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Linking in Vitro Effects and Detected Organic Micropollutants in Surface Water Using Mixture-Toxicity Modeling

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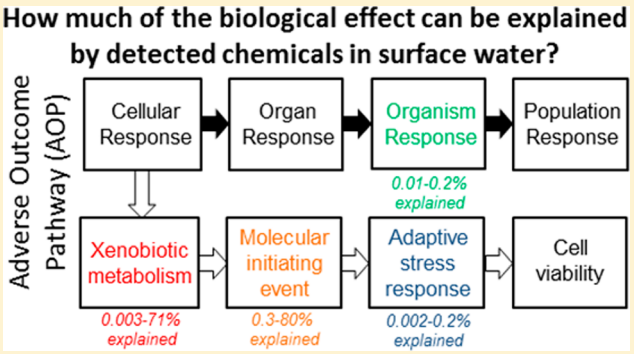
Supporting Information

ABSTRACT: Surface water can contain countless organic micropollutants, and targeted chemical analysis alone may only detect a small fraction of the chemicals present. Consequently, bioanalytical tools can be applied complementary to chemical analysis to detect the effects of complex chemical mixtures. In this study, bioassays indicative of activation of the aryl hydrocarbon receptor (AhR), activation of the pregnane X receptor (PXR), activation of the estrogen receptor (ER), adaptive stress responses to oxidative stress (Nrf2), genotoxicity (p53) and inflammation (NF-κB) and the fish embryo toxicity test were applied along with chemical analysis to water extracts from the Danube River. Mixture-toxicity modeling was applied to determine the contribution of detected chemicals to the biological effect. Effect concentrations for between 0 to 13 detected chemicals could be found in the literature for the different bioassays. Detected chemicals explained less than 0.2% of the biological effect in the PXR activation, adaptive stress response, and fish embryo toxicity assays, while five chemicals explained up to 80% of ER activation, and three chemicals explained up to 71% of AhR activation. This study highlights the importance of fingerprinting the effects of detected chemicals.



INTRODUCTION

Human-impacted rivers can contain a complex mixture of micropollutants, such as pharmaceuticals, pesticides, and industrial compounds, as well as their transformation products.[1,2] The sources of these contaminants can include both point sources, such as wastewater effluent discharge, and diffuse sources, such as runoff from urban and agricultural areas.[3] Given the diversity of micropollutants in water, targeted chemical analysis alone is insufficient to detect all chemicals present in the aquatic environment. Bioanalytical tools complement chemical analysis because they can provide information about the biological effects of chemicals present in a sample and reveal the presence of active compounds not detected by targeted analysis.

In vitro bioassays based on various cellular response pathways, including induction of xenobiotic metabolism, receptor-mediated effects, adaptive stress responses, and cytotoxicity, have been applied to detect the presence of micropollutants in water samples.[4−6] Although the activation of these endpoints does not necessarily translate into higher-level effects, biological response at the cellular level is a key step in the adverse outcome pathway.[7] Furthermore, bioassays indicative of xenobiotic metabolism and repair and defense mechanisms can be applied as sensitive tools to detect the presence of micropollutants because effects in these endpoints often occur at lower concentrations than those causing cell death or damage.

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Bioanalytical tools also have the advantage that they can take into account mixture effects among chemicals rather than focusing on individual chemicals. The mixture effects that occur among chemicals can be categorized as concentration addition or independent action for chemicals acting according to the same or a different mode(s) of action, respectively, both of which assume no interaction among the mixture components, or synergism or antagonism, where the mixture components can interact.[8] For environmental samples, such as surface water, which can contain many chemicals at low concentrations, synergism is rare; instead, concentration addition has been suggested as a conservative approach to evaluate the mixture toxicity of multicomponent mixtures not only for receptor-mediated effects[9] but also for adaptive stress responses[10] and cytotoxicity.[11] Mixtures that act in a concentration additive manner can be described using the bioanalytical equivalent concentration (BEQ) concept, which represents the concentration of a reference compound that elicits an equivalent response in a particular assay as the sample and can be determined from both bioassays and chemical analysis. By comparing the BEQ from bioanalysis (BEQ_{bio}) and the BEQ from chemical analysis (BEQ_{chem}), it is possible to determine the contribution of detected chemicals to the biological effect.[12] This approach has been applied to a wide range of water types including surface water,[13,14] wastewater,[6,14,15] recycled water,[16] and swimming-pool water.[17]

In this study, a suite of bioanalytical tools was applied to water samples from the human-impacted Danube River. The BEQ concept was utilized as a simple mixture effect prediction model to determine the contribution of detected chemicals to the biological effect. The battery of bioassays follows previous recommendations on the selection of sensitive indicator bioassays that cover endpoints related to different stages of cellular toxicity pathways, including the induction of xenobiotic metabolism and receptor-mediated effects representing important molecular initiating events, as well as adaptive stress responses and cytotoxicity or other apical endpoints.[4] Bioassays indicative of specific modes of action, such as estrogenic activity, have previously shown that a small number of chemicals often explain a high proportion of the biological effect in wastewater,[14,16,18] but less is known about the explanatory power of known chemicals in other endpoints.

Activation of the ligand-dependent transcription factor aryl hydrocarbon receptor (AhR) was assessed using the CAFLUX assay.[19] Although most applications focus on dioxin-like compounds, which are unlikely to be found in the water phase due to their hydrophobicity, around 16% of the 320 environmental compounds examined by Martin et al.[20] were found to induce AhR-dependent gene expression. Activation of the pregnane X receptor (PXR), which is an important factor in xenobiotic metabolism regulation, was assessed using the HG5LN-hPXR assay, and 73% of chemicals studied in Martin et al.[20] activated PXR. Activation of the estrogen receptor (ER) was assessed using the reporter gene MELN assay. A suite of bioassays indicative of adaptive stress responses reacting to oxidative stress (ARE-*bla*), genotoxicity (p53RE-*bla*), and inflammation (NF-κB-*bla*) were also included. Adaptive stress response pathways are activated to restore the cell to homeostasis after damage.[21] The oxidative stress response is mediated by Nrf2 and the antioxidant response element,[22] and 26% of the 1859 chemicals in the United States Environmental Protection Agency (USEPA) ToxCast database were active in the ARE-*bla* assay.[23] The p53 response is activated after DNA damage, leading to either repair or apoptosis,[24] and activation of p53 can indicate the presence of genotoxic carcinogens.[25] Approximately 15% of chemicals in the ToxCast database were active in the p53RE-*bla* assay.[23] The NF-κB pathway is an important driver of the inflammatory response and can target cytochrome P450s, cytokines, and apoptosis regulators,[21] and 3% of chemicals in the ToxCast database induced a response in the NF-κB-*bla* assay.[23] Finally, the fish embryo toxicity (FET) test using zebrafish was applied complementary to the cell-based bioassays because it can provide information about the organism-level response. Apical endpoints in this test include embryo coagulation and lack of heartbeat,[26] and a recently published database containing 641 chemicals showed that 74% of reviewed chemicals caused mortality in the FET test.[27]

