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Battery of In Vitro Bioassays: A Case Study for the Cost-Effective and Effect-Based Evaluation of Wastewater Effluent Quality

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Abstract: Wastewater treatment plants (WWTPs) represent an important input of contaminants in the environment. Therefore, it is critical to continuously monitor the performance of WWTPs to take appropriate action and avoid an influx of contaminants in the environment. In this study, a battery of seven in vitro bioassays covering a selected spectrum of toxicity effects is proposed for quality control of wastewater effluents. The bioassays address mixture toxicity, which is the combined adverse effect of multiple contaminants and can act as an early warning system. The proposed battery was applied to samples from 11 WWTPs of representative technology from the Danube River Basin (DRB). The order of toxic effects in terms of extent of exceedance of effect-based trigger values (EBTs) was PAH (PAH activity) > PXR (xenobiotic metabolism) > ER_{α} (estrogenic activity) > $PPAR_{\gamma}$ > Nrf_2 (oxidative stress) > anti-AR > GR. A mitigation plan for WWTP operators based on EBT exceedance is proposed. This study demonstrates that the proposed effect-based monitoring battery is a complementary tool to the chemical analysis approach. A regular application of such time- and cost-effective bioanalytical tools in the WWTPs of the DRB is proposed to provide a 'safety net' for aquatic ecosystems.

Keywords: Danube River Basin; wastewater treatment plants; organic micropollutants; effect-based trigger values; toxicity endpoints; endocrine-disrupting compounds; mixture toxicity; environmental risk assessment; risk management; drinking water

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1. Introduction

Effluent discharge from wastewater treatment plants (WWTPs) is a significant pollution point source for contaminants of emerging concern (CECs) in surface water [1]. This phenomenon was observed in the Danube River Basin (DRB), the most international river basin crossing the territory of 19 countries [2], where a number of CECs were determined, including compounds under regulatory scrutiny or of high environmental concern, such as per- and polyfluoroalkyl substances (PFAS), industrial chemicals, and pesticides [3,4]. The loads and types of pollutants into the environment have been escalating due to population growth and increasing introduction of new chemicals to the market [5].

With continuous discharge into the environment, many compounds, especially persistent compounds, bioaccumulate in food webs and can reach higher predators including humans [6,7], whereas more polar compounds can be found in drinking water. For many chemicals present in small quantities that are often considered harmless when evaluated

individually, there is an increasing concern about their toxic effects when assessed as a mixture [8,9]. Some classes of contaminants, such as estrogens, often occur at low concentrations, but they entail high toxicological concern for aquatic biota [10]. It was suggested that the current version of the Urban Waste Water Treatment Directive (UWWTD) is not 'in phase' with such environmental concerns [11]. Improved pollution prevention measures are essential and require proper and efficient quality control of WWTP effluents.

The conventional approach to monitoring WWTP effluent is chemical screening of a few preselected contaminants. Holistic broad screenings have also been conducted and reported in the literature [12]. Such approaches provide a chemical profiling of WWTP effluents and enable the evaluation of removal efficiencies [13]. Removal efficiency can be evaluated with carefully selected chemical indicators that are regularly found in WWTP influents and cover a broad range of physicochemical properties and biodegradability [14]. Nonetheless, a prerequisite of the screening approach is a highly standardized laboratory with advanced analytical instruments such as liquid and gas chromatography coupled with high-resolution mass spectrometry (LC- and GC-HRMS) [15,16]. Moreover, a considerable amount of time and resources (such as chemical reagents) is required for the application of this approach. Bioassays are time- and cost-effective analytical tools that can complement chemical screening approaches for the evaluation of wastewater effluent quality [17]. The application of wisely selected bioassays could act as an early warning system for the monitoring of wastewater quality.

The use of a battery of bioassays with reference to effect-based trigger values (EBTs) can serve as a 'safety net' for WWTP effluents [11]. This approach assesses several toxicity effects, such as estrogenic and antiandrogenic activities of a mixture of substances that occur in wastewater samples [18]. However, toxicity drivers may be compounds that differ from those used as performance indicators in bioassays. Toxicity may be caused by the cocktail effects of many chemicals at low concentration and depends on the type of input received by the WWTP, such as municipal wastewater or industrial wastewater (cocktail) [19]. Nonetheless, there is currently no regulation addressing the risk assessment of mixtures of CECs in WWTP effluents [20]. Effect-based risk assessment provides additional toxicity information on the chemical composition of wastewater, which could provide key information in the regulatory context.

