chemical analyses in monitoring programs, representing only a tiny proportion of the more than 100,000 organic chemicals of non-natural origin that are registered in the EU and may enter the aquatic environment (Schwarzenbach et al., 2006). Analysing stressor-response-relationships based on the concentrations from such a selective list strongly underestimates the effects of chemicals ignoring the vast majority of potentially toxic chemicals. Advanced approaches, thus, cope with complex mixtures (Altenburger et al., 2019) and use effect-based together with component-based tools for the assessment of chemicals (e.g., Brack et al., 2019; Posthuma et al., 2019). Such procedures, however, have hardly been applied in comprehensive studies on multiple (i.e., chemical and non-chemical) stress on aquatic ecosystems.

Another shortcoming refers to the ecosystem response variables. Established indicators for ecological health, e.g. within the EU-Water Framework Directive (EU-WFD), are often underrepresented concerning (i) measures for ecological processes and functions as important components of ecosystem integrity (e.g., Tilman et al., 2014; von Schiller et al., 2017) and (ii) micro-organisms, which often mediate essential ecological functions such as carbon mineralization or nutrient uptake (von Schiller et al., 2017). Most established monitoring programs focus on community-based metrics of macrofauna and plants. Thus there is a risk that relevant stressors will be overlooked when appropriate receptors within the ecosystems, such as densities and functions of microorganisms, are ignored. Nevertheless, the potential of ecological functions (often mediated by microorganisms) in aquatic ecosystems assessment and stressor diagnosis is increasingly recognized (e.g., Gessner and Chauvet, 2002; Young et al., 2008; Feio et al., 2010; Arroita et al., 2019). Such functional indicators could potentially complement existing community-based indicators and could enhance their diagnostic potential. They have substantial advantages, e.g., in the assessment over various time scales as well as by integrating over various spatial scales. They can also link ecological attributes to ecosystem services for humankind (von Schiller et al., 2017). Expanding the response variables towards functional variables has the potential to improve the diagnosis of relevant stressors in multi-stressor contexts (e.g., Clapcott et al., 2010, 2012).

In this study we aimed to disentangle the effects of multiple chemical and non-chemical stressors and identify relevant stressors, which substantially affect ecological response variables in a complex environmental setting along a longitudinal land use gradient. For this purpose, we used a well-characterized river model site, i.e. the Holtemme River (Germany) as part of the Terrestrial Environmental Observatories network (TERENO) of the German Helmholtz-Association (Zacharias et al., 2011; Wollschläger et al., 2017). The Holtemme originates from a near-natural national park and faces a longitudinal gradient of stressors typical for rivers in populated areas along the downstream river stretch, including urban and agricultural impacts, morphological alteration and wastewater effluents. We assumed that by applying

multi-target analysis of both chemical and non-chemical stressors in combination with component-based risk assessment as well as a range of both functional and structural ecological metrics for microbes and macrofauna, we will be able to disentangle stressor-response relationships, which are not detectable by standard assessment methods.

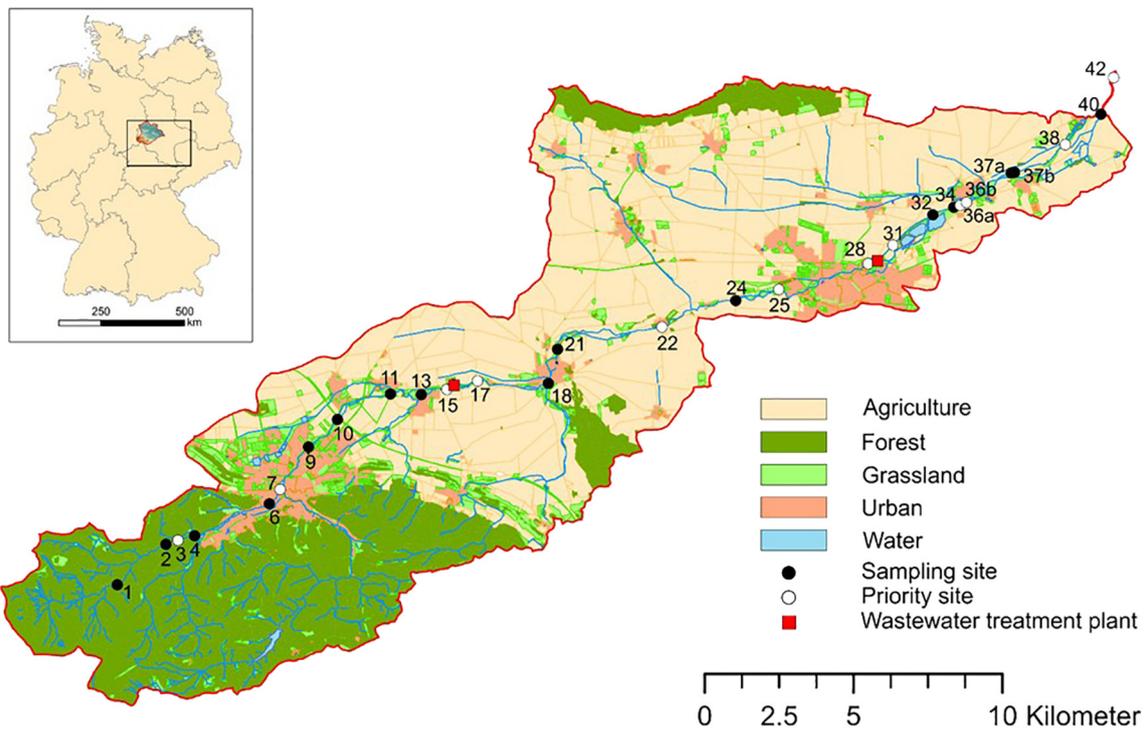
## 2. Study sites

The Holtemme is a third-order river that originates in the Harz National Park (Germany) at 862 m a.s.l. and flows after 47 km into the Bode River. The size of the catchment is 282 km<sup>2</sup> (Müller et al., 2015) with geology dominated by Mesozoic rocks covered with Tertiary and Quaternary sediments (Schuberth, 2008). Land use in the catchment reflects the typical progression from near-natural forested headwaters to agricultural and urban areas in the downstream reach (Fig. 1). Hence, riverine hydromorphology is almost natural in the upstream reach but highly altered in the downstream reach due to channelization, riparian clearcutting and incision in urban and agricultural areas. Besides potential diffuse sources such as adjoining agricultural land, the river receives substantial inputs of nutrients as well as a large range of pollutants from point sources. These are two wastewater treatment plants (WWTP) having a population equivalent of more than 60,000 each and from tributaries draining agricultural areas (Fig. 1). Furthermore, a stormwater drainage systems discharges untreated urban water at times of heavy rain events shortly upstream the second WWTP in Halberstadt. With this incremental increase of human stressors, the Holtemme River is a typical example of a multiple-stressed ecosystem. Hence, the river was chosen as a model site to study the effects of the longitudinal increase of human stressors on ecosystem structure and functioning within the TERENO network (see also Kamjunke et al., 2013; Müller et al., 2015; Inostroza et al., 2017; Beckers et al., 2018, 2020; Kamjunke et al., 2019; Fink et al., 2020).

## 3. Methods

Two longitudinal sampling campaigns at 28 sites along the river continuum were conducted from 6. to 10. October 2014 as well as from 5. to 10. October 2015. Sampling dates were chosen to represent baseflow conditions considering upstream and downstream reaches of potential point and diffuse source entries of pollution and nutrients (Fig. 1). From the 28 sites located in the river, we selected 12 sites as priority sites for the sampling of more complex biological and functional variables (Table 1). The priority sites were chosen to reflect the longitudinal development of human impacts and were thus installed upstream and downstream of major point sources (WWTPs, stormwater drainage) and tributaries, reaches with changing land use but also sites with existing gauging stations to allow for the calculation of nutrient and pollutant loads. In 2014, construction works were performed within the river between the sampling points 22 and 28, at 24.4 and 31.5 km from the first sampling point. This resulted in an increased water turbidity due to sediment mobilization.

As potential stressor variables, we quantified hydromorphological and land use parameters, micropollutants (toxicity), nutrients and well as physicochemical and biotic parameters (see Table 2 and the two following subsections for details). With respect to the response variables we quantified ecological community structures and functions across different organism groups. These include macroinvertebrate and fish communities as typical indicators in monitoring programs, e.g. within the EU-WFD, as well as chlorophyll concentration as proxy for algal biomass and eutrophication. As functional variables we quantified heterotrophic processes including bacterial production and extracellular enzyme activity (EEA) as measures for the utilization of dissolved organic matter as well as leaf litter degradation in coarse mesh bags as proxy for the utilization of particulate organic matter.



**Fig. 1.** Location of the study area in Germany and land use in the catchment of the Holtemme River. The location of the two waste water treatments plants (WWTPs) as well as regular and priority sampling sites are marked.

**3.1. Catchment and hydromorphology**

Land use information at each sampling site was extracted from CORINE 2000 land cover data using ArcGIS 10.5 (ESRI 2010,

Environmental Systems Research Institute, Redlands, CA). We used data provided by the local environment authority (LHW, 2019) to assess the status of riverine hydromorphology. Hydromorphological data are based on mappings of flow velocity parameters (e.g. diversity of flow)

**Table 1**

Sites at the Holtemme River sampled for environmental variables as well as biological and functional variables (Priority sites). For each site, geographical location and the distance from the first sampling point is given. The first sampling point is located 3.7 km downstream from the source of the Holtemme.

No	Name	Priority site	Latitude	Longitude	Distance [km]
1	UPS Hotel Steinerne Renne		51°48'16.7"N	10°41'58.8"E	0.00
2	Steinerne Renne		51°49'01.5"N	10°43'23.0"E	2.24
3	MOBICOS Wernigerode	x	51°49'04.3"N	10°43'43.9"E	2.70
4	UPS Braunes Wasser		51°49'11.0"N	10°44'13.2"E	3.42
6	DS Braunes Wasser		51°49'17.6"N	10°46'51.1"E	4.22
7	UPS Zillierbach	x	51°50'03.8"N	10°46'44.4"E	6.98
9	UPS RW drainage		51°50'49.7"N	10°47'29.6"E	8.74
10	UPS mill canal		51°51'20.2"N	10°48'20.1"E	10.19
11	UPS Barrenbach		51°51'48.1"N	10°49'52.8"E	12.35
13	UPS Silstedter Bach		51°51'53.4"N	10°50'46.2"E	13.68
15	UPS WWTP Silstedt	x	51°51'54.1"N	10°51'31.0"E	14.54
17	DS WWTP Silstedt	x	51°51'59.3"N	10°52'47.0"E	16.17
18	UPS Derenburg		51°52'00.4"N	10°54'25.0"E	18.15
21	DS Derenburg		51°52'37.5"N	10°54'46.7"E	19.61
22	Pegel Mahndorf	x	51°53'06.2"N	10°57'47.2"E	23.81
24	DS Stroebecker Fliess		51°53'34.9"N	10°59'56.0"E	26.73
25	UPS Halberstadt	x	51°53'48.2"N	11°01'11.3"E	28.39
28	DS RW drainage Hbs	x	51°54'11.2"N	11°3'35.83"E	31.86
31	DS WWTP Hbs	x	51°54'25.8"N	11°04'12.6"E	32.54
32	UPS Alte Holtemme		51°55'11.4"N	11°05'39.4"E	34.74
34	UPS Asse		51°55'21.9"N	11°06'25.8"E	35.69
36 a	UPS Weir Gr. Quenstedt	x	51°55'24.3"N	11°06'36.3"E	35.91
36 b	DS Weir Gr. Quenstedt	x	51°55'24.3"N	11°06'36.3"E	35.92
37 a	UPS Weir Ermsleben		51°55'58.3"N	11°08'3.40"E	37.97
37 b	DS Weir Ermsleben		51°55'58.3"N	11°08'3.40"E	37.98
38	MOBICOS Nienhagen	x	51°56'29.7"N	11°09'31.1"E	40.01
40	UPS Salzgraben		51°57'01.6"N	11°10'33.4"E	41.69
42	Mouth	x	51°57'47.8"N	11°10'57.7"E	43.35

