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Article

Comparative Evaluation of Toxicity End Points in Unicellular Bioassays for Environmental Pollutant Assessment

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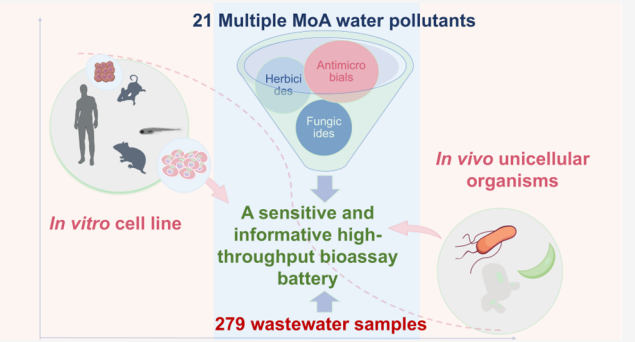
ABSTRACT: Aquatic environments contain mixtures of contaminants, of which many cannot be detected through conventional chemical monitoring. Effect-directed analysis (EDA) uses bioassays to detect bioactive compounds and relies on sensitive small volume assays. The aim of this study was to evaluate the sensitivity of three unicellular species (*Raphidocelis subcapitata*, *Escherichia coli*, and *Saccharomyces cerevisiae*) and five vertebrate cell lines to 21 chemicals with diverse modes of action. The algae assay exhibited the highest sensitivity, detecting toxicity in >80% of the chemicals, while yeast was the least responsive. Subsequently, 279 wastewater samples were screened using algae and three cell lines. Algae detected toxicity in >92% of the samples, while the cell line viability assays responded to 21−53% of the samples. Bioavailability modeling revealed that medium composition significantly influenced chemical partitioning. Algae assays that performed in protein- and lipid-free medium were more sensitive to lipophilic compounds than assays using heterotrophic cells. We hypothesize that medium-driven differences in bioavailability contribute to the higher sensitivity of algae, even to pharmaceuticals with no known algal targets. Combining algae and DR-EcoScreen cell assays captured 96.4% of detected toxicities in environmental samples. This combination offers a high-throughput, cost-effective strategy for screening environmental samples for bioactive substances.



KEYWORDS: *cellular bioassay, unicellular organisms, in vitro, sensitivity, bioavailability*

INTRODUCTION

Chemical pollutants in the environment pose significant challenges to ecological health and public safety. These pollutants include both naturally occurring and anthropogenically introduced substances. However, current environmental monitoring practices often fail to account for the full spectrum of toxicity, as only a fraction of observed effects can be explained by routinely monitored chemicals.[1−3] To address this gap, broader screening tools are necessary to identify unknown or unmonitored toxicants. Effect-Directed Analysis (EDA) is proposed as such a tool, combining fractionation techniques with target and nontarget screening for chemicals with bioassays to identify the key toxicants driving adverse effects on biological processes.[4,5]

Escher et al.[6] categorized bioassays by their ability to detect toxicity explained by known chemicals. Category 1 assays, which often target specific biological mechanisms such as hormone receptor binding, can typically explain a high fraction of monitored bioactivity, as few highly potent chemicals are often responsible for the effect. Category 2 assays, on the other hand, such as those measuring oxidative stress or cytotoxicity, typically measure the cumulative effects of a large number of weakly active substances, of which many are not identified and quantified by chemical analysis. Hence, the known chemicals therefore tend to explain a lower fraction of the measured bioactivity. Currently, category 1 bioassays are generally considered more suitable for EDA,[6,7] as they allow for a clearer linkage between observed effects and individual chemicals, whereas broad-spectrum bioassays tend to produce complex toxicity profiles that hinder the identification of specific drivers. A well-established example of such a targeted bioassay is the estrogen receptor (ER) assay, which has proven effective in identifying estrogenic compounds in environmental samples.[7,8] However, heavy reliance on mechanism-specific bioassays can also limit the EDA's scope, as it reduces the ability to detect toxicants that act through poorly understood or nonspecific mechanisms.

Some bioassays do not fall clearly into either category, as their classification may vary depending not only on the measured end point but also on the type of environmental sample.[6] For example, the AhR assay is considered category 1

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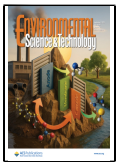

in sediment samples, where dioxin-like compounds dominate, but shifts to category 2 in water samples, which usually contain complex mixtures of weakly potent chemicals. Furthermore, receptor activity in complex samples may be masked by cytotoxic effects, meaning that the absence of a specific biological response does not necessarily imply the absence of the corresponding toxicant.

Recent advances in data-driven approaches, particularly the integration of large toxicological data sets with data characterizing chemical compositions, have significantly improved the feasibility of broad-spectrum bioassays by enabling more effective linkages between toxicity data and chemical occurrences.[9,10] In the light of these developments, there is a need for small-scale bioassay batteries capable of detecting chemicals with unknown modes of action to complement the specificity of receptor-based bioassays. This battery should be able to capture a wide range of toxicological modes of action (MoA) while maintaining high sensitivity to low concentrations. It should be small in scale, as concentrated sample volumes of e.g., wastewater are often limited; and rapid, to enable high-throughput screening.

Cells from vertebrates cultured in vitro are essential for detecting disturbance of biological pathways present only in vertebrates including mammals, which may not necessarily be present in single cell organisms.[4] These assays allow the for testing sensitivities across various species and tissue types, each with potentially distinct biochemical pathways and receptors that respond differently to chemical exposures.[11−14] For instance, human breast cancer cell lines are commonly used due to their high sensitivity to endocrine-disrupting chemicals, such as xenoestrogens.[15,16] Cytotoxicity, reflected by a reduction in cell viability, is typically used as a general measure of cellular dysfunction, which reflects a broad range of toxicological effects.[17] Cell viability can be measured on various different parameters, but Lundqvist et al.[18] highlighted that 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based cytotoxicity assays are particularly sensitive for detecting cellular dysfunction. The MTS assay measures mitochondrial metabolic activity as a proxy for the number of viable cells after 24 h of exposure and is therefore influenced by both specific toxic effects—such as interference with DNA replication or cell division—and more general stress responses, including oxidative stress, altered enzyme activity, or the disruption of energy metabolism. As such, the assay reflects a combination of specific and baseline toxicity.

Unicellular organisms, such as algae, bacteria, and fungi, are well-suited for toxicological assays due to their small size, which enables efficient testing with minimal sample volumes. These organisms represent diverse and distinct cell types, having diverged early in evolutionary history.[19,20] Their evolutionary separation makes them valuable in environmental risk assessments, as they offer insight into how different types of cells representing different ecologically distinct organism groups respond to environmental contaminants. Moreover, since unicellular organisms are often both the first step of food-webs and are vital for decomposition of organic matter, their responses to chemical substances have a high ecological relevance.[21,22]

Beyond the choice of test organism, understanding the mechanisms underlying bioassay sensitivity—such as chemical uptake and cellular processing—is essential for interpreting test results. Bioassay sensitivity is influenced by several factors including bioavailability, toxicokinetic and toxicodynamic processes.[23,24] Hence, the bioavailability of a chemical in the experimental setup determines the chemical fraction available for cellular uptake, thereby affecting the exposure level.[25] Then the ease by which the chemical crosses the cell membrane determines how readily it can enter the cells, while the cells' ability to biotransform the chemical—either by detoxifying it or converting it into more reactive forms—affects the magnitude of reactive chemical inside the cell. Finally, the presence of biological targets within the cells that are susceptible to the chemical determines whether a specific toxic effect will be triggered.[26]

The objective of this study was to construct an optimized bioassay battery for the preliminary screening of environmental samples, aimed at capturing the widest possible range of toxicological effects at minimal cost. This required identifying the most sensitive bioassays across a diverse set of chemicals and environmental samples, followed by optimizing the design of the bioassay battery based on the relationships among selected assays. To achieve this, growth rates in three unicellular organisms—*Raphidocelis subcapitata* (algae), *Escherichia coli* (bacteria), and *Saccharomyces cerevisiae* (fungi)—and cell viability in five cell-based assays using vertebrate cells from fish, mouse, hamster, and two human-derived cell lines were evaluated as toxicological end points.[27] These bioassays were first tested against 21 chemicals with distinct modes of action, commonly found in wastewater and of which some was previously assessed by Altenburger et al.[28] in other assays. Following the initial screening, a subset of bioassays was selected based on sensitivity, reproducibility, and ability to capture a broad spectrum of toxicological responses. This subset was exposed to 279 Wastewater Treatment Plant (WWTP) samples from Denmark, primarily effluents, to assess their sensitivity and performance when exposed to real environmental samples. The relationships among the selected bioassays were then analyzed to identify complementary assays, reduce redundancy, and enhance the overall efficiency of the bioassay battery.

