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Integrated Exposure and Algal Ecotoxicological Assessments of Effluents from Secondary and Advanced-Tertiary Wastewater-Treatment Plants

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Abstract: The great concern over the environmental impact of wastewaters has led to the designing of advanced treatment processes to upgrade conventional treatment plants and achieve a significant reduction of contaminants in receiving waters. In the present study we combined chemical and ecotoxicological analyses, aiming to evaluate the reduction of toxicity effects associated with the removal of micropollutants and to define the contribution of the detected compounds to the overall toxicity of the mixtures in a series of wastewater effluents collected from a secondary treatment (OUT 2) and from a tertiary activated carbon treatment (OUT 3) plant. The target compounds were selected after a screening procedure among pharmaceuticals, musk fragrances, and trace metals. The classical algal growth inhibition test was conducted on the original effluent samples and on different fractions obtained by solid-phase extraction (SPE) treatment. A good accordance was found between the removal of toxicity (30%–80%) and organic compounds (70%–80%) after the tertiary treatment, suggesting its high efficiency to improve the wastewater quality. The discrepancy between the contribution to the overall toxicity of the nonadsorbable compounds (i.e., inorganic or very polar organic compounds) as experimentally measured by the SPE bioassays (18%–76%) and calculated by the concentration addition approach (>97%) could be mitigated by including the bioavailability correction in metal-toxicity modeling of wastewater mixtures. For the organic compounds, the toxic equivalency method enabled us to quantify the portion of toxicity explained by the detected chemicals in both OUT 2 (82%–104%) and OUT 3 (5%–57%), validating the selection of the target molecules. The applied integrating approach could be implemented by the inclusion of both additional target chemicals and toxicity endpoints. *Environ Toxicol Chem* 2022;41:2404–2419. © 2022 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

Wastewater discharge is one of the most significant sources of pollutants for water bodies in urban environments. All of the regulations provide mitigation measures based on mandatory emission limit values or discharge permits on a target list of pollutants. As a complementary action, some countries have added ecotoxicological tests on wastewaters to control their toxicity. In Italy ecotoxicity tests with *Daphnia magna*, *Allivibrio fischeri*, and *Pseudokirchneriella subcapitata* on municipal discharges have been introduced by Italian Decree 152/2006

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(Italian Decree, 2006). Both approaches have intrinsic limits because, on the one hand, the monitoring of a target list of compounds cannot guarantee that all hazardous substances are covered; but, on the other hand, toxicological tests are not able to provide information on the toxicity drivers.

Some protocols have been developed to integrate chemical and ecotoxicological information. The first protocol was proposed by the US Environmental Protection Agency (USEPA) and is known as toxicity identification evaluation (TIE; USEPA, 1991), which evolved in the more complex scheme known as effect-directed analysis (Brack, 2003; Hecker & Hollert, 2009). Both protocols include a fractionation step of the environmental samples, followed by ecotoxicological tests on the single fractions (Burgess et al., 2013). This approach is going to become more rapid, effective, and automated by including in vitro or in vivo test batteries and applying advanced analytical technologies such as high-resolution mass spectrometry (HRMS; Brack et al., 2016). Biological analysis is used in both cases to trigger the chemical screening for the identification of the toxic compounds in the positive fractions.

Effect-based methods have been included in the European strategy against chemical pollution in water ecosystems (Wernersson et al., 2015), but they still need to integrate with the chemical data to identify the actual responsible chemicals of the sample toxicity. The relevance of the complementary approach is largely documented in the literature (Neale et al., 2017).

For the effect assessment of wastewater mixtures, whole-organism toxicity tests offer the advantage of in vivo evaluation of the integrated effects of all mixture components to key aquatic organisms. One of the fundamental bioassays recommended for the characterization of complex mixtures and wastewaters is the algal growth inhibition test (OSPAR Commission, 2005), which is included in the base set of tests for toxicity assessment in connection with classification and risk assessment of chemicals (Organisation for Economic Co-operation and Development [OECD], 2001). The effectiveness and sensitivity of algal assay for the ecotoxicological studies are mainly linked to its distinctive feature of being a chronic short-term test, able to provide integrated responses of millions of individuals, over several generations, within only 72 h of exposure. The freshwater alga *P. subcapitata*, which is a conventional test species in ecotoxicological assays, has proved to be highly sensitive to various chemicals, including pharmaceuticals and complex environmental samples (Xin et al., 2021).

In the present study we aimed to couple the exposure assessment, obtained from the chemical measurements, with the ecotoxicological assessment, carried out by a classical algal chronic test, of effluents from a municipal wastewater-treatment plant (WWTP); samples were collected at the outlets of both a conventional secondary treatment and a pilot plant which employs a dispersed activated carbon adsorption as a tertiary polishing treatment for contaminants of emerging concern (CEC).

The list of CEC to be monitored, together with other chemical parameters, has been selected after a screening procedure carried out by HRMS.

The chronic algal bioassay was chosen because it is sufficiently sensitive to detect the nonacute levels of effect that are likely expected in treated wastewaters. The algal assay was set up within a complex experimental framework including the evaluation of the toxicity effects measured in the original wastewater samples tested as a whole and in separated fractions obtained by solid-phase extraction (SPE). To further assess the role of trace elements, their toxicity bioavailability was assessed with ad hoc experimental and modeling studies.

The concentration addition model was adopted to integrate the chemical and ecotoxicological test results and identify the respective contribution of each chemical to the mixture toxicity, thus finding the most likely toxicity drivers, which are the chemicals showing the highest relative toxicity contribution. Introduced by Loewe and Muischneck (1926), the concentration addition model allows us to predict the toxicity of the entire mixture by summing up the toxicities of each individual component, assuming that each component contributes to the overall toxicity proportionally to its concentration in the mixture and its individual potency and has the same mode of action (Boedeker et al., 1993). The concentration addition model has been widely applied to study the ecotoxicology of complex mixtures of personal care products, including both pharmaceuticals and musk fragrances (Backhaus & Karlsson, 2014; Carusso et al., 2018; Ofrydopoulou et al., 2022; Schnell et al., 2009). Other studies demonstrated a potential use of concentration addition also for metal mixtures (Gopalapillai & Hale, 2016), even though it is needed to take into account that the model may present different predictive abilities depending on the metal species in a mixture and on the possibly occurring interactions between metal ions and the other mixture components (Liu et al., 2017).

To quantify the actual contribution of the individual organic compounds analyzed to the measured toxicity in the SPE extracts, the concentration addition model was implemented using the toxicity equivalency (TEQ) concept (Escher et al., 2008), which allows us to express the total toxicity of a mixture in terms of an equivalent concentration of a reference compound (TEQ_{bio}). The TEQ_{bio} can be compared to the TEQ_{chem}, which is the toxicity calculated by summing up the product of the measured concentration of the detected chemicals in the mixture and their relative potency with respect to the reference compound. This comparison enabled definition of the percentage of the observed toxic effect that could be explained by the detected chemicals as in the case of a study conducted on three Swiss WWTPs (Neale et al., 2017). That study demonstrated that only a small portion of the effect was explained by the measured concentration of chemicals and thus highlighted the importance of combining bioassays with chemical analysis to get a more comprehensive picture of the micropollutant mixture. Such an approach is typically applied to toxicants acting via a specific mechanism of action common to all compounds present in the mixture; however, it has proved useful also when not only one dominant but multiple modes of action determine nonspecific effects, such as algal growth inhibition, that may contribute to the overall toxicity (Escher et al., 2008).

In the present study the results allowed us to (1) characterize the levels of effect and exposure of two differently treated effluent wastewaters; (2) measure the capability of a tertiary activated carbon treatment plant to further reduce effluent toxicity with respect to a conventional one; (3) discriminate the role of the two classes of chemicals, organic compounds and trace elements, in affecting the overall toxicity on algae in the secondary and tertiary effluents; (4) estimate the total mixture toxicity and the contribution of each class of compounds; (5) highlight the importance of including the bioavailability of trace metals in the mixture toxicity concentration addition modeling; and (6) find a link between the determined concentrations of the organic chemicals and the algal toxicity effects measured in the extracted fraction of organics to quantify the amount of the observed toxic effect that can be explained by the detected chemicals.