**Table 2**  
Environmental variables sampled to describe stressor intensities at the Holtemme River.

Category	Variable	Unit	Mean (range)	Included into analysis of			
				Algae	Functions	Inverteb.	Fish
Hydromorphology	Canopy cover	Class [1–5] <sup>a</sup>	2 (1–5)	X	X		
	Flow diversity	Class [1–7]	5 (1–7)	X		X	X
	Overall status	Class [1–7]	5 (3–7)	X	X	X	X
	Status, river bed	Class [1–7]	5 (2–7)	X	X	X	X
	Substrate diversity	Class [1–5]	5 (2–7)	X		X	X
	Shannon habitat diversity	unitless	1.25 (0.20–1.99)			X	X
Land use	Arable	%	29 (0–58)	X	X	X	X
Micropollutants	Toxic units algae	unitless	$1.2 \times 10^{-2}$ ( $3.3 \times 10^{-6}$ – $3.8 \times 10^{-2}$ )	X	X		
	Toxic units <i>Daphnia</i>	unitless	$7.8 \times 10^{-2}$ ( $2.8 \times 10^{-5}$ – $3.7 \times 10^{-1}$ )			X	
	Toxic units fish	unitless	$2.1 \times 10^{-3}$ ( $2.7 \times 10^{-5}$ – $6.9 \times 10^{-3}$ )				X
Nutrients	Nitrate-N	mg L <sup>-1</sup>	2.6 (0.2–4.0)	X	X	X	X
	Soluble reactive phosphorous	mg L <sup>-1</sup>	0.05 (0.00–0.12)	X	X	X	X
Physico-chemistry	Distance from source	km	25.8 (3.7–47)	X	X	X	X
	Oxygen concentration	mg L <sup>-1</sup>	10.5 (9.4–12.3)			X	X
Biotic	Biofilm algae density	µg Chl cm <sup>-2</sup>	3.9 (0.2–11.3)		X		
	Macroinvertebrate abundance	ind. m <sup>-2</sup>	551 (120–1477)		X		

<sup>a</sup> Canopy cover: 1 = no, 2 = single trees, 3 = some trees or bushes, 4 = scattered forest, 5 = complete.

and morphological parameters (e.g. planform pattern, variability of cross-section by width/depth, longitudinal continuity and habitat composition) (Gellert et al., 2014). The status of each parameter is rated on a 1 (very good) to 7 (bad) scale. The national method is also the official approach to assess the hydromorphological status of streams and rivers according to the EU-WFD (Kampa and Bussetini, 2018). From this data, we extracted the overall hydromorphological status, the hydromorphological status of the river bed as well as flow and substrate diversity. In addition, we used the proportional distribution of benthic habitats to calculate habitat diversity based on the Shannon-Weaver index. Canopy cover was estimated on-site and classified into five status classes ranging from 1 (no trees) to 5 (complete canopy cover) (Table 2).

### 3.2. Physicochemical variables, micropollutants and toxicity

Conductivity and water temperature were measured with multiparameter probes (YSI 6600-V2) during water sampling. Water samples for nutrient concentrations were taken at the 28 sampling sites with a buried polypropylene sampler DIN 38401-15 (2010). Sample preparation and analyses followed the methods described by Kamjunke et al. (2015b) and Müller et al. (2018).

Concentrations of dissolved organic matter (DOM) were determined after high-temperature combustion (DIMATOC 2000, Dimatec Analysetechnik GmbH, Essen, Germany) and data for 2014 were taken from Kamjunke et al. (2019). Fluorescence excitation emission matrices (EEMs) were collected using a spectrofluorometer (AQUALOG, HORIBA Jobin Yvon, USA). Humification index (HIX), and fluorescence index (FI) were calculated as described in Kamjunke et al. (2019). HIX is associated with the degree of condensation of fluorescent molecules and lower H/C ratios whereas low and high FI values indicate strong and weak terrestrial source contribution, respectively.

Trace elements were analysed according to DIN EN ISO 11885 and the norm EN ISO 17294-2:2017-01 (see Appendix Table S1 for data and further details on the method). Analysis of micropollutants by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) focused on 149 organic compounds including pharmaceuticals and personal care products (PCPs), pesticides, biocides and industrial compounds. Details on water sampling, chemical analysis of organic micropollutants and risk assessment are provided elsewhere (Beckers et al., 2018; Beckers et al., 2020) and in the Appendix (Table S2). Additionally, data on coumarin compounds including 4-methyl-7-diethylaminocoumarin (C47), 4-methyl-7-ethylaminocoumarin (C47T1) and 4-methyl-7-aminocoumarin (C47T2) were provided by Muschket et al. (submitted).

Concentrations of organic micropollutants were transformed into toxic units (TU) for algae, *Daphnia* as a representative for crustaceans and fish according to:

$$TU_i = MEC/LCx_i \text{ or } ECx_i \quad (1)$$

where MEC is the measured environmental concentration for an individual compound, *i*, LC is the lethal concentration and EC is the effect concentrations obtained from Busch et al. (2016). For each sampling point and organism group, sum toxic units (sumTUs) were calculated and compounds contributing strongest to the total toxicity were identified (i.e., risk driving compounds) by calculating the contribution of an individual compound to the sumTUs using:

$$\%i = (TU_i / \sum TU) 100\% \quad (2)$$

The sublethal antiandrogenic effects of coumarin compounds detected in the Holtemme River were assessed by calculating TUs (Eq. (1)) and flutamide equivalents (FEq) as:

$$FEq = EC50_{\text{flutamide}} * C_i / EC50_i \quad (3)$$

The effect concentrations were determined in a rapid androgen disruption adverse outcome reporter (RADAR) assay using medaka eleuthero-embryos (Muschket et al., 2018). Flutamide was used as a reference androgen antagonist.

In order to perform bioassays at selected sites, 50 L water samples were collected by on-site large volume solid-phase extraction (Schulze et al., 2017) at five sampling sites (i.e., points 3, 15, 17, 25, and 38, Table 1). The samples were prepared for bioassays according to Väitalo et al. (2017). Zebrafish embryo tests (ZFET) and algae assays were performed according to OECD Test Guideline 236 and Altenburger et al. (1990), respectively. The water samples were tested at four different relative enrichment factors (REF) in the ZFET and five REFs in the algae assay up to a maximum REF of 200. No clear toxicity profiles could be obtained for sampling point 38.

### 3.3. Bacterial production and extracellular enzyme activity

Production of planktonic as well as biofilm bacteria was measured using the leucine technique (Simon and Azam, 1989) as described by Kamjunke et al. (2015a). Triplicate 5 mL aliquots (planktonic) or pebbles of about 1 cm length (biofilm) were spiked with <sup>14</sup>C leucine (12.2 MBq µmol<sup>-1</sup>, Sigma, 50 nM or 5 µM final concentration, respectively). Samples were incubated in the laboratory at in situ temperature for 1 h in the dark on a shaker. Biofilms were removed from pebbles by

ultrasonication for 1 min (20 kHz, 20%; HTU Soni130, Heinemann, Germany). Radioactivity of filters was measured using a Liquid Scintillation Analyzer (2300 TR, Packard).

Extracellular enzyme activities were measured in 2015 only. They were determined with the fluorescence tracers (4-methylumbelliferyl (MUF), 7-amido-4-methylcoumarin (AMC)) according to Hoppe (1983) and Karrasch et al. (2003). We quantified the degradation rates of  $\alpha$ -D-glucosidase,  $\beta$ -D-glucosidase, phosphatase, L-leucine-aminopeptidase,  $\beta$ -D galactosidase, and N-acetyl- glucoseaminidase in surface water samples. As sum parameter over all extracellular enzymatic activities, we calculated the average over the normalized activity of six enzymes. For this purpose, we calculated the normalized activity for each of the six enzymes separately as contribution of the activity (a) measured at each point to the maximal activity ( $a_{\text{point}} / a_{\text{max}}$ ). The original data for each enzyme and further details on the methods are presented in the Appendix Table S3.

### 3.4. Benthic and planktonic autotrophic biomass

For benthic (biofilm) algae, chlorophyll *a* – content, dry mass, ash free dry mass (AFDM) and elemental composition of carbon, nitrogen and phosphorus (C/N/P) were analysed using standard methods (see Kamjunke et al., 2015b). In brief, defined areas of biofilm were scraped off with a scalpel and stored at  $-20^{\circ}\text{C}$ . Afterwards, lipophilic pigments were extracted in 90% of acetone and analysed using reverse-phase-HPLC as described in Schmitt-Jansen and Altenburger (2008). The total chlorophyll *a* – content of planktonic samples was analysed using the FluoroProbe (Fa. Bbe Moldaenke). DM and AFDM were analysed by drying, weighing, combusting (5 h at  $540^{\circ}\text{C}$ ) and reweighing of the samples. Elemental molar ratios (C/N and C/P) were calculated after analysis according to standard methods (DIN EN 1484 1997; ISO 6878 2004).

### 3.5. Leaf litter decomposition

Invertebrate-mediated leaf litter decomposition, an important ecosystem function in streams, was assessed by filling coarse mesh bags (pore size 5 mm) with  $3.8 \pm 0.1$  g freshly fallen and air-dried alder (*Alnus glutinosa*) leaves. Four replicate bags were exposed at the 12 priority sites (see Table 1) and retrieved after 6 weeks to determine dry mass (48 h at  $60^{\circ}\text{C}$ ) and ash free dry mass (4 h at  $500^{\circ}\text{C}$ ). Four additional bags were used to determine initial mass corrected for handling and leaching (within 24 h) losses. Breakdown rate (k, not corrected for temperature) was calculated using an exponential decay model  $M_t = M_i e^{-kt}$ , with  $M_t$  as mass at time *t* and  $M_i$  as initial mass.

### 3.6. Macroinvertebrates and fish

Benthic macroinvertebrates were sampled from the 12 priority sites with a hand net (25 cm  $\times$  25 cm frame, 500  $\mu\text{m}$  mesh) following a standardized multi-habitat sampling approach (Haase et al., 2004). Briefly, habitat composition at each site was visually estimated and 20 replicates were taken reflecting the resulting proportion of habitat types. Macroinvertebrates were counted and sorted alive into major taxonomic groups and preserved in 96% ethanol. The ecological status based on macroinvertebrates (Böhmer et al., 2004) was calculated using the ASTERICS software (Furse et al., 2006). ASTERICS was also used to calculate the metric “SPEAR<sub>pesticides</sub>” from the pesticide-specific bioindicator system SPEAR (SPECies At Risk) (Liess and Von der Ohe, 2005).