We propose the following hypotheses: 1) Different bioassays will show variable sensitivity to specific chemicals depending on the primary MoA and the biological targets present in each assay. For example, algae are expected to be most sensitive to chemicals that disrupt photosynthesis, yeast will be most sensitive to fungicides, *E. coli* will be most sensitive to antibiotics, and vertebrate cell assays will be most sensitive to pharmaceuticals aimed at affecting biosynthetic pathways in mammalian organisms. 2) The composition of the media and the surface-to-volume ratio of the test wells could influence the bioavailability of the chemicals to the cells, affecting how much of the added substance is accessible for cellular interaction and thereby assay sensitivity. 3) Cells in in vitro assays, removed from the protective surfaces of their host organisms (e.g., skin or gut barriers), are expected to absorb bioavailable chemicals more readily through their membranes compared to unicellular organisms, which have evolved to survive with direct exposure to chemicals in the external medium. The unicellular organisms may therefore have protective cell walls that vary by species and may influence their uptake.[29−31] 4) Cells that can efficiently biotransform and excrete chemicals will have lower internal concentrations and thus exhibit lower sensitivity to their toxic effects.

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Environ. Sci. Technol. 2025, 59, 18042−18055

Table 1. Overview of the Chemicals Included in This Study, Arranged Alphabetically by Chemical Name, Highlighting Their Category, Use, and Biochemical Mode of Action (MoA), along with Key Physicochemical Properties[ab]

Chemical	Category	Use	Biochemical MoA	Log $D_{lip/w}$	Log $D_{BSA/w}$	Solubility (mgL^{-1})	Max Conc. (mgL^{-1})
1,3-diphenylguanidine	Rubber accelerator (guanidine)	Vulcanization in rubber industry	Modulation of enzyme activity[38]	2.46	2.42	1.0 at 25 °C	9
Amitriptyline	Pharmaceutical (tricyclic)	Treatment of depression and anxiety disorders	Blocking of monoamine reuptake; potential membrane disruption[39]	3.60	3.60	9.71 at 24 °C	9
Bisphenol A	Industrial chemical (phenol)	Manufacture of plastics and resins	Endocrine disruption[40]	4.19	4.19	300 at 25 °C	35.46
Ciprofloxacin	Pharmaceutical (fluoroquinolone)	Treatment of bacterial UTIs and GI infections	Inhibition of DNA gyrase and topoisomerase IV[41]	1.64	1.26	30,000 at 20 °C	24.75
Clarithromycin	Pharmaceutical (macrolide antibiotic)	Treatment of respiratory and skin infections	Inhibition of bacterial protein synthesis[42]	1.00	3.00	1.69 at 25 °C	7.5*
$Cu(H_20)_2Cl_2$	Metal catalyst and antimicrobial agent	Used in paints and antifoulants	Disruption of microbial metabolism[43]	-	-	757000 at 25 °C	238
Cyprodinil	Fungicide (pyrimidine derivative)	Crop protection	Inhibition of fungal protein biosynthesis[44]	3.73	2.98	13 at 25 °C	26.82
Diazinon	Insecticide (organophosphorus)	Pest control (neuroactive)	Inhibition of acetylcholinesterase[45]	3.25	2.85	40 at 25 °C	25.38
Diclofenac sodium salt	Pharmaceutical (NSAID)	Treatment of pain and inflammation	Inhibition of cyclooxygenase (COX-1/COX-2)[46]	2.64	4.20	5000 at 25 °C	70.2
Diuron	Herbicide (phenylurea)	Weed control in crops and noncrop areas and in paints and antifoulants	Inhibition of photosystem II[47]	3.80	3.80	42 at 25 °C	27.36
Erythromycin	Pharmaceutical (macrolide antibiotic)	Treatment of respiratory/soft tissue infections	Inhibition of bacterial protein synthesis[42]	−1.24	−0.93	4.2 at 25 °C	4.5*
Isproturon	Herbicide (phenylurea)	Selective weed control in cereals and in paints and antifoulants	Inhibition of PSII (photosynthesis)[47]	3.03	3.03	70 at 20 °C	63
Methyl methanesulfonate	Chemical reagent (DNA-alkylating agent)	DNA-damaging agent in research	Alkylation of DNA, causing strand breaks[48]	−0.66	−0.57	150 at 25 °C	116.46
Propiconazole	Fungicide (triazole)	Prevention of fungal diseases in crops and in paints and antifoulants	Inhibition of ergosterol biosynthesis[49]	2.23	2.23	110 at 25 °C	126*
Sulfamethoxazole	Pharmaceutical (sulfonamide)	Treatment of urinary/respiratory infections	Inhibition of dihydropteroate synthase[50]	1.03	1.07	610 at 37 °C	225
Terbuconazole	Fungicide (triazole)	Protection of crops and turf against fungi and in paints and antifoulants	Inhibition of ergosterol biosynthesis[49]	4.13	4.13	36 at 20 °C	234*
Terbutryn	Herbicide (triazine)	Controlling weeds in crops and water bodies and in paints and antifoulants	Inhibition of PSII (photosynthesis)[47]	2.90	2.90	25 at 20 °C	45*
Terbutylazine	Herbicide (triazine)	Weed control in crops	Inhibition of PSII (photosynthesis)[47]	3.25	3.25	5.0 at 20 °C	0.06
Triclosan	Biocide (phenolic)	Antimicrobial in personal care products	Disruption of the electron transport chain in mitochondria[51]	3.28	3.28	10 at 20 °C	41.4*
Triphenyl phosphate	Flame retardant and plasticizer	Added to polymers to reduce flammability	Inhibits acetylcholinesterase[52]	3.73	3.73	1.9 at 25 °C	27.36
$ZnCl_2$	Metal salt	Galvanization and chemical reagent and in paints and antifoulants	Denaturation of proteins and disruption of metabolism[53]	/	/	Very soluble in water	239

[a]These include the partition coefficients between lipid and water (log Dlip/w), the partition coefficient between protein and water (log DBSA/W) at pH 7.4 (obtained from the UFZ-LSER tool database), the water solubility (in mg L^{-1} from PubChem), and the highest concentration tested in the bioassays (Max Conc, mg L^{-1}). [b]Asterisks (*) indicate nominal concentrations potentially exceeding reported water solubility under the given test conditions (see Materials and Methods).