In the present study, a battery of seven in vitro bioassays was applied to samples from 11 WWTPs of representative technology from the Danube River Basin (DRB). The application of bioanalytical tools to wastewater monitoring was previously proposed and applied to samples from 12 WWTPs in the DRB [21]. The present investigation of other WWTPs from the DRB serves as a feasibility study to provide evidence for the application of bioanalytical tools in the regulatory context. Results of this study were compared to EBT values found in the literature, and the extent of exceedance was calculated. The aims of this study are to (1) demonstrate the feasibility of the application of effect-based methods—a battery with seven in vitro bioassays—to evaluate mixture toxicity effects of wastewater in the DRB, (2) propose action plans at the WWTP operator level for prioritized cases of risk assessment (with exceedance of EBTs), and (3) provide a future outlook for the wider application of effect-based bioanalytical monitoring tools for quality control of wastewater.

2. Materials and Methods

2.1. Sampling

The 11 studied WWTPs studied (Figure 1) were selected by the Pressures and Measures Expert Group of the International Commission for the Protection of the Danube River (ICPDR). Selection was based on the dominant technology of the country and the size of population served, with the aim of obtaining a representative and holistic view of the pollution status of the WWTPs. Detailed information about the location, daily flow, treatment technologies, and capacity (population equivalent) of the studied WWTPs is included in Table S1 of the Supplementary Materials. Certified clean polycarbonate bottles were utilized to collect 24 h composite effluent wastewater samples [14]. Samples were

collected under the Joint Danube Survey 4 (JDS4) sampling campaign during dry weather and under normal WWTP operating conditions on 26 August 2019. Sample 1 L aliquots were processed for the analysis by in vitro bioassays. After arrival to the laboratory, all samples were processed immediately.

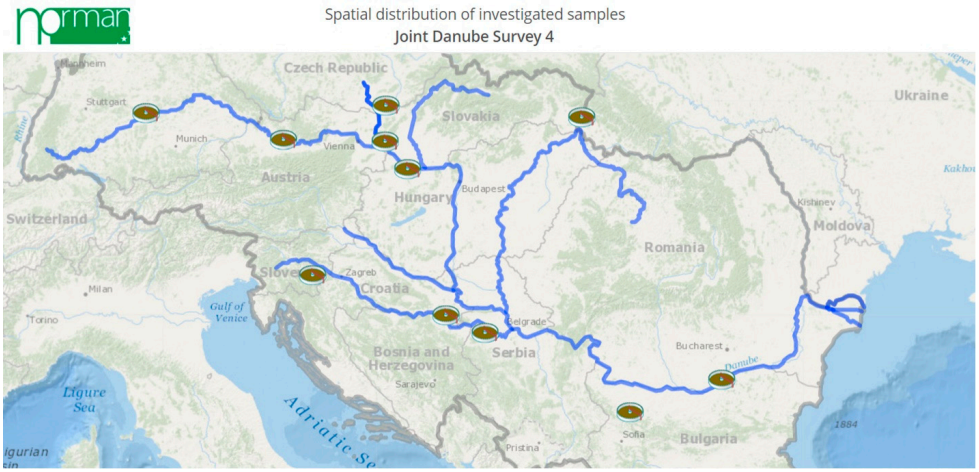

Figure 1. Spatial distribution of the investigated wastewater effluent samples. An online interactive map is available at https://norman-data.eu/JDS4_Samples (accessed on 23 November 2022).

2.2. Effect-Based Monitoring Battery of Bioassays

CALUX® bioassays (Chemical Activated Luciferase eXpression; BioDetection Systems BV, Amsterdam, the Netherlands) were applied for the analysis of effluent wastewater samples. Sample preparation was performed following fully validated methods and standard operational procedures, which are described in detail elsewhere [21]. Briefly, optimized solid-phase extraction (SPE) was applied to extract wastewater samples. The enriched extract with a mixture of compounds was exposed to genetically modified cell lines. The cell lines utilized in CALUX® bioassays were incorporated with firefly luciferase gene, which is coupled with responsive elements (Res) such as reporter genes. The presence of specific compounds triggers the activation of such Res and consequently initiates the creation of luciferase, which emits light in the presence of an appropriate substrate. Under such a mechanism, the amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compounds. CALUX® bioassays have been proven to be among the best bioanalytical tools to discriminate between receptor-based toxic effects in aquatic samples including wastewater [22,23], such as xenobiotic metabolism (e.g., binding to peroxisome proliferator-activated receptor gamma ($PPAR_\gamma$)) and hormone receptor-mediated effects (e.g., estrogenic (ER_α) effect) [23,24].