The fish community was only sampled in 2014 from the 12 priority sites following the national sampling protocol (Diekmann et al., 2005). Briefly, reaches of  $\sim 100$  m were sampled at each site with electrofishing over the entire distance. Collected individuals were determined and length and individual weight were measured. The ecological status

based on fish was calculated using the fIBs software (Diekmann et al., 2005).

### 3.7. Statistical analyses

To test which variables of stream habitat structure, nutrient status, and catchment land use were correlated with target biological structures and functions, we chose predictor (stressor) variables based on their likely mechanistic influence on the response variables. For each of the response variables and each of the stressor variables we conducted a Spearman rank correlation test using the “cor.test” function in the stats package within R with the method “spearman” and approximate *p*-values (version 3.6.1, R language for statistical computing (R Core Team, 2019)). Using the “false discovery rate” approach (Benjamini and Yekutieli, 2001), we corrected for the increased chance of falsely rejected null hypotheses which is induced by multiple comparisons. We did the correction with the *p.adjust* function of the stats package in R.

For the stressor-response combinations, which revealed significant correlations, we assessed the details of the relationship by using linear regressions. Because we assumed that multiple stressors may have redundant effects on the same response variable, we used Akaike's information criterion (AIC) to develop parsimonious models with a good model fit. Here, the AIC provides a trade-off between goodness of fit and model simplicity. To develop the models, we used the “ols\_step\_both\_aic” function of the olsrr package in R (Hebbali, 2020). The function added and removed predictive variables from the set of variables which were significantly correlated to the metric in question in a stepwise process, until no further minimizing of the AIC was possible (Venables and Ripley, 2002). Before creating linear models, we checked that the relationships were linear. In some cases the stressors had to be log-transformed to reach linearity of the regressions. The residuals of the regressions were homoscedastic and normal distributed. An overview of environmental predictors used in the individual analyses is given in Table 2.

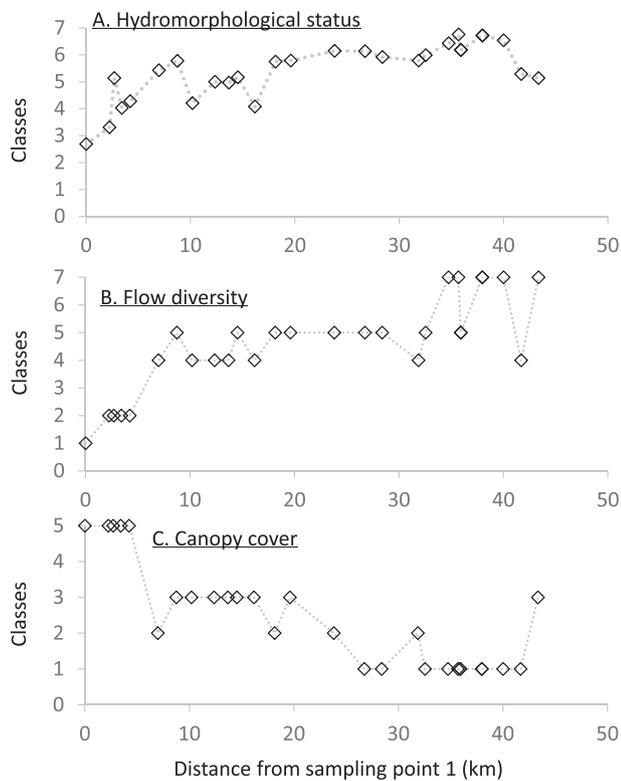
Since all statistical approaches assumed independence of the samples of metrics along the stream, we assessed the autocorrelation of each metric separately for each sampled year (2014 or 2015). We decided that the observations along the Holtemme River are best represented by a one-linear up- to downstream vector, equal to a time series, with approximately equal distances between the sampling sites. Based on this, we used the “acf” function in R (stats package) to test for autocorrelations. Overall, we did 29 autocorrelation tests for the 19 metrics. Of the 29 autocorrelation tests conducted, only 3 tests revealed an autocorrelation higher than expected for white noise (Appendix Fig. S1). This was the case for planktonic algal density in 2015 (but not 2014), SPEAR<sub>pesticides</sub> index for macroinvertebrates in 2014 (but not 2015) and fish Shannon diversity in 2014. Since we only found an autocorrelation in very few cases and often only in one of the two measured years, we deem strong influence on correlations and regressions unlikely.

## 4. Results

### 4.1. Drivers and stressors

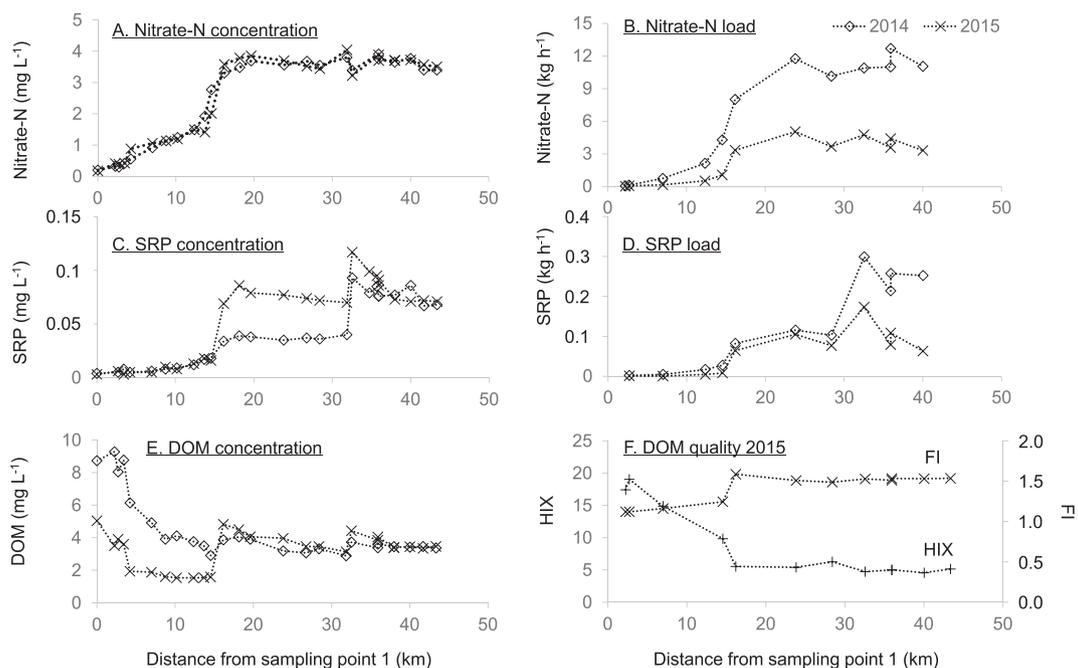
#### 4.1.1. Hydromorphology and riparian zone

The upstream section of the Holtemme River (km 0–4.2 from first sampling point) was characterized by near-natural conditions with high flow and substrate diversity and 100% canopy cover (Fig. 2). After entering the city of Wernigerode (at sampling point 7, “UPS Zillierbach”, Table 1), the overall hydromorphological status changed to “strongly modified” (status class 5) mainly due to river straightening, lower flow and substrate diversity, as well as increasing light exposure due to reduced riparian vegetation. After passing through subsequent sub-catchments dominated by agricultural land use, the overall



**Fig. 2.** Status classes of the (A) overall hydromorphological status, (B) flow diversity and (C) canopy cover along the river course. Canopy cover refers to a scale from 1 (highest) to 5 (lowest), while the other parameters refer to a scale from 1 to 7.

hydromorphological status decreased in the lower reaches of the river to the lowest status class 7, i.e. “heavily modified” with strongly reduced flow diversity and no canopy cover.



**Fig. 3.** Longitudinal gradient of nutrients and dissolved organic matter (DOM). (A, B) Nitrate-N (Müller et al., 2018), (C, D) soluble reactive phosphorus (SRP) and (E, F) DOM during the samplings in 2014 (from Kamjunke et al., 2019) and 2015. The panels on the left side (A, C, E) represent concentrations, whereas the upper two panels of the right side (B, D) represent loads. The lower left panel (F) represents the fluorescence (FI) and humification (HIX) index of the DOM as measure for the DOM quality in 2015. For further details on molecular DOM composition see Kamjunke et al. (2019).

#### 4.1.2. Nutrients and organic carbon

Nitrate-N concentrations (Müller et al., 2018) were low ( $0.2 \text{ mg L}^{-1}$ ) at the uppermost sampling point and increased with downstream distance, until reaching stable concentrations between 3.3 and  $4.0 \text{ mg L}^{-1}$  at km 16.17 from the first sampling point in both years (Fig. 3a). Nitrate-N concentrations were strongly correlated with agricultural land use ( $\rho = 0.82$ ,  $p < 0.001$ ) in the catchment area (see also Müller et al., 2018 for nitrate-N source apportionment based on stable isotope analyses). Moreover, inputs from the first WWTP increased nitrate-N concentrations by  $1.1 \text{ mg L}^{-1}$ , whereas nitrate-N concentrations were diluted after the effluent of the second WWTP. Spatial nitrate-N concentration patterns were identical between the two years, but nitrate-N load differed significantly due to lower discharges in 2015 (Fig. 3b). This pattern differed for soluble reactive phosphorus (SRP), which significantly increased in concentration with WWTP effluent and showed nearly identical loadings but different concentrations between the two years at least in the upper 30 km of the study section (Fig. 3c, d).

The DOM concentration was initially high and strongly decreased within the uppermost 5 km stream section (Fig. 3e; see Kamjunke et al., 2019 for data of 2014). This decrease was associated with a drop in the HIX, indicating decreasingly humic and predominantly recalcitrant material with downstream distance. In the downstream reaches, significant increases in total DOM quantity were registered downstream of the WWTPs in both years and for both WWTPs. These increases in quantity were associated with increases in the FI, particularly at the first WWTP, indicating increases of microbial degradable material.

#### 4.1.3. Trace element concentration

All (trace) element concentrations detected in 2015 were generally below critical values according to the German surface water directive (OGewV, 2016; see Appendix Table S1). Nevertheless, variations with implications for the source and control of elements could be detected. The subcatchment of the tributary “Braunes Wasser” differs from the headwaters of the Holtemme, resulting in enhanced concentrations of K, Na, Ca, Mg, Cl and  $\text{SO}_4$  downstream the confluence (4.22 km from

first sampling point) in relation to the sampling point upstream (3.42 km from first sampling point). Electric conductivity increased between the two sampling points from 138 to 422  $\mu\text{S cm}^{-3}$ . A second important source for trace elements were the WWTPs. After the effluent from the first WWTP, the concentrations of K and Na increased threefold, concentrations of Mo sixfold, Ni twofold and Rb fourfold. Effects of the second WWTP were distinctly smaller (Appendix Table S1).

4.1.4. Micropollutants and potential toxicity

In total, 116 out of 149 analysed micropollutants were detected in the combined data sets from both sampling campaigns. The detected compounds included 32 pharmaceuticals, 54 pesticides, 9 biocides, 13 industrial compounds, 4 artificial sweeteners and 2 PCPs as well as caf-

feine and cotinine. Concentrations of micropollutants for each sampling site during the sampling campaign in 2014 and 2015 are presented in the Appendix Table S2. The description and discussion of organic micropollutant concentrations and associated risks focuses on the results from the campaign in 2015 as not all compounds were analysed in the first campaign.

