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Mixtures of Chemical Pollutants at European Legislation Safety Concentrations: How Safe Are They?

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ABSTRACT

The risk posed by complex chemical mixtures in the environment to wildlife and humans is increasingly debated, but has been rarely tested under environmentally relevant scenarios. To address this issue, two mixtures of 14 or 19 substances of concern (pesticides, pharmaceuticals, heavy metals, polyaromatic hydrocarbons, a surfactant, and a plasticizer), each present at its safety limit concentration imposed by the European legislation, were prepared and tested for their toxic effects. The effects of the mixtures were assessed in 35 bioassays, based on 11 organisms representing different trophic levels. A consortium of 16 laboratories was involved in performing the bioassays. The mixtures elicited quantifiable toxic effects on some of the test systems employed, including i) changes in marine microbial composition, ii) microalgae toxicity, iii) immobilization in the crustacean *Daphnia magna*, iv) fish embryo toxicity, v) impaired frog embryo development, and vi) increased expression on oxidative stress-linked reporter genes. Estrogenic activity close to regulatory safety limit concentrations was uncovered by receptor-binding assays. The results highlight the need of precautionary actions on the assessment of chemical mixtures even in cases where individual toxicants are present at seemingly harmless concentrations.

Key words: bioassays; effects; mixtures; ecotoxicology; biomarkers

ABBREVIATIONS

DEQ	Diuron equivalent
E2	17β-estradiol
EE2	Ethinylestradiol
EEQ	Estradiol equivalent
EQS	Environmental Quality Standard
AA-EQS	Annual average EQS
MAC-EQS	Maximum allowed concentration EQS
WFD	Water Framework Directive
ER	Estrogen receptor

In Europe, as in most other industrialized regions of the world, diverse classes of chemical pollutants are released into the aquatic environment, mainly from agriculture, industry, medical facilities, and household waste. The European Union (EU) Directive 2000/60/EC (Water Framework Directive, WFD) has established a strategy for water protection that includes specific measures for pollution control to achieve good ecological and chemical status at the European level. Good chemical status is defined in terms of compliance with the safety limit concentration for substances of concern (Environmental Quality Standards, EQS) which are aimed to ensure that they do not cause any harmful effects to or via the aquatic environment. For technical and economic reasons, there is a tendency to limit chemical analysis to already regulated substances that are known to pose a threat to humans or aquatic organisms. However, environmental samples are usually very complex and can contain numerous natural and anthropogenic chemicals, even though most are present at very low concentrations. When assessing the chemical status of an aquatic environment, the individual substance EQS values are considered as safety limits, disregarding the very likely scenario of a combined action of co-occurring pollutants. Although it has been assumed that safety factors applied to the derivation of EQS values protect against the combined action of pollutants, there has been a growing concern from both scientists and the public regarding this issue. In response, the European Commission has issued a communication on combination effects of chemicals (COM 2012-252) asking for a stronger effort to ensure that the risks associated with chemical mixtures are properly understood and assessed. Biological based assays (bioassays) offer the possibility to monitor the overall response from multiple chemicals in an environmental sample and assess the impact on different levels of biological organization, such as community, population, individual and/or sub-organism levels. However, different bioassays are rarely tested on identical samples and therefore available information on the comparability, complementarity, and potential uses of the different bioassays is severely lacking.

To address the challenges posed by mixtures of pollutants to the water quality monitoring, artificial mixtures were created and effects measured using diverse bioassays, including non-OECD standards, to investigate the response to identical samples.

Two mixtures were prepared, Mix14 and Mix19, with 14 and 19 substances of concern, respectively, at concentrations equivalent to the Annual Average Environmental Quality Standard (AA-EQS). The substances were selected to include a wide range of chemical groups with known toxicological effects. Mix14 contained priority substances (PSs) whose quality standards were taken from European legislation (COM 2011-876, 2008/105/EC, 2013/39/EU3), whereas Mix19 contained five additional emerging pollutants that may become PSs in the future, selected by taking into account their prevalence in European surface waters (Loos *et al.*, 2009, 2013) and their known effects.

Thirty five *in vitro* and *in vivo* bioassays routinely used by the participating laboratories were performed. The selection of bioassays took into account the endpoints and trophic levels commonly used for the risk assessment of chemicals under European legislation (EC 1907/2006), whereas other bioassays measured endpoints associated with the expected mode of action of substances present in the mixtures. The assessed endpoints included acute toxicity (in microalgae, bacteria, yeast, amoeba, nematode, and cell lines), immunotoxicity in fish, fish embryo toxicity (FET), frog teratogenicity, estrogenic activity, the response of several molecular biomarkers in transgenic bacteria, yeast and nematode, and gene expression analysis of molecular biomarkers in cell lines. The tests were carried out using 11 organisms from different trophic levels, microcosm, several cell lines, and biomarker reporter systems.

To our knowledge, this is the first time that such a complex mixture, harboring different classes of chemicals at regulatory safety concentrations, has been tested using such a broad range of bioassays and test organisms. This paper describes the outcome of this exercise, focusing specifically on the results of the bioassays that exhibited a significant quantifiable effect of the mixtures at concentrations considered safe for each compound.

MATERIALS AND METHODS

Preparation of Reference Mixtures

Mixtures Mix14 and Mix19 contained the chemicals listed in Table 1 at concentrations equivalent to the AA-EQS, which for simplification is designated from now on as EQS. For each mixture, 1000-fold concentrated reference materials were prepared, with organic compounds in methanol and inorganic chemicals in 2% nitric acid. Additional 10,000-fold concentrated reference materials were prepared for Mix14 to allow the assessment of effects at a wider range of concentrations. The chemicals used for the preparation of the reference mixtures were of ≥98% purity, whereas for BaP and DEET the purity was ≥96 and ≥97%, respectively.