Seven bioassays previously proposed in a joint position paper of the NORMAN Association and Water Europe [11] were applied in the investigation of the wastewater extracts: ER_α, antiandrogenic (anti-AR), glucocorticoid (GR), $PPAR_\gamma$ receptor ($PPAR_\gamma$), polycyclic aromatic hydrocarbon activity (PAH), oxidative stress (Nrf_2) and pregnane X receptor (PXR). The reference compounds for the 7 bioassays were 17ß-estradiol for ER_α, flutamide for anti-AR, dexamethasone for GR, rosiglitazone for $PPAR_\gamma$, B[a]P for PAH, curcumine for Nrf_2, and nicardipine for PXR. The responses from the CALUX® bioassays were benchmarked against the lowest EBTs [25,26] reported in the literature, as shown in Table 1.

Table 1. Performance indicator bioassays and their effect-based trigger values (EBTs).

Activity	LOD *	LOQ *	EBT Value
Estrogenic (ER_α)	0.02 ng 17ß-Estradiol-eq/L	0.05 ng 17ß-Estradiol-eq/L	0.1 ng 17ß-Estradiol-eq/L
Antiandrogenic (anti-AR)	1.90 µg Flutamide-eq/L	5.76 µg Flutamide-eq/L	14 µg Flutamide-eq/L
Glucocorticoid (GR)	0.02 ng Dexamethasone-eq/L	0.05 ng Dexamethasone-eq/L	100 ng Dexamethasone-eq/L
$PPAR_\gamma$ receptor ($PPAR_\gamma$)	10.1 ng Rosiglitazone-eq/L	31 ng Rosiglitazone-eq/L	10 ng Rosiglitazone-eq/L
PAH activity (PAH)	0.32 ng B[a]P-eq/L	0.96 ng B[a]P-eq/L	6.2 ng B[a]P-eq/L
Oxidative stress (Nrf_2)	1.45 µg Curcumine-eq/L	4.4 µg Curcumine-eq/L	10 µg Curcumine-eq/L
Pregnane X receptor (PXR)	0.74 µg Nicardipine-eq/L	2.2 µg Nicardipine-eq/L	3 µg Nicardipine-eq/L

* LOD and LOQ are based on processing of 1 L samples preconcentrated to 25 µL DMSO.

The bioassay responses show the effects of the samples as bioanalytical equivalents (BEQs) of the reference compounds [27]. For example, signals from the ER_α bioassay on wastewater samples are benchmarked against the signals from 17ß-estradiol references to establish the BEQ. Exceedance of EBT values is observed when the signal from sampled wastewater is stronger than the signal from 0.1 ng/L of 17ß-estradiol (for estrogenicity). In order to establish the potential risk of adverse effects of the sampled wastewater to ecosystems, the extent of exceedance (EoE) of EBT values was computed by dividing the BEQ of the wastewater samples by the EBTs. A putative action plan based on the EoE of EBT values was suggested [11,21], as shown in Table 2, which proposes actions to be taken by WWTP operators.

Table 2. Proposed actions in case of exceedance of EBTs.

EoE	Mitigation Plans Proposed for WWTP Operators
Below 1	-No further action
Between 1 and 3	-Perform quality check of data; -Monitor every 3 months for 1 year until EoE is below 1.
Between 3 and 10	-All actions of the category above; -Resample and reanalyze immediately to confirm exceedance of EBT; -Quantify toxicity drivers.
Between 10 and 100	-All actions of the category above; -Enhance program for source identification; -Monitor the distribution system closer to the point of exposure to confirm attenuation of CECs and to confirm the magnitude of assumed safety factors associated with removal efficiency, dilution, and post treatment.
More than 100	-All actions of the category above; -Consult the local environmental authorities immediately to determine the required response action; -Confirm plant corrective actions through additional monitoring to establish an EoE at least below 100.