Only a few micropollutants were detected in the headwater region up to sampling point 15 upstream of the first WWTP. Still, even at upstream sampling point 3, 36 compounds were detected belonging to the group of pharmaceuticals, PCPs, industrial compounds, pesticides and biocides. In the headwater region, the highest concentrations were detected for the artificial sweeteners saccharin and cyclamate (1229 ng L<sup>-1</sup> and 1275 ng L<sup>-1</sup>, respectively).

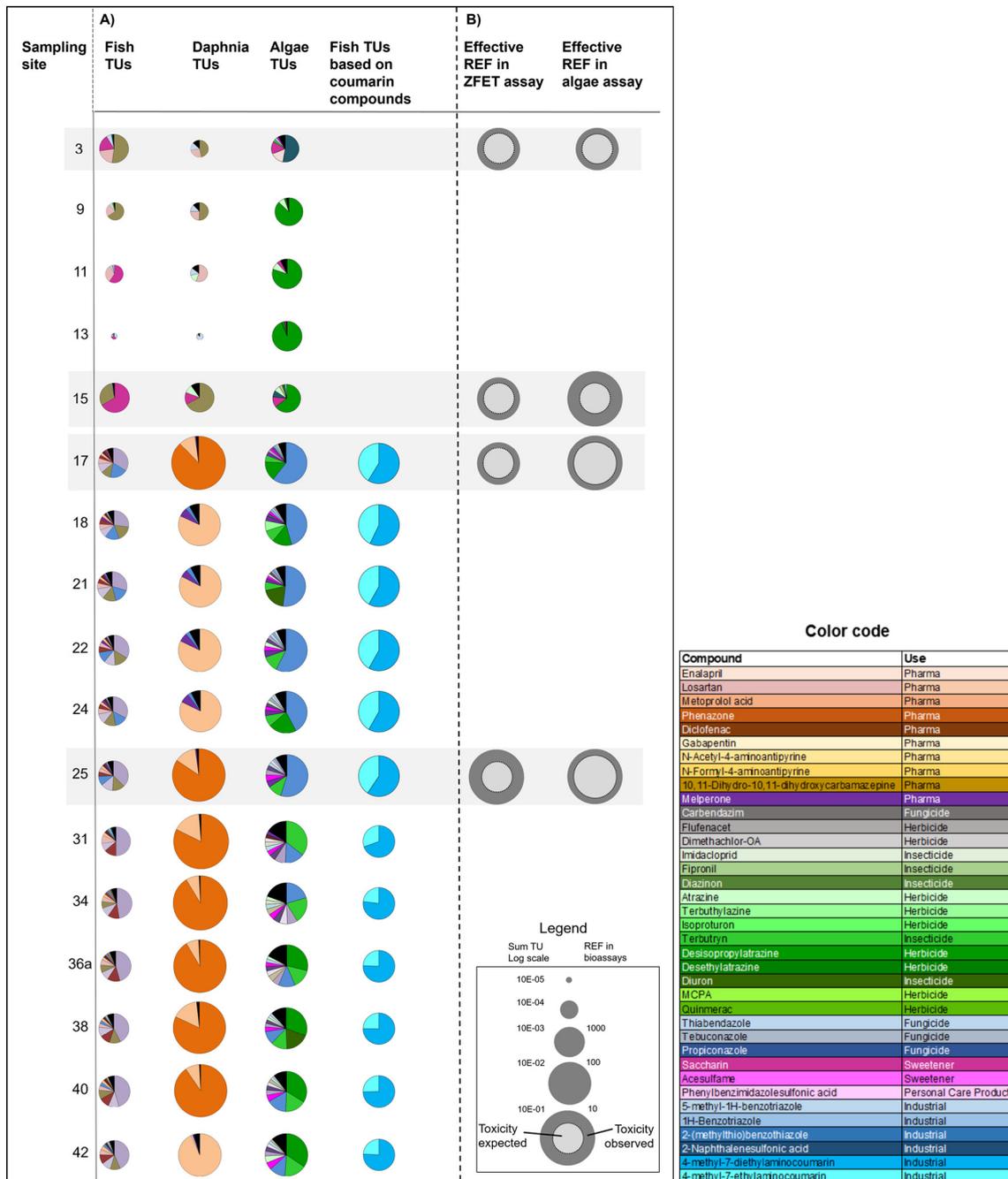
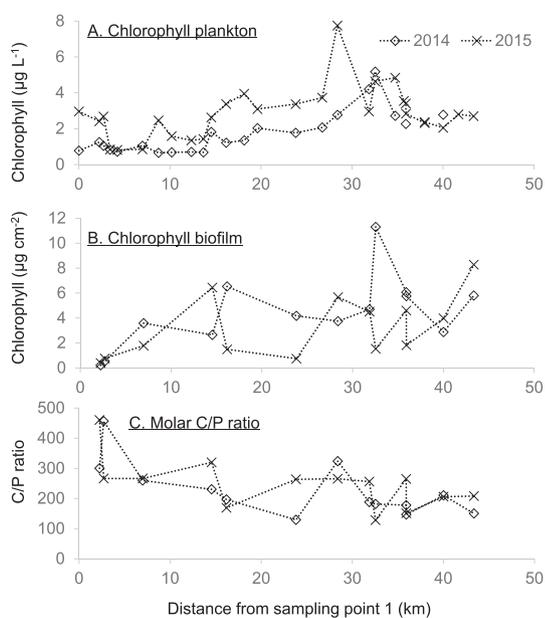


Fig. 4. (A) Longitudinal profile of sum toxic units (sumTUs) calculated for acute risk to fish, *Daphnia* and algae based on effect concentrations from Busch et al. (2016). Toxic units (TUs) for sublethal effects in fish based on antiandrogenic coumarin compounds 4-methyl-7-diethylaminocoumarin and the metabolite 4-methyl-7-ethylaminocoumarin. (B) Effective relative enrichment factor (REF) for toxicity of enriched water samples determined with the zebrafish embryo test (ZFET) and algae growth assay, respectively. Pharma = pharmaceuticals.



**Fig. 5.** Longitudinal gradients of planktonic and biofilm algae. (A) Suspended chlorophyll *a* concentrations as measure for planktonic algal density, (B) biofilm associated chlorophyll *a* concentrations as measure for biofilm algae (C) molar carbon to phosphorus ratios (C:P) of the biofilms.

Overall, the number and the concentrations of micropollutants increased dramatically with the effluents of the two WWTPs (i.e., sampling points 17 and 31).

For most of the compounds belonging to the group of pharmaceuticals, industrial compounds or PCPs, similar concentrations were observed at both WWTPs. Notable exceptions were the antidepressant melperon and the coumarin compounds C47, C47T1 and C47T2, which showed a specific input from the first WWTP.

Pesticide concentrations were rather low in the Holtemme River during this sampling campaign (i.e., ranging from 0 to 24 ng L<sup>-1</sup> in the main river) except for the metazachlor ESA (up to 190 ng L<sup>-1</sup>) and the wood protection products propiconazole (up to 1346 ng L<sup>-1</sup>), tebuconazole (up to 252 ng L<sup>-1</sup>) and thiabendazole (up to 41 ng L<sup>-1</sup>). Like pesticides, biocides were detected in low concentrations. Highest concentrations were observed for carbendazim and terbuthryn (up to 12 ng L<sup>-1</sup> and 28 ng L<sup>-1</sup>, respectively).

The potential risk of acute effects in aquatic organisms by the detected organic micropollutants was characterized using TUs. Fig. 4 shows sumTUs and effective REF determined in the bioassays for the sampling campaign in 2015 considering only the sampling sites in the

Holtemme River and disregarding the tributaries. The highest sumTU values were 0.007, 0.37 and 0.038 for fish, *Daphnia* and algae, respectively. The risk for all organisms was very low in the headwater region. Only at sampling point 3, a slightly higher risk for crustaceans compared to the subsequent sites and distinct risk drivers for algae were determined (Fig. 4a). The sumTUs increased by at least one order of magnitude downstream of the WWTPs for *Daphnia* and algae (i.e., sampling points 17 and 31). Sublethal risks for fish due to antiandrogenic coumarin compounds were observed downstream of the first WWTP and sumTUs decreased by an order of magnitude with the effluent of the second WWTP. Similarly, sum flutamide equivalents of C47 and C47T1 were highest downstream of the first WWTP (i.e., 1.6) and were decreased by a factor 5 at the river's mouth (i.e., 0.3) (Appendix Table S2).

The risk for crustaceans was driven by two individual compounds diazinon and fipronil. Acute toxicity was lowest for fish among all three organism groups and was attributed to a more complex mixture of risk driving compounds including the pharmaceutical transformation products of metamizol (i.e., *n*-formyl-4-aminoantipyrine and *n*-acetyl-4-aminoantipyrine), the fungicide propiconazole and the biocide carbendazim. The main risk drivers for algae downstream of the first WWTP included the fungicide propiconazole, the biocides terbuthryn and diuron and the legacy pesticide atrazine and its transformation products.

The calculated sumTUs were compared to the effective REF determined in the bioassays (Fig. 4b). The calculated sumTUs for fish and algae were one to two orders of magnitude lower than the determined effective REFs in the bioassays (Fig. 4b).

## 4.2. Ecological responses - status and functioning

### 4.2.1. Benthic and planktonic algae

Planktonic algae (Fig. 5a) hardly exceeded concentrations of 5 µg Chl *a* L<sup>-1</sup> (exception: 5.2 µg Chl *a* L<sup>-1</sup> at km 32.54 in 2014 and 7.8 µg Chl *a* L<sup>-1</sup> at km 28.39 in 2015). At this low level, planktonic algal density peaked in the middle reaches (km 16.2 to km 35.9), whereas densities were always below 3 µg Chl *a* L<sup>-1</sup> in the upper and lower reaches in both years. Overall, algae densities were significantly correlated to several stressor variables (see Table 3) with arable land use, sumTUs for algae, SRP and distance from source contributing to explain the final regression model (Table 4). Biofilm algae reached chlorophyll concentrations between 0.2 and 11.3 µg Chl *a* cm<sup>-2</sup> in 2014 and 0.4 and 8.3 µg Chl *a* cm<sup>-2</sup> in 2015 (Fig. 5b). In both years, values were low for the upper two sites within the forest and increased downstream. No consistent longitudinal pattern occurred in either year in the lower reaches. However, biofilm algal concentrations were correlated to both distance from source and share of arable land use. Both variables remain in the parsimonious regression model (Tables 3, 4). Nevertheless, no significant

**Table 3**

Results of Spearman correlation analysis for the dependency of algae-related metrics (columns) on different predictor variables (rows). Values represent the SPEARMAN correlation coefficient ( $\rho$ ) plus the number of cases in brackets. P-values were adjusted for increased false detection rate based on 50 multiple pairwise comparisons in this table (Benjamini and Yekutieli, 2001).