The current study aimed to assess what fraction of the biological effects of the cellular toxicity pathway can be explained by the quantified chemicals with available effect data. We used samples from a large water body with high dilutions and low levels of micropollutants and stemming from very diverse sources to test the hypothesis that in these water types, there is typically not a dominant chemical or chemical group; instead, the effects are largely driven by the mixture effects of many chemicals. Large-volume solid-phase extraction (LVSPE) water extracts from the Danube River were analyzed in the bioassays introduced above to determine BEQ_{bio}. The effect analysis was complemented with targeted chemical analysis of 272 water relevant chemicals, including pesticides, pharmaceuticals, artificial sweeteners, steroidal hormones, and industrial compounds. The target list is by no means comprehensive but is based on previous targeted and nontargeted analysis of the Danube River.[28] Effect concentrations for the individual detected chemicals were collected from the literature to determine BEQ_{chem}. By comparing BEQ_{bio} and BEQ_{chem}, it was possible to determine the extent to which the detected chemicals contributed to the mixture effect in each bioassay.

EXPERIMENTAL SECTION

Sampling. Sampling occurred during the third Joint Danube Survey (JDS3)[29] between August and September of 2013 using LVSPE (Maxx GmbH, Rangendingen, Germany).[30] The sampling locations, which included both the Danube River and its tributaries, are shown in Table S1 and Figure S1, along with detailed information about sample enrichment and extraction. Briefly, up to 500 L of water was passed through a stainless-steel chamber containing neutral sorbent Chromabond HR-X, anionic exchanger Chromabond HR-XAW, and cationic exchanger Chromabond HR-XCW (Macherey-Nagel, Dueren, Germany). After extraction, each solid phase was freeze-dried and then extracted with solvents, and the eluates were combined. The sample aliquots were reduced to dryness via rotary and nitrogen evaporation prior to shipping and then resuspended in either DMSO or methanol, depending on the assay.

Chemical Analysis. Target-screening analysis of the JDS sample extracts for 264 chemicals was performed by liquid chromatography high-resolution mass spectrometry (LC−HRMS) using an Agilent 1200 LC coupled to a Thermo LTQ Orbitrap XL. Analysis was run in both positive- and negative-mode electrospray ionization. For further details, see Hug et al.[31] A total of eight steroidal hormones and industrial phenolic compounds were analyzed by LC−MS/MS using an Agilent 1260 LC coupled to a ABSciex QTrap 6500 instrument operated in negative-mode electrospray ionization. Further details are

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Table 1. Overview of Bioassays Used in the Current Study

endpoint	assay	method reference	positive reference compound	maximum REF	data evaluation method	EC or LC value
activation of AhR	CAFLUX	Nagy et al.[19]	2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)	500	linear concentration-effect curve	EC_{10}
activation of PXR	HG5LN-hPXR	Lemaire et al.[43] Creusot et al.[15]	SR 12813[a]	500	linear concentration-effect curve	EC_{10}
activation of ER	MELN	Balaguer et al.[44] Kinani et al.[45]	17β-estradiol	500	linear concentration-effect curve	EC_{10}
oxidative stress response	ARE-*bla*	Invitrogen[46]	*tert*-butylhydroquinone (tBHQ)	500	linear concentration-effect curve	$EC_{IR1.5}$
p53 response	p53RE-*bla*	Neale et al.[47]	mitomycin	500	linear concentration-effect curve	$EC_{IR1.5}$
NF-κB response	NF-κB-*bla*	Jin et al.[48]	tumor necrosis factor alpha (TNFα)	250	linear concentration-effect curve	$EC_{IR1.5}$
mortality	Fish embryo toxicity (FET)	OECD[26]	3,4-dichloroaniline	1000	log−logistic concentration-effect curve	LC_{50}

[a]Tetraethyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate.

provided in Section S2 and Table S2. A full list of analyzed chemicals is provided in Table S3, along with method detection limits (MDL) for each chemical in units of nanogram per liter. For mixture modeling, the detected chemical concentration was converted to molar units.

Bioanalysis. Information about the studied bioassays and the derivation of effect concentrations can be found in Table 1 and Section S3. The data was expressed in units of relative enrichment factor (REF), which takes into account the sample enrichment by LVSPE and dilution in the assays. The data was expressed as concentration causing 10% effect (EC_{10}), effect concentration causing an induction ratio (IR) of 1.5 ($EC_{IR1.5}$), or concentration causing 50% mortality (LC_{50}). Linear concentration-effect curves were used to determine EC_{10} and $EC_{IR1.5}$, while LC_{50} was evaluated using log−logistic concentration-effect curves.[4] Cytotoxicity was assessed in parallel for the AhR, ER, oxidative stress response, p53 response, and NF-κB assays, and cell viability EC_{10} values were derived from log−logistic concentration-effect curves.[32]

Bioanalytical Equivalent Concentrations. The LC and EC values were converted to BEQ_{bio} using eq 1 with the LC_{50} or EC_{10} or $EC_{IR1.5}$ value of the reference compound (ref) and the matching LC_{50} or EC_{10} or $EC_{IR1.5}$ value of the extract only.

