Effect-based nationwide surface water quality assessment to identify ecotoxicological risks

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ABSTRACT

A large portion of the toxic effects observed in surface waters cannot be attributed to compounds regularly measured by water authorities. Hence, there is an urgent need for an effect-based monitoring strategy that employs bioassays to identify environmental risks. The aim of the present study was to perform an effect-based nationwide water quality assessment to identify ecotoxicological risks in a wide variety of surface waters. At 45 locations silicone rubbers and polar organic chemical integrative samplers were exposed to surface water for 6 weeks. Alongside the passive samplers an *in-situ* daphnid test was performed. Subsequent to field exposure, accumulated compounds were extracted from the passive samplers after which a battery of *in vivo* and *in vitro* bioassays was exposed to the extracts. The bioassay battery was selected such that it could identify the risks posed by a wide range of chemical pollutants and their transformation products, while simultaneously allowing for targeted identification of groups of compounds that cause specific effects. Bioassay responses were compared to effect-based trigger values to identify potential ecotoxicological risks at the investigated locations. Responses were observed in all bioassays, and trigger values were exceeded in 9 out of the 21 applied assays, allowing for ranking of the investigated locations based on ecotoxicological risks. No relationship between land use and the identification of ecotoxicological risks was observed. Based on the results, considerations regarding future improvements of effect-based monitoring are given. It is concluded that effect-based water quality assessment allowed prioritization of sites based on ecotoxicological risks, identified the presence of hazardous compounds regardless of being listed as priority substances, and meanwhile could prevent costly chemical analysis at sites with low ecotoxicological risks.

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1. Introduction

According to the European Union (EU) Water Framework Directive (WFD)(The European Parliament and the Council of the European Union, 2013), chemical water quality is determined by monitoring surface waters for the presence of 45 (groups of) priority substances. However, the use of many of these compounds is restricted or banned and, as a result, concentrations of priority substances in European waters are decreasing (Altenburger et al., 2015; Fliedner et al., 2016). Simultaneously, industries have

switched to a plethora of alternative compounds, which may enter the aquatic environment. Hence, the priority substances list is outdated, as the selected compounds are frequently absent nowadays, while the compounds present are not listed as priority substances (Busch et al., 2016; Schriks et al., 2010a; Schwarzenbach et al., 2006). Consequently, when toxic effects are observed in surface waters, these can often not be attributed to compounds measured by water authorities (Altenburger et al., 2015; Neale et al., 2015). Risks of pollutants to freshwater ecosystems are thus caused by mixtures of a myriad of (un)known, unregulated and unmonitored compounds (Daughton, 2005). Understanding of these risks requires a paradigm shift, that allows for new holistic monitoring methods that do not solely depend on chemical analysis of priority substances, but contrastingly consider biological effects first (Hamers et al., 2018; Leusch et al., 2014; van der Oost

et al., 2017a, b). Therefore, there is a need for an effect-based monitoring strategy that employs bioassays to identify environmental risk (Brack et al., 2017; Wernersson et al., 2015).

Bioassay responses to surface water samples are caused by the combined action of mixtures of all bioavailable (un)known compounds and their metabolites present, thereby overcoming the limitations posed by chemical analysis of a limited number of target compounds (Brack et al., 2017; Neale et al., 2015). Indeed, the applicability and reproducibility of a battery of bioassays to identify ecotoxicity in regular water quality monitoring has been shown in recent years (Blackwell et al., 2019; Di Paolo et al., 2016; Hamers et al., 2018; Jia et al., 2015; Leusch et al., 2014; Novák et al., 2018; van der Oost et al., 2017a). The ecotoxicity profiles of the surface water samples that are generated by such a bioassay battery allow for calculation and ranking of a cumulative ecotoxicological risk for the selected locations. Subsequently, at locations where risks are identified, it becomes relevant to investigate the drivers of the observed effects. The aim of the present study was therefore to identify ecotoxicological risks in an effect-based nationwide water quality assessment in a wide variety of surface waters in The Netherlands.

The success of effect-monitoring relies largely on the ease of use, endpoint specificity and scale of the used bioassays, as well as on the ability to interpret the measured responses. To ensure sensitivity to a wide range of potential stressors, while still providing specific endpoint sensitivity, the present study employed a previously successfully implemented bioassay battery including *in situ* whole organism assays as well as laboratory based whole organism *in vivo* and mechanism specific *in vitro* assays (van der Oost et al., 2017a). Adverse effects in the whole organism assays point to general toxic pressure and represent a high ecological relevance. *In vitro* or small-scale *in vivo* assays that target highly specific molecular initiating events allow for focused identification and subsequent confirmation of (groups of) toxic compounds (Brack et al., 2016; Escher et al., 2018; Neale et al., 2017). The identification of ecotoxicological risks from bioassay battery responses follows from the comparison of bioanalytical signals to previously determined thresholds, defined as effect-based trigger values (EBT), that differentiate between acceptable and poor water quality (Tang et al., 2013). Recently van der Oost et al. (2017b) and Escher et al. (2018) derived EBTs for a variety of bioassays commonly applied in surface water quality assessment.