	Planktonic algal density (µg Chl L <sup>-1</sup> )	AFDM biofilme (mg cm <sup>-2</sup> )	Algae density biofilm (µg Chl. cm <sup>-2</sup> )	C/N molar ratio biofilm	C/P molar ratio biofilm
Distance from source	0.60 (51)***	0.58 (26)**	0.57 (26)**	0.39 (26)	-0.63 (26)**
Arable land use (%)	0.64 (48)***	0.68 (24)**	0.61 (24)**	0.53 (24)*	-0.5 (24)*
Canopy cover (class)	-0.6 (51)***	-0.38 (26)	-0.35 (26)	-0.11 (26)	0.4 (26)
Hydromorphological status, overall (class)	0.54 (51)***	0.25 (26)	0.23 (26)	0.25 (26)	-0.39 (26)
Hydromorphological status, river bed (class)	0.53 (51)***	0.4 (26)	0.38 (26)	0.41 (26)	-0.53 (26)*
Flow diversity (class)	0.48 (51)**	0.56 (26)**	0.56 (26)*	0.44 (26)	-0.45 (26)*
Substrate diversity (class)	0.44 (51)**	0.36 (26)	0.36 (26)	0.36 (26)	-0.42 (26)
Nitrate-N (mg L <sup>-1</sup> )	0.58 (51)***	0.34 (26)	0.4 (26)	0.25 (26)	-0.47 (26)*
Soluble reactive phosphorus (mg L <sup>-1</sup> )	0.77 (50)***	0.27 (25)	0.28 (25)	0.07 (25)	-0.55 (25)*
Toxic units algae	0.67 (38)***	0.38 (22)	0.36 (22)	0.17 (22)	-0.52 (22)*

AFDM: ash free dry mass.

\*  $p < 0.05$

\*\*  $p < 0.01$

\*\*\*  $p < 0.001$

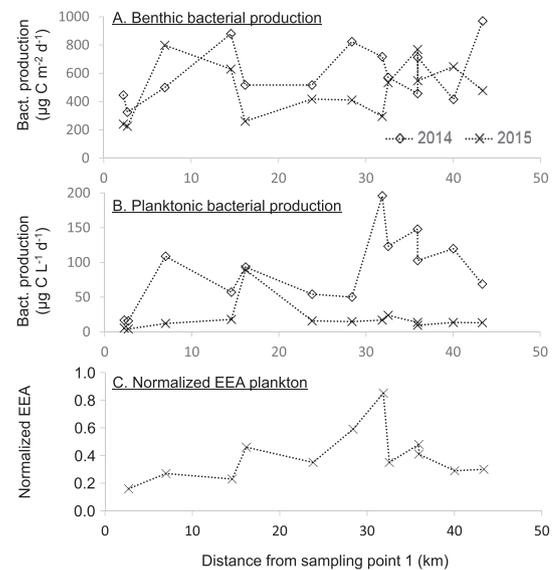
correlation between biofilm algal concentrations and inorganic nutrients (neither nitrate-N nor SRP) was observed (Spearman  $p > 0.05$ ).

The C/P ratio of the biofilms generally decreased with distance from source from values of 300 and 457 (km 2.24 and 2.70, respectively) in 2014 and 460 and 266 (km 2.24 and 2.70, respectively) in 2015 to values of 150 (2014) and 208 (2015) at the river mouth (km 43.35). This resulted in a significant negative correlation of the C/P ratio with distance from source (Spearman:  $\rho = -0.63, p < 0.01$ ) and a significant negative correlation with SRP concentration (Spearman:  $\rho = -0.55, p < 0.05$ ) and sumTUs for algae (Spearman:  $\rho = -0.52, p < 0.05$ ), with the latter two variables being related to the point source WWTP. Both sumTUs for algae as well as share of arable land use explain the linear model (Table 4). Within this overall trend, the two WWTPs, which were the dominant sources of SRP, decreased the C/P ratio of the biofilm algae consistently in both years (Fig. 5c). Such a consistent effect in the biofilm quality was not found in the biofilm quantity (Chlorophyll, AFDM), which was mostly explained by arable land use. Here, sewage efflux resulted either in increases (in 2014 at both sewage plants) or decreases (in 2015 at both sewage plants) of the quantity. No significant change with distance from source was found in the C/N ratio of the biofilms (Spearman:  $\rho = 0.39, p > 0.05$ , Table 3), which ranged from molar C/N ratios between 6.0 and 12.6.

#### 4.2.2. Ecological functions: heterotrophic microbial activity

Bacterial production (BP) showed different patterns for both planktonic and biofilm bacteria with biofilm bacteria being generally less variable over space and time (Fig. 6a, b). While both parameters showed consistently low values at the upper sites within the forest, values increase at the first urban site and showed no clear trends downstream. Correspondingly, hardly any significant global correlation was found between planktonic or benthic bacterial production and environmental variables (Table 5). However, clear local responses to point sources were detected. The effluent of the first WWTP resulted in a consistent increase in planktonic BP and a decrease in local (biofilm) BP in both years. This pattern was not visible at the second WWTP. Here, planktonic BP (and benthic BP in 2015) already increased above the WWTP, coinciding with an urban stormwater drainage system.

Normalized extracellular enzyme activity (EEA) in 2015 also showed low values in the upper sites above the input of the first WWTP similar to the findings for planktonic BP in 2015 (Fig. 6c, Appendix Table S3). The average normalized activity showed a first (smaller) peak at the first WWTP and then a strong peak with highest values at the urban



**Fig. 6.** Longitudinal gradient of microbial activity. (A) Biofilm bacterial production, (B) planktonic bacterial production (values for 2014 from Kamjunke et al., 2019) and (C) enzyme activity (average normalized value. See Appendix Table S3 for details on single enzymes).

stormwater drainage system just upstream of the second WWTP. A small peak was again recorded in the regulated section upstream of the weir.

#### 4.2.3. Ecological functions: Leaf litter decomposition

Leaf litter decomposition showed a unimodal response to river distance with highest values at km 22 and lowest values at km 7 and 38. Leaf litter decomposition was loosely (though non-significantly) correlated to macroinvertebrate density (Spearman:  $\rho = 0.75, p > 0.05$ ) while no such coupling was recorded for any other variable (Table 5).

#### 4.2.4. Macroinvertebrates: densities, diversity and activity

The invertebrate abundance was low ( $< 280 \text{ ind. m}^{-2}$ ) at the two upper forested sites compared to the urban site (km 6.98) in both years (Fig. 7a), while Shannon diversity was high ( $> 2.5$ ) at those sites in both years (Fig. 7b). Abundance increased sharply with increasing

**Table 4**

Parsimonious linear models describing the metrics, which have exhibited a significant correlation to the environmental variables (Table 3; and 6). Some independent variables have been ln-transformed to fulfil the assumptions of linear models. MIV = macroinvertebrates; arable = arable land use (% of hydrological catchment); toxic units (unitless) = either algae, *Daphnia* (MIV) or fish; distance = Distance from source (km);  $O_2$  = oxygen concentration ( $\text{mg L}^{-1}$ ); nitrateN = nitrate-N concentration ( $\text{mg L}^{-1}$ ); SRP = soluble reactive phosphorus concentration ( $\text{mg L}^{-1}$ ). See Table 2 for further definitions of environmental variables.

Metric type	Metric	Parsimonious linear model	F	R <sup>2</sup>
Algae-related	Planktonic algal density ( $\mu\text{g Chl L}^{-1}$ )	$1.6 + 0.07^* \text{arable} - 18.54^* \text{toxic units} + 34.69^* \text{SRP} - 0.11^* \text{distance}$	$F_{4,32} = 13.25$	0.62***
	AFDM, biofilms ( $\text{mg cm}^{-2}$ )	$0.17 - 0.02^* \text{arable} + 0.06^* \text{distance}$	$F_{2,21} = 8.60$	0.45**
	Algae density, biofilms ( $\mu\text{g Chl. cm}^{-2}$ )	$0.29 + 0.04^* \ln(\text{arable} + 1)$	$F_{1,22} = 6.72$	0.23*
	C/N molar, biofilms	$8.20 + 0.03^* \text{arable}$	$F_{1,22} = 5.92$	0.21*
	C/P molar, biofilms	$312.68 - 3004.59^* \text{toxic units} - 1.27^* \text{arable}$	$F_{2,18} = 6.85$	0.43**
MIV-related	Abundance ( $\text{ind. m}^{-2}$ )	$3332.10 - 265.8^* O_2$	$F_{1,23} = 6.63$	0.224*
	Shannon diversity	$3.47 - 1.58^* \text{toxic units} - 0.24^* \text{substrate diversity}$	$F_{2,19} = 4.15$	0.31*
	SPEAR pesticides	$66.52 - 35.34^* \ln(\text{toxic units} + 1) + 0.80^* \ln(\text{arable} + 1) - 7.98^* \text{nitrateN}$	$F_{3,17} = 19.78$	0.78***
	Status class	$-2.35 - 0.36^* \ln(\text{arable} + 1) + 0.70^* \text{nitrateN} + 1.36^* \ln(\text{distance} + 1) + 2.62^* \ln(\text{toxic units} + 1) - 0.46^* \ln(\text{SRP}) - 0.17^* \text{flow diversity}$	$F_{6,14} = 27.03$	0.92***
Fish-related	Shannon diversity	$-0.34 + 0.10^* \text{distance} - 0.04^* \text{arable}$	$F_{2,7} = 249.0$	0.98***
	Contribution, 0+ fish (% of community)	$38.45 - 500.89^* \text{SRP}$	$F_{1,9} = 13.16$	0.59**

\*  $p < 0.05$

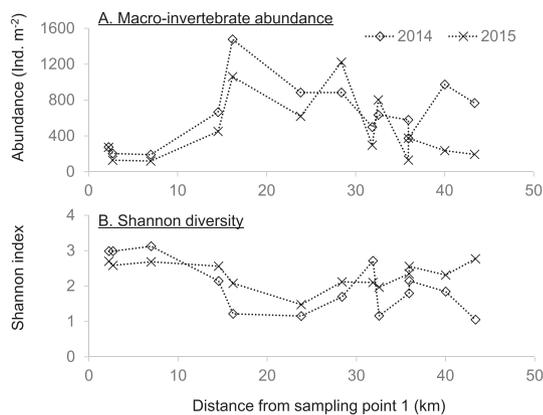
\*\*  $p < 0.01$

\*\*\*  $p < 0.001$

**Table 5**

Results of Spearman correlation analysis for the dependency of metrics related to ecological functions (columns) on different predictor variables (rows). Values represent the SPEARMAN correlation coefficient ( $\rho$ ) plus the number of cases in brackets. All relationships in this table were non-significant ( $p > 0.05$ , adjusted for increased false detection rate based on 40 multiple pairwise comparisons in this table (Benjamini and Yekutieli, 2001)).