MATERIALS AND METHODS

Chemical Selection and Exposure. We selected chemicals to encompass a broad range of MoA commonly observed in aquatic environments (Table 1). The selection enable a comparative evaluation of how each bioassay might respond differently to chemicals with defined MoA, in line with hypothesis 1. Our selection strategy included reference chemicals from Altenburger et al.:[28] diazinon, diclofenac, bisphenol A, propiconazole, triphenyl phosphate, diuron, triclosan, and cyprodinil. Additionally, we incorporated chemicals based on frequency of environmental occurrence in Danish WWTPs[32−35] and having well-defined MoA in their target organisms. Clarithromycin, ciprofloxacin, erythromycin, sulfamethoxazole, amitriptyline, and methylmethanesulfonate were included to assess antimicrobial activity in bacterial assays; terbutryn and isoproturon represent herbicide; 1,3-diphenylguanidine is a tire manufacturing chemical, and Cu^{2+} and Zn^{2+} are metal ions frequently utilized in paints and antifoulants. Triclosan, recognized as both an antimicrobial and a mitochondrial electron transport disruptor was included for its broad toxicological profile.[36]

To facilitate comparisons, each chemical in Table 1 is categorized by catagory and use and primary biochemical MoA. Given that many compounds do not fit neatly into a single category, this classification serves as a general guideline. All chemicals were purchased through Sigma-Aldrich, and stock solutions were made in methanol (≥99.9%, HPLC grade, Sigma-Aldrich). The final methanol concentration in the test media was kept at or below 0.1% (v/v) for algae and cell-based assays and below 0.5% (v/v) for yeast and *E. coli* assays. For the algae assays, where the medium contains only mineral

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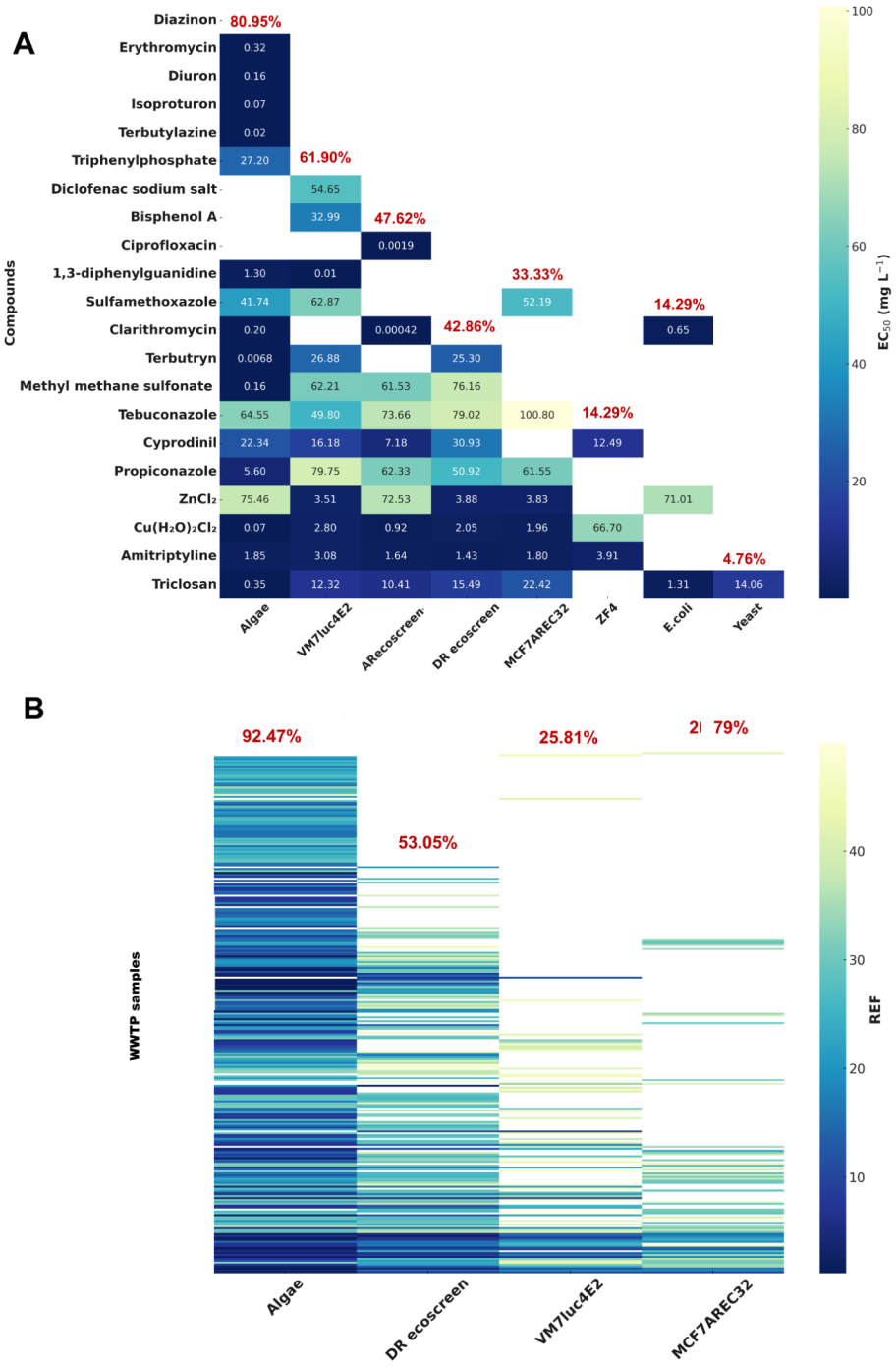

Figure 1. Heat map of toxicity given as EC_{50} for the specific chemicals across the eight bioassays (A) and unknown chemical mixtures of wastewater samples (B) across four bioassays. The *y*-axis represents chemicals or wastewater samples ordered with the most frequently detected chemicals/samples at the bottom of the axis, while bioassay names are given at the *x*-axis. EC_{50} values for the chemicals and samples are given and depicted by color intensity in the heat map, with the color scale expressed in units of mg L^{-1} for tested chemicals and REF for wastewater. White areas indicate no observed toxicity at the highest concentration tested. The red percentage represents the frequency of toxicity observed in the 21 chemicals and 279 WWTP sample tested. We established a threshold of REF 50 for an EC_{50} value, above which a sample is considered nontoxic. The data are based on fitted concentration−response curves (eq 1).

nutrients (Supporting Information, sheet "Bioassays info."). The highest test concentration was selected based on reported water solubility of the chemical (Supporting Information, sheet "Chemical info."). In contrast, the media used for yeast, *E. coli*, and cell-based tests typically have higher organic content, which can significantly increase the actual solubility of certain chemicals compared to pure water (Supporting Information, sheet "Bioassays info."). Consequently, some nominal concentrations exceeded literature water-solubility values (marked with an asterisk in Table 1). However, if no precipitation was observed under test conditions, we proceeded with these concentrations to ensure a sufficiently broad coverage of potential toxic effects.

We acknowledge that a more quantitative estimation of solubility in test media could be achieved using partitioning-based approaches, such as that described by Fischer et al.

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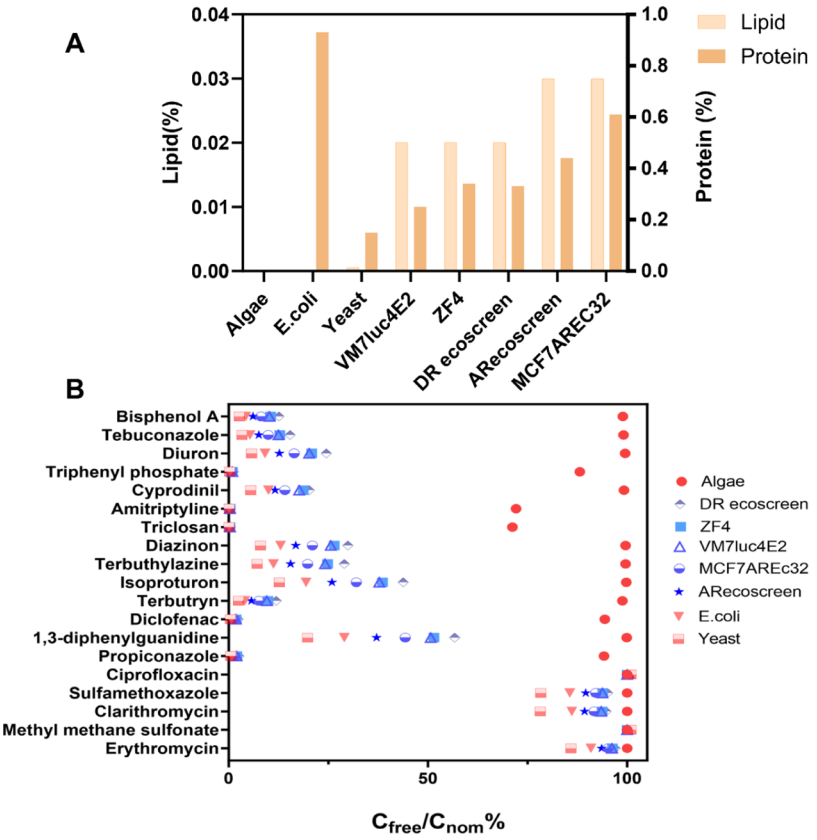

Figure 2. Comparative analysis of media composition and the resulting chemical bioavailability. Panel A presents the composition of protein (light yellow) and lipid (dark yellow) in the media used for the eight bioassays. Panel B shows the ratios of free concentration to nominal concentration (C_{free}/C_{nom}) for the test chemicals across the eight bioassays, effectively representing bioavailability in each system. The chemicals on the Y axis have increasing lipophilicity (log Dlip/$w_{pH7.4}$ obtained from the UFZ-LSER tool database) from bottom to top. The panel uses a gradient of red hues for unicellular organisms tested in vivo and gradient of blue for different cell lines tested in vitro, allowing for an intuitive comparison of bioavailability across systems.