The short-term stability of the organic reference materials was assessed according to an isochronous study (ISO Guide 35, 2006) in order to simulate problematic transport or storage conditions with a reference temperature of −20°C and a test temperature of 24°C for up to 8 weeks. During the isochronous study, no significant degradation was observed in all the reference materials produced and dispatched, as checked by applying a two-tailed t-test with 99% as confidence level (for details, see Supplementary Materials and Methods). The organic and inorganic reference materials were transported in dry ice and stored in all laboratories under the reference temperatures of −20°C and 4°C, respectively. It was therefore assumed that the reference mixtures used by the different laboratories were identical, at least until reconstitution. Mixtures or solvent control (SC) (methanol and 2% nitric acid) was directly diluted into bioassay media following a common protocol and tested at final concentrations of 1× and 10×EQS for Mix14 and 1×EQS for Mix19, unless stated otherwise.

Marine Microcosm

Seawater (SW) was collected at the middle of the Gulf of Trieste (45° 32' 55, 68" N, 13° 33' 1, 89"E) at depth of chlorophyll maximum on 18 July 2013. Sampling was performed using a Niskin sampler and the SW was immediately pre-filtered through a 53-μm acid-washed Nitex filter to remove larger phytoplankton grazers. All samples were kept at environmental temperature, protected from light, and brought to the Marine Biology Station, Piran within 1 h after sampling. The time zero sample was taken before distributing the water into acid-washed and sterilized 1-l bottles. Each exposure mixture was added directly to 1 l of SW and triplicates were generated for each treatment. At the same time, two sets of controls were prepared in triplicate: SC (0.1% methanol (v/v) and 0.002% nitric acid in 1-l SW) and SW without any addition. All bottles were incubated in a thermostatic room at constant temperature (15°C) and day/night light conditions. The pH was adjusted to standard SW pH (8.3) with 0.1-M NaOH. After 6, 12, 24, and 48 h of exposure, equal volumes were taken from each of the triplicate bottles for bacterial production and phytoplankton pigment analyses.

Bacterial production was measured as protein synthesis rates of plankton bacteria population using the ^{3}H-leucine incorporation method (Smith and Azam, 1992) and expressed as the number of cells/l/h, using 20-fg C bacterium^{-1} as the conversion factor.

The qualitative and quantitative analyses of phytoplankton pigments in the water samples were determined using a reverse-phase HPLC (high performance liquid chromatography) method (Barlow *et al.*, 1993). Water samples were filtered through Whatman GF/F filters, extracted in 90% acetone, sonicated and centrifuged for 10 min at 4000 rpm to remove particles. The supernatant was mixed with 1-M ammonium acetate (1:1), the pigments were separated by RP-HPLC using a 3-μm C18 column (Pecosphere, 35 × 4.5 mm, Perkin Elmer) and detected by absorbance at 440 nm using a diode array detector. The data were statistically evaluated using two-way ANOVA.

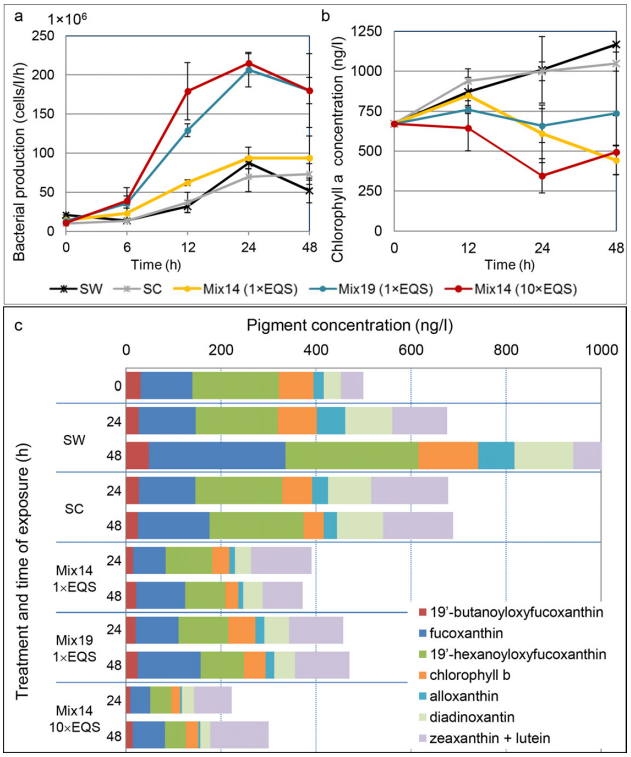


FIG. 1. Marine microcosm. Effect of the chemical mixtures on the natural phytoplankton and bacterioplankton community. Endpoints measured were bacterial production (a), chlorophyll *a* concentration (b), and other phytoplankton pigments (c). For comparison, identical SW samples have been left untreated (SW) or were exposed to SC. Error bars represent the standard deviation ($n = 3$).

Freshwater Microalgae

Cultures of three microalgal species in exponential growth phase were exposed to the test mixtures and the effects on growth rate and photosynthesis (for freshwater algae only) were assessed. SC at equivalent dilutions as the reference mixtures was tested in parallel. The tests were conducted with three replicates for each treatment. Sigmoidal curves were fitted to the data with GraphPad Prism 5 Software (La Jolla, CA, USA). The EC_{50} and EC_{10} values were calculated from the fit.

Pseudokirchneriella subcapitata cultures with a cell density of 2×10^5 cells/ml were exposed to samples in 96-well plates according to Escher *et al.* (2008). The two mixtures were tested at concentrations ranging from 0.03× to 100×EQS for Mix14 and from 0.8× to 100×EQS for Mix19. Diuron was used as a reference compound and the data expressed as diuron-equivalent concentration (DEQ), by multiplying the relative potencies of the photosystem II (PSII) inhibitors diuron, atrazine, isoproturon, and simazine with their known concentration in the mixture (Vermeirssen *et al.*, 2010).