MATERIALS AND METHODS

Plant description and sampling

Effluent samples were collected from a municipal WWTP located in the outer belt of Milan, Lombardy region, northern Italy, serving approximately 120,000 population equivalents. After primary sedimentation, the secondary treatment process was performed by activated sludge with enhanced biological nitrogen removal and simultaneous phosphorus precipitation.

Part of the secondary effluent outflowing from the conventional WWTP feeds a pilot plant employing a tertiary advanced treatment based on an Actiflo® Carb system. This patented Veolia technology consists of a precontact tank where powdered activated carbon (PAC) is dosed, followed by coagulation, flocculation, and lamellar settler, where microsand is added to enhance the sedimentation of suspended solids and PAC. The system works with an inlet flow rate that varies between 4 and 9 m^3/h, which can be adjusted according to the different tested operating conditions of PAC dosage, as summarized in Table 1. The PAC, supplied by Jacobi Carbons Italia, was a meso-macroporous lignite coal–based carbon with an iodine number equal to 1160 mg/g.

During the winter 2020–2021 we collected sixteen 5-h composite samples from the outputs of the conventional plant (OUT 2) and from the outputs of the pilot plants (OUT 3) with a delay of 30 min between OUT 2 and OUT 3 to consider the pilot plant hydraulic retention time. We tested eight different configurations of the pilot plant in 2-consecutive-day replicates (Table 1). The tested configurations differed for some parameters such as flow rate, virgin PAC dosage, and total PAC concentrations. Our study is only a part of a more complex experimental design for the optimization of the pilot plant where chemical concentrations were measured. Our aim in the present study was to test algal toxicity on a representative part of the whole collected samples to understand if the adopted ecotoxicological test was sensitive enough to the variations in the pilot plant configurations.

Wastewater quality parameters (pH, total organic carbon, total suspended solids, total phosphorus) were regularly determined in the 5-h composite samples by standard methods (Supporting Information, Table SM1.1). All samples were filtered on-site with glass microfiber filters (grade GF/F, mean porosity 0.7 μm) and poured into amber glass bottles, which were transported under refrigeration to the analytical laboratories as soon as possible for the subsequent chemical analyses and ecotoxicological tests.

Selection of monitored compounds by suspect screening

The selection of organic compounds to monitor was based on the results of previous surveys and of a preliminary suspect screening investigation on conventional WWTP effluent (OUT 2). The experimental setting for suspect screening is presented in Supporting Information, Section SM1.1. Twenty-four-hour

TABLE 1: Configurations of the Actiflo Carb pilot plant[a]

Configuration[b]	Day–replicate	Flow rate (m^3/h)	Virgin PAC dosage (mg/L)	Nominal PAC concentration (g/L)[c]	Measured PAC concentration (g/L)[d]
1	a	8.6	10	0.51	0.41
	b				0.41
2	a	8.6	5	0.28	0.29
	b				0.31
3	a	6	10	0.56	0.42
	b				0.41
4	a	6	20	0.29	0.48
	b				0.45
5	a	8.6	10	0.51	0.33
	b				0.36
6	a	8.6	10	0.51	0.46
	b				0.42
7	a	6	20	0.29	0.55
	b				0.53
8	a	8.6	5	0.28	0.32
	b				0.23

[a]Each sample was collected in 2-consecutive-day replicates (a and b).
[b]For all configurations: coagulant ($FeCl_3$ 41% commercial solution) dose = 7 mgFe/L, flocculant (powdered cationic polyelectrolyte) dose = 1 mg/L.
[c]Sum of virgin PAC + recirculated PAC.
[d]Estimated from the total suspended solids mass balance.
PAC = powdered activated carbon.

time-proportional composite WWTP effluents were collected by an automatic sampler for 7 consecutive days; 0.5 L for each sampling day was extracted by SPE on Oasis® HLB cartridges (200 mg/6 ml; Waters). The extracts were subjected to suspect analysis by liquid chromatography (LC)–HRMS, using a Q-Exactive Focus (Thermo Fisher Scientific). Using this approach, we were able to screen for the presence of a list of more than 60 suspect compounds, chosen among the most monitored emerging compounds in regulatory and environmental monitoring programs (Supporting Information, Table SM2.1), and to make a semiquantitative assessment of their concentrations (Supporting Information, Table SM2.2). The compounds to be included in the final experimental list were chosen on the basis of their (1) semiquantitative concentrations measured in the effluents of the selected WWTP (Supporting Information, Table SM2.3); (2) refractoriness to removal in the treatment processes (Supporting Information, Table SM2.2); (3) belonging to different therapeutic classes for pharmaceuticals or uses for industrial chemicals (Table 2); and (4) representativeness of different physicochemical properties (e.g., octanol–water partitioning coefficient and the environmental half-lives; Supporting Information, Figure SM2.1). According to the above list of criteria, the suspect screening procedure led to the selection of 14 polar molecules: 12 pharmaceuticals, one industrial compound (methyl-benzotriazole), and one pharmaceutical transformation product (gabapentin-lactam) formed from the antiepileptic gabapentin. To the final target list of organic compounds (Table 2) we added four polycyclic musk fragrances (PMFs) and one PMF metabolite which have been already detected at high concentrations in a previous survey of WWTP effluents in the same geographical area (Rusconi et al., 2017) and confirmed by Tasselli et al. (2021). Six trace elements (Cd, Cr, Cu, Ni, Pb, Zn) were also included according to the current Italian (Italian Decree, 2015) and European legislation (European Commission, 2013).

TABLE 2: Selected target organic compounds

Compound	Chemical Abstracts Service no.	Category of use
Amisulpride	298-46-4	Antidepressant drug
Ofloxacin	84057-84-1	Antibiotic quinolone
Sulfamethoxazole	15307-86-5	Antibiotic sulfonamide
Metoprolol	22071-15-4	Beta-blocker drug
Lamotrigine	71675-85-9	Antiepileptic drug
5-Methyl-benzotriazole	138402-11-6	Industrial product
Gabapentin-lactam	479-92-5	Transformation product
Azithromycin	37350-58-6	Antibiotic macrolide
Propyphenazone	64744-50-9	Analgesic-antipyretic drug
Carbamazepine	136-85-6	Antiepileptic drug
Irbesartan	723-46-6	Cardiovascular drug
Ketoprofen	82419-36-1	Anti-inflammatory drug (NSAID)
Clarithromycin	81103-11-9	Antibiotic/macrolide
Diclofenac	83905-01-5	Anti-inflammatory drug (NSAID)
Celestolide (ADBI)	13171-00-1	Musk fragrance
Galaxolide (HHCB)	1222-05-5	Musk fragrance
Galaxolidone (HHCB-lactone)	507442-49-1	Musk fragrance
Phantolide (AHMI)	15323-35-0	Musk fragrance
Tonalide (AHTN)	1506-02-1	Musk fragrance

NSAID = Non-steroidal anti-inflamatory drug; ADBI = 4-acetyl-6-tert-butyl-1, 1-dimethyl indan; HHCB = 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8 hexamethylcyclopenta(g)-2-benzopyran; AHMI = acetyl hexamethyl indan; AHTN = 6 acetyl-1,1,2,4,4,7-hexamethyl tetralin.

Analytical determinations

Details of the analytical methods and their validation can be found in Supporting Information, Section SM1.2. Briefly, trace elements were analyzed by inductively coupled plasma–optical emission spectrometry after microwave-assisted digestion of the samples with HCl. The PMFs were determined by gas chromatography–MS according to the method of Tasselli & Guzzella (2020). The 14 polar organic compounds selected by suspect screening were analyzed by LC-MS/MS after SPE extraction on Oasis HLB cartridges (200 mg/6 ml; Waters).

Biological assays

The algal toxicity test was performed following the OECD guideline (OECD, 2011), and the green alga *P. subcapitata*, from the Italian National Research Council's culture collection, was used as the test organism. The basic method of the algal growth inhibition test was applied by setting up four different experiments, each aimed at highlighting a specific aspect of the sample toxicity.