(2019).[37] However, application of this method requires detailed compositional data (e.g., protein and lipid content) for each test medium, which was not fully available in our study. Specifically, the protein and lipid content of the *E. coli* and yeast media was calculated based on general information provided by the manufacturers (Supporting Information, sheet "Bioassays info."), rather than detailed knowledge of chemical composition. For consistency across assays, we therefore chose to rely on reported water solubility values and visual assessment of precipitation under test conditions.

Wastewater Sample Collection and Preparation. Wastewater samples, focusing on effluents, were collected from two WWTPs—Vand Center Syd and BIOFOS—between April 2021 and February 2024. The total number of samples was 279, composed of 254 effluent samples plus 25 influent samples. The detailed sampling and sample preparation protocols were described in Tisler et al.[32] and Kilpinen et al.[35] Briefly, 1.5 L of each sample was adjusted to pH 6.5 using LC-MS grade formic acid and ammonium hydroxide, then vacuum-filtered through Whatman GF/F (0.7 μm) and GF/A (1.6 μm) glass microfiber filters. The filtered samples were stored at 4 °C until solid-phase extraction (SPE).

SPE was performed using multilayer cartridges, each composed of 200 mg Superclean ENVI-Carb, 275 mg Oasis HLB (Waters, Taastrup, Denmark), and 275 mg Isolute ENV$^+$ (Biotage, Uppsala, Sweden). Before packing, the adsorbent materials were cleansed twice with methanol (1:10 material-to-MeOH ratio) using ultrasonication. The cartridges were then assembled, loaded with filtered samples, and eluted with 12 mL methanol. The eluate was evaporated to approximately 200 μL, and the volume was adjusted to 3 mL, giving an enrichment factor of 500. Extracts were stored at −18 °C until further analysis.

In Vivo Toxicity. *Algae Growth Inhibition Test.* The algae growth inhibition assay was conducted using *Raphidocelis subcapitata* (Akershus, Norway, 1959, NIVA-CHL-1) according to OECD guideline 201[54] with slight modifications.[4,55] The composition of algae media is shown in the sheet "Bioassays info" of Supporting Information. The algae were cultured in 20 mL transparent glass vials at 20 °C, under a light intensity ranging between 80 and 100 μmol m^{-2} s^{-1} in a climate chamber. To maintain the cells in suspension and ensure sufficient CO_2 exchange, the vials were placed on an orbital shaker at approximately 200 rounds minute^{-1}.

Toxicity tests were performed in 96-well plates, which were first immersed in LC-MS grade methanol and ultrasonicated twice (5 min each). Subsequently, the plates were immersed in LC-MS grade water under 80−100 μmol m^{-2} s^{-1} light for 1 week.

To minimize potential solvent effects, the highest concentration of each chemical (or wastewater extract) was added to the wells and air-dried for 12 h and then resuspended in 400 μL algae medium. Seven 1:2 serial dilutions were prepared by sequentially mixing and transferring 200 μL of medium from

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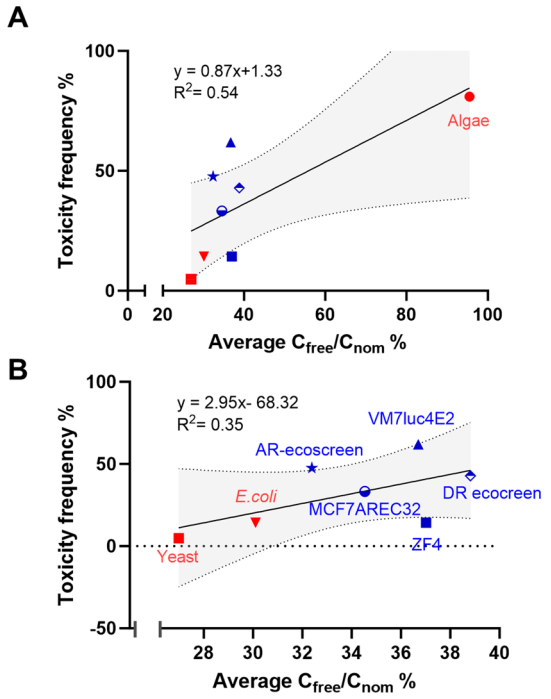

Figure 3. Relationship between toxicity frequency and average C_{free}/C_{nom}, considering all eight bioassays (A) and excluding algae (B). Unicellular organisms and cell lines are represented by red and blue, respectively. The shaded area illustrates the 95% confidence interval of the regression line.

each well. Each concentration was tested in five intraplate replicates, while negative and solvent controls were tested in four and ten replicates, respectively. A positive control (terbutryn) was included once per week to verify assay performance over time. The assay was initiated by adding 150 μL of algae culture ($1-3 \times 10^4$ cells mL^{-1}, $Abs_{648\ nm}$ = 0.7−1) per well. Absorbance at 684 nm was measured at the start and up to five times until 48 h using a SpectraMax i3 reader (Molecular Devices). The linear relationship between the logarithm of absorbance and time was verified on controls to ensure the algae grew exponentially, and that the relative growth rate exceeded 0.5 d^{-1}. If these conditions were not met, the test would be repeated. pH was measured at the beginning and end in both control wells and the highest concentration wells. The algae were tested at a maximal relative enrichment factor (REF) of 30 for the WWTP samples.

Yeast and E. coli Growth Inhibition Test. The growth inhibition assay for yeast (*Saccharomyces cerevisiae*, strain BY4741) and *Escherichia coli* (strain OP50) were performed using similar methodologies. Each microorganism was initially streaked onto nutrient-rich agar plates (yeast extract peptone dextrose (YPD), Sigma-Aldrich, Product No. Y1375) for yeast and Luria−Bertani (LB) broth (Sigma-Aldrich, Product No. L3022) for *E. coli* (sheet "Bioassays info." of Supporting Information for media details). Yeast cultures were incubated at 30 °C for 48 h, while *E. coli* was incubated at 37 °C until visible colonies appeared.

Single colonies were transferred to 200 mL of autoclaved YPD or LB broth in Erlenmeyer flasks. These cultures were grown at 30 °C for yeast and 37 °C for *E. coli*, with 150 rpm orbital shaking for 24 h. One day prior to the experiment, 6 μL of yeast culture and 10 μL of *E. coli* culture were each inoculated into fresh media (6 mL YPD or 10 mL LB broth) and incubated an additional 24 h to promote robust growth.

At the onset of the test, yeast and *E. coli* cells were centrifuged and resuspended in fresh media. Chemicals and solvent controls were prepared at the required concentrations in the respective media in 4 mL HPLC-vials, and 180 μL was pipetted into each well of a 96-well microtiter plate. The test was initiated by adding 20 μL of microbial suspension, adjusting the final cell density to between 7×10^6 and 10^7 cells mL^{-1}. Each concentration had five intraplate replicates; negative controls and solvent controls had four and ten intraplate replicates, respectively. There was no consistent positive control included, as no standardized or widely accepted positive control currently exists for growth inhibition in these systems. Absorbance at 600 nm was recorded using a SpectraMax i3 microplate reader at regular intervals: every 15 min for yeast and every 20 min for *E. coli*, over a total duration of 8 h for yeast and 12 h for *E. coli*, respectively. pH was measured in control wells and at the highest tested concentration at the start and end of the test. The logarithm of absorbance over time was plotted to determine the growth rate during the exponential phase in the controls, and growth rates of all treatments were determined within that time interval.