$$BEQ_{bio} = \frac{LC_{50}(ref)}{LC_{50}(extract)} \text{ or } \frac{EC_{10}(ref)}{EC_{10}(extract)} \text{ or } \frac{EC_{IR1.5}(ref)}{EC_{IR1.5}(extract)} \quad (1)$$

The BEQ concept has been typically applied to log−logistic concentration-effect curves; however, for many environmental samples, linear concentration-effect curves may be more suitable for data evaluation. This is because environmental samples may only induce low effects and to obtain the 50% effect concentration one would have to either use an unfeasibly high enrichment factor or extrapolate the concentration-effect curves. For linear concentration-effect curves to remain valid, they should reach no more than 20 to 30% effect or have an IR no greater than 5 to ensure that they remain in the linear range of the curve. Linear concentration-effect curves have previously been shown to be a robust data evaluation method for environmental samples, individual chemicals, and chemical mixtures.[10] It must be stressed that the BEQ concept is only valid if the slopes of the sample and reference compound are parallel in log−logistic concentration-effect curves.[33] However, parallel slopes are not a requirement for linear concentration-effect curves with a common intercept at the effect axis because the BEQ is the ratio between concentrations at a given effect level and is, therefore, independent of the effect level. The EC value from a linear concentration-effect curve was calculated using eq 2 using the example of EC_{10}, but the same equation is applicable for EC_{20}; for example, with 20% used instead of 10% or $EC_{IR1.5}$ with an IR of 1.5 as the effect benchmark. BEQ can then be calculated using eq 3, and although this example is for EC_{10}, this ratio is constant across the entire linear concentration-effect curve range.

$$EC_{10} = \frac{10\%}{slope} \quad (2)$$

$$BEQ_{bio} = \frac{EC_{10}(ref)}{EC_{10}(extract)} = \frac{10\%}{slope(ref)} \cdot \frac{slope(extract)}{10\%} = \frac{slope(extract)}{slope(ref)} \quad (3)$$

To calculate BEQ_{chem}, it was first necessary to determine the relative effect potency (REP_i) of the detected chemicals (i). Because the EC values for the detected chemicals were generally provided as EC_{50} values in the literature, it was necessary to use EC_{50} values derived from log−logistic concentration-effect curves for the AhR, PXR, and ER assays. REP_i was calculated using eq 4, with the LC_{50}, EC_{50}, or $EC_{IR1.5}$ value of the reference compound and the matching LC_{50}, EC_{50}, or $EC_{IR1.5}$ value of detected chemical i.

$$REP_i = \frac{LC_{50}(ref)}{LC_{50}(i)} \text{ or } \frac{EC_{50}(ref)}{EC_{50}(i)} \text{ or } \frac{EC_{IR1.5}(ref)}{EC_{IR1.5}(i)} \quad (4)$$

All LC values for the detected chemicals in the FET test were collected from Scholz et al.,[27] and the EC values were collected from the peer-reviewed literature (AhR, PXR, and ER assays) or the ToxCast database (oxidative stress response, p53 response, and NF-κB assays),[23] which includes over 1800 compounds in over 800 different assays. All ToxCast data was re-evaluated to determine $EC_{IR1.5}$ using linear concentration-effect curves. Because each chemical in the ToxCast database was run multiple times, it was possible to determine the mean $EC_{IR1.5}$ value and the associated standard deviation. BEQ_{chem} was

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calculated for each JDS sample using REP_i and the detected concentration (M) (eq 5). The variability associated with BEQ_{chem} for the chemicals present in the ToxCast database was assessed using error propagation. EC and LC values collected from the literature generally did not include standard deviation, so it was not possible to determine the variability associated with BEQ_{chem} for the AhR, PXR, ER, and FET assays.

$$BEQ_{chem} = \sum_{i=1}^{n} REP_i \cdot C_i \quad (5)$$

RESULTS AND DISCUSSION

Chemical Analysis. Of the 272 analyzed chemicals, 94 were detected at least once in the 22 JDS samples. The number of chemicals detected at each site ranged from 20 to 64. The sum of the molar concentration and number of detected chemicals at each site are shown in Figure 1A, with the concentrations in pM for each of the detected chemicals at the different sampling sites shown in Table S4. The most frequently detected chemicals were the artificial sweetener acesulfame, the industrial compounds triphenylphosphine oxide and 2-benzothiazolesulfonic acid, and the antimicrobial sulfamethoxazole, which were present at detectable levels at all studied sites. In all but one tributary, other common wastewater micropollutants, including carbamazepine and its transformation products, the corrosion inhibitors benzotriazole and methylbenzotriazole, the artificial sweeteners cyclamate and sucralose, and several herbicides and transformation products (metolachlor, isoproturon, atrazine, and terbuthylazine-2-hydroxy) were detected. The antidiabetic pharmaceutical metformin was found at the highest concentrations, with concentrations up to 7.6 nM. Overall, chemical contamination was relatively low, with none of the detected chemicals exceeding the Water Framework Directive environmental-quality standards.[34]