Overall toxicity. All OUT 2 and OUT 3 samples were tested following the standard protocol, as described in Supporting Information, Section SM1.3. All wastewater samples, prior to use for preparing the test cultures, were spiked with the same nutrient concentration used to prepare the control in standard water. This enrichment was aimed at reducing the possible fluctuations of the nutrient content from one effluent to another and eliminating false negative results due to low nutrient concentrations. The effect of the increased trophic level resulting from the enrichment of already P-rich waters (Supporting Information, Table SM1.1) was minimized by measuring algal growth within a short time (72 h), that is, before the differentiation of the nutritional responses (USEPA, 1985). The 72-h cell number (cells per milliliter) was used to calculate the toxicity effect as a percentage of growth inhibition (I) of the inoculated samples relative to the control cultures with the following equation:

$$\text{Toxicity effect (\%I)} = \left(1 - \frac{\text{Growth}_{\text{sample}}}{\text{Growth}_{\text{control}}}\right) \times 100 \quad (1)$$

In Equation 1, $\text{Growth}_{\text{sample}}$ is the 72-h cell number (cells per milliliter) measured in the effluent samples and $\text{Growth}_{\text{control}}$ is the 72-h cell number (cells per milliliter) measured in the standard control medium.

Toxicity of organic compounds (SPE-enrichment test and SPE-eluate test). For the testing of the organic fraction of both OUT 2 and OUT 3, after the SPE treatment, eluates in

MeOH were solvent-exchanged with dimethylsulfoxide (DMSO) to obtain different wastewater extract concentrations. The concentration was expressed as a relative enrichment factor (REF), calculated from the product of the dilution factor of the bioassay by the enrichment factor of the SPE-extracted sample in the following equation:

$$\text{REF} = \text{Dilution factor}_{\text{bioassay}} \times \text{Enrichment factor}_{\text{SPE}} \quad (2)$$

The entire procedure is described in Supporting Information, Section SM1.3. Algal assays were run following the same procedure as for the overall toxicity test but using a smaller test solution, which allowed us to test up to six serial dilutions with a concentration range from REF = 1 to REF = 80. Solvent controls with the same DMSO concentration as the samples were set up in parallel and used for the calculation of toxicity.

The 72-h percentage inhibition values were processed using the statistical software ProbAlg (Puddu, 1989) to determine the dose–response curve by Probit analysis and then calculating the median effective concentration (EC50), in REF units.

Based on the TIE Phase I procedure (USEPA, 1991), the aqueous eluates of five configurations of both OUT 2 and OUT 3 (1, 2, 4, 6, 8) were collected downstream of SPE cartridges and tested for toxicity together with the respective original samples. The detoxification effect following the SPE treatment was evaluated by comparing the toxicity effects of the original sample with the toxicity effects of the SPE-eluate samples, and it was quantified as the toxicity reduction percentage (%TR), using the following equation:

$$\text{Toxicity reduction (\%TR)} = \frac{(\%I_{\text{original}} - \%I_{\text{SPE eluate}}) \times 100}{\%I_{\text{original}}} \quad (3)$$

In Equation 3, $\%I_{\text{original}}$ is the overall toxicity as measured in the effluent sample and $\%I_{\text{SPE eluate}}$ is the toxicity effect as measured in the eluate samples downstream the extraction treatment. Eluates from the SPE extraction of ultrapure water were used as procedural blanks (Blank SPE).

Water effect ratio procedure and trace element bioavailability. The toxic bioavailability of trace elements was investigated by setting up additional experiments based on the water effect ratio (WER) procedure (USEPA, 1994). The WER procedure defines the water chemistry effect by measuring the differences between the toxicity of a given trace element simultaneously diluted in a laboratory and in the studied water. In our study, a model compound (copper) was spiked at one known and toxic concentration of 30 µg/L (Mingazzini & Palumbo, 2004) both in the standard water and in the wastewater samples. Both treatments were tested in parallel with their respective control solutions, consisting of the same standard and wastewater samples without copper addition. The toxicity effect measured in each of the tested samples, that is, the percentage toxicity of Cu (Cu-%I), was calculated from the following equation:

$$\text{Cu-\%I} = \frac{\text{72-h Growth}_{\text{C}} - \text{72-h Growth}_{\text{C+Cu}}}{\text{72-h Growth}_{\text{C}}} \times 100 \quad (4)$$

In Equation 4, 72-h Growth_{C} is the 72-h cell number (cells per milliliter) as measured in the copper nonspiked water and 72-h $\text{Growth}_{\text{C+Cu}}$ is the 72-h cell number (cells per milliliter) as measured in the copper spiked water.

The copper WER (Cu-WER), which is a proxy for the copper bioavailability, was calculated as the ratio between the Cu-%I of the standard water and the Cu-%I of the wastewater sample by the following equation:

$$\text{WER} = \frac{\text{Cu-\%I}_{(\text{standard water})}}{\text{Cu-\%I}_{(\text{wastewater sample})}} \quad (5)$$

The trace element bioavailability experiment was carried out on selected OUT 2 and OUT 3 wastewater samples (Configurations 4–8). In addition, in two cases (Configurations 6 and 8), Cu was added to the samples downstream of the SPE treatment.

The bioavailability of some trace elements (Cu, Ni, Zn, Pb) was also predicted by the Bio-met bioavailability tool, a user-friendly biotic ligand model (Peters et al., 2020), considering the specific physical–chemical features of waters. The model used is a free online resource available at www.bio-met.net.

Mixture toxicity evaluation

Identification of contribution based on the concentration addition model. The ecotoxicological data of each target compound, that is, the algal EC50, selected from literature reviews and electronic databases as detailed the Supporting Information, Section SM1.4 and Table SM1.2, were used with individual measured concentrations in ponderal units (MEC_i) to estimate the cumulative mixture toxicity by summing up the individual toxic units (TUs) calculated by the following equation:

$$\text{TU}_i = \frac{\text{MEC}_i}{\text{EC50}_i} \quad (6)$$

where MEC_i is the measured concentration of a given compound i and EC50_i is the corresponding algal EC50.

By comparing the TU_i of each compound with the total toxicity of the mixture (sum of toxic units [STU]), the percentage contribution of each compound can be calculated by the following equation:

$$\text{STU(\%)} = \frac{\text{TU}_i}{\text{STU}} \times 100 \quad (7)$$

TEQ concept. Based on the procedure of Escher et al. (2008), all of the toxicity effects measured in the SPE-enrichment bioassay were expressed as TEQ_{bio}, which was calculated using one reference compound with the following equation:

$$TEQ_{bio} = \frac{EC50_{ref}}{EC50_{sample}} \quad (8)$$

In Equation 8, $EC50_{ref}$ is the algal EC50 of one reference compound and $EC50_{sample}$ is the toxic concentration of the organic extracts in REF units. We selected the reference compound among the 19 organic compounds listed in Table 2 mainly based on the substance that was most toxic toward algae and that showed the highest STU (percentage). See Supporting Information, Section SM1.7, for major details.

Further, to assess the contribution of the detected chemicals to the measured effect, TEQ_{bio} was compared to the effects predicted from the detected chemicals, that is, TEQ_{chem}. First, the relative effect potency (REP) of each detected chemical (i) was calculated by dividing the effect concentration value of the reference compound (ref) by the effect concentration value of the detected chemical as in the following equation:

$$REP_i = \frac{EC50_{ref}}{EC50_i} \quad (9)$$

The toxic equivalent concentration of a given compound ($TEQ_{chem,i}$) is calculated as the product of the REP_i and the concentration of the compound (MEC_i) by the following equation:

$$TEQ_{chem,i} = REP_i \times MEC_i \quad (10)$$

The TEQ_{chem} of the mixture was calculated by summing up the $TEQ_{chem,i}$ by the following equation:

$$TEQ_{chem,mixture} = \sum TEQ_i \quad (11)$$

RESULTS

Chemical concentrations in wastewaters

Table 3 shows the average concentrations of the analytes in the OUT 2 and OUT 3 samples. The concentrations of analytes in all samples are reported in Supporting Information, Table SM1.3 and in Figures 1 and 2. All results are reported for the eight configurations as the mean value of 2-day replicates.

In the secondary effluent, the average concentrations of some organic compounds such as galaxolide (5100 ng/L) and its metabolite galaxolidone (1200 ng/L) exceeded 1 µg/L. Apart from these compounds, those with the highest concentrations in OUT 2 samples were, in decreasing order, irbesartan, a cardiovascular drug (710 ng/L); diclofenac (642 ng/L); 5-methyl-benzotriazole (551 ng/L); azithromycin (406 ng/L); gabapentin-lactam (283 ng/L); carbamazepine (243 ng/L); and tonalide (225 ng/L). All of the other pharmaceuticals have a mean concentration <200 ng/L, whereas celestolide and phantolide were close to the detection limits. For the trace elements, the highest concentrations were measured for Zn (39 µg/L) and Ni (11.5 µg/L).