	Average normalized EEA activity	Planktonic bacterial production ( $\mu\text{g C L}^{-1} \text{d}^{-1}$ )	Biofilm bacterial production ( $\mu\text{g C cm}^{-2} \text{d}^{-1}$ )	Leaf mass loss rate (g org. matter $\text{d}^{-1}$ )
Distance from source	0.34 (12)	0.22 (26)	0.36 (26)	0.05 (11)
Arable land use (%)	0.56 (11)	0.26 (24)	0.29 (24)	-0.05 (10)
Canopy cover (class)	-0.47 (12)	-0.26 (26)	-0.34 (26)	-0.07 (11)
Hydromorphological status, overall (class)	0.26 (12)	0.18 (26)	0.27 (26)	0.07 (11)
Hydromorphological status, river bed (class)	0.21 (12)	0.16 (26)	0.32 (26)	0.12 (11)
Nitrate-N ( $\text{mg L}^{-1}$ )	0.7 (12)	0.31 (26)	0.14 (26)	-0.05 (11)
Soluble reactive phosphorus ( $\text{mg L}^{-1}$ )	0.49 (12)	0.13 (25)	0.03 (25)	0.32 (10)
Toxic units algae	0.58 (10)	0.3 (22)	-0.01 (22)	-0.14 (8)
Biofilm algae density ( $\mu\text{g Chl cm}^{-2}$ )	0.15 (12)	0.43 (26)	0.4 (26)	0 (11)
Macroinvertebrate abundance ( $\text{ind m}^{-2}$ )	0.46 (12)	0.53 (26)	0.03 (26)	0.75 (11)



**Fig. 7.** Longitudinal gradient of the macro-invertebrates. (A) Abundance and (B) Shannon diversity for both years.

anthropogenic impact to km 14.54 and again under the impact of inputs from the first WWTP with peak abundances of 1477 and 1061  $\text{ind. m}^{-2}$  in 2014 and 2015, respectively, at km 16.17. At the same time, Shannon diversity dropped at both sites. From km 16.17 onwards, both abundance and diversity showed variable patterns with slightly decreasing abundance and increasing diversity with river length. The inputs of

the second WWTP again resulted in an increasing abundance and decreasing diversity in both years. Contrasting patterns in both years were observed at the river mouth with increasing abundances and decreasing diversity in 2014 but not in 2015. Shannon diversity showed strongest correlations with both substrate diversity (Spearman  $\rho = -0.53$ ,  $p < 0.05$ ) and sumTUs for *Daphnia* (Spearman  $\rho = -0.58$ ,  $p < 0.05$ ) with both variables contributing the final linear model (Table 4). Macroinvertebrate abundance showed hardly a significant correlation to any stressor variable with the exception of a significant correlation to oxygen ( $\rho = -0.47$ ,  $p < 0.05$ ) (Table 6).

The ecological status based on macroinvertebrates was “very good” to “good” at the upper sites and declined downstream. The “poor” status remained for all downstream river reaches except the “bad” status upstream and the “moderate” status downstream of the weir (Fig. 8a). The ecological status as an integrative metric was significantly correlated to many variables (Table 6), among which share of arable land use (Spearman:  $\rho = 0.88$ ,  $p < 0.001$ ), nitrate-N concentrations (Spearman:  $\rho = 0.74$ ,  $p < 0.001$ ) and sumTUs for *Daphnia* (Spearman:  $\rho = 0.78$ ,  $p < 0.001$ ) had the strongest explanatory power (Table 6). These variables together with distance from source, SRP and flow diversity explain status class for macroinvertebrates in the linear model (Table 4).

The metric SPEAR<sub>pesticides</sub> showed strongest negative correlation with the point-source (WWTP) related variables sumTU for *Daphnia* (Spearman:  $\rho = 0.84$ ,  $p < 0.001$ , Fig. 8b) and SRP concentrations

**Table 6**

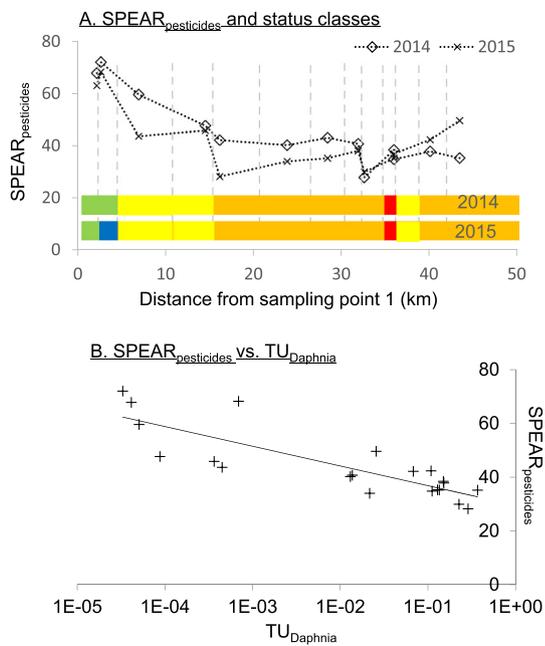
Results of Spearman correlation analysis for the dependency of metrics related to macroinvertebrates (MIV) and fish (columns) on different predictor variables (rows). Values represent the SPEARMAN correlation coefficient ( $\rho$ ) plus the number of cases in brackets. P-values were adjusted for increased false detection rate based on 44 multiple pairwise comparisons for macroinvertebrates and on 55 multiple pairwise comparisons for fish (Benjamini and Yekutieli, 2001).

	MIV abundance ( $\text{ind. m}^{-2}$ )	MIV Shannon diversity	SPEAR pesticides for MIV	Status class MIV	Fish abundance ( $\text{ind./100 m}$ )	Fish biomass (g wet weight /100 m)	Fish Shannon diversity	Contribution of 0 + fish (% of community)	Status classes fish (FIBs)
Distance from source	0.2 (26)	-0.4 (26)	-0.57 (26)**	0.66 (26)**	-0.36 (11)	-0.13 (11)	0.99 (11)***	-0.74 (11)	0.14 (11)
Arable land use (%)	0.23 (24)	-0.43 (24)	-0.57 (24)*	0.88 (24)***	-0.57 (10)	-0.22 (10)	0.94 (10)**	-0.78 (10)	0.28 (10)
Hydromorphological status, overall (class)	0.09 (26)	-0.33 (26)	-0.53 (26)*	0.49 (26)*	-0.08 (11)	-0.02 (11)	0.45 (11)	-0.64 (11)	0.14 (11)
Hydromorphological status, river bed (class)	0.07 (26)	-0.37 (26)	-0.52 (26)*	0.57 (26)**	-0.2 (11)	-0.13 (11)	0.74 (11)	-0.66 (11)	0.04 (11)
Flow diversity (class)	0.31 (26)	-0.48 (26)*	-0.45 (26)*	0.57 (26)**	-0.09 (11)	0.08 (11)	0.79 (11)*	-0.42 (11)	-0.06 (11)
Substrate diversity (class)	0.33 (26)	-0.53 (26)*	-0.49 (26)*	0.58 (26)**	-0.02 (11)	0.24 (11)	0.53 (11)	-0.43 (11)	0.24 (11)
Shannon habitat diversity	0 (26)	0.07 (26)	0.18 (26)	-0.22 (26)	0.04 (11)	0.21 (11)	-0.54 (11)	0.27 (11)	0.19 (11)
Nitrate-N ( $\text{mg L}^{-1}$ )	0.23 (26)	-0.39 (26)	-0.60 (26)**	0.74 (26)***	-0.34 (11)	-0.14 (11)	0.70 (11)	-0.75 (11)	0.5 (11)
Soluble reactive phosphorus ( $\text{mg L}^{-1}$ )	0.29 (25)	-0.45 (25)*	-0.82 (25)***	0.62 (25)**	-0.47 (11)	0.04 (11)	0.84 (11)*	-0.90 (11)**	0.3 (11)
Oxygen concentration ( $\text{mg L}^{-1}$ )	-0.47 (25)*	0.36 (25)	0.39 (25)	-0.24 (25)	-0.36 (10)	-0.71 (10)	0.28 (10)	-0.03 (10)	-0.49 (10)
Toxic units <i>Daphnia</i>	0.43 (22)	-0.58 (22)*	-0.84 (22)***	0.78 (22)***	-	-	-	-	-
Toxic units fish	-	-	-	-	-0.17 (11)	0.29 (11)	0.61 (11)	-0.75 (11)	0.58 (11)

\*  $p < 0.05$

\*\*  $p < 0.01$

\*\*\*  $p < 0.001$



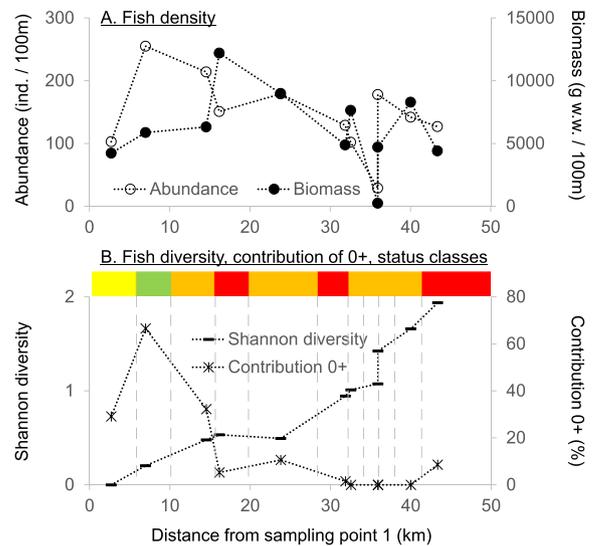
**Fig. 8.** Ecological status according to macroinvertebrates and trait-based SPEAR<sub>pesticides</sub> index displaying the contribution of sensitive species to the total community for both years. (A) Longitudinal development. The colour bar correspond to the ecological status based on macroinvertebrates and colours refer to: blue: “very good”; green: “good”; yellow: “moderate”; orange: “poor”; red: “bad”. (B) Relation between SPEAR<sub>pesticides</sub> and toxic units for *Daphnia* (Spearman:  $\rho = 0.84$ ,  $p < 0.001$ ).

(Spearman:  $\rho = 0.82$ ,  $p < 0.001$ ). Besides toxic units, other stressor variables were correlated to SPEAR<sub>pesticides</sub> (Table 6) and both share of arable land use and nitrate-N concentrations contribute to explain SPEAR<sub>pesticides</sub> in the parsimonious linear model (Table 4). The highest contribution of sensitive species was recorded in the near-natural headwaters (Fig. 8a). The contribution of sensitive species decreased with urban impact at 7 and 14.5 km distance from the first sampling point and then dropped sharply downstream of the WWTP inputs between km 14.5 and 16.2. Such a drop with WWTP impact was recorded in both years and for both WWTPs (Fig. 8a).

#### 4.2.5. Fish fauna: densities, diversity, reproduction and status

Total fish abundance and total fish biomass were not systematically related to any environmental variable (with the exception of a significant relationship between fish biomass and oxygen concentration, Table 6). However, with WWTP effluents, total abundance decreased and total biomass increased. By far the lowest values of both abundance and biomass were recorded in the upstream section of the weir (km 35.91) while the downstream section (km 35.92) showed regular densities. Distinct patterns were recorded for the contribution of young of year fish (0+) to total fish abundance (Fig. 9b). Here, only the three upper sites (km 2.7, 6.98 and 14.54) showed high contributions to the total fish abundance of 29, 67 and 32%, respectively. The contribution dropped to 5% after passing the first WWTP and again dropped to 0% at all sites downstream of the second WWTP (with the exception of 9% at the river mouth (km 43.35)). Overall, the contribution of 0+ fish to the total fish community showed significant relationships only to the point-source (WWTP) related variable SRP (Spearman:  $\rho = -0.90$ ,  $p < 0.01$ ; see Table 6 for further details).

Shannon diversity of the fish community continuously increased with distance from source irrespective of impacts from 0 at km 2.7 (brown trout as single species) to 1.9 at the river mouth (Fig. 9b) with a very strong relationship between diversity and distance from source (Shannon:  $\rho = 0.99$ ,  $p < 0.001$ ) and with significant correlations to



**Fig. 9.** Longitudinal development of the fish community in 2014. (A) Fish density, i.e. abundance and biomass. (B) Shannon diversity and contribution of young of the year fish (0+) to the total fish abundance. The colour bar correspond to the ecological status based on fish and colours refer to: green: “good”; yellow: “moderate”; orange: “poor”; red: “bad”.

other variables being related to distance. Both distance from source and share of arable land use explain fish Shannon diversity in the linear model (Table 4). Ecological status according to the assessment tool FIBS was “good” or “moderate” at the upper two sites whereas it was “poor” to “bad” at the other downstream sites (Fig. 9b) and not significantly related to any stressor variable.