In Vitro Toxicity. *Cell Mediums and Maintenance Procedure.* Embryonic Zebrafish Fibroblast Cell Line (ZF4, CVCL_3275) were cultured in Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM-F12) with phenol red, 10% fetal bovine serum (FBS), 1% penicillin−streptomycin (pen−strep), 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, and 1200 mg L^{-1} sodium bicarbonate, at 28 °C, 5% CO_2, subcultured weekly (1:10) with 0.25% trypsin in Phosphate-Buffered Saline (PBS). The Chinese Hamster Ovary cell line AR-EcoScreen GR-KO M1(AR-EcoScreen) was obtained from the Japanese Collection of Research Bioresources (JCRB), maintained in phenol-red-free DMEM-F12 with 10% dextran charcoal-FBS, 1% pen−strep, 1% L-glutamine, 50 μg mL^{-1} Zeocin, and 25 μg mL^{-1} Hygromycin B Gold (InvivoGen, USA). The mouse hepatocellular carcinoma cell line (DR-EcoScreen, JCRB1630) was grown in medium consisting of α-Minimum Essential Media (α-MEM), 5% FBS, 2% pen−strep, 1% L-glutamine and 150 μg mL^{-1} Hygromycin B Gold. The experimental medium consisted of α-MEM, 5% FBS, and 1% pen−strep. Human breast carcinoma cell line (MCF7AREc32) donated by Prof. R. Wolf (University of Dundee) was propagated in DMEM GlutaMAX (4.5 g L^{-1} D-glucose) with 10% FBS and 1% pen−strep, excluding Geneticin during the test phase. Finally, the human breast carcinoma cell line (VM7Luc4E2) provided by Prof. M. Denison (University of California), was cultured initially in RPMI 1640 with L-glutamine, 8% FBS, 0.9% pen−strep, and 0.55 mg mL^{-1} Gentamicin. Prior to toxicity testing, the medium was switched to DMEM (4.5 g L^{-1} glucose) with 4.5% dextran−charcoal FBS for 48−72 h.

General Cell Maintenance. Except for ZF4 (28 °C), all lines were kept at 37 °C with 5% CO_2. Media were refreshed 2−3 times per week. Adherent cells were subcultured using 0.05% trypsin−EDTA (Gibco). All reagents and media, unless specified, were from Gibco, Thermo Fisher Scientific.

Cell MTS Viability Testing. Cells were seeded into transparent 384-well plates (Costar, Corning) at line-specific densities (e.g., app. 5200 cells well^{-1} for MCF7 AREc32 and 4000 cells well^{-1} for AR-EcoScreen, GR-KO M1, and DR-

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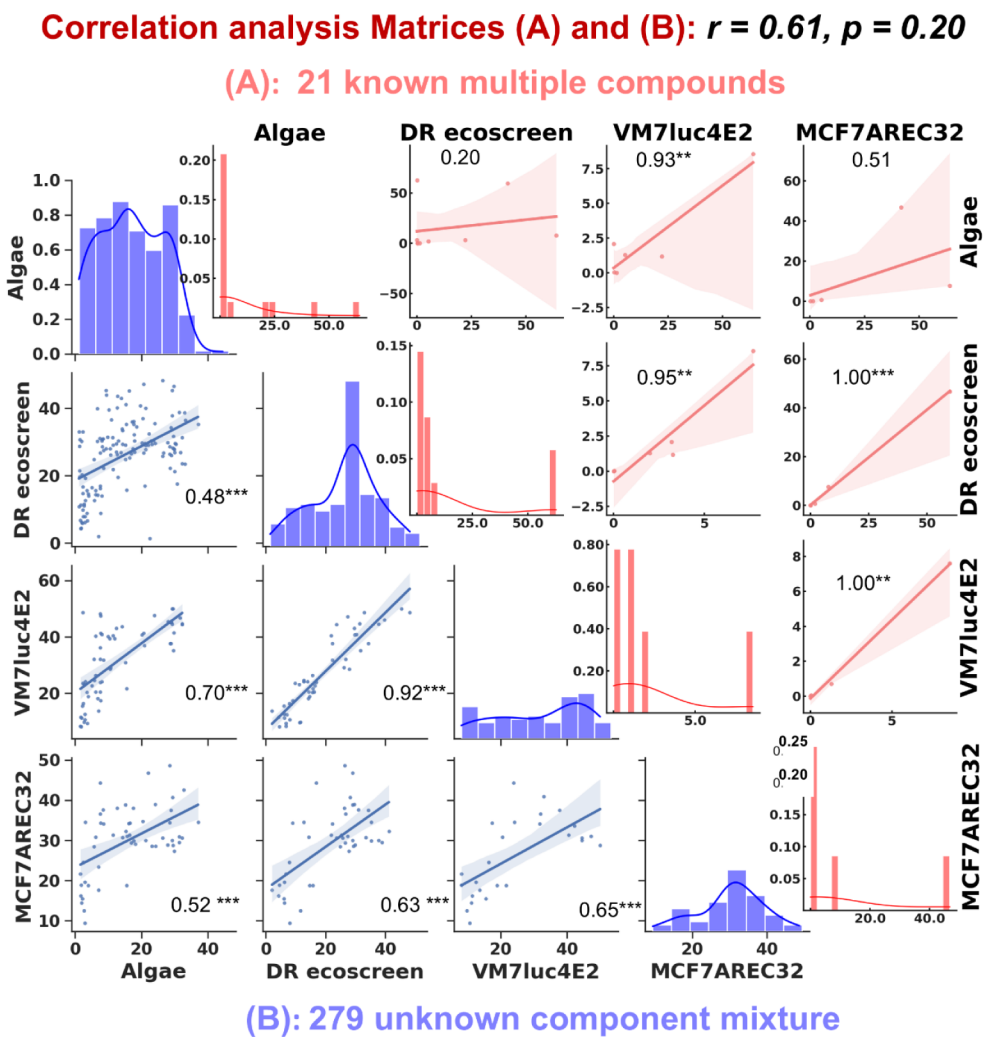

Figure 4. Correlation analysis of four bioassays for 279 wastewater samples and 21 individual chemicals. The lower triangle (A) shows the wastewater sample results, with histograms on the diagonal displaying frequency distributions and kernel density estimation trend lines. Scatter plots in each cell illustrate pairwise relationships, featuring actual data points and regression lines with confidence intervals. Red numbers represent Pearson correlation coefficients (−1 to 1), with values near 1 indicating a strong positive correlation and values near −1 indicating a strong negative correlation. Asterisks denote significance levels, with more asterisks indicating higher significance. The upper triangle (B) depicts the individual chemical tests, with each cell containing a Pearson correlation coefficient.

EcoScreen, 16000 cells well^{-1} for VM7luc4E2 and 2500 cells well^{-1} for ZF4). After 24 h of incubation, test chemicals, solvent controls (0.1% MeOH, matching highest chemical concentration), and a positive control (10% DMSO) were added. Each test chemical and the positive control were tested in four intraplate replicates, while the solvent control was included in eight intraplate replicates. Following another 24 h (or 48 h for ZF4) of exposure an MTS-based assay (CellTiter 96 AQueous One Solution, Promega) was performed according to the manufacturer's instructions. Ten μL of MTS reagent (~17% v/v of the well volume) was added, and plates were incubated for 30−60 min at 37 °C, 5% CO_2, until color development was sufficient. Absorbance at 490 nm was recorded with a Spark Multimode Microplate Reader (Tecan, Austria). Relative cell density was calculated by normalizing each well's absorbance to the mean of the solvent control wells. The WWTP samples were tested at a maximum relative enrichment factor of 25.