In the tertiary effluent OUT 3, galaxolide was the only compound present at concentrations exceeding 1 µg/L (1131 ng/L), whereas the pharmaceutical with the highest concentration was still irbesartan (713 ng/L). All of the other compounds were present at concentrations <100 ng/L, apart from galaxolidone (211 ng/L), diclofenac (204 ng/L), and the transformation product gabapentin-lactam (150 ng/L). Regarding the other PMFs in the tertiary effluent, tonalide was present at a mean value of only 27 ng/L, whereas celestolide and phantolide concentrations were lower than the limit of detection (LOD).

Again, for the trace elements, the highest concentrations were determined for Zn (33 µg/L) and Ni (8.3 µg/L).

The removal of organic compounds in the tertiary effluent is on the order of 80% for fragrances and 70% for pharmaceuticals estimated from the averages of the total sum of compounds in each class for the whole series of experiment (Supporting Information, Figure SM1.1). On the contrary, for trace elements a removal of <20% has been calculated (Supporting Information, Table SM1.3; Figure 2). For a full assessment of the trace element removal rate of the pilot plant, the trace metal contribution from the chemicals used in the treatment process (mainly coagulants), which were not investigated in the present study, should be considered too.

TABLE 3: Average ($n = 8$) concentrations and relative standard deviations of the analytes in the OUT 2 and OUT 3 samples[a]

	OUT 2		OUT 3	
Compound	Mean (ng/L)	RSD	Mean (ng/L)	RSD
Amisulpride	20.1	45.1	1.0	216.0
Ofloxacin	77.6	49.2	6.1	139.7
Sulfamethoxazole	83.3	53.7	27.5	105.5
Metoprolol	118.8	66.5	4.6	230.6
Lamotrigine	63.1	72.2	8.6	162.3
5-Methyl-benzotriazole	550.9	52.3	62.3	140.9
Gabapentin-lactam	282.8	31.9	149.8	51.8
Azithromycin	405.8	69.4	47.4	180.3
Propyphenazone	15.6	36.1	4.8	75.3
Carbamazepine	243.5	29.7	39.3	129.5
Irbesartan	709.6	30.1	313.7	68.2
Ketoprofen	57.4	55.7	26.1	74.0
Clarithromycin	73.3	25.7	17.0	102.8
Diclofenac	642.1	31.1	203.5	87.3
Celestolide	5.5	16.6	<LOQ	
Galaxolide	5118.4	29.0	1130.9	80.4
Galaxolidone	1201.4	23.6	211.3	97.2
Phantolide	<LOQ		<LOQ	
Tonalide	224.6	30.9	27.0	87.7
Cd	626	47	655	50
Cr	<LOQ		1266	41
Cu	<LOQ		2091	21
Ni	11,498	60	8352	53
Pb	2211	27	2525	44
Zn	38,981	45	32,689	24

[a] The data from 2-day replicates were first averaged and then, from the eight obtained values, mean and RSD were derived.
RSD = relative standard deviation; LOQ = level of quantification.

Biological assays

Overall toxicity. The samples of the eight configurations collected at the outlets of both the biological treatment (OUT 2) and the tertiary treatment (OUT 3) were toxicity-tested using the algal growth inhibition responses as a direct measure of the

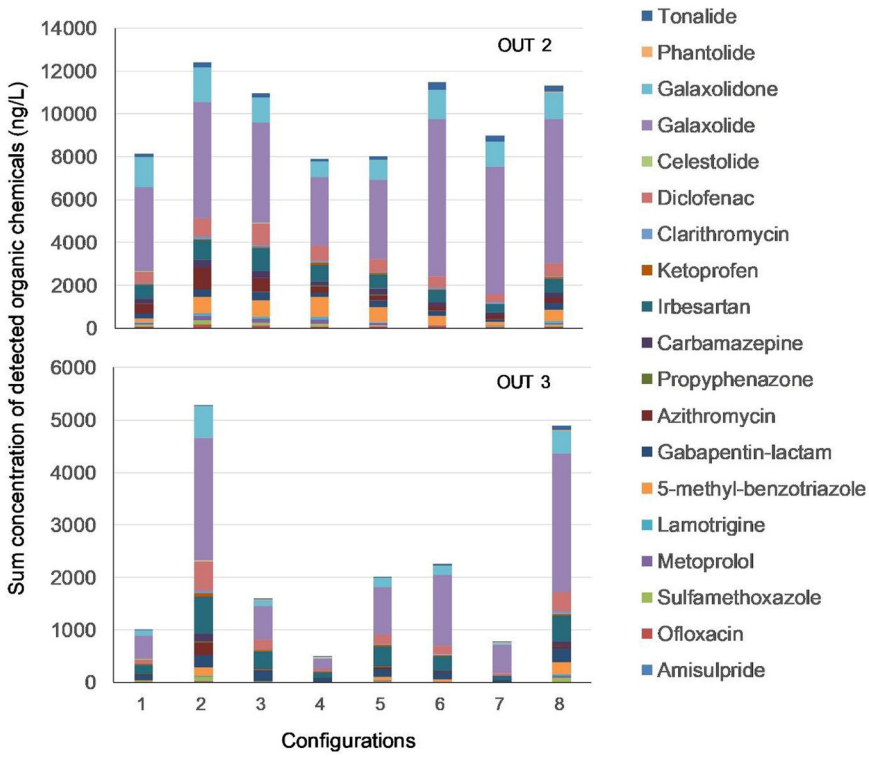

FIGURE 1: Concentrations of organic compounds averaged on all of the samples of the secondary (OUT 2) and tertiary (OUT 3) effluents.

toxic effects of all chemicals present in the mixture as a whole. In the following, the toxicity effects of the original samples without any treatment beside filtration will be referred as the *overall toxicity*.

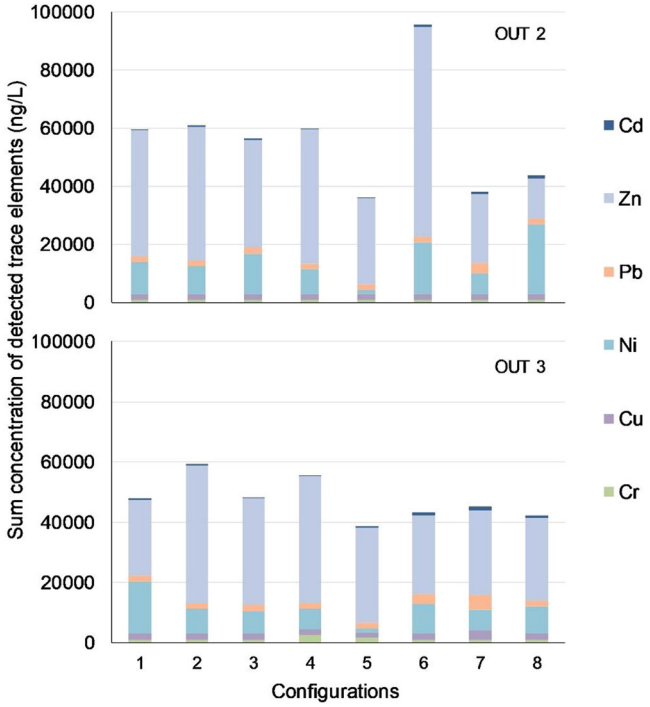

FIGURE 2: Concentration of trace elements averaged on all of the samples of the secondary (OUT 2) and tertiary (OUT 3) effluents.

All of the acceptance criteria for the algal bioassay were met, and all of the test results can therefore be considered valid (Supporting Information, Section SM1.5 and Table SM1.4).

The algal growth responses at 72 h are shown in the same Supporting Information, Table SM1.4, for each day's replicate, while the overall toxicity, as 72-h percentage growth inhibition, is shown in Figure 3 and in Supporting Information, Table SM1.5. For both effluents a good overlap of the responses between the two replicates of the same sample was observed. Evident toxic effects, that is, above the significance threshold (10%I), were always measured in the secondary effluent (Figure 3). The percentage inhibition values of all samples was >19%I. The average overall toxicity of 25(±5)%I suggests the constant presence in the secondary effluent of nonbiodegradable compounds acting as algal growth inhibitors.