PSII inhibition was measured via the effective quantum yield method using a Maxi-Imaging PAM (pulse amplitude modulation, IPAM) (Walz, Effeltrich, Germany) as described previously (Escher *et al.*, 2008) after 2- and 24-h of exposure. Algae growth was measured by absorbance (685 nm) in a microtiter plate photometer (Synergy 4, Biotek, Winooski, VT) after 2-, 20-,

TABLE 1. Composition of Chemicals in the Reference Mixtures

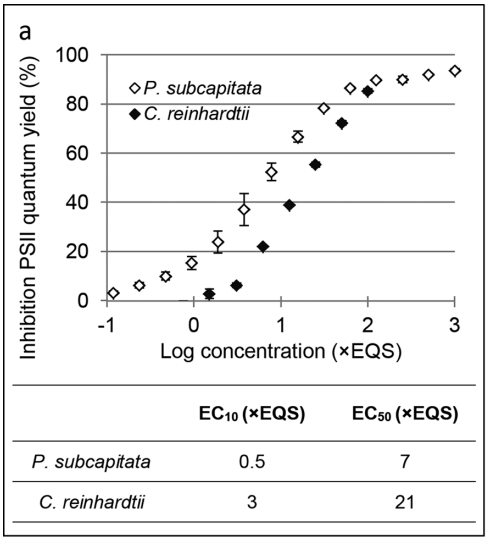
Substances	CAS[b]	Use	Mode of action/reported effects	AA-EQS (μg/l)
Atrazine	1912-24-9	Herbicide	Photosystem II inhibitor	0.6[c]
Benzo[a]pyrene (BaP)	50-32-8	By-product of incomplete combustion of organic material	Intercalation of BaP metabolites in DNA causing mutagenesis, carcinogenesis	0.00017[c]
Cadmium	7440-43-9	Industrial by-product; used in metal plating and to make pigments, batteries, and plastics.	Indirect formation of reactive oxygen species, depletion of glutathione, lipid peroxidation	0.08[c]
Chlorfenvinphos	470-90-6	Insecticide	Inhibition of cholinesterase activity	0.1[c]
Chlorpyrifos	2921-88-2	Insecticide	Inhibition of cholinesterase activity	0.03[c]
DEHP	117-81-7	Plasticizer	DNA damage, carcinogenicity	1.3[d]
Diclofenac	15307-79-6	Pharmaceutical pain killer; non-steroidal anti-inflammatory drug (NSAID)	Can cause adverse hepatic effects in certain organisms	0.1[d]
Diuron	330-54-1	Herbicide	Photosystem II inhibitor	0.2[c]
17β-estradiol	50-28-2	Natural estrogen	Natural estrogen	0.0004[d]
Fluoranthene	206-44-0	Product of incomplete combustion	Causes mutagenesis, carcinogenesis	0.0063[c]
Isoproturon	34123-59-6	Herbicide	Photosystem II inhibitor	0.3[c]
Ni	7440-02-0	Industry, preparation of alloys	Depletion of glutathione levels, binds to sulfhydryl groups of proteins, carcinogenicity	4[c]
4-nonylphenol	25154-52-3	Mostly used for the production of surfactants (nonylphenolethoxylates)	Endocrine disruptor	0.3[c]
Simazine	122-34-9	Herbicide	Photosystem II inhibitor	1[c]
Carbamazepine[a]	298-46-4	Pharmaceutical (anti-epileptic, mood-stabilizing drug)	Teratogenicity	0.5[e]
Sulfamethoxazole[a]	723-46-6	Pharmaceutical (antibiotic)	Interferes with folic acid synthesis	0.6[e]
Triclosan[a] (Irgasan)	3380-34-5	Anti-bacterial and antifungal agent used in cosmetics and detergents	Inhibition of cellular efflux pumps	0.02[e]
N,N-diethyl-m-toluamide (DEET)[a]	134-62-3	Insect repellent	Affects insect odorant receptors, inhibits cholinesterase activity (nervous system)	41[e]
Bisphenol A[a]	80-05-7	Plasticizer	ER agonist	1.5[e]

[a]Used only in Mix19 (in addition to the other chemicals also present in Mix14).
[b]Chemical Abstracts Service.
[c]According to European Directive 2013/39/EU.
[d]Taken from COM 2011-876.
[e]Proposal from Ecotox Centre, Switzerland.

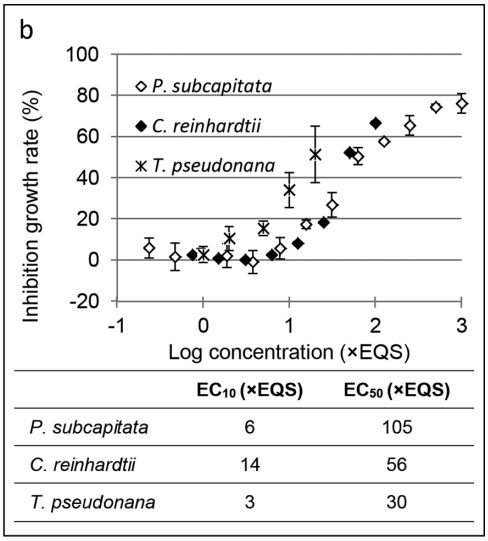
and 24-h exposure. Freshwater algal growth inhibition measurements with *P. subcapitata* were performed by three laboratories for longer exposure times (72 h and 96 h) with Mix14 (1× and 10×EQS) and Mix19 (1×EQS).

Chlamydomonas reinhardtii (CC-125, wild-type mt+137c) was cultured in Talaquil medium, as reported previously (Pillai *et al.*, 2014). The growth conditions were 25°C with constant agitation and illumination of 100 μmol photon m^{-2} s^{-1}. *C. reinhardtii* (2.5×10^5 cells/ml) were exposed to Mix14 for 24 h in a total volume of 20 ml. A dose-dependent response of Mix14 ranging from 0.7× to 100×EQS was investigated. The growth rate was estimated by measuring the cell numbers by CASY counter (Roche Innovatis AG, Switzerland). The photosynthetic yield was determined after 2 h and 24 h with PhytoPAM (Heinz Wald Gmbh, Germany).