A comparison of the different bioassay methods and cell characteristics (cell size, cell type (eukaryotic or prokaryotic), and outer membrane composition) is presented in sheet "Bioassays info." of Supporting Information.

Data Analysis. The relative growth rates during the exponential phase for the algae, yeast, and *E. coli* growth inhibition assays were calculated in Excel. Differences among treatments were then tested by an analysis of variance (ANOVA), followed by Fisher's Least Significant Difference test for multiple pairwise comparisons using the *agricolae* package in *R* (version 1.3.1093). A significance level of $p < 0.05$ was applied. If there was no difference between treatments, the sample was denoted nontoxic. Media and solvent controls were pooled for further analysis after confirming no significant difference using ANOVA. For samples with significant effects with increasing concentration, the relative growth rates as a function of chemical concentration were fitted with a three-parameter log−logistic function:

$$y = \frac{d}{1 + \left(\frac{x}{e}\right)^b} \quad (1)$$

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Environ. Sci. Technol. 2025, 59, 18042−18055

where y represents the growth rate, d is the upper limit defined by the growth rates of the untreated controls, x is the concentration of the chemical given in mg L^{-1} or in REF values for the WWTP samples, while e is the 50% effect concentration (EC_{50}), and b is proportional to the slope around e.

The cell viability data were fitted by a two-parameter model fixing the upper limit d to 1. The curves were fitted using the *drm* package in *R*. Data visualization and correlation analysis between biological tests were achieved using *R* and *GraphPad* (version 9.3.1, GraphPad Software, Boston, Massachusetts, USA).

Modeling Free Concentration. To compare bioavailable concentrations across the various chemicals and tests, we applied the mass balance models described by Fischer et al.[56] The bioavailable concentration (C_{free}) was derived from the nominal concentration (C_{nom}) by accounting for partitioning into lipids and proteins. Thus, C_{free} is the fraction truly available to interact with biological targets, whereas C_{nom} refers to the initial, unadjusted concentration in the test system. The ratio of C_{free}/C_{nom} is the fraction of the nominal concentration that remains unbound in the system.

To capture chemical distribution across different biological matrices, we used two partition coefficients. Both coefficients were obtained from the UFZ-LSER tool,[57] which relies on linear solvation energy relationships (LSERs) and considers multiple physicochemical properties (e.g., lipophilicity, hydrogen bonding, and electronic characteristics) to predict distribution behavior. According to the UFZ-LSER tool's documentation, log $D_{lip/W;pH\ 7.4}$ represents how a chemical partitions between a standardized "lipid-like" phase and water at pH 7.4, intended to approximate a biological membrane or similar lipophilic environment.[56] We also used log $D_{BSA/W}$ to evaluate binding to bovine serum albumin (BSA), thus capturing partitioning into serum proteins at pH 7.4.

The UFZ-LSER tool predicts log $D_{lip/W}$ and log $D_{BSA/W}$ at pH 7.4 because pH 7.4 is close to the pH value in human tissues and aligns with the buffered conditions of the nutrient medium of the cell assays. Although the initial pH in the cultures of algae (n = 12), yeast (n = 21), and *E. coli* (n = 21) were approximately 8.10 ± 0.19, 7.60 ± 0.15, and 6.96 ± 0.18, respectively, we used pH 7.4 for the initial calculations to maintain consistency across assays. Over the course of the experiments, metabolic activities such as photosynthesis and respiration resulted in final pH values of approximately 9.19 ± 0.18, 6.90 ± 0.17, and 6.82 ± 0.19, respectively, which may have influenced the log D dissociable chemicals. Nonetheless, these pH variations were not incorporated into the current model.

We calculated C_{free}/C_{nom} for each chemical, as this ratio remains constant regardless of the nominal concentration. The distribution coefficients of the chemicals, the compositional details of the biological test media, cell densities, and nominal concentrations were used as input parameters to derive the resulting values. In our calculations, a uniform default cell model was applied, and only the cell numbers were adjusted, as detailed information on the lipid and protein composition of the cells was not available. The Excel file used for these calculations is provided by Fischer et al. (sheets "Calculation templates 1 and 2" of Supporting Information), and the detailed parameter settings of the input file are described in sheet "Bioavailability modeling" of Supporting Information.

Results and Discussion. *Sensitivities to Selected Chemicals.* The test of 21 chemicals with different MoA uncovered significant variability in responses across the eight bioassays (Figure 1A, sheet "$EC_{50;nom}$" of Supporting Information). The algae test was the most sensitive, with 17 chemicals producing concentration−response curves, resulting in a toxicity frequency of 80.95%. In contrast, the fungal representative, yeast, was the least sensitive assay being sensitive to the biocide triclosan only (EC_{50} = 14.06 ± 3.43 mg L^{-1}). The in vitro assays using human (VM7luc4E2), hamster (AR-EcoScreen), mouse (DR-EcoScreen), and another human cell line (MCF7AREC32), had an intermediate sensitivity, with toxic effects observed in 61.90%, 47.62%, 42.86%, and 33.33%, respectively. In contrast, *E. coli* and the zebrafish cell line (ZF4) demonstrated minimal responses, each detecting toxicity in only three chemicals (toxicity frequency = 14.29%). Based on detection frequency and EC_{50} values, and within the scope of the tested compounds and experimental conditions, the sensitivity hierarchy was algae > human cells > rodent cells > fish cells > *E. coli* > yeast. Algae showed the lowest EC_{50} values for 11 of the 20 chemicals toxic in at least one assay, often surpassing other assays by 1−5 orders of magnitude. Interestingly, several chemicals, including ciprofloxacin, diclofenac, and bisphenol A, were nontoxic in algae but exhibited significant toxicity in mammalian cell assays, reflecting complementary sensitivity across different bioassays.

Chemical Mechanisms Driving Bioassay Sensitivity. The significant variability in toxicity profiles across bioassays reflects, in part, the presence of chemicals with diverse MoA among the selected compounds. For instance, diazinon did not induce any response, which is consistent with its MoA as an acetylcholinesterase inhibitor—an enzyme absent in the unicellular organisms or cell lines used in our assays.[58] This illustrates a key limitation of the current test battery in capturing neurotoxic effects, which are of high concern in environmental toxicology. While we conducted preliminary tests with *Daphnia magna*, a model organism known for its sensitivity to neurotoxicants, the assay has not yet been optimized for high-throughput application and was therefore excluded from this study. Nonetheless, incorporating such assays in future work will be important to improve the coverage of neurotoxicity-related mechanisms.

Herbicides such as diuron, isoproturon, and terbutylazine exhibited specific toxicity in algae, aligning with our expectation that algae would be most sensitive to photosystem II inhibitors. These herbicides block the electron transport of photosystem two, a process essential to algae survival. Terbutryn, another photosystem II inhibitor, was also toxic in human cell assays but at concentrations approximately 200-fold higher than in algae. This suggests potential secondary modes of action, such as inducing oxidative stress or inflicting membrane disruption, as highlighted by Brown et al.[59] Oxidative stress is also the main adverse effect of triclosan, a mitochondrial electron transport decoupler, exhibiting broad toxicity across all assays except the zebrafish cells. The reduced toxicity in the zebrafish cell assay could be attributed to the lower incubation temperature used, which slows metabolic rates, delaying triclosan uptake and activation.[60] Additionally, fish cells may rely on more widely distributed detoxification mechanisms, including cytochrome P450 enzymes, which are expressed across multiple tissues and play a key role in metabolizing triclosan. This broad enzyme distribution enhances triclosan metabolism and excretion, reducing its intracellular accumulation and toxic effects.[61,62] Measurements

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Environ. Sci. Technol. 2025, 59, 18042−18055

of internal triclosan concentrations over time would, however, be needed to confirm these hypotheses of why fish cell lines are not susceptible to triclosan in this study.