An additional limitation of the present chemical water quality assessment is that grab sampling is commonly used for surface water sample collection. Yet, concentrations of compounds typically vary over time and therefore grab sampling only provides a snapshot of the chemical make-up of a water body (Jones et al., 2015). Passive sampling can overcome these limitations by exposing a sorbent to the target environment for several weeks to months, accumulating compounds from the water over time (Vrana et al., 2005). In this way, passive sampling integrates fluctuations in compound concentrations in time, and simultaneously enriches surface water samples to an extent that (bio)analytical detection limits become very low. Current limitations of passive sampling in water quality assessment are the compound selectivity of the receiving phase and the challenge of precisely determining the sampled volume of water (Roll and Halden, 2016). Nonetheless, the advantages of passive sampling compared to grab sampling outweigh its disadvantages, and passive sampling is increasingly applied as a valuable tool in the monitoring of environmental contaminants. Hence, the combination of passive sampling and effect-monitoring allows for time-integrated and reliable surface water quality assessment, that considers effects of all sampled (un) known compounds, regardless of priority lists.

In the present study, silicone rubber (SR) and polar organic chemical integrative sampler (POCIS) passive samplers were applied at 45 surface water locations. Alongside the passive samplers an *in-situ* daphnid test was performed. Subsequent to field exposure, accumulated compounds were extracted from the passive samplers after which a battery of *in vivo* and *in vitro* bioassays was exposed to the extracts. Bioassay responses were compared to effect-based trigger values to identify potential ecotoxicological risks at the investigated locations. Finally, responses were related to surrounding land use, water body morphology and WFD ecological water quality assessment scores.

2. Material & methods

2.1. Sampling sites

Sampling sites were selected in collaboration with 12 Dutch waterboards and the Dutch national water authority. Sites were classified based on the major surrounding land use or potential source of pollution. When classification of a location was not possible due to the diffuse or variable nature of contamination, it was assigned to the category "complex". This resulted in the classification of 45 surface water locations into six categories (Fig. S1): reference (n = 5), urban (n = 7), wastewater treatment plant effluent impacted (WWTP; n = 7), horticulture (n = 7), mixed agriculture (agri mix; n = 7) and complex (n = 12).

2.2. Deployment, extraction and estimating sampled volumes of passive samplers

2.2.1. Passive sampling devices

Silicone rubber (SR) sheets, with a weight of 20 g per set of six sheets, spiked with performance reference compounds (PRCs), were obtained from Deltares (Utrecht, The Netherlands) and applied for the sampling of nonpolar compounds.

POCIS, containing 0.22 g Oasis HLB sorbent, were obtained from Exposmeter (Tavelsjö, Sweden) and applied for the sampling of compounds in the more polar range. No sampler pre-treatment was required, and the samplers were transported to the study sites in their original airtight packaging.

2.2.2. Field deployment of passive samplers

SR sheets and POCIS were deployed simultaneously at each sampling location in cages to attach and protect the passive samplers during the exposure period. Cages were secured to the bottom or to the embankment to avoid loss of samplers and to ensure permanent inundation. Per location, six SR sheets and four POCIS were exposed for a period of six wk. After exposure, the samplers were transported to the laboratory and stored at −20 °C until extraction.

2.2.3. Extraction of silicone rubber

SR sheets were cut into small pieces and put in precleaned thimbles of a Tecator Soxtec Avanti 2050 extraction system. Extractions were performed in 80 mL of a MeOH:acetonitrile (1:2 v/v) mixture with boiling stones. The extraction program was as follows: 120 min boiling at 180 °C, 30 min rinsing, 5 min recovery, and 1 min drying. Cooled extracts were filtered over glass fiber filters and collected in 250 mL glass bottles. Extraction jars were rinsed twice with 10 mL of extraction mixture. Extracts were evaporated by TurboVap II Zymark at 45 °C to approximately 5 mL, transferred quantitatively (rinsed twice with 5 mL extraction mixture) to 15 mL conical tubes, evaporated under nitrogen, and finally the end volumes were filled up to exactly 10 mL with extraction mixture.

2.2.4. Extraction of POCIS

To enable elution, the sorbent between the POCIS membranes was transferred quantitatively into an empty solid phase extraction (SPE) column with a polyethylene frit. Columns were dried under vacuum extraction, followed by centrifugation (2000 rpm, 15 min) under nitrogen flow. Dry SPE columns were eluted three times with 3 mL of acetone, with 5 min equilibration time between elutions. Eluates were collected in 10 mL conical tubes, and the end volumes were filled up to exactly 10 mL with acetone.

2.2.5. Estimation of sampled water volumes

2.2.5.1. Silicone rubber. SR sheets were spiked with PRCs with a wide hydrophobicity range (biphenyl D10 and polychlorinated biphenyl (PCB) congeners 1, 2, 3, 10, 14, 21, 30, 50, 55, 78, 104, 145, and 204) that do not occur in Dutch surface waters. For PRC chemical analysis, SR extracts were transferred to petroleum ether by adding 2 mL extract to 40 mL petroleum ether and concentrated with Kuderna Danish at $80\,°C$. The petroleum ether extract was cleaned up with aluminium oxide and silica gel column chromatography. The cleaned extract was evaporated to exactly 2 mL and analysed with an Agilent 7890 Triple Quadrupole gas chromatography mass spectrometer (GC-MS/MS) equipped with Edwards pump. Quantification was performed using an external calibration series of 6 concentrations. The rate of PRC dissipation was used to calculate the exchange rates (R_S values, in L/day) of the samplers (Booij and Smedes, 2010). Subsequently, 50% of this calculated R_S was used as a provisional estimation of the average extracted water volume per day as described by van der Oost et al. (2017a).