A toxic effect was also detected downstream from the tertiary treatment in the OUT 3 samples, although, as expected, at a lower level of toxicity (Figure 3). While in three cases (1, 4, and 7) the inhibition values remained even lower than 5%I, in the other configurations, the values fell within the range 10%–25%I. The overall mean toxicities in the tertiary effluent (mean 12 [±8]%I) were significantly lower in the secondary effluent (Student t test, $p = 0.003$). The results highlight the effectiveness of advanced wastewater treatment to improve the quality of discharged effluents and the sensitivity of the algal test to detect significant differences even at low levels of exposure.

Toxicity of organic compounds—SPE extracts. To compare the concentrations of organic compounds with the toxicity

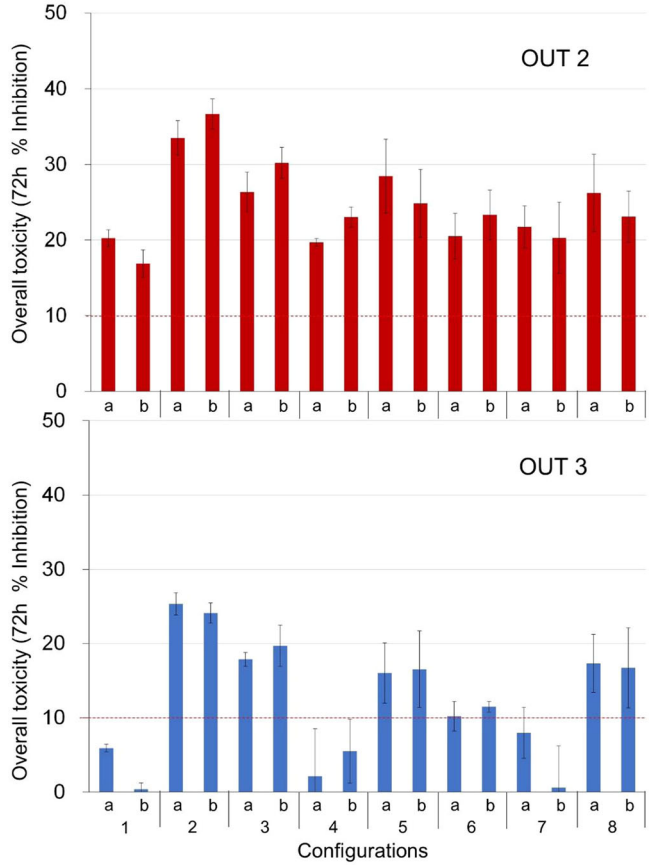

FIGURE 3: Overall toxicity as measured in the 2-day replicates (a, b) of the eight configurations from both the secondary (OUT 2) and tertiary (OUT 3) effluents. Toxicity values are reported for each day replicate as mean (±SD) between three replicates of test cultures. Red line is the significance toxicity threshold (10% inhibition).

attributable to this class of chemicals, the algal tests through the enrichment sample procedure were carried out and the EC50, expressed as an REF, was determined.

The test validity criteria of biomass development and growth rate of all control cultures were met (Supporting Information, Table SM1.6). For all tested samples, a high proportionality was detected between the enrichment factors and the growth responses so that the percentage inhibition values were spread over the large range of effect from 0% to 95% (Supporting Information, Figure SM1.2 and Table SM1.7). This result guaranteed the possibility of calculating a robust EC50 value for all samples (Table 4).

The EC50s of the secondary effluent had a limited variability (13.3–25.1 REF), and the average was 19.2 (±3.7; relative SD [RSD] = 19.2%) REF. The low variability indicated that the contribution of the extracted organic compounds to the whole toxicity of the secondary effluent was quite constant over the experimental campaign. A greater variability of EC50 values was observed in the tertiary effluent samples, with EC50s ranging from 22 to 46.5 REF and an average of 31.9 (±8.8; RSD = 26.8%) REF.

The extracted organic compounds of the tertiary effluent had a mean toxicity toward algae significantly lower than that of the secondary effluent (Student *t* test, $p = 0.0018$), supporting the general detoxification capacity of the PAC treatment.

TABLE 4: Median effective concentrations as relative enrichment factor with 95% fiducial limits, indicated as + and −, in the OUT 2 and OUT 3 extracted samples

	EC50 and fiducial limits 95%						
OUT 2	REF	−	+	OUT 3	REF	−	+
1	19.7	18.2	26.4	1	27.2	25.0	30.6
2	15.5	10.7	22.9	2	30.6	24.1	39.2
3	13.3	8.8	19.8	3	23.6	19.3	28.5
4	19.7	15.1	24.4	4	28.4	26.6	30.9
5	25.1	22.3	28.2	5	35.85	32.2	40.2
6	21.0	14.2	30.9	6	46.54	40.1	55.0
7	21.7	16.3	27.5	7	40.61	36.4	46.7
8	17.8	12.9	23.0	8	22.00	16.4	28.0

EC50 = median effective concentration; REF = relative enrichment factor.

Toxicity of organic compounds—SPE eluates. A strategy to evaluate the role of organic micropollutants as causative toxicants was to test the SPE-eluate samples collected downstream from the passage of the original wastewaters into the SPE cartridges during the extraction of the effluents. The experiment was conducted on five samples (configurations 1, 2, 4, 6, 8) using only 1 day's replicate, of both OUT 2 and OUT 3. The 72-h growth responses of all eluate samples, including the procedural blanks, are shown in Supporting Information, Table SM1.4, for each day's replicate. The percentage growth inhibition measured on the SPE eluates was compared with the overall toxicity of the corresponding samples in Supporting Information, Figure SM1.3 and Table SM1.8, where the toxicity reduction (percentage) is also reported.

In OUT 2, an important toxicity reduction after the SPE treatment (from 40% to 86%) was measured in most of the samples. It is interesting to note that, apart from Configuration 1 samples, the residual toxicities measured in the eluate samples were steadily approximately 10%I (mean 11.2 ± 1.1%I) and can be attributed to inorganic and very polar or soluble organic compounds not retained during SPE extraction. By contrast, the difference between the overall and the SPE-eluate toxicities, varying from 9%I to 23%I, likely represents the contribution of the organic compounds adsorbed on SPE.

In OUT 3 (Supporting Information, Figure SM1.3), it was difficult to evaluate the toxicity reduction because the overall toxicities were mostly <10%I, which can be considered a sensitivity limit of the test. The toxicity reductions were effectively measured in two configurations (65% in Configuration 2, 62% in Configuration 8), both characterized by significant overall toxicity values.

Trace element bioavailability. To investigate the impact of water chemistry on the bioavailability of the dissolved trace elements, ad hoc spiking experiments, using Cu as the model compound, were performed on some samples from secondary and tertiary effluents, as well as on SPE eluates.

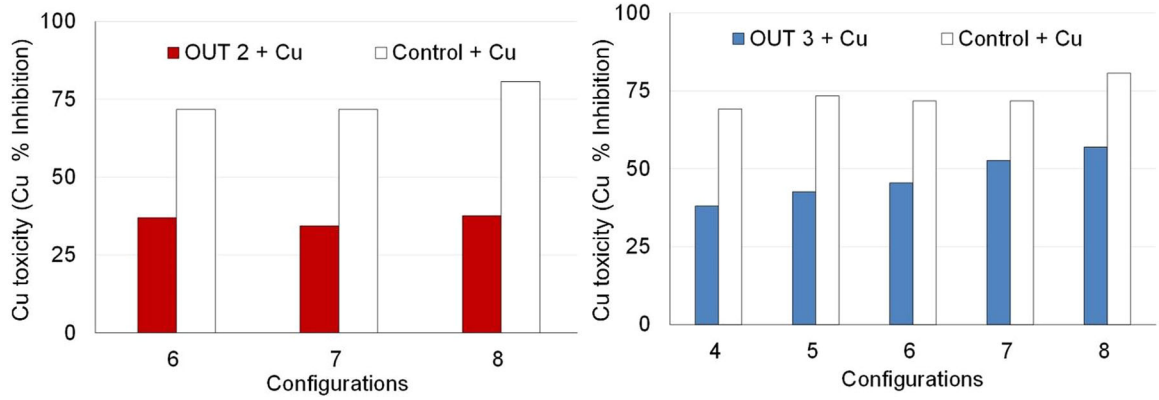

FIGURE 4: Copper toxicity of secondary (OUT 2 + Cu) and tertiary (OUT 3 + Cu) effluents compared to standard water (Control + Cu).