Contrary to expectations, both *E. coli* and yeast demonstrated low sensitivity to the respective compound classes. This lack of response to ciprofloxacin and sulfamethoxazole—both antibiotics with known activity against Gram-negative bacteria—further confirms the unexpectedly low sensitivity of the *E. coli* assay.[63] Similarly, yeast exhibited limited responses to the fungicides tested. In contrast, algae and mammalian cells showed strong responses to several of these compounds. Among the antibiotics, erythromycin was toxic only to algae, clarithromycin affected both algae and bacterial systems, and ciprofloxacin was toxic exclusively to hamster cells (AR-EcoScreen). The observed algal sensitivity to macrolides such as erythromycin and clarithromycin may be explained by their interaction with algal ribosomes, which share structural similarities with bacterial ribosomes.[64,65] The selective toxicity of ciprofloxacin in mammalian cells has been noted in previous research, with hypotheses pointing to possible off-target effects on DNA synthesis pathways.[66,67] The fungicides propiconazole and tebuconazole were highly toxic to algae and showed moderate toxicity to mammalian cells but had little to no effect on yeast. The intended mode of action of azole fungicides is the inhibition of a specific cytochrome P450 monooxygenase (CYP51) in fungi active in the synthesis of ergosterol.[49] A similar mode of action may underlie the toxicity observed in algae, as their CYP51 homologues are structurally and functionally similar to those in fungi.[68] Inhibiting these enzymes can disrupt membrane formation and inhibit algal growth. The high toxicity observed in mammalian cells may be linked to the promiscuous binding of azoles to cytochrome P450 enzymes involved in in different biosynthetic pathways such as e.g., steroidogenesis, disrupting metabolic processes and hormonal balances, as reported in studies on reproductive toxicity and human liver cells.[69−71] This could explain the toxicity observed in VM7Luc4E2 and MCF7AREc32, both of which are breast cancer cell lines with hormone-responsive characteristics. The lack of effect on yeast is surprising, as we later found that prochloraz induced responses in the yeast assay with EC_{50} values around 50 mg L^{-1} (unpublished data). Prochloraz is an azole fungicide with a similar MoA as propiconazole and tebuconazole but generally being effective at lower concentrations.[72] Hence, as an EC_{50} value of 50 mg L^{-1} is high for such a potent fungicide, it is likely that yeast either does not take up the fungicides well or metabolize them so rapidly that the less potent propiconazole and tebuconazole never reach internal effective concentrations within the tested concentration range. Cyprodinil, despite having an unclear mode of action, has been reported to exhibit broad toxicity, with proposed mechanisms including interference with cellular respiration or membrane function,[73] which corresponds well with it being effective in five of the eight bioassays.

Pharmaceutical chemicals were expected mainly to have effects on mammalian or vertebrate cell lines, but the results showed clear toxic effects in the algae, which may reflect unintended targets being present in algae. These findings are supported by Figure S1, which shows that across 70 chemicals from various classes of environmental pollutants tested in algal, EC_{50} values deviated from baseline toxicity predictions not only for herbicides, but also for a large proportion of the pharmaceuticals (Figure S1). Amitriptyline, an antidepressant targeting serotonin and norepinephrine transporters, displayed comparable toxicity in algae and vertebrate cells, an unexpected finding given the absence of these transporters in algae. This indicates that amitriptyline may affect other vital transporters or pathways necessary for algal growth, for example efflux pumps as suggested by Munoz-Bellido et al.[74] to explain their antimicrobial activity. The high sensitivity of algae to antidepressants was also found by Minguez et al.,[75] who tested three antidepressants in seven marine bioassays (two algae species, two crustaceans, oyster larval development and metamorphosis and a mollusk cell line) and found the two algae species and the oyster larvae to be the most sensitive.[74] Similarly, methylmethanesulfonate (MMS), a genotoxic agent, exhibited extreme toxicity in algae, about 400 times higher than in other assays. The heightened sensitivity of algae to MMS may result from their rapid DNA synthesis and relatively inefficient DNA repair mechanisms.[76]

Metals such as copper and zinc exhibited toxicity across six of the eight assays, likely due to their disruption of enzymatic functions essential for a wide range of metabolic pathways. Barber et al.[77] explicitly highlighted that copper toxicity induces oxidative damage and dysfunction in cellular metabolism and enzymatic systems.

Among the remaining chemicals, bisphenol A and diclofenac exhibited limited activity, being toxic only in human cell assays at high concentrations (EC_{50} = 33 mg L^{-1} and 55 mg L^{-1}, respectively), likely reflecting nonspecific narcotic effects or membrane disruption.[78] Similarly, 1,3-diphenylguanidine and triphenyl phosphate primarily showed toxicity in algae and one mammalian assay, possibly due to enzyme inhibition or oxidative stress pathways.[79]

The data clearly illustrates that the mode of action highlighted for the intended use of specific products are rarely the only relevant mechanism, as many chemicals exhibit toxicity in unexpected organisms. In our tests, algae not only showed high sensitivity in terms of frequency of effects, but also often had the lowest effective concentrations for a chemical or sample compared to the other assays (Figure 1, sheet "EC50-nom" and "Toxdata WWTP" of Supporting Information).

Sensitivities to Wastewater Samples. Based on the results of the sensitivity ranking, we chose the algae test and three of the cell lines which were also transfected to induce a receptor, to test 254 WWTP effluents and 25 influent samples (Figure 1B, sheet "Toxdata" of Supporting Information). The algae assay was also the most sensitive to the WWTP samples showing EC_{50} values from REF 1.16 to 44.49 (mean ± stdev: REF 16.91 ± 9.53) in 258 samples out of 279 samples. In comparison, the DR-EcoScreen assay, which assesses toxicity in murine cells, recorded EC_{50} values for 148 samples (mean ± stdev: REF 26.58 ± 11.19), with REF values ranging from 1.41 to 48.18 while VM7luc4E2 and MCF7AREC32, detected toxic effects in only 72 and 58 samples, respectively, highlighting the differential sensitivity for cytotoxicity of mammalian cell lines to the wastewater contaminants tested. The results on the WWTP samples confirmed the results on the individual chemicals of algae being the most sensitive bioassay, but also that the cell lines were more sensitive in some cases where algae were not. By combining the algae assay with all three mammalian cell line assays, the toxicity detection frequency increased from a maximum of 92.47% (algae alone) to 98.65%.

Chemical Bioavailability and Cell Surfaces. Our findings partially supported the hypothesis 1 that bioassay sensitivity depends on the mode of action and biological

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Environ. Sci. Technol. 2025, 59, 18042−18055

targets of the chemicals, at least for the sensitivity of algae to herbicides. However, deviations observed for antibiotics and fungicides suggest that mechanisms affecting bioavailability, uptake, biotransformation and off-target effects may play a more significant role than initially anticipated. Medium composition affecting chemical bioavailability has been suggested to affect variability in assay sensitivity,[56,80] though the specific impact was not quantified. We therefore quantified the chemicals bioavailability across the different assay systems to elucidate its potential influence on toxicity outcomes.

The percentages of protein and lipid within the different media and the media effect on the freely available fraction for each tested chemical is shown in Figure 2. For in vivo unicellular organisms, the algae medium contains only essential inorganic nutrients, reflecting its exclusive reliance on inorganic nutrients and light for growth and C_{free}/C_{nom} is therefore close to 100% for most chemicals (Figure 2B). In contrast, the medium for *E. coli* including amino acids, peptides, and carbohydrates, had the highest protein content (0.93%) among all media, while lipid content was the lowest (0.0028%). Yeast had a protein content in the low range of the cell assays (0.15% as compared to 0.25−0.61% for the cell assays), while the lipid content of 0.056% was intermediate between the *E. coli*and the cell assays, which all ranged from 0.02 to 0.03% (Figure 2A). The different contents of lipids and proteins in the growth media resulted in large variabilities in bioavailability expressed as C_{free}/C_{nom}. In Figure 2B, where chemicals are arranged from low to high lipophilicity ($logD_{pH7.4}$) along the *Y*-axis, a clear trend is observed: as lipophilicity increases, bioavailability decreases across all media, and the gap between algae medium and other media grows. For low-lipophilicity chemicals such as erythromycin, MMS, ciprofloxacin, sulfamethoxazole, and clarithromycin (log $D_{lip/w}$: −1.24−1.64), bioavailability remains consistently high across all media ($C_{free}/C_{nom} \approx 80-100\%$), resulting in minimal differences in chemical availability between the algal medium and the more complex mammalian or bacterial assay media. However, the substantial variability observed in EC_{50} values across assays—such as, MMS (EC_{50} from 0.16 mg L^{-1} to 76.16 mg L^{-1}) and clarithromycin (EC_{50} from 0.00042 mg L^{-1} to 0.2 mg L^{-1})—suggests that bioavailability alone does not account for the observed toxicity differences. Additional factors such as differences in cellular uptake, target site accessibility, detoxification and repair capacity and efflux mechanisms likely also contribute to these discrepancies.