Thalassiosira pseudonana (strain CCMP 1335) was obtained as axenic culture from the Provasoli-Guillard National Center for



	EC_{10} (×EQS)	EC_{50} (×EQS)
P. subcapitata	0.5	7
C. reinhardtii	3	21



	EC_{10} (×EQS)	EC_{50} (×EQS)
P. subcapitata	6	105
C. reinhardtii	14	56
T. pseudonana	3	30

FIG. 2. Cytotoxicity to microalgae. Dose response curves of Mix14 were generated for the inhibition of photosynthesis after 2-h exposure (a) and inhibition of growth after 24-h exposure (b) of the freshwater microalgae *P. subcapitata* and *C. reinhardtii* and the growth of marine diatom *T. pseudonana*. The x-axis is displayed as concentration of Mix14, in terms of EQS. The EC_{10} and EC_{50} values obtained from the fit of the data are shown for each of the endpoints. No effect from exposure to the solvent was observed for any of the organisms. Error bars represent the standard deviation, $n = 3$.

Culture of Marine Phytoplankton (CCMP, West Boothbay Harbour, Maine, USA) and cultured in artificial seawater (ASW-f/2) at 16°C and photoperiod 13/11-h light/dark. *T. pseudonana* cultures were synchronized according to Hildebrand *et al.* (2007) and exposed to the mixtures at cell density of 1×10^6 cells/ml in a total volume of 20 ml. A dose-dependent response of Mix14 ranging from 1× and 20×EQS and Mix19 at 1×EQS were investigated after 24, 48, and 72 h. Cell densities were determined by measuring the absorption at 450 nm using a microplate spectrophotometer (Biorad, Hercules, CA) and used to calculate growth rates and growth inhibition, as previously described (Bopp and Lettieri, 2007).

Daphnia Magna *Acute Immobilization test*

The test followed the ISO 6341 (2012) standard method. Five newly hatched neonates (age <24 h) were placed in glass beakers (100 ml) and exposed to the mixtures in the dark at 18–22°C. Four replicates were made per treatment (i.e., 20 animals per treatment and 20–40 animals in the control group). The number of immobile animals was counted after 24 and 48 h. Potassium dichromate was used as a reference compound, with an EC_{50} of 1.8 mg/l (95% CI, 1.7–1.9 mg/l), fulfilling the validity criteria in the ISO standard of an EC_{50} between 0.9 and 2.4 mg/L22.

The concentration-response relationships were calculated with the ToxCalc software (Ver 5.0) (Tidepool) with maximum likelihood logit regression.

D. Magna *Reproduction Test*

The test followed the OECD Test No. 211 (2012) and the ISO 10706 (2001) guidelines, with newly hatched daphnids placed separately in glass beakers. Exposure to the mixtures, control, and solvent occurred at 21 ± 1°C and photoperiod 16/8-h light/dark (10 animals per condition). During 21 days of exposure, the survival and the reproduction were monitored. Exposure mixtures were changed three times a week and daphnids were fed with green algae (Pseudokirchneriella, Chlorella, and Scenedesmus spp.). Offspring produced by parent animals were counted and removed. Survival of parent animals and the number of live offspring were evaluated and expressed as a percentage of control.

Mean, standard deviation, and the number of replicates were used for statistical evaluation using GraphPad QuickCalc on-line software, and statistical significance of differences between control and exposure mixtures was tested by unpaired t-test.

FET Test

The FET test was conducted according to the OECD TG. 236 (2013) and the ISO 15088 (2008) guidelines with zebrafish (*Danio rerio*) embryos. Fertilized eggs were exposed to the mixtures under static conditions for 5 days: 10 embryos per 40-ml media and three replicates per treatment in two independent experiments. Embryos were monitored daily for mortality, the number of hatched embryos, type of deformities (head, tail deformities, absence of gas bladder) and the number of defected embryos, underdeveloped embryos and length.

Statistical evaluation of the data was done by ANOVA followed by Dunnett and Fisher LSD *post hoc* test (for data in individual experimental runs). Homogeneity of variance and normality were tested by Levene and Shapiro-Wilk tests, respectively. Nonparametric Kruskal-Wallis test was used for data without normal distribution and a Chi-square test was used for testing differences in frequencies. Statistica for Windows (StatSoft) and Microsoft Excel were used for calculations.

Frog Embryo Teratogenesis Assay Xenopus

The test followed the ASTM E 1439-98 (1998) guideline and was performed under constant temperature (20°C) and low light. *Xenopus laevis* adults were maintained in 20-l plastic tanks in dechlorinated tap water (males and females together, four animals per tank) and were fed with a mixture of ground beef liver, lung, and heart with gelatin and reptile multivitamin mix. Room and water temperature was 19°C, 12-h day/night rhythm.

Two breeding pairs were placed in separated plastic tanks equipped with bottom plastic nets, thermostats set to 23°C, and bubblers. Both males and females were stimulated with human chorionic gonadotropin (females 500 IU and males 300 IU) in the form of Pregnyl 5000 (N.V. Organon, Holland) injected into the dorsal lymph sac. Eggs were staged according to Nieuwkoop and Faber (1994). After reaching stage 46, normally cleaving embryos

were manually collected from the tank with a plastic dropper and placed in sterile plastic Petri dishes for the exposure to the mixtures or SC, in five replicates, each containing 30 embryos in 10 ml of solution. Solutions were changed every 24 h, and dead embryos were removed. After 96 h, embryos from each dish were moved to test tubes and anesthetized with 5 ml of 100-mg/l tricainemethanesulfonate, and then fixed with 5 ml of 3% formaldehyde. The embryos were observed with a light microscope, digitally photographed, and measured with QuickPhoto MICRO software. The parameters evaluated in this test included mortality, embryo length, and the number and type of malformations and were assessed according to the Atlas of Abnormalities (Bantle, 1991).