Figure 4 compares the Cu toxicity (Cu-%I) measured in Cu-spiked wastewater (OUT 2 and OUT 3 + Cu) and in Cu-spiked standard water (Control + Cu).

An average Cu-%I value of 73.4 (±4.3) was calculated for the standard water. By contrast, much lower Cu-%I values were measured in both effluent types. In the three OUT 2 samples Cu exerted only half of the toxic effect as that in the standard water, with Cu-%I varying from 34%I to 38%I (SD = 1.7) and average Cu-WER = 2.16. This effect was slightly less pronounced in OUT 3 samples. In all samples Cu toxicity was always >38%I, even exceeding 50%I in Configurations 7 and 8. The Cu-%I in the tertiary effluent is on average 1.6-fold lower than in the standard control (average Cu-WER = 1.55), indicating the persistence of an important water-chemistry effect of lowering the bioavailability of trace elements and reducing the toxicity even after the activated carbon treatment. The decrease of Cu toxicity in both effluents compared to the standard water can be interpreted in terms of bioavailability; thus, the WER could be considered a descriptor of the changes of metal bioavailability.

Additional tests with spiking of Cu in the SPE eluates showed the role of wastewater organic matter in regulating the trace element toxicity, which was observed to increase following the conceivable reduction of the organic matter from the secondary to the tertiary effluents, to the SPE eluates, up to the control medium, which is completely lacking in organic substance. The results are shown in Supporting Information, Section SM1.6 and Figure SM1.4.

DISCUSSION

Wastewater toxicity

The alga *P. subcapitata* showed great sensitivity toward the highly different levels of contamination as depicted by the parallel chemical analysis of the wastewater samples from the conventional and tertiary treatment plants. Numerous cases of conventional plants characterized by persistent algal toxicity <30% effect in the effluent samples were also described in the review by Völker et al. (2019), focusing on the toxicity removal by secondary or advanced-tertiary treatments. The overall measured toxicities in the samples from the conventional plant, steadily around the mean of 25(±5)%I, are in good agreement with those measured in effluents from biological activated sludge plants in earlier studies where quite similar levels of toxicity toward algae (23%I) were found in a municipal treatment plant in the federal state of Upper Austria (Latif & Licek, 2004).

In agreement with the lower chemical contamination measured in the OUT 3 samples, the algal test highlighted an improvement of the quality of the wastewaters after the treatment with PAC. By comparing the overall toxicity values of the two effluents and keeping in mind that the wastewater feeding the tertiary PAC pilot plant is outflowing from the conventional plant, it was possible to estimate the effectiveness of the tertiary treatment in removal of the residual toxicity. The average difference between the toxicity of OUT 2 and OUT 3 is 54(±12)%, with values ranging from a minimum of 30% to a maximum of >80% (Supporting Information, Table SM1.5), mostly depending on the dosage of fresh activated carbon (milligrams per liter) used in the pilot plant (Table 1). The highest percentage difference, that is, the highest detoxifying effect, was associated with a high dosage of fresh PAC (up to 20 mg/L), while, on the contrary, in Configurations 2 and 8, where the lowest PAC dosage (5 mg/L) was applied, the minimum percentage differences were found.

In general, a dosage of PAC between 10 and 20 mg/L was reported to be sufficiently efficient at removing micropollutants (Boehler et al., 2012; Serrano et al., 2011). More specifically, a dosage of 14 mg/L proved to be effective at reducing the algal toxicity by up to 84% (Margot et al., 2013), in compliance with our findings.

The lower REF, which caused 50% growth inhibition, in OUT 2 than in OUT 3 (Table 4) suggested that a significant contribution to algae toxicity of the organic compounds, which were adsorbed on SPE cartridges, was present in the secondary effluents.

To estimate the percentage contribution of adsorbable organic compounds, we expressed the percentage inhibition of the SPE extract with a REF = 1, that is, reconstructing the original dilution. At the same time, toxicity tests carried out on the SPE eluate, that is, the nonretained fraction after the SPE treatment, allowed us to estimate the contribution from nonadsorbable compounds (i.e., inorganic or very polar organic compounds). The sum of the toxicity values of the SPE extracts

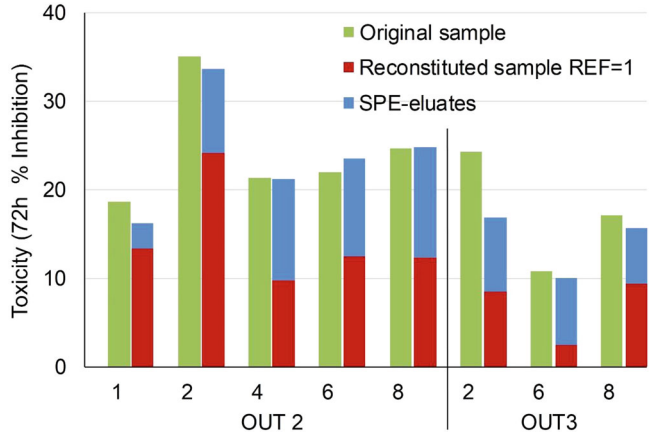

FIGURE 5: Comparison of the overall toxicity (expressed as 72-h percentage inhibition) with the sum of the toxicity measured in the solid-phase extraction (SPE) extracts (reconstituted to REF = 1) and in the SPE eluates. REF = relative enrichment factor.

(reconstituted to REF = 1) and of the SPE eluates significantly reproduced the overall toxicity of the original sample (paired *t* test at $p = 0.704$), showing that this approach can be used to estimate the percentage contribution of organic (from 23.8% to 82.3%) and nonadsorbable compounds (from 17.7% to 76.3%) to the overall toxicity (Figure 5 and Table 5). Samples with overall toxicity values lower than the significance level of 10%I were not included in the calculation of the contribution.

Application of the concentration addition model

The integration of chemical concentrations and ecotoxicological test results was carried out by modeling the mixture toxicity using the concentration addition method.

In both OUT 2 and OUT 3, the STU turns out to be greater than unity, ranging from 1.7 to 4.8. This means that the chemical mixture concentration in the effluent is approximately from two to four times higher than that which causes an inhibition effect of 50%. On the contrary, the measured overall toxicities were all <50%I, ranging from 10.8%I to 35.1%I (Table 5), highlighting a toxicity overestimation by the concentration addition model which is in accordance with a critical review on the ecotoxicology of pharmaceutical mixtures (Backhaus, 2014). The overestimation of concentration addition was also described at low effect levels for metal mixture toxicity toward higher plants and daphnids, on average with a factor 1.4–3.6 (Nys et al., 2017).

The relevance of each chemical class affecting the mixture toxicity was identified by calculating the percentage STU (Table 5; Supporting Information, Table SM1.9). Analyzing the estimated toxicity of both OUT 2 and OUT 3, it is evident that the greatest contribution is provided by trace metals, which always accounted for >97% of the STU.

The role of trace metals dominating the predicted toxicity of the mixtures is also shown in Supporting Information, Figure SM1.5, which depicts the toxic unit distribution of all of the detected chemicals in the effluent wastewater samples. In both OUT 2 and OUT 3, Zn alone seems responsible for >75% of the expected mixture toxicity. The first five compounds explain up to 98% of the STU, while the contribution of the other compounds seems negligible.