2.2.5.2. POCIS. While standardized protocols for the determination of sampled volume of passive samplers have been described for nonpolar samplers, no such consensus has yet been reached for polar passive samplers (Harman et al., 2011). This is partly due to the different nature of polar and nonpolar passive samplers, and the processes that hence dictate the uptake of polar compounds in passive samplers (Harman et al., 2012). Sampling rates for polar compound uptake in POCIS in stagnant to near stagnant water have been reported in the range from 0.001 to 2.46 L/day, with an average sampling rate of 0.18 L/day (Harman et al., 2012). Hence, to compare bioassay effects between sites, in the present study the same estimated average sampled volume of 0.18 L/day was applied to determine the concentration factor of all field deployed POCIS.

2.3. Bioassay battery

Whole organism bioassays and antibiotics WaterSCAN assays were performed at the Waterproef Laboratory (Edam, The Netherlands). In vitro CALUX assays were performed at the Bio-Detection Systems laboratories (Amsterdam, The Netherlands). Passive sampling extracts were converted to other solvents before exposure in the bioassays. More information on bioassay analytical details and solvent transfer is given in the supplementary information (pages S2-5). An overview of the employed bioassays, their endpoints, and their respective units of effect expression and EBTs is given in Table 1.

2.3.1. Daphnia in situ exposure

Daphnids were exposed to the surface water at 33 of the 45 study sites in glass jars. Field exposure was carried out during the first or second week of the passive sampler deployment. The survival of the in situ exposed daphnids was recorded after 1 wk of exposure. An observed mortality of 20% was used as trigger value for potential ecological effects (van der Oost et al., 2017b).

2.3.2. Whole organism bioassays

For the whole organism bioassays, SR passive sampler extracts were subjected to three bioassays. As these whole organism bioassays have no specific target compound group, toxicity is expressed as toxic units (TU), rather than reference compound equivalents. Herein, one TU represents the dilution at which the extract causes 50% effect for the respective endpoint of the test (EC_{50}).

2.3.2.1. Daphnia 48 h immobilization. The Daphnia 48 h immobilization assay was performed according to the Organisation for Economic Co-operation and Development (OECD) standard 202 (OECD Environmental Health and Safety, 2004), with reduced test volumes. EC_{50} values (volume percentage) were determined by nonlinear regression analysis with a log-logistic model by the statistical program SPSS (IBM Analytics). Bioassays were considered valid if >90% of the daphnids in the negative controls were mobile at the end of the test.

2.3.2.2. Algatox. The inhibition of algal growth was determined according to OECD standard 201 (OECD Environmental Health and Safety, 2006), with reduced test volumes, based on Peterson et al. (2005). After 72 h, exponential algal growth curves were determined to assess the percentage of growth inhibition compared to controls. Algal growth rate in the controls was required to reach 0.92/d, according to the OECD standard. The EC_{50} values were calculated using sigmoidal dose–response curves with variable slopes.

2.3.2.3. Microtox. The Microtox® test was performed by exposing the bioluminescent marine bacterium Aliivibrio fischeri to a dilution range of the passive sampler extracts. Toxicity was determined by quantifying inhibition of the luminescence produced by A. fischeri exposed to the extracts after 5, 15, and 30 min of exposure. Microtox Omni software (version 1.18) was used for determination of the TU values.

2.3.3. CALUX assays

Passive sampler extracts were analysed by a panel of in vitro CALUX® bioassays. Specific CALUX assays were performed on either polar or non-polar extracts, as suggested by van der Oost et al. (2017a). SR extracts were subjected to DR, PAH, PPARγ, Nrf2, PXR and p53 (with and without S9 metabolism) assays and POCIS extracts were subjected to ERα, anti-AR and GR assays, according to previously described protocols (Hamers et al., 2006; Murk et al., 1996; Sonneveld et al., 2004; Van Der Linden et al., 2008). The DR CALUX assay was performed without a sulfuric acid clean up step to eliminate PAHs and isolate the dioxins and dioxin-like polychlorinated biphenyls. To rule out confounding influences, cells were also monitored for cytotoxicity, which resulted in additional data for cytotoxicity caused by both polar and non-polar passive sampler extracts. The effects of the extracts were expressed as bioanalytical equivalents (BEQs) of the reference compounds (Table 1).

2.3.4. Antibiotics activity assay

Activities of 5 classes of antibiotics in the POCIS extracts were determined with the WaterSCAN assay, obtained from RIKILT (Wageningen, The Netherlands). The test system comprised 5 plates (details outlined in Pikkemaat et al., 2008): tetracyclines (T), quinolones (Q), β-lactams and macrolides (B + M), sulphonamides (S), and aminoglycosides (A). After incubation of the test plates, antibiotic activities were estimated and expressed as BEQ concentrations of the reference compounds (Table 1).

Table 1
Bioassay battery applied to assess toxicity at 45 surface water locations in The Netherlands. Effect-based trigger values (EBT) were previously defined by Escher et al. (2018) (PAH, anti-AR and ER CALUX) and van der Oost et al., 2017b.