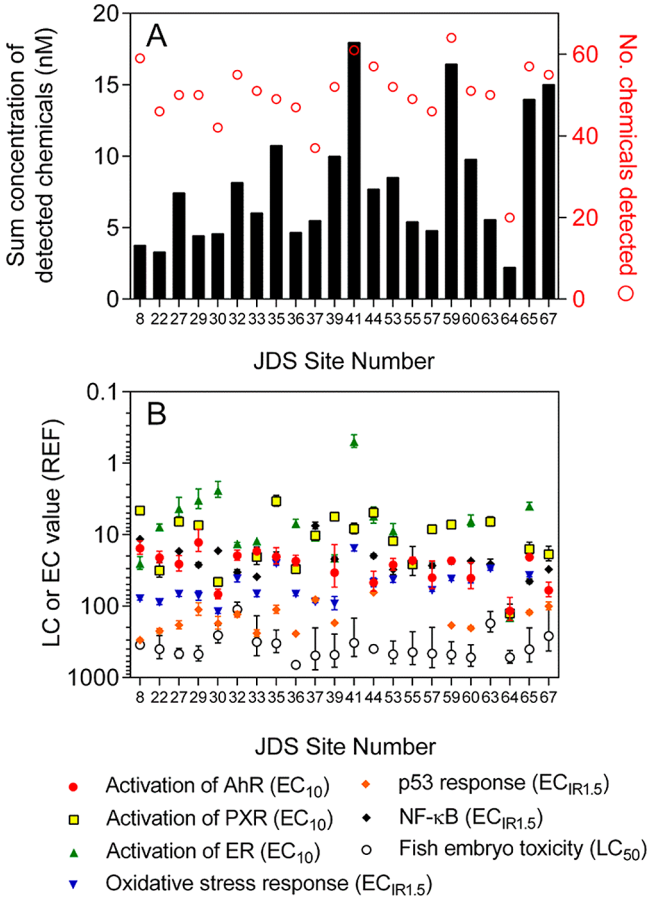

Figure 1. (A) The sum molar concentration of chemicals detected at each JDS site (black bars), along with the number of chemicals detected at each site (red circles), and (B) LC or EC values for all samples in units of relative enrichment factor (REF).

Bioanalysis. The EC and LC values for the different JDS water samples are shown in Figure 1B and Table S5, with the concentration-effect curves for all assays shown in Figure S2. The assays indicative of activation of ER, activation of PXR, activation of AhR, and the NF-κB response tended to be the most responsive, followed by the oxidative stress response. The p53 response occurred at higher effect concentrations. The least responsive assay was the FET test, which required a REF of 100 to 500 for 50% mortality or a REF of 50 to 300 for 10% mortality.

Although most samples did have a response in the assays, the effects were relatively low, with the EC values for the oxidative stress and AhR assays similar to previously benchmarked EC values for surface water.[35] Kittinger et al.[36] also only detected minimal effects in Danube River samples when assessing genotoxicity. Furthermore, ER activation, when expressed as BEQ_{bio} (0.02−1.1 pM), was lower than generally observed in wastewater effluent[37] due to dilution in the river, although one contaminated site, JDS 41 (BEQ_{bio} 4.7 pM), was identified by this assay.

JDS 64 had the lowest sum chemical concentration, and this corresponded to no effect at the maximum REF for the oxidative stress response, p53 response, and NF-κB assays, and only minimal effects at high concentrations in the other assays. Cell viability was assessed in parallel for most cell-based assays, and in most cases, there was negligible cytotoxicity in the studied concentration range. However, cytotoxicity did mask other endpoint manifestations at high REFs in some samples for the AhR (JDS 41 and 63), ER (JDS 35, 55, 57, 59, 63 and 67), oxidative stress response (JDS 55 and 67), p53 response (JDS 41, 55, 57 and 63), and NF-κB (JDS 36 and 41) assays. Hence, it was not possible to derive EC values for induction for these particular samples, but EC_{10} values for cytotoxicity were calculated and are included in Table S5. JDS 41, which had the highest effect in the ER activation and oxidative stress response assays and was cytotoxic in several other assays, was the most polluted site with the highest amount of total detected chemical concentration. Overall, there was no significant relationship between effect and sum detected chemicals at each site for the different assays.

For mixture modeling, the EC and LC values were converted to BEQ_{bio} using the respective reference compounds for each assay (Table 2, Table S5). Although the EC or LC values give an indication of the sensitivity of the assay, BEQ_{bio} converts the effect into the concentration of reference compound that would elicit the same response as the sample mixture. Furthermore, the BEQ concept simplifies mixture-toxicity modeling.[10,11]

Bioanalytical Equivalent Concentration from Chemical Analysis. Prior to calculating BEQ_{chem}, the published literature and ToxCast database were searched for EC or LC values for the detected chemicals. For each assay, between 0 and 13 literature EC or LC values could be found for the 94 detected chemicals (Table S6). Using the literature EC or LC values for each chemical and the EC or LC value of the assay reference compound, the REP_i was calculated using eq 4 (Table 3).

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Table 2. BEQ_{bio} and BEQ_{chem} Values for Each Sample in the Different Bioassays with the Percentage of Effect That Can Be Explained by the Detected Chemicals