## 5. Discussion

Here, we present a longitudinal approach with a comprehensive dataset on both potential stressors as well as response variables across trophic levels and across community structures and functions. Even though co-correlation of stressors appeared, which is typical for almost all real-world settings under multiple stress, specific response trends were observed across the different metrics, allowing the identification of major stressors and the likely cause-and-effect chain with respect to attributes at ecosystem levels. The effects of four stressor categories were detected, i.e. (i) hydromorphological alterations, (ii) elevated nutrient levels and eutrophication, (iii) stress related to elevated organic matter levels and enhanced secondary production and (iv) micropollutants and toxicity-related stress. The effects are discussed separately below before potential interconnections between stressors are addressed.

### 5.1. Hydromorphological alterations

The Holtemme River exhibited a near-natural hydromorphology in the forested headwaters with high flow diversity. Flow diversity decreased rapidly with hydromorphological alterations when the Holtemme River enters the urban area. However, concentrations of micropollutants were low in this first urban section, upstream of the first WWTP, which enabled to disentangle the effects of hydromorphological alterations from co-varying chemicals in this upper urban reach. The transition from forest to urban was associated with decreases in the ecological status of macroinvertebrate and fish confirming their sensitivity towards hydromorphological alterations (e.g., Eloşegi et al., 2010). This is also supported by the significant contribution of flow diversity to the prediction of macroinvertebrate status class as revealed by the linear model. However, this integrative metric

is also explained by several other variables (see discussion in section *Potential interconnections between stressor variables*).

Among the general ecological metrics, Shannon diversity of macroinvertebrates decreased with hydromorphological degradation when reaching the urban area, an effect which was stronger in 2014 compared to 2015. Over all data, macroinvertebrate Shannon diversity was correlated to hydromorphological indices and especially substrate diversity contributes to explain macroinvertebrate Shannon diversity in the linear model (Table 4). This relationship is probably due to the decreasing niche diversity associated to the decreasing diversity of hydromorphological habitats (Peipoch et al., 2015). This pattern was not observed for the fish community. Here, fish Shannon diversity was not obviously related to any stressor but strongly positively correlated with the distance of the sampling site from the source. The fish community usually changes with regard to both species composition and diversity along large-scale longitudinal gradients in a river continuum (Vannote et al., 1980). However, the Holtemme River is less than 50 km long and belongs entirely to the trout and grayling zone. With distance from the mouth we found a decrease in trout abundance and grayling was completely absent. Instead, the higher fish diversity in downstream reaches was mainly due to fish species such as Prussian carp (*Carassius gibelio*), which indicates modifications of the natural fish community. This is also reflected in the poor to bad ecological status based on the fish community (Fig. 9), which compares local community composition to a natural river type- and zone-specific fish community. Therefore, this strong relationship of fish diversity with distance from source and its independence from hydromorphological variables (Table 6) suggest that the diversity of this highly mobile group is strongly controlled by immigration from the river mouth rather than local hydromorphology (Stoll et al., 2014).

Abundances neither of fish nor of macroinvertebrates were negatively affected by hydromorphological alterations in the headwaters and macroinvertebrate density even increased in both years with decreasing hydromorphological quality in the headwaters, a phenomenon which is probably explained by increases in the resource loading (see section *Elevated organic matter levels and enhanced secondary production*). Algal density as well as ecological functions were hardly affected by hydromorphological alterations. The abundance and activity are probably stronger controlled by small scale heterogeneity (e.g., Risse-Buhl et al., 2020) and organic and inorganic resources (see below) than by modification in the macro-structure of the river.

A strong signal in some metrics was related to the impoundment of the river at the weir (km 35.91 from first sampling point). Fish abundance and biomass responded most strongly with strong declines in the regulated upstream part of the weir, likely due to emigration of riverine fish from the lentic conditions. Furthermore, status class according to macroinvertebrates dropped from “poor” to “bad”, showing its sensitivity towards flow regulation.

Taken together, clear effects of hydromorphological alterations were recorded particularly by general status classes for macroinvertebrates and macroinvertebrate diversity. Furthermore, fish densities were clearly affected by river regulation. The effects of hydromorphological alterations on microbes and ecological processes (i.e., bacterial production, enzyme activity and leaf litter degradation) were rather low.

## 5.2. Elevated nutrient levels and eutrophication-related stress

The present study and the associated stable isotope-based study on nitrate origin in the Holtemme River by Müller et al. (2018) on the same sampling campaigns could clearly follow the nutrients from their sources to the effect on algal growth. Nutrient concentrations were low in the forested headwaters and increased in the downstream part of the river. Agriculture played a dominant role as a nitrate-N source in explaining this increase (Müller et al., 2018). For SRP, the strongest increases were related to the two WWTPs. These different origins, diffuse (nitrate) versus point (SRP) sources, also match with the

different concentration/ loading relationships for nitrate versus SRP in the present study (Fig. 3), with similar concentration devolution for nitrate and similar loading devolution between the years but much more discharge-dependent concentrations for SRP (see Musolff et al., 2017).

Nutrient effects on the observed algal biomass differed for planktonic and benthic algae. Densities of planktonic algae usually increase with developmental time linked to water residence time and thus increase with distance from source (e.g., Reynolds and Descy, 1996). Corresponding to these expectations, concentrations of planktonic algae as found here were generally low compared to large rivers (e.g., Hardenbicker et al., 2016) and increased with distance downstream. The overall moderate increase of planktonic algae density in the lower parts might also be slightly enhanced in 2014 due to the construction works (between 28.4 and 31.5 km from first sampling point), which could have resulted in re-suspension of benthic algae. Benthic algae (biofilms) by contrast provide a better indicator for the local growth conditions. In our study, benthic algae biomass was low only at the sites in the forested headwaters under low nutrient concentrations and high shading. It appears likely that algae growth was resource limited under these low concentrations of SRP (below  $10 \mu\text{g L}^{-1}$ ). As the ratio of inorganic N versus P was far higher than the Redfield ratio (16:1; Hillebrand and Sommer, 1999), P is most likely the limiting nutrient in the Holtemme River. There is no strict threshold for nutrient limitation in autotrophic biofilms. However, SRP concentrations below  $30 \mu\text{g L}^{-1}$  were associated to strong changes in riverine community of autotrophic biofilms in comparison to communities exposed to higher concentration (Bowes et al., 2012). Besides nutrient concentrations, light exposure altered by riparian vegetation also has a strong impact on autotrophic biofilm density (Stovall et al., 2009). Bowes et al. (2012) highlighted the potential of shading to significantly reduce the autotrophic biofilm densities on top of nutrient reduction and Greenwood and Rosemond (2011) showed that increases in nutrient concentrations within a moderate range had only little effect on benthic algae communities under intact canopy cover. This co-limitation by light and nutrients explains why very low densities of autotrophic biofilms were found only when low SRP concentrations coincided with shading due to full canopy cover. The urban sites upstream of the first WWTP showed similarly low SRP concentrations but higher light availability from scattered riparian vegetation. Here, we observed already distinctly higher densities of benthic algae compared to the upper forested sites. Apart from the very low densities in the forested headwaters, autotrophic biofilm biomass showed hardly any consistent relationship to SRP but large variation between sites, which is probably related to the heterogeneous environment in streams (Palmer and Poff, 1997). This suggests that resource limitations on benthic algae biomass occurs only at very low SRP concentrations.

Even though total biomass of benthic algae showed no consistent trend in the pronounced SRP gradient, algae composition might change significantly. We observed that stoichiometric composition, particularly the carbon to phosphorous ratio (C/P), decreased with increasing SRP concentrations, with consistently negative responses with enhanced SRP concentrations due to the WWTPs as well as in an overall negative correlation between the C/P ratio of the biofilms and the SRP concentration. Such a response in biofilm C/P stoichiometry to SRP concentration is common (e.g., Hill et al., 2011; Kamjunke et al., 2015b) even though strong shifts in the community composition can lead to opposite effects (Iannino et al., 2020). In the present study, also sumTUs for algae contribute to explain the C/P ratio. This is probably explained by the expected effects of SRP on the C/P ratio, as both SRP and toxic risk drivers co-correlate and predominantly come from WWTPs as a joint point source. At the same time, light exposure due to a loss of riparian vegetation can lead to elevated C/P ratios due to enhanced primary production at limiting P concentrations (Fanta et al., 2010). In our study, the forested sites had the highest C/P ratios indicating P-limitation (Hillebrand and Sommer, 1999) and suggesting that P-limitation had a stronger (positive) effect on the C/P ratio of the biofilms overriding

the potentially negative effect of shading. The C/P ratio, thus, seems to be an appropriate indicator for enhanced P loading on aquatic ecosystems. As nutrient stoichiometry is an important measure for food quality, effects of enhanced SRP concentration on the stoichiometry of the biofilm can potentially also affect higher trophic levels within the river food web (e.g., Iannino et al., 2019).

The absolute values of benthic (biofilm) algae biomass were relatively low in the range of oligotrophic (upper reference sites of the Holtemme) to mesotrophic (rest of the Holtemme) river according to the suggestion of Dodds and Smith (2016). Biofilm algae usually show distinct seasonal patterns in biomass but also in its dominant control mechanisms (e.g., Rosemond et al., 2000) with peak densities during spring and autumn (e.g., Olapade and Leff, 2005; Ledford et al., 2017). Furthermore, autotrophic biofilms can strongly be affected by flood events with increased turbidity and relocation of the river bed (Biggs and Smith, 2002). Given such seasonal and event-driven dynamics, our results must be contextualized as reflecting stable, base-flow conditions during autumn. Nevertheless, under such conditions, we could show that nutrient limitation for algae biomass only occurs under very low SRP concentrations, which highlights the role of shading in eutrophication control. Even though SRP concentrations hardly affected algae biomass at moderate to high levels, they still altered the nutritional quality of the biofilm algae.

### 5.3. Elevated organic matter levels and enhanced secondary production

Our longitudinal study includes detailed analyses of the DOM quantity and quality (see Kamjunke et al., 2019 for details on molecular DOM composition during the sampling campaign based on high-field FTICR mass spectrometry and NMR spectroscopy), bacterial activity as well as quantitative data of higher trophic levels, allowing us to evaluate DOM origin, dynamics and effects in the stream.

The DOM profiles showed pronounced shifts from the headwater region to the lower reaches. The headwaters were characterized by high DOM quantities with relatively high degree of humification and low amount of freshly produced material as indicated by the indices HIX and FI (McKnight et al., 2001; Wickland et al., 2007). DOM was characterized by high abundances of rather unsaturated CHO compounds indicating terrestrial origin and low modification by microbial activity (Kamjunke et al., 2019). This DOM composition showed strong shifts along the downstream passage with decrease of average oxidation and unsaturation followed by an increased relative abundance of CHNO and CHOS compounds as indicators for agriculture and waste water, respectively (Kamjunke et al., 2019). The degree of humification decreased strongly downstream while the FI increased indicating a higher biological activity and bioavailability of the DOM. WWTPs effluent and stormwater drainage systems enhanced these trends.