	Bioassay	Endpoint	Reference compound	EBT	Unit
in situ	Daphnia *in situ*	Mortality	n/a	20	% mortality
in vivo non-polar	Daphniatox	Mortality	n/a	0.05	TU
	Algatox	Algal growth inhibition	n/a	0.05	TU
	Microtox	Luminescence inhibition	n/a	0.05	TU
in vitro CALUX non-polar	cytotox nonpolar	Cytotoxicity	n/a	0.05	TU
	DR	Dioxin (-like) activity	2,3,7,8-TCDD	50	pg TEQ/L
	PAH	PAH activity	benzo(a)pyrene	6.21	ng BEQ/L
	PPARγ	Lipid metabolism inhibition	rosiglitazone	10	ng REQ/L
	Nrf2	Oxidative stress	curcumin	10	μg CEQ/L
	PXR	Toxic compound metabolism	nicardipine	3	μg NEQ/L
	p53 -S9	Genotoxicity	actinomycin D	0.005	ng AEQ/L
	p53 + S9	Genotoxicity (after metabolism)	actinomycin D	0.005	μg CEQ/L
in vitro CALUX polar	cytotox polar	Cytotoxicity	n/a	0.05	TU
	ER	Estrogenic activity	17β-estradiol	0.1	ng EEQ/L
	anti-AR	Antiandrogenic activity	flutamide	14.4	μg FEQ/L
	GR	Glucocorticoid activity	dexamethasone	100	ng DEQ/L
in vitro antibiotics polar	T	Bacterial growth inhibition (Tetracyclines)	oxytetracycline	250	ng OEQ/L
	Q	Bacterial growth inhibition (Quinolones)	flumequine	100	ng FEQ/L
	B + M	Bacterial growth inhibition (β-lactams and Macrolides)	penicillin G	50	ng PEQ/L
	S	Bacterial growth inhibition (Sulfonamides)	sulfamethoxazole	100	ng SEQ/L
	A	Bacterial growth inhibition (Aminoglycosides)	neomycin	500	ng NEQ/L

2.4. Data analysis

Bioassay effects were expressed as BEQ/L by using the estimated sampled water volumes of the respective passive samplers to determine the concentration factor of the used extracts. Subsequently, bioassay effects were compared to previously defined EBTs. EBTs from Escher et al. (2018) were utilized when available, and when the used reference compounds matched those applied in the current study. This was the case for the PAH, *anti*-AR and ER CALUX assays. For all other applied bioassays, EBTs from van der Oost et al. (2017b) were used (Table 1).

Average numbers of EBT exceedances per land use category were tested for equality of variances using a F-test, and subsequently differences between land use were tested for significance using a Two-sample T-test assuming equal variances ($\alpha = 0.05$). Statistical analyses were performed in Excel for Mac version 16 (Microsoft).

Multivariate analysis was applied to gain insight in the relationship between the surrounding land use, water type and ecological water quality and the bioanalytical responses. Only the tests that showed a response above the respective EBT were included. The total bioanalytical dataset consisted of 9 responding bioassays and 45 locations. Alongside this response matrix, two location variables were included in the multivariate analysis: A measure of ecological quality, expressed as WFD ecological quality assessment scores for macrofauna (EQR mafa), obtained from the Dutch waterboards, and the water type of the locations, expressed as ditch, pond or lake for lentic waters, and stream, channel or river for lotic waters. Missing values in the dataset were substituted with the average response value for each bioassay, to minimise their effect on multivariate analysis outcome. Bioassay responses were transformed to a logarithmic scale and the resulting dataset was ordinated by redundancy analyses (RDA) in CANOCO 4.2 for Windows (Ter Braak, 1990, 1988). The data analyses are fully described by Verdonschot and Ter Braak (1994). An unrestricted permutation test was used to test the validity of the total ordination as described by Ter Braak (1990) and Verdonschot and Ter Braak (1994).

3. Results

### 3.1. Bioassay battery responses to passive sampler extracts

Passive samplers for polar and non-polar compounds were successfully exposed at 45 surface water locations. During extraction, POCIS extracts were lost for three sampling locations, resulting in an incomplete dataset for these locations. Therefore, these locations were excluded from the comparison of EBT exceedances per location. All bioassays met their respective validity criteria. Responses were observed in all 21 bioassays, but for each bioassay there were clear differences in the strength of the responses between the locations. A representative example of the 21 bioassays is given in Fig. 1, which depicts the estrogen receptor (ER) CALUX responses to the POCIS extracts. ER responses were observed at all but one location, with only a non-detect at one of the reference locations. The intensity of the response was highly variable for the different locations per land use, with the highest response at one of the urban locations. On average, the highest responses were observed at urban (0.40 ng EEQ/L), complex (0.38 EEQ/L) and WWTP (0.36 EEQ/L) locations, while the lowest responses were observed at the reference locations (0.13 EEQ/L). This is also reflected by the percentage of EBT exceedances per land use category, where EBT exceedance for the ER CALUX assay was observed at the majority of urban (71%), complex (89%) and WWTP (86%) locations, while the EBT was exceeded at 40% of the reference locations. Responses in the other 20 bioassays are listed in Table S1. This information was subsequently used to calculate the number of EBT exceedances per location.