A significant discrepancy was found between the contributions of the chemical classes (organic and inorganic) as modeled by the toxic unit approach and those measured by the algal test (Table 5). Compared to the experimental findings provided by the algal testing, the contribution of trace elements is overestimated, while the contribution of the organic

TABLE 5: Comparison of the overall toxicity (as 72-h percentage inhibition) with the sum of the toxicity values of the solid-phase extraction (SPE) extracts (reconstituted to REF = 1) and of the SPE eluates

	Experimental toxicity					Modeled toxicity		
	Original sample	Reconstituted sample	SPE-eluates	%	%	Sum of toxic	Contribution of each class of chemicals to the total sum of toxic unit (%STU)	
Configuration	Overall toxicity (%I)	Organic toxicity (%I)	Inorganic[a] toxicity (%I)	contribution Organics	contribution Inorganics[a]	units (STU)	Organics	Inorganics (trace elements)
OUT 2								
1	18.6	13.4	2.9	82.3	17.7	3 .0	1.4	98.6
2	35.1	24.3	9.6	71.7	28.3	3.2	2.0	98.0
4	21.4	9.8	11.6	45.8	54.2	3.1	1.6	98.4
6	21.9	12.5	11.2	52.8	47.2	4.8	1.0	99
8	24.7	12.4	12.6	49.6	50.4	1.7	2.8	97.2
OUT 3								
1	3.1	0.3	0.20	ND	ND	2.1	0.2	99.8
2	24.7	8.6	8.4	50.3	49.7	3.1	1.0	99.0
4	3.8	0.1	4.2	ND	ND	2.9	0.1	99.9
6	10.8	2.4	7.7	23.8	76.3	2.1	0.5	99.5
8	17.0	9.4	6.4	59.4	40.6	2.1	1.2	98.8

[a]Including very polar and soluble organic compounds (nonadsorbable compounds).
The percentage contribution of adsorbable (called "organic") and nonadsorbable compounds (called "inorganic" but including very polar and soluble organic compounds) to the calculated toxicity is also reported. The results are compared to the predicted toxicity calculated by the concentration addition model.
ND = contribution not determined for overall toxicity <10% inhibition; REF = relative enrichment factor.

contaminants is insignificant in the concentration addition estimation. The reasons behind these discrepancies are multiple. A source of overestimation is the substitution of concentrations below the LOD with the value LOD/2. This approach was guided by a precautionary intent (Finizio et al., 2022) because the nondetection of a compound does not mean that it is necessarily absent (Menz et al., 2017). The case of Cu is critical because most of the measured concentrations were below the LOD, which is even higher than its predicted-no-effect concentration (PNEC; Supporting Information, Table SM1.2): the substitution of censored data with LOD/2 made Cu the second and third toxicity driver among the metals in OUT 2 and OUT 3, respectively (Supporting Information, Figure SM1.5).

Furthermore, the standard algal toxicity test measures a nonspecific endpoint, such as growth inhibition, which is not linked to any specific mechanism of action and provides an integrated response of the totality of compounds interacting in the mixture, including by-products or transformation products or undetected compounds. Instead, the concentration addition model assumes that the components of the mixture, sharing the same mode of action, do not interact (Backhaus, 2014). Some authors described the difficulties of studying the metal mixture with the concentration addition approach because it does not account for the interactions among the mixture components, such as uptake competition or complexing to organic ligands, which could reduce the metal bioavailable concentration (Gopalapillai & Hale, 2016; Nys et al., 2017). Bioavailability of metals in our effluents has been evaluated by experimental tests (see above, *Trace element bioavailability*) and modeling, and the results are discussed below (see *Toxicity of trace elements and their bioavailability*).

Finally, the main reason for the discrepancy is that the studied mixture as a whole was probably too heterogeneous to approximate an estimation of the algal toxicity using the concentration addition model. Instead, in many cases more successful findings were previously reported for less heterogeneous mixtures of wastewaters containing only one class of chemicals, such as pharmaceuticals (Backhaus et al., 2000; Menz et al., 2017; Neale et al., 2017).

Given these discrepancies, it is legitimately questionable why the alternative model to concentration addition, that is, independent action, was not used to predict the mixture toxicity. In contrast to concentration addition, the independent action model assumes a dissimilar mechanism of action of all mixture components, which produce statistically independent responses (Escher et al., 2021). The mixture effect could be calculated from the effect that each component would singly cause at the concentration at which it is present in the mixture (Carusso et al., 2018). Thus, application of the independent action strictly requires knowledge of the individual mechanism of action as well as of the individual concentration–response curves for all of the mixture components. Unfortunately, it was impossible for us to get experimental dose–response curves for each compound; therefore, we could not use the independent action alternative model. We were not even able to access the information on the mode of action of each mixture component. As supported by the literature, following a precautionary principle, if no mode of action information is available, the concentration addition method should be preferred over the independent action approach (Backhaus, 2014; Junghans et al., 2006).

To model the contribution of analyzed organic compounds, we adopted the TEQ approach (Escher et al., 2008) to quantify the actual contribution of the analytically detected organic chemicals to the observed biological effects as measured by algal toxicity testing of the organic extracts, as described in the following section.

Toxicity of organic compounds estimated as TEQ concentrations

The TEQ approach is a special case of the concentration addition model, largely adopted as a quantitative tool to evaluate the biological responses of chemical mixtures (Escher et al., 2021) and to find a link between the observed ecotoxicological effects and the measured chemical concentrations in the same extracted organic fraction. As detailed in Supporting Information, Section SM1.7, we selected clarithromycin as the reference compound, to be used to translate the effects measured in the organic extracts in TEQ_{bio} and to model the toxicity effects of the detected compound organic chemicals calculating the TEQ_{chem} (Supporting Information, Table SM1.11). The predicted $TEQ_{chem,mixture}$ values were compared to the TEQ_{bio} to assess which amount of the observed toxic effects could be explained by the detected organic chemicals (Table 6).

It can be observed that in the secondary wastewater samples a large part of the total TEQ_{bio} (82%–104%) can be explained by the TEQ_{chem}, and the two variables are well correlated (Supporting Information, Figure SM1.6). This result supports the reliability of the concentration addition model to predict the mixture toxicity of the organic compounds present in the extracted samples. Also, it highlights that the selection of target compounds, carried out by suspect screening, was sufficiently representative of the actual contamination of the investigated wastewaters in terms of algal toxicity.

In the tertiary effluent, the portion attributable to the detected chemicals (5.1%–56.5%) is lower and more variable than in OUT 2. Only in Configuration 2 did the contribution of the analyzed chemicals reach 90%.

The plots of the individual TEQ_{chem} (Figure 6, left panel) clearly show how the analytical picture of the secondary and tertiary wastewater configurations could significantly vary if the concentration of each detected chemical is normalized to the REP. Compared to Figure 1, reporting the measured concentrations, the predominance of compounds in the mixture is completely overturned. The antibiotic clarithromycin was firmly the dominant compound, accounting for 70%–84% in OUT 2 and for 49%–80% in OUT 3 (Figure 6, right panel). Clarithromycin is followed by galaxolide (9%–20% in OUT 2 and 10%–40% in OUT 3) and galaxolidone (3%–7% in OUT 2 and 3%–5% in OUT 3). This is undoubtedly due to the highest toxic effect of clarithromycin toward algae, its EC50 of 2 μg/L (Guo et al., 2015) being the lowest effect concentration value among

TABLE 6: TEQ_{bio} and $TEQ_{chem,mixture}$ for OUT 2 and OUT 3 configurations and contribution of the detected organic chemicals to the measured biological effect (percentage explained effect)

	OUT 2							
	1	2	3	4	5	6	7	8
TEQ_{bio} (ng/L)	101.5	129.2	150.4	101.6	79.6	95.1	92.1	112.1
$TEQ_{chem,mixture}$ (ng/L)	86.6	128.3	123.5	100.6	64.3	99.8	81.9	98.3
Effect (%)	85.3	99.3	82.1	99.0	80.8	104.9	88.9	87.7
	OUT 3							
	1	2	3	4	5	6	7	8
TEQ_{bio} (ng/L)	73.4	66.2	84.9	70.3	55.8	43.0	49.2	90.9
$TEQ_{chem,mixture}$ (ng/L)	9.0	59.9	12.1	3.6	15.5	20.8	3.7	51.4
Effect (%)	12.3	90.5	14.2	5.1	27.8	48.4	7.5	56.5

TEQ_{bio} = toxic equivalency of a mixture in terms of an equivalent concentration of a reference compound; TEQ_{chem} = toxic equivalency calculated by summing up the product of the measured concentration of the detected chemicals in the mixture and their relative potency with respect to the reference compound.

all those considered in our study, as reported in Supporting Information, Table SM1.2, where it is also classified as very toxic for aquatic organisms. Particularly for green algae, this compound must be considered as one of the most problematic pharmaceuticals (Sharma et al., 2021) because it has a specific inhibiting action on bacterial 70S ribosomal proteins, which are present in a homologous form in the chloroplast ribosomes of green algae (Villain et al., 2016). Because of its high ecotoxicity, clarithromycin, as well as the other macrolide antibiotic azithromycin, has been included in the first watch list as a substance to be monitored in all European Union member states for future revisions of the priority substances list (Guo et al., 2020). These results are consistent with those of previous studies, which also found high antibiotic toxicity mainly caused by macrolides (Markert et al., 2020; Tousova et al., 2018). Those studies highlighted also the effects of antibiotic drugs in multicomponent mixtures, which could even produce a strong synergism resulting in a toxicity increase up to an order of magnitude (Tousova et al., 2018). Azithromycin, together with carbamazepine, were among the major contributors to TEQ_{chem}, even with contributions always <4% (Supporting Information, Table SM1.12). On the contrary, irbesartan and diclofenac, which were both present at high concentrations in both effluents, gave a negligible contribution to the algal toxicity. Even if diclofenac is not highly toxic to algae, it was always present in the secondary wastewater sample at very high concentrations, up to one order of magnitude above its PNEC value, indicating a very