Differences from controls were analyzed by ANOVA followed by Dunnett and Fisher Least Significant Difference *post hoc* test and the results controlled by nonparametric Kruskal-Wallis test.

In vitro *Human Estrogen Receptor Transactivation Assays*

The detection of (anti)estrogenic activity by the ER-CALUX, the MELN, and the Yeast Estrogen Screen (YES) assays is based on stably transfected transcriptional activation of responsive elements (luciferase for the two former assays and β-galactosidase for the last). The results in these tests were expressed as EC_{50} (the concentration causing 50% of the maximum effect) as well as estradiol equivalent (EEQ) concentration, which were derived from chemical and bioassay data (Vindimian *et al.*, 1983).

ER-CALUX. The reference mixtures were reconstituted in MQ water, subjected to solid phase extraction, and diluted in dimethyl sulfoxide (DMSO) prior to the exposure. Human U2-OS osteosarcoma cells stably transfected with estrogen receptor alpha (ERα) were seeded into 96-well plates in DMEM/F12 medium (without phenol red) and supplemented with stripped serum. After 24 h of incubation (37°C, 5% CO_2), the medium was replaced by medium containing the water extracts (1% DMSO). A dose-dependent response ranging from 1× to 1000×EQS was investigated for Mix14 and from 1× to 100×EQS for Mix19. After 24 h of incubation, the medium was removed and the cells were lysed in 30 μl of Triton-lysis buffer. The amount of luciferase activity was quantified using a luminometer (MicroLumat Plus, Berthold Technologies, Switzerland). All plates included a dose-response curve of the reference compound 17β-estradiol. All mixtures and estradiol were analyzed in triplicates. Only test concentrations where no cytotoxicity was observed using a microscope were used for quantification of the response (Van der Linden *et al.*, 2008). The data were evaluated by fitting a dose-response using GraphPad Prism 5 Software (La Jolla, California, USA).

MELN assay. The MELN cell line was obtained by stable transfection of MCF-7 human breast cancer cells with ERα (Balaguer *et al.*, 2001). Cells were seeded into 96-well plates at a density of 50,000 cells/well in phenol red free DMEM supplemented with 3% stripped serum. After 24 h of incubation (37°C, 5% CO_2), the mixtures, the reference compound 17β-estradiol, and SC were added in fresh medium. A dose-dependent response ranging from 0.12× to 475×EQS was investigated for Mix14 and from 0.08× to 26×EQS for Mix19. After overnight exposure (18 h), 0.3mM of D-luciferin was added to the wells. After 5 min, the luminescence signal was measured in living cells for 2 s/well using a luminometer (μBeta, Wallac). All mixtures, estradiol, and SC were analyzed in triplicates. Modelling of dose-response curves was done using the Regtox Microsoft Excel macro based on the Hill equation model.

YES assay. The YES was performed according to Routledge and Sumpter (1996) with recombinant yeast *Saccharomyces cerevisiae* provided by John Sumpter (Brunel University, Uxbridge, UK). At test initiation, 1:2 dilution series of the reference substance 17β-estradiol, the mixtures, and SC (ethanol) were pipetted into triplicate wells on 96-well plates and the solvent was evaporated completely under sterile conditions. Suspension with 4×10^7 yeast cells was seeded on the test plate (200 μl/well) and incubated at 30°C. After 72 h, cell density ($OD_{620\,nm}$) and color change ($OD_{540\,nm}$) were measured using a plate reader (Synergy 2, Biotek). A dose-dependent response ranging from 0.8× to 1000×EQS was investigated for Mix14 and from 0.8× to 100×EQS for Mix19. The data were fitted to a sigmoidal curve with GraphPad Prism 5 Software (La Jolla, CA, USA). The fit provided the EC_{10} and EC_{50} as well as EEQ.

In vitro *Human ERα Competition Assay*

To test the binding ability of the recombinant receptor we used the PolarScreen ERα competitor green assay developed by Life Technologies, with a recombinant wild-type ERα ligand binding domain ($wtER\alpha^{LBD}$) (Ferrero *et al.*, 2014). The assay is based on the displacement of the Fluormone ES2 from the ER receptor by competitor molecules and a consequent decrease in the maximum fluorescence signal. The intensity of the fluorescence polarization (P) signal was measured with an Infinite 200 Pro multimode plate reader (Tecan).

A dose-dependent response ranging from 0.01× to 200×EQS was investigated for Mix14 and from 0.001× to 20×EQS for Mix19. 17β-estradiol was used as a reference compound. The data were fitted to a sigmoidal one site competition four parameters logistic curve with OriginPro Software. The fit provided the IC_{50} (concentration of test compound required to reduce the maximum polarization value at 50%) as well as EEQ. IC_{50} values were obtained by the average of at least four different experiments.

Zebrafish Embryo Estrogenic Activity Assay

The estrogenic potency of the mixtures was assessed by the *in vivo* test EASZY (Detection of Endocrine Active Substances acting through human ER, using transgenic cyp19a1b-GFP zebrafish embryos) (Brion *et al.*, 2012). Newly fertilized zebrafish eggs were exposed to the mixtures for 96 h under static condition. A range of three dilutions was tested, from 0.04× to 4×EQS for Mix14 and from 0.04× to 0.4×EQS for Mix19, with 17α-ethinylestradiol (EE2) (0.05nM) as a reference compound. Three independent experiments were performed. At the end of each experiment, the fluorescence of each living zebrafish embryo was acquired using a fluorescence microscope and quantified using ImageJ. The data (expressed as mean fold induction above control) were analyzed to determine the estrogenic activity of each mixture using a parametric two-way ANOVA and post-hoc test using R statistical software.