These changes in molecular and fluorescent characteristics were mirrored by pronounced effects on the microbial activity although with different patterns for the different response variables (i.e., benthic bacterial production, planktonic bacterial production and extracellular enzymatic activity, EEA). Generally, local variability was high in all three metrics leading to hardly any significant correlation with stressor variables. However, the data do show some distinct patterns associated with anthropogenic activity. All metrics were relatively low in the headwaters and generally enhanced downstream, probably due to a mixture of direct (DOM import) and indirect (related to enhanced primary production, Carr et al., 2005, see section *Elevated nutrient levels and eutrophication-related stress*) effects of anthropogenic activity. Wastewater effluent was in turn associated with major effects. Interestingly, EEA was enhanced with the first WWTP effluent and then again (and most strongly) shortly before the effluent of the second WWTP after passing a stormwater drainage system which is a source of untreated wastewater at times of strong surface runoff (Beckers et al., 2018). EEA, thus, responded to both treated and (distinctly stronger) untreated wastewater. It seems to act specifically and appears to be

a good functional indicator for wastewater impact, which can help detecting the stimulating effects of wastewater in contrast to the suppressing (toxic) effects (see section *Micropollutants and toxicity related stress* and Karrasch et al., 2019).

The bacterial production results showed similar positive responses to wastewater for planktonic bacteria but contrasting responses for biofilm bacteria. In both years, the activity of biofilm bacteria decreased pronouncedly with the first WWTP and also with the effluent of the stormwater drainage system. Benthic bacteria were shown to be less dependent of DOM quality in the water column (Kamjunke et al., 2015a). This might be due to their association with benthic algae, which represents autochthonous substrate sources. Furthermore, local (benthic) bacteria might respond stronger to chronic toxic effects of the wastewater than floating (plankton) bacteria. Tlili et al. (2017) reported long-term responses of benthic bacterial communities to WWTP mixtures.

On higher trophic levels, macroinvertebrate abundance also increased initially with increasing anthropogenic activity and peaked with the effluent of the first WWTP in both years. This suggests at least in the headwaters a relationship to the overall productivity of the system, which was enhanced by anthropogenic activity. In the lower half of the river, macroinvertebrates were more variable between sites and years and macroinvertebrate abundance was not consistently related to any stressor. However, we found a strong positive trend between macroinvertebrate abundance and leaf litter decomposition. Leaf litter decomposition is a key function in aquatic ecosystems and suggested as functional indicator to assess ecosystem health (e.g., Young et al., 2008; Woodward et al., 2012; von Schiller et al., 2017) although its relationship to environmental stressors and other variables is complex. Increasing nutrient concentrations have been reported to increase the rates of leaf litter decomposition from low to moderate levels and decrease the rate at high levels (e.g., Gulis et al., 2006; Woodward et al., 2012). In contrast, chemical stressors, habitat homogenization and increased salinity have been reported to decrease leaf litter decomposition (Rasmussen et al., 2012; Schäfer et al., 2012) and Schäfer et al. (2007) demonstrated a negative relationship between shredder-mediated leaf processing with the SPEAR index. Our study could not confirm such findings. Instead, the positive trend between macroinvertebrate abundance and leaf litter decomposition suggests that decomposition rates in the coarse mesh bags are predominantly explained by invertebrate quantity (Graça, 2001). Here, the stimulating effects of human activity (e.g., by increased resources and corresponding primary and bacterial production) override the potentially negative effects on invertebrate quantity (e.g. due to loss of habitat diversity or toxicants).

Together, the data of the sampling campaign (including Kamjunke et al., 2019) on DOM, heterotrophic activity and densities of heterotrophs across guilds show significant anthropogenic effects on the carbon dynamics, carbon processing within the food web and associated effects of enhanced secondary production.

### 5.4. Micropollutants and toxicity related stress

Micropollutants are increasingly recognized as a risk for aquatic communities (e.g., Liess and Von der Ohe, 2005; Malaj et al., 2014; Schäfer et al., 2016; Münze et al., 2017), however, studies which link micropollutants to effects on the ecosystem level (i.e., metrics derived from community structures or ecosystem functions) are still rare. Here, we considered both micropollutants as well as trace elements as sources of toxicity related stress. As the concentrations of trace elements were generally low with respect to critical values, e.g. with respect to the surface water directive (OGewV, 2016, compare Appendix Table S1), we focus the discussion on micropollutants.

The by far largest sources for micropollutants were the effluents from the two WWTPs: Similar concentrations of most compounds downstream of both WWTPs indicated that these are commonly used

chemicals in households and urban environments. Increased levels of sumTUs were determined for *Daphnia* and algae downstream of the WWTPs (Fig. 4a, Appendix Table S2). SumTU values ranging from  $10^{-2}$  to  $10^{-1}$  as predicted for *Daphnia* have been linked to short-term and long-term effects on the aquatic invertebrate community structure (Liess and von der Ohe, 2005). Several risk drivers detected here are used in urban or private settings as biocides and have been banned from agriculture, e.g. diazinon has been banned in 1995 and atrazine has been banned in 1990 (BVL, 2009). Trace amounts of atrazine are continuously observed and may originate from groundwater infiltration, which resulted in the observed risk for algae in the headwater region (Vonberg et al., 2014). Carbendazim as an important risk driver for acute risk for fish was still registered as a biocide during the time of sampling, e.g. in outdoor paints (BAuA, 2019). Given the base flow conditions during our study in autumn, it is not surprising that we did not detect peak concentrations of pesticides from agriculture, which are mainly applied in spring and summer and exported to the aquatic ecosystem especially by surface runoff (Leu et al., 2004).

We observed a decreasing contribution of pesticide-sensitive species within the invertebrate community with increasing toxic risk. The SPEAR<sub>pesticides</sub> index clearly dropped consistently for both years downstream of the WWTPs at both locations and showed an overall strong correlation with TU<sub>Daphnia</sub>. It is likely that the loss of sensitive species contributes to the overall loss of Shannon diversity. SPEAR<sub>pesticides</sub> also shows tight correlations with other point-source related variables, especially SRP. However, it is unlikely that the loss of sensitive species is explained by the non-toxic SRP. Regarding integrative metrics for the ecological status, the status classes for invertebrates dropped from “moderate” to “poor” at the first WWTP and remained at “poor” with the effluent of the second WWTP. This pattern was consistent in both years and TU<sub>Daphnia</sub> contributes to explain macroinvertebrate status classes among other stressor variables supporting the conclusion that toxicants emitted from the WWTPs affect the ecological status.

In addition to macroinvertebrates, the fish community also showed remarkable patterns relative to toxicants. Especially the contribution of young fish to the total fish community sharply dropped after the effluent of the first WWTP and remained at a low level downstream. The status of the fish community (software FiBs) dropped from “poor” to “bad” with the effluent of the first WWTP but was not significantly related to TU<sub>fish</sub>. A previous study conducted at the Holtemme River highlighted the high potential sublethal risk on behaviour, reproduction or growth of fish due to constantly emitted micropollutants (Beckers et al., 2018). The coumarin compounds C47 and C47T1 have been identified as potent antiandrogenic compounds in an in vivo fish assay using water extracts from the Holtemme River downstream of the first WWTP by Muschket et al. (2018). Muschket et al. (2018) observed antiandrogenic effects even without enrichment of the water sample. In this study, the coumarin compounds were detected in lower concentrations (Appendix Table S2, Fig. 4a). Yet, they pose a constant, chronic and specific burden for fish in addition to other constantly emitted compounds representing a baseline exposure. Against this background and by considering the negative effect of the WWTPs on young fish as observed here, it may be hypothesized that micropollutants emitted constantly from WWTPs, especially the coumarin compounds, may impact the fish community and its reproduction in the Holtemme River. Further in-depth studies are necessary to establish cause-effect-relationships between this chemical exposure and effects on organisms, communities and ecological functions in the field.

### 5.5. Potential interconnections between stressor variables

As discussed in the previous sections, our comprehensive dataset enabled us to draw likely cause-and-effect chains from the driver and corresponding stressor variable to the effects in the ecosystems for different stressor categories and to identify appropriate response variables that indicate specific stressors. However, some response variables

show significant correlation to several stressor variables and are explained by multiple variables in the linear model. This was especially true for the status classes of macroinvertebrates, which were explained by six variables in the linear regression model (Table 4). Here, variables for nutrient concentrations (both SRP and nitrate-N as well as share of arable land use, which is linked to nutrient concentrations, Müller et al., 2018), toxic stress (sumTUs) and hydromorphological alterations (flow diversity) contribute to explain macroinvertebrate status classes. Furthermore, the model has a very high explanatory power ( $R^2=0.98$ ). This finding supports the value of this integrative metric as measure for general degradation. However, it also shows that this metric alone is not suitable as indicator to identify specific stressors.

The SPEAR<sub>pesticides</sub> index as trait-based metric indicates the contribution of sensitive species towards pesticides (Liess and Von der Ohe, 2005). In our study, SPEAR<sub>pesticides</sub> is strongly linked to sumTUs for *Daphnia*, which predominantly originate from the WWTPs. However, also oxygen concentration, share of arable land use and nitrate-N explains SPEAR<sub>pesticides</sub> in the regression model. At least with respect to share of arable land use and nitrate-N (which is linked to share of arable land use; see Müller et al., 2018), it is possible, that SPEAR responds to pesticides from agriculture. As our study was performed in autumn, we did not measure significant amounts of pesticides from agriculture. However, SPEAR as a community-based indicator might still be affected by pesticide applications in spring and summer.

The multi-variable control of phytoplankton is difficult to explain, as the floating phytoplankton is expected to be predominantly controlled by water residence time and thus by distance from source (see discussion in subsection *Elevated nutrient levels and eutrophication-related stress*). It appears likely, that the variables share of arable land use as well as SRP and sumTUs for *Daphnia* (both increase with WWTP impact in the middle and lower reaches) contribute to explain phytoplankton due to their co-correlation with distance from source.

Our study design is not suitable to detect the types of interactions between stressors. This would require experimental testing (e.g., Graeber et al., 2017; Beermann et al., 2018; Polst et al., 2018) in addition to the field data analyses as performed here. However, by revealing likely interconnections between stressors, our study provides a set of different stressor and response variables as relevant candidates for an experimental testing of interactions in multi-factorial experiments in the future. This would ideally be performed in mesocosms connected to the Holtemme (Fink et al., 2020).

## 6. Conclusion

In the present study we quantified a variety of both stressor variables and ecological response variables, including effect based metrics for micropollutants and state-of-the-art measures for ecosystem analyses besides established indicators of the EU-WFD monitoring. This comprehensive dataset together with the detailed analyses of drivers from the same sampling campaigns (esp. Beckers et al., 2018, 2020; Müller et al., 2015; Kamjunke et al., 2019) allowed us to disentangle effects of multiple stressors and identify likely cause-and effect chains from drivers and corresponding stressor variable to the effects in the ecosystems. With this respect, the dataset demonstrates the relevance of four major stressor categories (see subchapters above) and reveals promising stressor and response variables as potential indicators to detect effects on the ecosystem.

Given the complexity of multiple stress in real world settings, it is increasingly important to combine different state-of-the-art tools for stressor diagnosis and ecosystem analyses, which are up to now often applied in separate studies, at model sites. Such sites for intense interdisciplinary research as established at the Holtemme River are, besides experimental approaches and data-driven analyses on larger scales, one important cornerstone for understanding the real-world effects of multiple stressors and to develop improved diagnostic indicators.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2020.144324>.

## Declaration of competing interest

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.

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