3. Results and Discussion

3.1. Wastewater Monitoring with Battery of Bioassays

The proposed battery of bioassays was applied to the 11 wastewater effluent samples collected from WWTPs in Austria (AT), Bulgaria (BG), Czechia (CZ), Germany (DE), Croatia (HR), Hungary (HU), Romania (RO), Serbia (RS), Slovenia (SI), Slovakia (SK), and Ukraine (UA). Table 3 presents the results normalized to the respective limit of quantification (LOQ) of each bioassay, indicating how many times the observed signals were higher than the LOQ, which expresses the sensitivity of the bioassay. Results below LOQ are presented as 0.5-fold LOQ (0.5). This table permits the comparison of the results for

the different locations of sampling and the magnitude of the dispersion of the results between the sampling points with reference to the sensitivity of the applied bioassays. The PAH activity (average fold times above LOQ for readings from 11 WWTPs: over 3000) and estrogenicity (ER_α, average fold times above LOQ: over 30) were the highest, both observed in all 11 studied samples. An extremely high PAH CALUX® signal was observed due to one extreme value (32292-fold times higher than LOQ) for wastewater from Donauwörth, Germany. This could be a result of a significant local contamination source with poor removal efficiency at the WWTP or a contaminated sample. Xenobiotic metabolism (PXR) and oxidative stress (Nrf_2) were observed in 9 and 10 samples, respectively.

Table 3. Normalized results of the battery of CALUX bioassays of the 11 studied effluent wastewater samples.

Effluent Wastewater Sampling Site	Anti-AR CALUX	ER_α CALUX	GR CALUX	$PPAR_\gamma$ CALUX	PAH CALUX	PXR CALUX	Nrf_2 CALUX
Asten, AT	2.6	26	1.5	0.5	115	10	9.5
Vratsa, BG	3.0	58	0.5	0.5	71	0.5	0.5
Hodonín, CZ	2.5	48	0.5	0.5	96	0.5	7.6
Donauwörth, DE	3.6	30	5.0	1.0	32,292	44	3.7
Županja, HR	0.5	30	0.5	0.5	92	27	4.4
Győr, HU	1.5	17	0.5	1.3	90	24	2.0
Giurgiu, RO	0.5	44	2.4	0.5	36	5.9	2.2
Šabac, RS	1.3	32	0.5	0.5	76	3.6	3.0
Novo Mesto, SI	0.5	5.0	0.5	0.5	58	4.4	1.9
Bratislava, SK	0.5	12	0.5	0.5	177	4.7	2.5
Uzhgorod, UA	1.3	38	0.5	0.5	104	5.0	2.0

The same methodology was applied to WWTP effluents collected in a previous sampling campaign that took place in 2017 [21]. The sample collected from the WWTP in Šabac, Serbia was monitored in both campaigns, and the results were consistent; a difference within a factor of five was observed for all bioassays. The PAH activity, ER_α, PXR, and Nrf_2 were the most frequently detected adverse effects in both sampling campaigns (22 WWTPs in total). In both campaigns, the GR and $PPAR_\gamma$ effects were low. These findings show the common contaminant profile in the effluents of the studied WWTPs in terms of toxicity effects.

3.2. Risk Assessment Based on Effect-Based Trigger Values

It should be noted that positive results of the bioassays above LOQ do not necessarily imply that the detected toxic effects are at a harmful level in the environment [21,28]. In order to perform environmental risk assessment, the results of the battery were benchmarked against the corresponding EBT value of each toxic effect listed in Table 1 [29]. Table 4 shows the measured EoE of the EBT values for each toxic effect in the studied samples, and the color-coding scheme outlined in Table 2 was adopted to facilitate visualization.

Table 4. Extent of exceedance (EoE) of EBT values and proposed action plan based on the signals of in vitro bioassays from analysis of JDS4 effluent wastewater samples.