ID	activation of AhR			activation of PXR			activation of ER			oxidative stress response			p53 response			fish embryo toxicity		
	BEQ_{bio} (M)	BEQ_{chem} (M)	% effect	BEQ_{bio} (M)	BEQ_{chem} (M)	% effect	BEQ_{bio} (M)	BEQ_{chem} (M)	% effect	BEQ_{bio} (M)	BEQ_{chem} (M)	% effect	BEQ_{bio} (M)	BEQ_{chem} (M)	% effect	BEQ_{bio} (M)	BEQ_{chem} (M)	% effect
8	4.40×10^{-14}	1.94×10^{-15}	4.4%	3.43×10^{-9}	6.51×10^{-13}	0.02%	9.61×10^{-14}	1.79×10^{-14}	19%	3.11×10^{-8}	1.10×10^{-11}	0.04%	1.51×10^{-10}	4.00×10^{-14}	0.03%	6.74×10^{-8}	2.66×10^{-11}	0.04%
22	3.28×10^{-14}	<MDL	–	4.98×10^{-10}	3.65×10^{-13}	0.07%	3.14×10^{-13}	1.45×10^{-14}	4.6%	2.75×10^{-8}	1.37×10^{-12}	0.01%	2.03×10^{-10}	4.53×10^{-15}	0.002%	5.88×10^{-8}	6.48×10^{-12}	0.01%
27	2.65×10^{-14}	1.44×10^{-15}	5.4%	2.40×10^{-9}	4.94×10^{-13}	0.02%	5.67×10^{-13}	1.04×10^{-14}	1.8%	3.59×10^{-8}	1.77×10^{-11}	0.05%	2.46×10^{-10}	6.39×10^{-14}	0.03%	5.07×10^{-8}	3.57×10^{-11}	0.07%
29	5.37×10^{-14}	<MDL	–	2.13×10^{-9}	4.31×10^{-13}	0.02%	7.44×10^{-13}	3.96×10^{-14}	5.3%	3.40×10^{-8}	1.45×10^{-11}	0.04%	4.04×10^{-10}	7.74×10^{-14}	0.02%	4.96×10^{-8}	5.04×10^{-11}	0.1%
30	1.01×10^{-14}	<MDL	–	3.45×10^{-10}	3.42×10^{-13}	0.10%	1.03×10^{-12}	4.32×10^{-14}	4.2%	2.05×10^{-8}	2.39×10^{-11}	0.1%	2.59×10^{-10}	3.88×10^{-14}	0.01%	9.20×10^{-8}	2.32×10^{-11}	0.03%
32	3.50×10^{-14}	6.38×10^{-15}	18%	$<3.17 \times 10^{-11}$	4.74×10^{-13}	–	1.82×10^{-13}	1.11×10^{-13}	61%	5.93×10^{-8}	1.27×10^{-10}	0.2%	3.47×10^{-10}	2.54×10^{-13}	0.07%	2.11×10^{-7}	1.31×10^{-10}	0.06%
33	4.05×10^{-14}	1.34×10^{-15}	3.3%	7.72×10^{-10}	4.82×10^{-13}	0.06%	1.98×10^{-13}	6.00×10^{-14}	30%	3.62×10^{-8}	5.19×10^{-11}	0.1%	1.89×10^{-10}	1.31×10^{-13}	0.07%	7.43×10^{-8}	6.90×10^{-11}	0.09%
35	3.36×10^{-14}	8.55×10^{-15}	25%	4.61×10^{-9}	6.45×10^{-13}	0.01%	cytotox	3.07×10^{-14}	–	9.58×10^{-8}	1.31×10^{-11}	0.01%	4.04×10^{-10}	3.69×10^{-14}	0.01%	7.08×10^{-8}	2.47×10^{-11}	0.03%
36	2.94×10^{-14}	<MDL	–	5.24×10^{-10}	3.57×10^{-13}	0.07%	3.47×10^{-13}	2.37×10^{-14}	6.8%	3.60×10^{-8}	1.69×10^{-11}	0.05%	1.87×10^{-10}	4.75×10^{-14}	0.03%	3.54×10^{-8}	2.57×10^{-11}	0.07%
37	$<1.25 \times 10^{-14}$	<MDL	–	1.53×10^{-9}	3.19×10^{-13}	0.02%	2.25×10^{-13}	1.68×10^{-14}	7.4%	2.79×10^{-8}	2.28×10^{-12}	0.01%	5.58×10^{-10}	2.45×10^{-14}	0.004%	4.80×10^{-8}	1.37×10^{-11}	0.03%
39	2.02×10^{-14}	1.02×10^{-14}	50%	2.84×10^{-9}	6.90×10^{-13}	0.02%	1.15×10^{-13}	4.63×10^{-14}	40%	2.63×10^{-8}	2.03×10^{-11}	0.08%	2.63×10^{-10}	6.67×10^{-14}	0.03%	4.90×10^{-8}	3.99×10^{-11}	0.08%
41	cytotox	1.91×10^{-14}	–	1.91×10^{-9}	4.84×10^{-13}	0.03%	4.56×10^{-12}	1.43×10^{-13}	3.1%	1.58×10^{-7}	1.25×10^{-11}	0.01%	cytotox	1.18×10^{-13}	–	7.22×10^{-8}	1.48×10^{-11}	0.02%
44	1.85×10^{-14}	1.08×10^{-14}	59%	3.22×10^{-9}	6.86×10^{-13}	0.02%	4.47×10^{-13}	3.06×10^{-14}	6.8%	5.20×10^{-8}	2.30×10^{-11}	0.04%	7.02×10^{-10}	1.57×10^{-13}	0.02%	5.93×10^{-8}	3.58×10^{-11}	0.06%
53	2.59×10^{-14}	1.48×10^{-15}	5.7%	1.28×10^{-9}	4.75×10^{-13}	0.04%	2.73×10^{-13}	3.68×10^{-14}	14%	5.69×10^{-8}	3.67×10^{-12}	0.01%	$<1.01 \times 10^{-10}$	2.91×10^{-14}	–	4.98×10^{-8}	1.83×10^{-11}	0.04%
55	2.99×10^{-14}	1.48×10^{-15}	5.0%	6.08×10^{-10}	5.16×10^{-13}	0.08%	cytotox	<MDL	–	cytotox	2.82×10^{-12}	–	cytotox	1.36×10^{-13}	–	5.32×10^{-8}	1.85×10^{-11}	0.03%
57	1.71×10^{-14}	1.22×10^{-15}	7.1%	1.88×10^{-9}	3.99×10^{-13}	0.02%	cytotox	1.22×10^{-14}	–	4.07×10^{-8}	2.45×10^{-12}	0.01%	cytotox	1.06×10^{-13}	–	5.10×10^{-8}	1.58×10^{-11}	0.03%
59	2.96×10^{-14}	2.05×10^{-14}	69%	2.21×10^{-9}	6.94×10^{-13}	0.03%	cytotox	4.53×10^{-14}	–	5.85×10^{-8}	2.38×10^{-11}	0.04%	2.45×10^{-10}	1.63×10^{-13}	0.07%	4.94×10^{-8}	9.91×10^{-11}	0.20%
60	1.69×10^{-14}	1.20×10^{-14}	71%	$<3.17 \times 10^{-11}$	4.42×10^{-13}	–	3.77×10^{-13}	1.19×10^{-15}	0.31%	5.50×10^{-8}	2.89×10^{-12}	0.01%	2.23×10^{-10}	1.04×10^{-13}	0.05%	4.52×10^{-8}	7.85×10^{-12}	0.02%
63	cytotox	1.25×10^{-14}	–	2.41×10^{-9}	1.15×10^{-12}	0.05%	cytotox	4.14×10^{-14}	–	8.11×10^{-8}	8.74×10^{-12}	0.01%	cytotox	2.16×10^{-13}	–	1.35×10^{-7}	5.18×10^{-11}	0.04%
64	5.87×10^{-15}	<MDL	–	1.25×10^{-10}	7.21×10^{-14}	0.06%	1.69×10^{-14}	1.36×10^{-14}	80%	$<4.99 \times 10^{-9}$	1.99×10^{-12}	–	$<1.01 \times 10^{-10}$	1.88×10^{-14}	–	4.49×10^{-8}	1.80×10^{-11}	0.04%
65	3.33×10^{-14}	7.48×10^{-15}	22%	9.97×10^{-10}	6.28×10^{-13}	0.06%	6.15×10^{-13}	4.76×10^{-14}	7.7%	6.53×10^{-8}	4.20×10^{-12}	0.01%	3.69×10^{-10}	1.29×10^{-13}	0.03%	5.85×10^{-8}	1.78×10^{-11}	0.03%
67	1.14×10^{-14}	2.20×10^{-15}	19%	8.43×10^{-10}	5.74×10^{-13}	0.07%	cytotox	3.12×10^{-14}	–	cytotox	2.91×10^{-11}	–	4.52×10^{-10}	1.42×10^{-13}	0.03%	9.00×10^{-8}	2.49×10^{-11}	0.03%