3.2. Effect-based trigger value exceedances per location

All locations caused the exceedance of at least one EBT in the bioassay battery. The sum of EBT exceedances per location and the resulting average number of EBT exceedances per land use category are depicted in Fig. 2. The variation between locations within a land use category was largest for horticultural locations, while urban locations showed the most consistent number of responses above
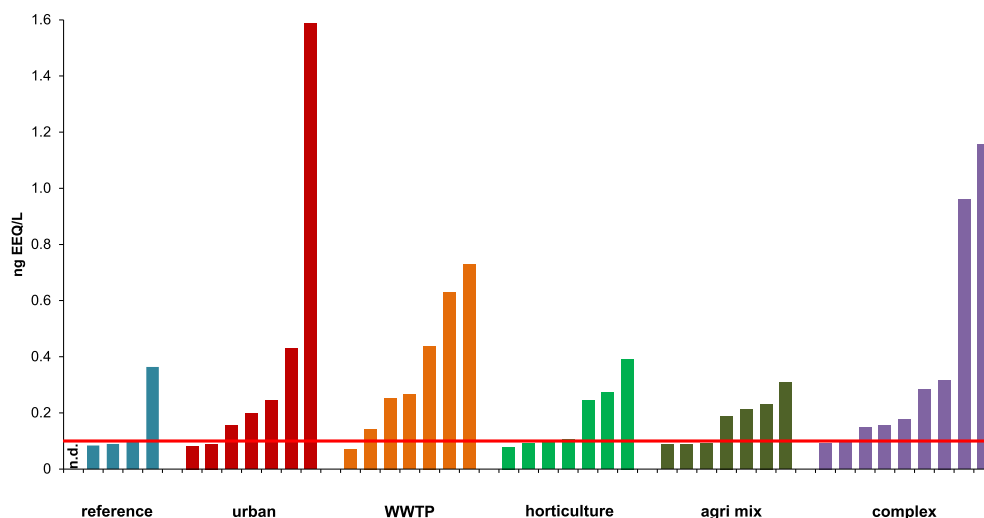
Fig. 1. Estrogen receptor chemically activated luciferase expression (ER CALUX) assay responses to POCIS passive sampler extracts from 42 surface water locations with different surrounding land uses expressed as 17β-estradiol equivalents (ng EEQ/L). The red line indicates the effect-based trigger value (0.1 ng EEQ/L). WWTP = wastewater treatment plant and agri mix = mixed agriculture, n.d. = no detected bioanalytical response. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
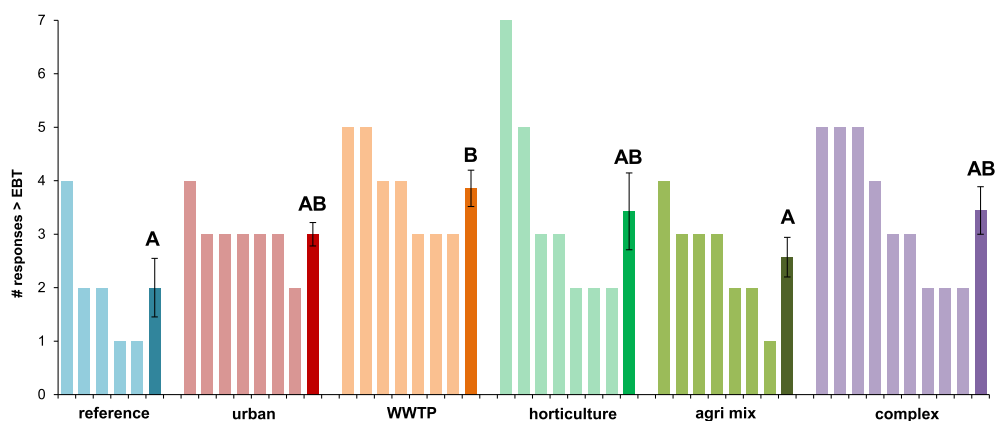

Fig. 2. Number of effect-based trigger value (EBT) exceedances per location (light bars) and average number of exceedances per land use category (dark bars, ±SE) of a panel of 21 bioassays at 42 surface water locations grouped by surrounding land use. WWTP = wastewater treatment plant and agri mix = mixed agriculture. Statistical differences between land use averages are indicated with letters (Two-sample Ttest assuming equal variances, $\alpha = 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the EBT per location. The lowest average number of EBT exceedances was observed at reference locations (2), and the highest number of EBT exceedances were observed at urban (3), WWTP (3.9), complex (3.4) and horticulture (3.4) locations, including one location with seven EBT exceedances in the latter. However, only at WWTP locations the average number of EBT exceedances was significantly higher than at the reference ($p < 0.01$) and mixed agriculture ($p < 0.05$) locations. The sum of EBT exceedances per site allowed for the ranking of sites based on ecotoxicological risk, where the sites with the highest number of EBT exceedances are assumed to be at the highest risk of surface water pollution (Table S1). EBT exceedances were observed for 9 out of the 21 applied bioassays: For the *in situ Daphnia* test and for 8 *in vitro* CALUX assays performed with both non-polar and polar extracts.

3.3. Ecotoxicological risk identification

Next, a heat map was constructed that visualizes the percentage of the investigated locations with EBT exceedance per land use category (Fig. 3). Interestingly, the EBT for the PAH CALUX assay

was exceeded at all the investigated locations, and hence this assay did not allow for differentiation in ecotoxicological effect identification between locations or land uses. Reference locations showed the lowest percentage of EBT exceedances, however ecotoxicological risk was not completely absent, with responses in PAH, Nrf2, PXR and ER CALUX tests. At urban locations, ecotoxicological risks were driven most strongly by PXR, ER and *anti*-AR activity. At WWTP locations, the most profound contribution to ecotoxicological risks was caused by PXR and ER activity. A less frequent, but nonetheless substantial contribution to ecotoxicological risks was observed for Nrf2 and *anti*-AR activity. Similarly, for horticultural locations, ecotoxicological risks were most frequently caused by PXR and ER, with a contribution of ER and *anti*-AR activity. However, risk indication was also frequently observed in the *Daphnia in situ* test, and polar extracts from horticultural locations were the only samples to cause cytotoxicity above the EBT. For mixed agricultural locations, risks were most frequently caused by ER activity, with less frequent contributions of PXR and *anti*-AR. Notably, mixed agricultural locations were the only ones to cause EBT exceeding PPARγ activity. Complex locations also showed EBT exceedances
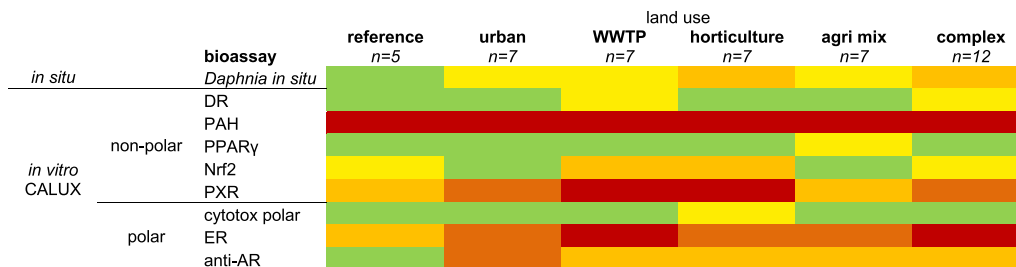
Fig. 3. Heat map depicting responses in the 9 bioassays that gave a signal above the EBT for 45 surface water locations with different surrounding land uses. Colours indicate the percentage of the investigated locations with EBT exceedance per land use category: green = 0%, yellow = 0—25%, light orange = 25—50%, dark orange = 50—75% and red = 75—100% of locations. Deviating number of samples per land use for *Daphnia in situ*: reference = 4, urban = 5, WWTP = 6, horticulture = 2, agri mix = 6 and complex = 10. For complex land use, n = 9 for polar CALUX assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