Escherichia Coli Bioluminescent Reporter Strains

A panel of 12 engineered bioluminescent microbial reporters was studied, each harboring a plasmid-born fusion of a stress responsive gene promoter (*recA*, *katG*, *micF*, *zntA*, *arsR*, *fabA*, *grpE*, *marR*, *cydA*, *sodA*, *yqjF*, and *soxS*; see Supplementary table 2) to a bioluminescence gene cassette (*Photorhabdus luminescens luxCDABE*) (van der Meer and Belkin, 2010).

The reporter strains were grown overnight in 170-μl lysogeny broth (LB) medium supplemented with 100-μg/ml ampicillin. The cultures were diluted 100-fold in M9 medium and regrown with shaking at 37°C for 3 h. Culture aliquots were transferred into an opaque white 96-well microtiter plate (Greiner Bio-One)

and diluted 1:1 with the mixture or the individual model chemical as a positive control (see Supplementary table 2). Each mixture was tested in a concentration series ranging from 0.08× to 5×EQS; additional concentrations up to 50×EQS were tested for Mix14.

Luminescence was measured at 37°C for 10-min intervals using a VICTOR^2 plate reader (Wallac, Turku, Finland) and displayed as arbitrary relative luminescence units (RLUs). Activity was calculated as the difference in the intensity of the signal in the presence and absence of the inducer (Δ RLU) (Belkin *et al.*, 1997). All experiments were carried out in duplicate and repeated at least three times. The lowest concentration detected was determined as the concentration at which the Δ RLU was >2, and was validated by the use of a paired t-test.

Caenorhabditis Elegans Bioluminescent Reporter Strains

Five *Caenorhabditis elegans* transgenic strains were used: *cyp-35A2* (58cop (25.3.47)), *mtl-2* (62cop (6.15.47)), *ugt-1* (59cop (8.13.47)), *gst-38* (54cop (7.7.47)), and *gcs-1* (23cop (5.23.47)). Each strain was dual-labeled, by linking the promoter of the biomarker to the coding region of a Red Fluorescent Protein (mCherry) and an invariant transmembrane vesicular GABA transporter, *unc-47,* to the coding region of a green fluorescent protein (GFP). All strains were maintained at 20°C on nematode growth medium (NGM) agar plates that were seeded with *Escherichia coli* (OP50).

The exposure mixtures and SC were prepared in OP50 and tested in parallel and BaP (100 μg/ml) and $CdCl_2$ (100μM) were used as positive controls for *cyp35A2* and *mtl-2*, respectively. NGM agar plates (20-ml volume) were inoculated with 200 μl of the spiked OP50 and the seeded plates were incubated at room temperature for 24 h (to allow for bacterial growth). All strains were aged synchronized, placed (as L1 larvae) on the NGM plates and exposed to the respective conditions for 48 h at 20°C. Single worms were picked onto a glass slide with a drop of M9 and immobilized with sodium azide (2%). Images were captured with a Nikon DS-2Mv digital camera and NIS-Elements F 2.20 software linked to a Nikon ECLIPSE TE2000-S-inverted microscope, using the filters G-2A (Ex 510nm–560nm) for mCherry and FITC (Ex 465nm–495nm) for GFP. The fluorescence intensities from 10 worms per condition were analyzed using ImageJ.

For the growth size assay, wild-type nematodes ($N = 10$ per condition) were plated on NGM plates (containing the OP50 medium with the mixtures) and maintained up to 120 h. Adult nematodes were transferred to new plates between 72 h and 96 h to remove hatched offspring. Images of worms were obtained using an inverted microscope and the flat volumetric surface area and length determined by tracing the nematodes using the Image-Pro Express software (Media Cybernetics, Inc.). Data obtained from the fluorescence experiments were analyzed using the one-way ANOVA followed by the Tukey's multiple comparison test for significant differences between the treatments. The phenotypic assays were assessed by means of the two-way ANOVA. All tests were executed with GraphPad Prism.

Gene Expression Analysis with Quantitative Real-Time PCR

Cell lines were from and maintained according to ATCC. Human epithelial cervix cells (HeLa) were cultured in Dulbecco's Modified Eagle Medium (DMEM) + 10% Fetal Bovine Serum (FBS). Chicken epithelial hepatocellular (LMH) cells were cultured in Waymouth's MB + 10% in 0.1% gelatin-coated flasks. Both cell lines were kept at 37°C, 5% CO_2. Zebrafish epithelial liver (ZFL) cells were cultured in 50% L-15/ 35% DMEM High glucose/ 15% Ham's F12 supplemented with 5% FBS, 15-mM HEPES, 0.15-g/l sodium bicarbonate, 1X Insulin-Transferrin-Selenium at 28°C and 3% CO_2. The exposure mixtures or solvent was reconstituted in MQ water and immediately before use mixed with cell culturing medium (1:4) to get the desired exposure concentration, with no effect on the pH of the cell culturing media. Cells were plated in 6- or 12-well plates, and after 18–20 h exposed to the mixtures. HeLa and LMH cells were treated for 24 h and ZFL for 40 h, $n = 4$.

Following exposure the cells were lysed and total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Germany) and quantified by Nano-Vue (GE Healthcare). cDNA synthesis followed the qScript cDNA synthesis kit (Quanta Biosciences) and real-time qRT-PCR of each sample was performed in triplicate using the KAPA SYBR FAST qPCR kit (Kapa Biosystems) on an Mx 3000P qPCR machine (Stratagene). The thermocycling conditions were as follows; denaturation 5 min at 95°C followed by 40 cycles of 95°C for 2 s and 60°C for 30 s. The obtained Ct values were normalized using *elongation factor 1 alpha 1* (*eef1a/1*) and relative gene expression was determined using the Δ Δ Ct method (Schmittgen and Livak, 2008). The primers used and the genes they are directed against are listed in Supplementary table 3. These included androgen receptor (AR), ERα, ER beta (ERβ), metallothionein (MT2A), cytochrome P450, family 1 subfamily A, polypeptide 1 (CYP1A1), glutathione S-transferase, cyclooxygenase-2 (COX2), interleukin-6 (IL6), interleukin-8 (IL8), and tumor suppressor protein (p53). Data variance were analyzed using the GraphPad Prism 5 software by one-way (ANOVA) followed by Dunnet post-test for multiple group comparison.