MDL: method detection limit; cytotox: induction was masked by cytotoxicity.

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Table 3. Relative Effect Potencies (REP_i) for the Detected Chemicals in the Different Assays

assay:	activation of AhR		activation of PXR		activation of ER		oxidative stress response		p53 response		fish embryo toxicity	
no. of chemicals:	3*		13		6		13		4		12	
ref compd:	2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)		SR 12813†		17β-estradiol		*tert*-butylhydroquinone (tBHQ)		mitomycin		3,4-dichloroaniline	
	detected chemical	REP_i	detected chemical	REP_i	detected chemical	REP_i	detected chemical[k]	REP_i	detected chemical[k]	REP_i	detected chemical[l]	REP_i
	carbaryl[a]	3.33×10^{-6}	bezafibrate[d]	6.21×10^{-4}	benzophenone-3[g]	7.04×10^{-7}	2,4-dinitrophenol	0.06	2,4-dinitro-phenol	1.09×10^{-3}	2,4-dinitro-phenol	0.49
	daidzein[b]	5.92×10^{-4}	bisphenol A[d]	4.63×10^{-3}	bisphenol A[e]	4.49×10^{-5}	2-phenylphenol	0.08	carbendazim	7.35×10^{-3}	atrazine	0.04
	terbuthylazine[c]	1.36×10^{-4}	carbamazepine[d]	4.61×10^{-4}	bisphenol S[h]	1.18×10^{-6}	bisphenol A	0.07	diclofenac	1.02×10^{-3}	caffeine	3.41×10^{-3}
			diclofenac[d]	1.02×10^{-3}	daidzein[i]	6.47×10^{-5}	carbaryl	1.33	genistein	1.26×10^{-3}	carbamazepine	0.05
			diuron[e]	2.61×10^{-3}	estrone[j]	0.02	chlorophene	0.11			carbaryl	0.18
			erythromycin[e]	2.92×10^{-5}	genistein[j]	5.35×10^{-4}	daidzein	0.07			chlorotoluron	0.45
			estrone[e]	3.92×10^{-3}			diclofenac	0.06			diclofenac	0.13
			isoproturon[e]	1.67×10^{-3}			genistein	0.74			genistein	0.62
			ketoprofen[d]	3.59×10^{-4}			metolachlor	0.35			metoprolol	1.22×10^{-3}
			metolachlor[f]	0.10			perfluoroheptanoic acid	0.03			p-nitrophenol	0.22
			prometryn[d]	8.71×10^{-3}			tri(butoxyethyl) phosphate	0.14			salicylic acid	0.07
			terbuthylazine[d]	3.76×10^{-3}			triclosan	0.18			triclosan	10.53
			triclosan[d]	0.02			triethyl citrate	0.04				

*REP_i data from AhR CALUX using mouse hepatoma cell line †Tetraethyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate [a]Denison et al.[49] [b]Long et al.[50] [c]Ghisari et al.[51] [d]Creusot et al.[15] [e]Creusot.[52] [f]Lemaire et al.[43] [g]Molina−Molina et al.[53] [h]Molina−Molina et al.[54] [i]Current study. [j]Pillon et al.[55] [k]USEPA ToxCast database.[23] [l]Scholz et al.[27]

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LC_{50} values at 48 h exposure for the FET test were collected for 12 chemicals from Scholtz et al.,[27] with REP_i calculated using the mean 3,4-dichloroaniline LC_{50} value from the same study. A total of 13 EC values were collected from the literature for the PXR assay, while six EC values were available for the ER assay. Although no EC values were available for the detected chemicals in the AhR CAFLUX assay, EC values were available for three of the detected chemicals (terbuthylazine, carbaryl, and daidzein) in the mouse AhR CALUX assay. Although these assays focus on the same endpoint (reporter gene expression), they utilize different animal cell lines (rat hepatoma versus mouse hepatoma), and previous work has shown species-specific differences in responsiveness to some AhR ligands.[38] To account for differences in sensitivity between the mouse and rat AhR models, we also collected TCDD EC values from each study to calculate REP_i rather than using the TCDD EC value from the current study.

EC values for the oxidative stress response, p53 response, and NF-κB assays were collected from the ToxCast database.[23] A total of 486, 278, and 62 chemicals in the ToxCast database were active in the oxidative stress response, p53 response, and NF-κB assays, respectively (Figure 2). Of the 94 chemicals

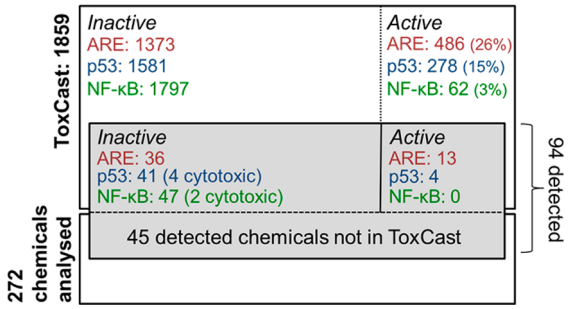

Figure 2. Overview of the active and inactive detected chemicals present in the ToxCast database in the oxidative stress response (ARE, red), p53 response (blue), and NF-κB response (green) assays.

detected in the JDS samples, 49 of these were also included in the ToxCast database. However, many of the detected compounds were not active in the assays, with 13 compounds active in the oxidative stress response assay, four compounds active in the p53 response assay, and none active in the NF-κB assay. $EC_{IR1.5}$ values for the detected chemicals in the oxidative stress response and p53 response assays were calculated from raw emission data available in the ToxCast MySQL database. To derive REP_i for the detected chemicals, we used experimental $EC_{IR1.5}$ values for reference compounds tBHQ and mitomycin.