Effluent Wastewater Sampling Site	PAH CALUX	ER_α CALUX	Nrf_2 CALUX	PXR CALUX	Anti-AR CALUX	$PPAR_\gamma$ CALUX	GR CALUX
Asten, AT	17.7	13.0	6.8	<LOD	1.6	<LOD	0.4
Vratsa, BG	11.0	29.0	<LOD	<LOD	1.9	<LOD	<LOD
Hodonín, CZ	14.8	24.0	<LOD	85.7	1.6	<LOD	<LOD
Donauwörth, DE	5000	15.0	29.0	41.7	2.2	63.0	1.2
Županja, HR	14.2	15.0	18.0	49.3	<LOD	<LOD	<LOD
Győr, HU	13.9	8.5	16.0	23.0	0.9	82.0	<LOD
Giurgiu, RO	5.6	22.0	3.9	25.0	<LOD	<LOD	0.6
Šabac, RS	11.8	16.0	2.4	34.3	0.8	<LOD	<LOD
Novo Mesto, SI	9.0	2.5	2.9	21.0	<LOD	<LOD	<LOD
Bratislava, SK	27.4	6.2	3.1	28.7	<LOD	<LOD	<LOD
Uzhgorod, UA	16.1	19.0	3.3	22.7	0.8	<LOD	<LOD
No. of samples with EoE > 1	11	11	9	9	4	2	1
Mean value	467	15	8	30	1	13	0.2
Standard deviation	1503	8	9	24	1	30	NA

All the WWTP effluents (100%) show exceedance of the EBT value of the PAH activity indicator and the estrogenic activity indicator (ER_α), and nine out of the eleven sampling points (80%) present exceedance of the EBT value of the oxidative stress indicator (Nrf_2) and the indicator related to the xenobiotic metabolism, whereas the indicator of antiandrogenic activity was exceeded at four sampling points, and those of $PPAR_\gamma$ and GR activity were exceeded at two and one sampling points, respectively. Therefore, the order of the toxic effects in terms of frequency of EBT value exceedance is PAH, ER_α > Nrf_2, PXR > anti-AR > $PRAR_\gamma$ > GR.

Furthermore, in terms of the extent of exceedance, the results demonstrate that most of the investigated WWTP effluents present considerable exceedance of the EBT value of the PAH activity (EoE >10), with one categorized in the fourth level of the action plan, exceeding the EBT value by 5000 times. This phenomenon can be attributed to a significant local contamination source. The order of the toxic effects in terms of extent of exceedance of EBT values was generated based on the mean values of the EoE, considering as zero the values < LOD. The order is PAH > PXR > ER_α > $PPAR_\gamma$ > Nrf_2, > anti-AR > GR.

Cases with exceedances of EBT are prioritized and highlighted in Table 4. Based on the EoE, an action plan at the WWTP operator level was proposed according to Table 2. Blue color in Table 4 indicates cases with EoE values between 1 and 3, for example, anti-AR activity in the effluent wastewater from WWTPs of Asten, Vratsa, Hodonín, and Donauwörth. A quality check of the data is proposed, together with a monitoring plan to be implemented every three months for one year until no exceedance of EBT value is detected.

Cases with EoE values between 3 and 10 are highlighted in green color, such as ER_α activity in the WWTPs of Győr and Bratislava, and PAH activity in the WWTPs of Giurgiu and Novo Mesto. For such cases, resampling and reanalysis are recommended to confirm the EoE and identify and quantify the toxicity drivers, in addition to the aforementioned actions proposed for cases with EoE values between 1 and 3.

Cases with EoE values between 10 and 100 are highlighted in orange in Table 4, such as PXR in all studied WWTPs except for Asten and Vratsa. On top of the actions proposed for the category with EoE values between 3 and 10, further actions are suggested for the orange highlighted cases: source identification and monitoring of the distribution system until attenuation of the toxicity drivers is confirmed.

The above results generated by the proposed battery of in vitro assays are indicative of the classes of the compounds that are related to the specific toxic effects. Additional investigation is required to reveal the toxicity-driving chemicals for these toxic effects. This investigation is challenging and time-consuming. However, the combination of in vitro

bioassays and non-target screening has been proven to be a key approach to identify estrogen-active contaminants in the environment [30]. In general, EBT value-based action plans entail quality checks on data, continuous monitoring, toxicity driver analysis, and mitigation programs. Among them, toxicity driver analysis could inform WWTP operators and regulators about the sources of contamination. The toxic units of the quantified pollutants could be related to the bioanalytical equivalent concentration derived from the bioassay tests using the component-based assessment (CBA) approach [31,32]. When the CBA method cannot explain the EBT exceedance measured from the bioassay, an effect-directed analysis (EDA) protocol could be performed for identification of toxicity drivers [33,34]. Law enforcement authorities could utilize such information to implement measures in line with the 'polluter pays' principle based on related regulations. The establishment of regulatory limits on the emission of selected contaminants could reduce the loads of prioritized chemicals that reach the WWTP, which is consistent with the Sustainable Development Goals of "clean water and sanitation" and "responsible consumption and production". Moreover, pretreatment methods could reduce the prioritized toxic effects of wastewater, such as the application of ozonation to reduce the estrogenic activity of influent wastewater [35].