most frequently for PXR and ER, with less frequent exceedances for *anti*-AR activity and the *Daphnia in situ* test. DR activity above the EBT was observed only at WWTP and complex locations.

3.4. Response frequency per bioassay

Responses of all bioassays were summarized to gain insight into which assays responded most frequently to the passive sampler extracts and were hence the main determinants of the detection of ecotoxicological risks (Fig. 4). Bioassay signals were categorized as no response, or a response below or above the EBT of that test. The frequency of effect detection in the bioassays ranged from largely no response (96% of locations) for the Algatox assay, to responses above the EBT at all locations for the PAH CALUX assay. The PAH, PXR, Nrf2 and DR CALUX assays showed a response at all locations, however with a varying frequency of responses above the EBT, with the most striking result for the PAH CALUX assay, for which the EBT was exceeded at all the investigated locations. Nine out of the battery of 21 bioassays showed responses above the EBT (Figs. 3 and 4). The other 12 assays gave no response above their EBT. Out of these, nine showed no bioanalytical response at all at more than 50% of the investigated locations. These were the GR CALUX and the five antibiotics assays which were exposed to the polar passive sampler extracts, and the whole organism Daphniatox and Algatox and the *in vitro* p53 CALUX assay with S9 metabolism exposed to the non-polar extracts.

3.5. Multivariate analysis

The ordination result of the RDA with land use as explaining variable is presented as a correlation biplot of bioassay responses, land use, and environmental quality scores (Fig. S1). The RDA revealed no significant variables in the dataset. Hence, land use, water type and the ecological quality score did not explain the variation observed in the bioassay battery responses.

4. Discussion

4.1. Effect-based identification of ecotoxicological risks

In the present study, an effect-based nationwide water quality assessment to identify ecotoxicological risks was performed. Effects were observed in all bioassays, and EBT exceedances were observed for 9 out of the 21 bioassays. The sum of EBT exceedances per site allowed for the ranking of sites based on ecotoxicological risk, rather than on the presence of a limited number of target compounds (Hamers et al., 2018), which can be considered as a proof of principle of effect-based water quality assessment. Subsequently, at locations where risks were identified, it becomes relevant to investigate the drivers of the observed effects.

The bioassays that showed responses above EBTs in the present study, and hence allowed the identification of ecotoxicological risks, were the DR, PAH, PPARγ, Nrf2 and PXR CALUX assays for non-polar extracts, the ER, *anti*-AR and cytotoxicity CALUX assays for polar extracts, and the *in situ Daphnia* assay. This is partly in line with previous findings by Escher et al. (2014) and van der Oost et al. (2017a), that identified high responses of *in vitro* assays for, amongst others, PAH, Nrf2, PXR and ER and *anti*-AR activity in surface water. Following from the observed CALUX responses, in the present study, risks were caused by both polar and non-polar
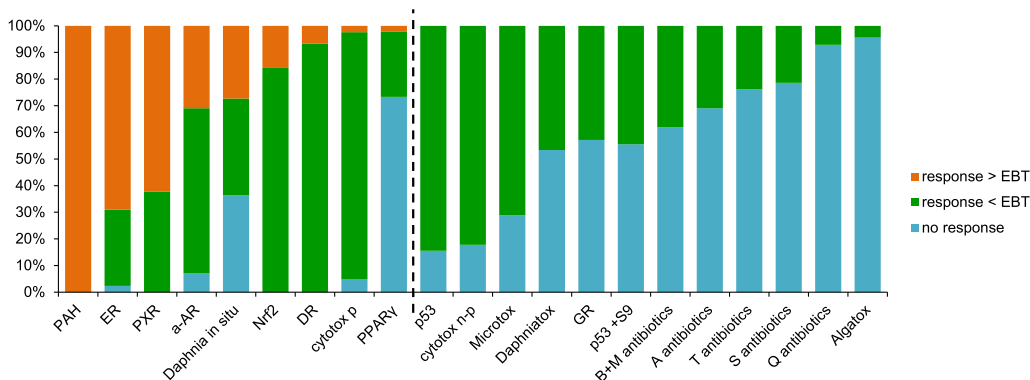
Fig. 4. Frequency of responses of a panel of 21 bioassays to passive sampler extracts from 45 surface water locations. Colours indicate the bioassay responses and EBT exceedances at the percentage of study locations. The dashed line indicates the division between bioassays with and without EBT exceedance in the present study. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