Using REP_i and the detected chemical concentration, BEQ_{chem} was calculated using eq 5 for each water sample (Table 2). BEQ_{chem} could not be calculated for some samples for the AhR and ER bioassays because none of the chemicals with literature EC values were detected in the samples. Furthermore, it was not possible to derive BEQ_{chem} values for the NF-κB assay because none of the detected chemicals were active, despite the NF-κB assay being one of the more responsive for the JDS samples (Figure 1B). Only 3% of the 1859 chemicals in the ToxCast database were active in the NF-κB assay, compared to 26 and 15% of chemicals in oxidative stress response and p53 response assays, respectively (Figure 2). The NF-κB assay has been used for water quality monitoring in only one study,[4] and it is still unclear what types of water-based pollutants induce a response in this assay.

Percent of Biological Effect That Can Be Explained by Chemical Analysis. The comparison between BEQ_{bio} and BEQ_{chem} for each assay is shown in Table 2, although the contribution of the individual detected chemicals to the biological effect is shown in Figure 3. For some JDS samples, it was not possible to determine the contribution of detected chemicals to the biological effect, and this was attributed to either cytotoxicity masking the manifestation of other endpoints, no effect at the maximum REF, or the active chemicals being below the MDL.

The BEQ_{chem} for AhR activation was calculated using only three chemicals, but they explained between 3 and 71% of the biological effect (Figure 3A). The effect was mostly driven by the phytoestrogen daidzein, which has previously been shown to be a weak AhR activator in mouse cells but not in human cells,[39] and the herbicide terbuthylazine. The insecticide carbaryl only explained 0.5% of the effect in JDS 67. Similarly, the BEQ_{chem} for activation of ER could explain up to 80% of the effect, with the hormone estrone and the phytoestrogen genistein contributing significantly. Estrogenic effects in wastewater are often explained by the presence of potent natural and synthetic estrogenic hormones, such as 17β-estradiol and 17α-ethinylestradiol,[14,18] but these compounds were below the detection limit in the current study. Previous studies have also attributed ER activation in river water to genistein.[40]

In contrast, the detected chemicals could explain less than 0.2% of the biological effect in the adaptive stress response assays, PXR assay, and the FET test (Figure 3). A number of studies have also shown similarly low contributions of detected chemicals to the oxidative stress response in a range of water types, including wastewater and pool water.[10,17,41] Genistein dominated the contribution of quantified chemicals to the biological effect for the oxidative stress response. Although carbaryl and the disinfectant chlorophene were detected in the JDS samples and are active in the oxidative stress response assay, they occurred only in samples in which cytotoxicity masked induction and could not be used to explain the biological effect. It has also been demonstrated that detected chemicals in surface water and wastewater can only explain a small fraction of PXR activity.[15] However, for this receptor, the use of the concentration addition model may be limited because it has been recently demonstrated that, due to a large ligand-binding pocket, PXR can stably bind binary mixtures of certain weakly active chemicals, which leads to synergistic activation of target genes.[42] The herbicide metolachlor mostly contributed to the effect in the PXR assay. The BEQ_{chem} versus BEQ_{bio} comparison for water samples has not been conducted previously for the p53 response or FET assays; thus, it was not possible to compare our results with the literature. Genistein and the industrial compound 2,4-dinitrophenol mostly contributed to the p53 response in samples collected from Austria to Serbia (JDS 8 to 39), while the fungicide carbendazim dominated the effect further downstream. Finally, 2,4-dinitrophenol and genistein together contributed up to 0.08% of the effect explained by quantified chemicals in the FET assay, though antimicrobial triclosan alone could explain up to 0.15% of the biological effect in JDS 59 (Figure 3).

The small contribution of detected chemicals to the biological response in the adaptive stress response and PXR assays is not surprising because many compounds can activate these endpoints, as discussed earlier. Furthermore, 471 out of 641 or 74%, of reviewed compounds in Scholz et al.[27] had a response in the FET test. Consequently, many compounds are active in these assays, and, although comparability may have been