One of the prerequisites for these impact-driven actions is the regulatory application of effect-based monitoring of wastewater. The proposed in vitro bioassays have been proven to provide important information about toxic effects of effluent wastewater from 11 WWTPs in this feasibility study and 12 other WWTPs in the DRB in a previous study [21]. This investigation provides key evidence that supports the regulatory application of bioanalytical tools in wastewater monitoring. Nonetheless, a wide range of human and ecological EBT values have been proposed by the scientific community for certain toxicity endpoints [36], of which the lowest ecological EBT values reported in the literature were adopted in this investigation. NORMAN and Water Europe proposed bioassays and EBT values for the application of a battery for the effect-based assessment for wastewater monitoring [11]. Harmonization of EBT values is a vital foundation for the application of bioanalytical tools in the regulatory context [37].

3.3. Wider Application of the Battery in Wastewater Quality Control

The chemical analysis of WWTP effluents provides the holistic occurrence profiles of CECs in the wastewater but not the cocktail effects of the mixture of CECs. The proposed battery of bioassays fills in this gap by detecting the presence of chemical groups that cause the studied toxicity endpoints [38]. This is a time- and cost-effective bioanalytical technique that could serve as an early warning system for wastewater quality control. The establishment of the relative effect potency (REP) values of the detected CECs (from chemical analysis) could serve as a bridge between chemical data and the toxicity effects measured in the bioassays [37,39]. Such combined biochemical analysis of wastewater effluents can provide key information about the toxicity status and sources of pollution in the matrix.

Moreover, the combined use of the bioanalytical tools and chemical analysis approaches can provide comprehensive profile of the performance of WWTPs in the removal of toxicity stressors [40]. Chemical data can be used for the evaluation of removal efficiency at WWTPs against chemical groups in terms of physiochemical properties [41]. The results of bioassays can reveal chemical groups (in terms of toxicity endpoints) that are not properly removed in the wastewater abatement process, which could be translated into particular chemicals with the identification of toxicity drivers [38,42]. Application of bioassays as a complementary tool to chemical analysis can contribute to a more complete understanding of the shortcomings of current wastewater treatment methods, which could lead to new proposals for improvement [24]. Furthermore, the application of bioassays and chemical analysis of WWTP sludge could help to characterize organic contaminants retained in the sludge and their toxicological effects [43]. Such analysis of WWTP sludge

can reveal the status of the removal process and the applicability of the sludge to the circular economy [44].

Further development of the proposed battery can make it a better effect-based monitoring tool for WWTP effluents and sludge. The inclusion of in vivo bioassays in the battery allows for the detection of more modes of action [23], as in vivo tests can address apical effects that are missed by in vitro tests [45]. A battery that includes both in vitro and in vivo assays can be used to screen for more toxicity effects in WWTP effluent samples [46], thereby serving as an enhanced 'safety net' for freshwater and/or marine ecosystems that accept the aforementioned effluents.

4. Conclusions

Daily composite wastewater effluent samples from 11 WWTPs selected by the ICPDR were collected and analyzed with a battery of seven bioassays. Risk assessment revealed that groups of CECs causing PAH activity, estrogenicity, xenobiotic metabolism, and oxidative stress remain present in the WWTP effluents in the DRB, with special concern related to those associated with PAH activity. Mitigation plans for WWTP operators were proposed based on the EoE of EBT values.

Overall, this study demonstrates the applicability of the effect-based monitoring approach of WWTP effluents using a battery of bioassays. The results of this study can inform WWTP operators about the toxicity of wastewater as a result of the cocktail effects of the mixture of substances, with action plans proposed based on the EoE. Bioanalytical effect-based monitoring serves as a complementary tool to the chemical analysis approach for WWTP effluents and sludge for the identification of pollution and evaluation of WWTP performance. This feasibility study provides key evidence for the regular monitoring of wastewater effluents by a battery of in vitro bioassays (once every six months) in the DRB. Further development of the battery by including in vivo assays could allow it to serve as a 'safety net' for aquatic ecosystems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15040619/s1, Table S1: Detailed information of the studied WWTPs.

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