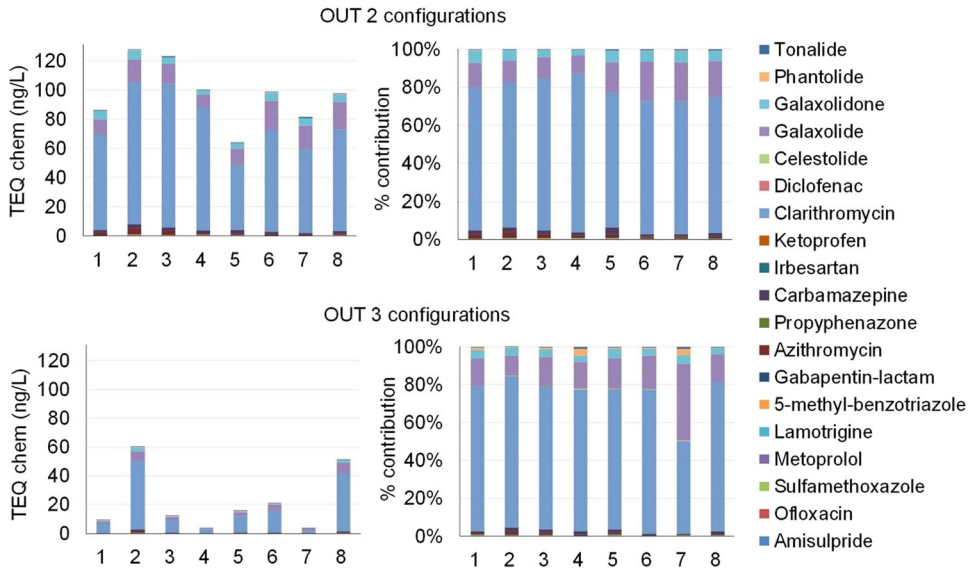

FIGURE 6: Toxic equivalency (left panel) and percentage contribution (right panel) for individual chemicals detected in the extracted organic fraction from OUT 2 and OUT 3 wastewater samples. TEQ_{chem} = toxic equivalency calculated by summing up the product of the measured concentration of the detected chemicals in the mixture and their relative potency with respect to the reference compound.

high potential risk for the aquatic environment. The demonstration of possible synergisms requires that the chemical composition be fully characterized, but this is very difficult in the case of complex matrices such as effluents.

Toxicity of trace elements and their bioavailability

The widely known complex interactions occurring among metals in complex mixtures could affect the reliability of the concentration addition approach. In fact, it is worth remarking that for trace metals the total STU is always higher than unity, ranging from 1.7 to 4.8 (Supporting Information, Table SM1.13), indicating again the overestimation of the concentration addition model for toxicity of multicomponent mixtures of metals to *P. subcapitata*.

Furthermore, there is a significant disagreement between the measured toxicity contribution from inorganics and the modeled one, which might be explained in terms of reduction of bioavailability of trace elements in organic-rich matrices such as wastewaters (see above, *Application of the concentration addition model*). To investigate the effect of the matrix on metal bioavailability, we tested two approaches, the experimental measurement of Cu-WER and the application of a simplified biotic ligand model (Bio-met) as suggested by the European Union (European Commission, 2021). The Cu-WER correction was specifically derived for algal tests but has been applied to all elements as a fixed factor, with a simplified but incorrect assumption that the correction developed for Cu is similar for the other metals, while Bio-met modeling was available only for four elements, Zn, Ni, Cu, and Pb, and the correction was derived for their overall toxicity.

We compared the impacts of the different approaches on the toxicity drivers and on the relative weight of metals in the overall sample toxicity. The application of both bioavailability corrections to the metal concentrations was effective at approximating the total STU to unity, but the reduction of the contribution of the trace elements to the total toxicity was very limited (Supporting Information, Tables SM1.14 and SM1.15). For what concerns the toxicity drivers, Zn was still the predominant element among the metals (Supporting Information, Table SM1.13). Its high contribution originates from the combination of its lowest value of algal EC50 with the steadily high concentrations of this element in all effluent samples, as illustrated above (see *Biological assays*). Besides Cu, whose contribution we discussed in the previous section, the other most significant component of the metal mixtures was Ni, which presents the same average contribution of approximately 10% STU in both treatment plants.

The increase of toxicity bioavailability in Cu-WER tests going from OUT 2 to OUT 3, and finally after the SPE treatment (Supporting Information, Figure SM1.4), suggests that part of the bioavailability reduction could be attributed to the dissolved organic fraction, which is not adequately modeled by the simplified biotic ligand model. Nevertheless, we cannot neglect the possible contribution of organic chelating ligands such as ethylenediaminetetraacetic acid or nitrilotriacetic acid, which are usually present at microgram per liter levels in wastewaters (Clara et al., 2012) and have been demonstrated to be the most important ligands for all of the metals considered in sewage effluents (Peters et al., 2014). The presence of water-soluble ligands, not adsorbed on lipophilic phases, could explain the low contribution of trace element fractions to the experimental overall toxicity in the algal tests.

CONCLUSIONS

The exposure and effect assessments showed the complexity of the analyzed mixtures. The algal toxicity tests performed on the whole mixture revealed the joint effects of all interacting contaminants, even accounting for any unidentified chemicals. The ecotoxicological tests carried out on the whole samples of outflows, of both secondary and tertiary treatments, allowed us to demonstrate that the applied tertiary treatment with dispersed active carbon is able to reduce the toxicity related to the adsorbable organic compounds, but a baseline toxicity attributable to nonadsorbable compounds (i.e., very polar or inorganic compounds) is always present and can be still measured as algal growth inhibition.

Furthermore, the additional tests carried out on separated fractions discriminated the role of adsorbable (mainly organic) and nonadsorbable (i.e., very polar or inorganic) toxicants affecting the overall toxicity. However, the biological approach alone could not be sufficient to define the main parameter responsible for the whole measured effects. By contrast, chemical analysis provided a picture of the complex chemical composition of the mixture, though limited to 25 target chemicals. Application of the concentration addition model to the chemical data highlighted the main drivers of toxicity, on which efforts to optimize tertiary treatments should be focused; but it was not able to model the whole toxicity of the sample.

The integration of chemical data with ecotoxicological tests, by calculating TEQ concentrations in specific tests on SPE concentrated samples, demonstrated that we can compare the mixture toxicity derived from chemical data with the measured toxicity of the ecotoxicological test. This comparison validated the selection of the target molecules, carried out by suspect screening with HRMS, by showing that the selected compounds are largely representative of the toxicity attributable to adsorbable organic compounds in the original samples.

These tools for integrating ecotoxicological tests and chemical data were demonstrated to be effective at managing the portion of toxicity attributable to organic compounds, but we found many discrepancies when we applied them to model the toxicity of trace metal mixtures. The complexity of the synergistic or competing interactions between elements and the presence of industrial or natural chelating molecules in discharges, which strongly impacted their bioavailability, still

represent challenges for the toxicity modeling of real samples, especially wastewaters.

We also must keep in mind that in the present study the ecotoxicological analysis was limited to only one test organism. Most importantly, in our study we used only one nonspecific endpoint, growth inhibition; hence, the observed toxicity could be the result of multiple effects, including those produced by compounds with modes of action different from those of the target compounds considered in our study. For example, the specific endpoint of photosynthesis inhibition, typically the mode of action of herbicides (Neale et al., 2017; Vermeirssen et al., 2010), could be overlooked by our approach. The inclusion of additional chemicals, for example, herbicides, in the chemical screening and the parallel performance of algal tests to determine the efficiency of photosynthesis could enhance our approach, enabling us to explain an even greater portion of the measured toxicity effect.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5424.

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