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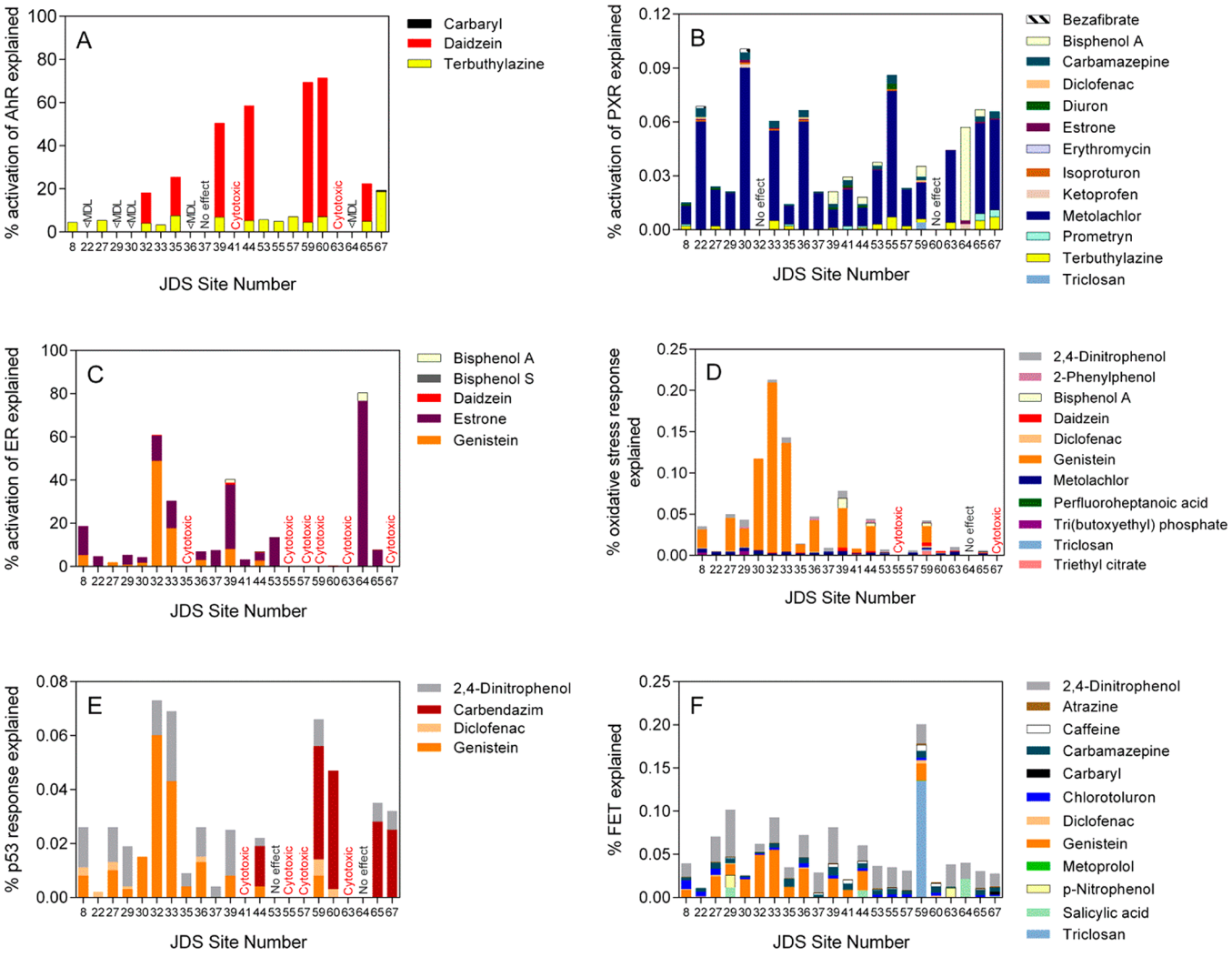

Figure 3. Percent of the biological effect explained by individual detected chemicals for (A) activation of AhR, (B) activation of PXR, (C) activation of ER, (D) oxidative stress response, (E) p53 response and (F) fish embryo toxicity (FET).

improved with more literature EC and LC values for the detected compounds, it is unlikely to have a significant influence on the comparison. In illustration of this point, if we assume that 74% of chemicals should have an effect in the FET test, then 70 of the 94 detected chemicals could be active in this assay. However, published LC values were only available for 12 of the detected chemicals. If we simply extrapolate the effect explained by the detected chemicals with available LC_{50} values in each sample to all 70 detected chemicals without considering differences in potencies, we can still only explain up to 1.6% of the effect. This example does not take into consideration any differences in mode of action or chemical potency but simply aims to illustrate the potential for many compounds to contribute to effect in apical endpoints.

Limitations and Outlook. There are some limitations associated with the current study. Primarily, improved understanding of the contribution of the detected chemicals to the biological effect is hampered by the lack of REP_i values for detected chemicals. Out of the 94 detected chemicals in the JDS samples, between 0 and 13 corresponding EC or LC values could be found for the different assays. Although the USEPA ToxCast program provides EC values for a large number of compounds, many of these are not typical water pollutants, and only 52% of chemical detected in the JDS samples were present in the ToxCast database. Many of the detected chemicals in the ToxCast database were not active in the adaptive stress response assays, but such information is not readily available for the other studied assays. However, this information is important as it makes a difference to the effect balance if a chemical's contribution is zero or if it is unknown. Consequently, fingerprinting the biological and toxicological effect(s) of commonly detected water pollutants is recommended to help fill in the knowledge gap. Furthermore, the available literature data stems from a number of different sources, and it is possible that the experimental protocols for the same assay may differ slightly, leading to potential differences in sensitivity or reproducibility. This limitation could be overcome by improved standardization of bioassays.

A specific limitation associated with the AhR assay is that the EC values available in the literature are based on the mouse AhR model, while BEQ_{bio} is based on the rat AhR model. Hence, potential differences in species sensitivity may be a source of variability for the comparison of BEQ_{bio} and BEQ_{chem}. A further limitation with using literature EC and LC values is that the error associated with the value is often not provided. This was the case for the AhR, PXR, ER, and FET assays, and consequently, it was not possible to calculate the error associated with the BEQ_{chem} values. It was possible to calculate the error associated with BEQ_{chem} for the oxidative stress response

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and p53 response assays because the $EC_{IR1.5}$ values used to derive REP_i were re-evaluated from a series of replicate experiments from the ToxCast MySQL database (standard deviations associated with BEQ_{chem} are provided in Table S7).

This study demonstrated the applicability of the BEQ concept to assess the contribution of detected chemicals to the biological effect of chemical mixtures present in the Danube River. Because the detected chemicals could not explain a significant proportion of the effect, particularly in the adaptive stress response, PXR, and FET assays, this supports the application of bioanalytical tools complementary to chemical analysis for water quality monitoring. Furthermore, because targeted chemical analysis was applied, we cannot exclude the fact that we may not have targeted the most relevant chemicals. Consequently, further identification using tools such as effect-directed analysis may provide improved understanding about chemical stressors in the Danube River.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b04083.

Figures showing the JDS sample sites and concentration-effect curves. Tables showing the Joint Danube Survey sampling sites and extracted water volumes, multiple-reaction monitoring transitions for LC-MS/MS, analyzed chemicals and their detection limit, EC and LC values for all samples in different bioassays, available literature EC and LC values, and BEQ_{chem} for oxidative stress and p53 response assays. Additional information about large-volume solid-phase extraction, LC-MS/MS analysis, and bioanalysis. (PDF)

Table showing detected chemicals and their method detection limit (MDL) in the different JDS samples. (XLSX)

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Notes

The authors declare no competing financial interest.

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