RESULTS

The effects of two chemical mixtures were assessed for a wide range of biological endpoints and organisms from different trophic levels (for a complete overview see Table 2).

Effect on a Marine Microcosm Composition

Natural bacterioplankton and phytoplankton communities were altered by both Mix14 and Mix19 mixtures. Bacterioplankton population exposed to Mix14 and Mix19 was able to grow at rates significantly higher ($p < 0.0001$) than SC and untreated SW (Fig. 1a). Conversely, after 24 h of incubation the phytoplankton biomass, expressed as chlorophyll *a* concentration, decreased significantly compared with both controls, where an increase (up to 900 ng/l) was recorded (Mix14 at 10×EQS $p < 0.0001$; Mix19 at 1×EQS $p < 0.003$; Mix14 at 1×EQS $p < 0.02$) (Fig. 1b). At the same time, the phytoplankton composition, assessed in terms of chemotaxonomic pigments, changed in Mix14 10×EQS, Mix14 1×EQS, and less in Mix19 10×EQS. A major decrease in pigment concentration was recorded for silicoflagellates (19′-butanoyloxyfucoxanthin), diatoms (fucoxanthin), prymnesiophytes (19′-hexanoyloxyfucoxanthin), but much less for cryptophytes (alloxanthin) and green algae (chlorophyll *b*) (Fig. 1c). A significant increase was observed only for cyanophytes (zeaxanthin + lutein) in all treatments.

Effects on Microalgae

The chemical mixtures induced acute toxicity in the three microalgae tested. The limit of detection of toxic compounds in the mixture was lower for PSII inhibition than growth (Fig. 2). PSII was significantly inhibited in the freshwater algae exposed for 2 h to Mix14, with EC_{50} at 7×EQS for *P. subcapitata* and 21×EQS for *C. reinhardtii* (Fig. 2a). A similar response was obtained for the exposure of *P. subcapitata* to Mix19, with EC_{50} at 13×EQS.

The growth rate of all three species was reduced in a dose-dependent manner (Fig. 2b) after 24-h exposure to Mix14, with

TABLE 2. Summary of Bioassays, Results, and Partner Laboratories in the EU-Wide Exercise

Organism/test	Biological endpoint	Exposure	Effects	EC_{50} (×EQS)	Comments
Microcosmos in marine water	Bacteria production and pigment concentration	6, 12, 24, 48 h	Increase in bacterioplankton decrease in phytoplankton	-	pH adjusted
Vibrio fischeri, Microtox EN ISO 11348-3	Inhibition bioluminescence	15, 30 min	No toxicity effect, stimulation of luminescence	-	pH adjusted
Escherichia coli (luminescent transgenic organisms)[a]	Induction of biomarkers	up to 800 min	Mix14: *zntA*, *arsR* induction Mix19:*cydA*, *micF* induction	-	-
Pseudokirchneriella subcapitata ISO 8692	Growth inhibition	24 h	Effect observed <10×EQS	105 (Mix14) 116 (Mix19)	72, 96 h tested in some labs
Pseudokirchneriella subcapitata	Inhibition of photosynthesis (PSII)	2 h	Effect observed <10×EQS	7.3 (Mix14) 12.6 (Mix19)	-
Chlamydomonas reinhardtii	Growth inhibition	24, 48, 72 h	Effect observed <10×EQS	56 (Mix14)	Mix19 tested only at 1×EQS
	Inhibition of photosynthesis (PSII)	2, 24 h	Effect observed <10×EQS	19.2 (Mix14)	
Thalassiosira pseudonana	Growth inhibition	24, 48, 72 h	Effect observed <10×EQS	28 (Mix14)	Mix19 tested only up to 2×EQS
Saccharomyces cerevisiae	Growth	8 h	No effect	-	-
	Genotoxicity	8 h	No effect	-	-
	Acute toxicity	4 h	Acute toxicity significant ($p < 0.05$) only >25×EQS	-	-
(Transgenic fluorescent)					
Daphnia magna	Acute immobilization	24, 48 h	Effect observed	7 (24 h)	Mix19 tested up to 2×EQS
EN ISO 6341			<10×EQS (Mix14)	3.4 (48 h)	
Daphnia magna	Reproduction test	21 days	100% mortality after 3 days at 10×EQS (Mix14)	-	No effect at 1×EQS with respect to SC
CSN ISO 10706					
Caenorhabditis elegans	Growth	120 h	Effect in development for Mix19 (1×EQS)	-	Growth uniform between exposures until 72 h, deviating after 96 h
	Lipid accumulation	48 h	Increased accumulation of lipids in storage compartments (Mix14 10×EQS)	-	Mix19 tested only at 1×EQS
	Pharyngeal pumping	48, 72 h	No effect on food intake (pharyngeal pumping)	-	-
	Movement	48, 72, 96 h	No significant trends in movement	-	-
Caenorhabditis elegans	Induction of several stress response proteins	48 h	Mix19 (1×EQS) induced expression of gst-38, involved in phase II detoxification	-	No effect on mtl-2, ugt-, gcs-1, and Cyp-35a2-