organic extracts. Several of these tests indicated risks at the majority of the studied locations. Most notably the PAH CALUX, which indicated ecotoxicological risks of polycyclic aromatic hydrocarbons (PAH) at all sites. This can in part be explained by the atmospheric origin of PAH loading to aquatic systems, causing the presence of PAHs even at locations with very limited anthropogenic pollution (Manoli and Samara, 1999). Interestingly, however, this was not the case for dioxins, which also partly find their way to the aquatic environment through atmospheric deposition (Kulkarni et al., 2008). In the present study, risk of dioxins was only observed for WWTP and complex locations, and infrequently at both. As for both groups of compounds the ultimate environmental sink is the sediment, which was not examined in the present study, this difference may be explained by the current emissions, which, in Europe, are more strongly regulated for dioxins and more common for PAHs (Kulkarni et al., 2008; Manoli and Samara, 1999).

The PAH and DR CALUX assays both target aryl hydrocarbon receptor binding, yet after different exposure times (4 vs. 24 h respectively), which affects the *in vitro* metabolization of PAHs (Pieterse et al., 2013). Since in the present study, water extracts subjected to the DR CALUX assay were not treated with a sulfuric acid clean up step to eliminate PAHs and isolate the dioxins and dioxin-like polychlorinated biphenyls, responses in the DR CALUX assay may be caused by stable PAHs that were not metabolized during the 24 h exposure. Thus, had the extracts been cleaned up with sulfuric acid, the three samples that showed EBT exceedance in the DR assay may well have lost their activity due to destruction of stable PAHs. This strengthens the observation that ecotoxicological risks in the investigated surface waters are much more common for PAHs than for dioxins.

Besides the ubiquity of PAHs in surface waters, the detection of ecotoxicological risk also depends on the EBT value used for this specific test. In the case of the PAH CALUX assay, this EBT value was obtained from the study by Escher et al. (2018), in which EBTs were derived by read across from existing EU WFD environmental quality standards (EQS). This resulted in an EBT value of 6.21 ng benzo(a)pyrene equivalents (BEQ) per litre, which is substantially lower than the EBT of 150 ng BEQ/L derived by van der Oost et al. (2017b). Had we applied the latter, the resulting detection of ecotoxicological risk caused by toxic PAH concentrations in surface water would have been markedly less dramatic, and would have resulted in an EBT exceedance at only a single location. However, the study by Escher et al. (2018) based their EBT on existing EQS values, about which a European wide consensus exists, and which reliably indicates ecotoxicological risks to aquatic communities. Hence, the dramatic EBT exceedance observed here may identify a serious risk posed by PAHs in the majority of waterbodies, even at locations with very few other anthropogenic pollution sources. Nonetheless, the profound influence of the value of the EBT for each bioassay on the detection of ecotoxicological risks should not be underestimated. This underlines the need for a standard procedure and consensus on EBT derivation and values for the successful application of effect-based monitoring strategies in water quality assessment.

4.2. Identification of location and land use specific ecotoxicological risks

Although several unique responses for the different land use and bioassay combinations were observed, no land use specific responses or patterns became apparent, and only small differences in EBT exceedances between land use types were found. This observation was corroborated by the outcome of the multivariate analysis, which revealed no significant effect of land use on the bioassay battery responses. The selected locations appear to suffer from the presence of complex mixtures of micropollutants, frequently caused by the same drivers. Hence, to identify pollution source specific drivers of ecotoxicological risks, in future research locations should be selected that better represent a single pollution source and that are more morphologically and biogeochemically similar to exclude confounding effects. Yet at the same time, these findings also confirm the complex nature of surface water pollution in large river deltas. This raises the question if categorizing sites into land use types is appropriate at all, and if alternatively, sampling sites may better be considered independent stochastic draws of diffuse pollution covering the industrialized world. When applying that paradigm, in the present study, several discriminating bioassays allowed for the identification of locations at risk from chemical stressors, and for the ranking and subsequent prioritization of the locations that were at the highest risk from micropollutants.

4.3. Considerations for improved effect-based monitoring

EBT exceedances were observed for 9 out of the applied 21 bioassays, indicating that 12 bioassays were less effective in elucidating ecotoxicological risks at the studied locations. The bioassays that were not discriminating for ecotoxicological risks were the *in vivo* whole organism bioassays, the antibiotics assays, and the p53 (with and without S9 metabolism), GR and cytotoxicity (for non-polar compounds) CALUX assays.

4.3.1. p53 and cytotoxicity CALUX assays

The p53 and cytotoxicity CALUX assays indicate risks at a high organisational level caused by all compounds in a water sample (Escher et al., 2018; Van der Linden et al., 2014; van der Oost et al., 2017b). Hence, signals above the EBT in these assays would imply far stretching ecological effects in the field (Maltby, 1999). Therefore, although these tests did not respond frequently or severely to surface water passive sampler extracts in the present study, the inclusion of such tests in future bioassay batteries is recommended given their ecological relevance. Yet, the inclusion of S9 metabolism in the p53 test can be debated. The S9 metabolism in this assay can elucidate the enzymatic activation of mutagenicity in the sample. However, given the time integrative nature of passive sampling (six weeks in the present study), metabolism and activation of more toxic or persistent metabolites is expected to occur in the field rather than in the laboratory, and the added value of *in vitro* metabolization is negligible. This was also illustrated by the much lower p53 test response after S9 metabolism in the present study. Hence, the p53 assay without S9 metabolism should be sufficient to assess mutagenicity of surface water samples in monitoring strategies that apply passive sampling techniques.