As lipophilicity increases, bioavailability in complex media decreases due to stronger binding with proteins and lipids. For high-lipophilicity chemicals such as bisphenol A, tebuconazole, and diuron (log $D_{lip/w}$: 3.25−4.19), C_{free}/C_{nom} values in algae medium remain close to 99%, while in complex media they range from 2.64% to 24.50%, leading to a pronounced gap of 75% to 96% in bioavailability between these assays and the algae assay. A few chemicals—such as triclosan, amitriptyline, triphenyl phosphate, diclofenac, and propiconazole—exhibit exceptionally low bioavailability in both complex media ($C_{free}/C_{nom} < 1\%$) and algae media ($71.20\% < C_{free}/C_{nom} < 88.10\%$). In complex media, sorption is primarily driven by binding to organic components present in the test media. In contrast, in the algae system, sorption occurs predominantly at the cellular level—on cell membranes and within cells—resulting in considerable sequestration in both media types. For instance, triclosan contains phenolic hydroxyl groups that confer strong affinity for lipoproteins and membrane lipids, resulting in extensive sorption and extremely low freely dissolved concentrations.[81] However, our current model does not differentiate between cell types and applies default composition parameters; therefore, the extent of this effect remains uncertain.

In addition to bioavailability and partitioning behavior, the chemical stability of test compounds—such as susceptibility to degradation, hydrolysis, or photolysis—may also influence the effective exposure concentrations and should be considered in future studies.

Finally, the experimental setup, including the type of test plates used, may theoretically also influence bioavailability due to differences in surface-to-volume ratio. However, literature suggests that surface effects are minimal across 96-well and 384-well plates as demonstrated by Huchthausen et al.[82] on nine chemicals with different physiochemical properties tested in both 96- and 384-well plates using both receptor end points and cytotoxicity end points, and with C_{free} measured using Solid-Phase Microextraction.[82] The authors found little systematic bias in terms of 384-well plates showing lower effects or measure C_{free}. We therefore conclude that the change in surface/volume ratio between the two well types play a minor role compared to the type of media used in determining the bioavailable fraction of the chemicals. In addition, tests comparing plastic and glass plates showed no significant differences in assay sensitivity for terbutryn (unpublished data), indicating that medium composition is the primary determinant of chemical availability.

To better understand the relationship between chemical bioavailability and bioassay sensitivity, we analyzed the correlation between the average toxicity frequency (y) and bioavailability (x) of the tested chemicals across the eight bioassays (Figure 3). The initial analysis revealed a linear relationship, described by the equation $y = 0.87x + 1.33$, with an R^2 value of 0.58 and a p-value of 0.036 (Figure 3A), with the correlation mainly being driven by the algae.

Removing the algae from the correlation decreased the R^2 value to 0.35 making the correlation nonsignificant (p value of 0.16), but still positive. Frequencies of detected toxicities are not very robust data when only 21 chemicals were included, but starting to compare EC-values based on C_{free} would be biased in this case, as the different organisms and cells have not been exposed to comparable C_{free} concentrations, hence, we find frequency of detected toxicity when exposed to similar nominal concentrations the best measure of sensitivity. Yeast, *E. coli* and the fish cell line ZF4 exhibited toxicity lower than the values predicted by the trend line (Figure 3A). These negative deviations may be attributed to structural and physiological factors for yeast and *E. coli*, such as the presence of rigid cell walls that limit chemical penetration and intrinsic tolerance mechanisms of these single cell organisms that reduce intracellular accumulation of toxicants.[83−85] The lower sensitivity of the ZF4 cells, however, compared to the mammalian cells could either be due to the test running at lower temperatures (24 °C as opposed to 37 °C for the mammalian cells), or that the specific cell line has efficient detoxification and cellular defense mechanisms, such as efflux pumps and enhanced metabolic detoxification, which actively reduce intracellular toxicant concentrations and mitigate toxic effects.[86,87]

In contrast, VM7luc4E2 showed a toxicity frequency of 61.90%, closely matching or slightly exceeding the predicted

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Environ. Sci. Technol. 2025, 59, 18042−18055

value at a bioavailability of 36.70%. This alignment suggests that VM7luc4E2's sensitivity is largely driven by bioavailability.

The difference in the sensitivity of the mammalian cell lines follow the bioavailability trend, with more sensitive cell lines growing in media giving a higher chemical bioavailability (Figure 3).

Correlating Bioassays: Optimization of the Bioassay Battery. To assess the complementarity of the bioassays used for pollutant detection, we conducted a correlation analysis among the four assays applied to both the 21 individual chemicals (A) and 279 wastewater samples (B), which is shown in Figure 4. The analysis revealed a strong correlation between the human cell line (VM7luc4E2) and the rodent cell line (DR-EcoScreen) (r = 0.92), which is likely due to their similar medium compositions (Figure 2A).

In contrast, the MCF7AREc32 cell line, which contains considerably more protein than the other cell lines, resulting in a lower chemical bioavailability (Figure 2B) and a resulting lower detection frequency for both individual chemicals and samples (Figure 1), did not correlate so well with the other two cell lines. While the MCF7AREc32 assay is a valuable tool in water quality assessments when transfected to express the Nuclear factor erythroid 2−related factor 2 (Nrf2) receptor, designed to detect a broad range of oxidative stress-inducing compounds via its modified receptor system, the end point of cytotoxicity is not well suited for EDA approaches. Therefore, we excluded it from our final bioassay battery to reduce redundancy.

When comparing the algae assay to the cell-based assays, the low correlations with both VM7luc4E2 (r = 0.70) and DR-EcoScreen (r = 0.48), indicate that the algae assay is likely to detect pollutants with different toxicity mechanisms compared to the cell lines. Given that DR-EcoScreen not only has a higher detection frequency than VM7luc4E2, but also exhibits the lowest correlation with the algae assay, we recommend DR-EcoScreen as the best complementary assay to the algae-based method. The detection frequencies for the various assays tested on environmental samples are illustrated in the Venn diagram shown in Figure S2.

The present study indicates that the sensitivity of unicellular assays, as well as cell cultures, is likely to be more affected by how the culture media affects bioavailability of chemicals, rather than on the biology of the organism or cell type. To fully verify this indication the bioavailable fraction of chemicals in the test systems should be measured e.g., with Solid-Phase Microextraction[82] and concentration−response curves based on measured chemical concentrations should be compared across species and cell types. However, even without this verification, the present study clearly shows that the algae assay is the most sensitive in picking up contaminants interfering with biological processes that can inhibit growth or cause cytotoxicity, possibly supplemented with DR-EcoScreen as a cell assay picking up chemicals with different MoA. These findings provide valuable data for data-driven EDA and support the development of sensitive and broadly responsive bioassay batteries with the potential to detect both known and previously unrecognized environmental contaminants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.5c04027.

Chemical properties, assay conditions, and toxicity data for 21 compounds and 279 wastewater samples, supporting sensitivity comparisons and bioavailability analysis (XLSX)

Algal toxicity deviations (Figure S1) and bioactivity overlap across bioassays (Figure S2) (PDF)

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Notes

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