4.3.2. Whole organism bioassays

For the whole organism bioassays, it can be argued that more sensitive alternatives should be developed and applied. For example, the Algatox assay showed no response to surface water extracts from approximately 95% of the locations in the present study. This is unexpected, as herbicides, that are the major target compound group of this bioassay, are the most frequently detected pesticide group in European surface waters (Booij et al., 2015; Schreiner et al., 2016). Recent work has shown that fluorescence based algal bioassays are efficient and effective in the assessment of toxicity to primary producers in regionwide screening efforts (de Baat et al., 2018; Novák et al., 2018; Sjollema et al., 2014). Hence, in the future, replacement of the Algatox assay with fluorescence based algal bioassays may result in more effective assessment of risks to primary producers in effect-based monitoring.

Finally, the applicability of the *in situ Daphnia* assay in

micropollutant effect monitoring should be questioned. Although it was responsive and discriminating in the present and previous studies (van der Oost et al., 2017a), it is nearly impossible to determine the contribution of micropollutants to the observed mortality. Exposure of daphnids in the field for seven days gives rise to a multitude of confounding factors including oxygen dynamics, food availability, pH, salinity and temperature, and unless the effects of these on daphnid mortality can be fully excluded, the outcome of the test cannot be considered indicative of micropollutant risk in surface water. Nonetheless, the added value of *in situ* or active biomonitoring approaches in water quality assessment strategies should not be underestimated, as they represent the most realistic exposure scenario available in the effect-based toolbox. Recently, promising strategies to differentiate between the effects of chemical exposure and confounding factors in active biomonitoring with invertebrates were described (e.g. Brettschneider et al., 2019).

4.3.3. Antibiotics and GR CALUX assays

The antibiotics and GR CALUX assays target specific groups of compounds, and their inclusion in bioassay batteries is only justified when there is an assumable occurrence and risk of these groups of compounds. Glucocorticoids mainly find their way into surface waters through industrial and hospital effluents. Glucocorticoid concentrations in such effluents are high, but decrease substantially after wastewater treatment (Schriks et al., 2010b; Van Der Linden et al., 2008). Hence, application of the GR CALUX assay in surface water monitoring is only marginally relevant, as the risk of glucocorticoids in surface waters is expected to be negligible. Therefore, this test can be omitted in future bioassay batteries to save costs, or be replaced with a more relevant endpoint to surface water toxicity like the *anti*-PR CALUX assay, for which a recently defined EBT value is available (Escher et al., 2018).

Contrasting to glucocorticoids, antibiotics are ubiquitous in NW European surface waters. They reach surface waters through diffuse input from the general public and the agri-food sector, giving rise to surface water concentrations that are expected to cause risks to bacteria, fungi and microalgae (Hernando et al., 2006; Kümmerer, 2009; Zhou et al., 2019). Hence, risks of antibiotics in the here tested surface waters are assumable, and the lack of effects above the EBT for the antibiotics assays in the present study is therefore unexpected. Given the low responsiveness of the here applied WaterSCAN antibiotics assay (Pikkemaat et al., 2008) and the ubiquity of antibiotics in surface waters, there is evidently a need for a more sensitive detection method for antibiotics residues. A potentially suitable alternative is the use of whole-cell based biosensors, in which, similar to CALUX assays, receptor binding mediated bioluminescence detects antibiotics activity at a sublethal level (Virolainen and Karp, 2014). However, this method is yet to be applied as bioanalytical tool in surface water antibiotics screening.

4.3.4. Sediments and metals

As of yet, successful effect-based monitoring efforts have been focused mainly on pollution of surface waters by organic compounds (Altenburger et al., 2019; Hamers et al., 2018; van der Oost et al., 2017b), while relatively little attention has been given to the inclusion of sediments as a relevant source of impaired ecological surface water quality. Sediments are the largest chemical repositories on earth where harmful compounds accumulate, thereby representing a significant threat to the health of aquatic ecosystems (Burton, 2013). Despite their relevant role in aquatic ecosystem health, sediments are often overlooked and understudied in regular water quality assessment strategies like the WFD (Borja et al., 2004).

Metal pollution is another relevant source of impaired ecological surface water quality that is currently largely overlooked in effect-based monitoring efforts. Metal pollution can have severe detrimental effects on water quality owing to its toxicity, frequency and abundance (Armitage et al., 2007; Sin et al., 2001). Only very rarely have the effects of metal pollution on aquatic ecosystems been studied using a combination of passive sampling and bioanalytical tools (Roig et al., 2011). Given the relevance of these pollution sources to aquatic ecosystem health, the development of integrative strategies that include the effect-based assessment of metal pollution as well as sediment quality would be a valuable addition to future research efforts.

4.4. Conclusions

Scientists and water authorities together are faced with the challenge of the increasing complexity of pollution in surface waters, and how to make the impact of this pollution on aquatic ecosystems measurable. Traditional chemical target analysis of a limited selection of pollutants has lost its relevance. Fortunately, the current availability and future development of a wide variety of alternative tools, in the form of effect-based methods, allows for a holistic interpretation of the harmful effects of all chemicals present in surface waters without individual identification of the causing compounds. It is likely that the debate on the most efficient and effective combination of effect-based tools in bioassay batteries, a conclusive approach to EBT derivation, as well as the regionwide implementation of the resulting monitoring strategies, will be ongoing for some time to come. Yet, at present, there is no practical limitation to the application of effect-based water quality assessment methods in regular water quality monitoring at a region- or nationwide scale.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.05.040.

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