TABLE 2. Continued

Organism/test	Biological endpoint	Exposure	Effects	EC_{50} (×EQS)	Comments
Dual-fluorescent transgenic organisms *Danio rerio* FET (EN ISO 15088)	FET	120 h	Malformations observed for Mix14 (10×EQS) and Mix19 (1×EQS)	-	Mix14 (1×EQS) no effect
Xenopus laevis FETAX ASTM E 1439-98	Frog embryo teratogenicity, embryo malformation	96 h	Mix14 (10×EQS): 62 ± 10%; Mix14 (1×EQS): 43 ± 12%; Mix19 (1×EQS): 34 ± 14% No effect on embryo length	-	15 ± 12% malformed embryos in SC
Dictyostelium discoideum (soil-living amoeba)	Lysosomal membrane stability	3 h	Effects statistically not different from the solvent	-	-
	Replication	24 h	No effect	-	-
Gasterosteus aculeatus (Three-spined stickleback) *Ex vivo* splenic leucocyte immune activities	Leucocyte distribution Cellular mortality Respiratory burst Lysosomal membrane integrity Phagocytosis activity	18 h	No effect on any of the endpoints tested	-	-
MTT assay, cell lines: RTG-2	*In vitro* cytotoxicity	72 h	No effect	-	-
		20 h	No effect	-	-
RPTEC/TERT1, HepG2, MCF7 HUVEC/TERT			No effect	-	-
Neutral red test H4IIE-luc cells	Acute cytotoxicity		No effect	-	-
xCELLigence Primary hepatocytes cultures, juvenile Atlantic salmon (*Salmo salar* L.)	Cytotoxicity system	up to 120 min	No effect	-	-
Atlantic salmon (*Salmo salar* L.)	ELISA (Vtg, Zrp regulation)	5 days	No effect	-	Maximum concentration tested was 0.16 EQS
	qRT-PCR (Vtg, ERα, Zrp) Regulation biomarkers	5 days	No effect	-	
HeLa, LMH, ZFL cells[b] Regulation biomarkers qRT-PCR	Gene expression	24, 40 h	HeLa: regulation of MT2A, AR, p53, GSTK1, IL6, IL8 LMH: regulation of IL8	-	No effect ZFL cells
YES	ER-binding activity	72 h	Activity measured for Mix14 and Mix19	92.3 (Mix14)	-

TABLE 2. Continued

Organism/test	Biological endpoint	Exposure	Effects	EC_{50} (×EQS)	Comments
				90.5 (Mix19)	
ER-CALUX		24 h	Activity measured for Mix14 and Mix19	4.9 (Mix14) 4.7 (Mix19)	-
ER-activated luciferase induction MELN cells		18 h	Activity measured for Mix14 and Mix19	34.2 (Mix14) 13.3 (Mix19)	-
wtERα^{LBD} binding assay		2 h	Binding measured for Mix14 and Mix19	IC_{50} 74.9 (Mix14) IC_{50} 7.8 (Mix19)	-
EASZY, *in vivo* transgenic zebrafish larvae		96 h	Activity measured for Mix14 above 4×EQS	-	-
PLHC-1 cells EROD induction	Dioxin-like activity	24 h	No effect	-	-
AR-CALUX	AR-binding activity	24 h	No effect	-	-
AR-activated luciferase induction MDA-kb2 cells		18 h	No effect	-	-
PPAR-CALUX	PPAR γ2-like activity	24 h	No effect	-	-
PXR-activated luciferase induction, HG5LN-PXR cells	PXR-binding activity	18 h	Effect >10×EQS	-	-

[a] All tested reporter genes are detailed in Supplementary table 2.
[b] All tested reporter genes are detailed in Supplementary table 3.

an EC_{50} of 30 (*T. pseudonana*) < 56 (*C. reinhardtii*) < 105 (*P. subcapitata*) ×EQS. The growth inhibition assays with *P. subcapitata* performed for 72 h and 96 h of exposure by other three laboratories measured no significant effect at 1×EQS for either Mix14 or Mix19, similar to the results obtained at 24-h exposure. Exposure to Mix14 at a higher concentration (10×EQS) in the three laboratories led to inhibition of *P. subcapitata* growth by 31, 13, and 14%, respectively.

Effects on D. Magna

The calculated EC_{50} values for acute immobilization at 24-h and 48-h exposure to Mix14 was 8× and 2.8×EQS, respectively (Fig. 3a). Additionally, the results with Mix14 at 10×EQS were comparable among the three laboratories performing the bioassay (Fig. 3b). Both mixtures at 1×EQS did not induce any significant effect on the acute immobilization of *D. magna* neither in the chronic reproduction test. However, exposure to Mix14 at 10×EQS proved to be highly toxic with longer exposure times leading to 100% mortality after 3 days.

Embryo Toxicity and Development

After exposure for 5 days, effects in several endpoints related to FET were observed at 1×EQS for Mix19 and 10×EQS for Mix14, as detailed in Table 3. Effects specifically observed included mortality, a change in the number of hatched embryos, head deformations, tail deformations, absence of gas bladder, generally underdeveloped embryos, and embryo length (examples shown in Fig. 4). On shorter times of exposure, only higher concentrations of the mixture triggered significant effects in FET, particularly in terms of the number of defective embryos after 72 h and in the number of hatched embryos after 96 h (Table 3).

The studied mixtures also impaired the development of frog embryo. Using the Frog Embryo Teratogenesis Assay Xenopus (FETAX), 43 ± 12% and 34 ± 14% malformed frog embryos were observed for exposure to 1×EQS of Mix14 and Mix19, respectively, whereas exposure to 10×EQS of Mix14 caused 62 ± 10% malformed embryos. The effects were significantly different from SC (ANOVA, Dunnett post-test, $p < 0.05$), which proved to be moderately toxic (15 ± 12% malformed embryos). The most commonly observed malformations in FETAX included incomplete gut coiling and skeletal malformations such as flexed and waivy tail (see Fig. 4 and Table 3). Eye deformities or thoracic edema were also recorded in lower frequency.

In the bioassays using the nematode *C. elegans*, growth was uniform among the different treatments with the mixtures or solvent during the first 72 h (namely the larval stages L1–L4), but started to deviate after worms had reached adulthood. Nematodes chronically exposed (from L1 stage) to Mix19 at 1×EQS were marked by a statistically significant reduction in final length after 120 h (see Supplementary fig. 2). Though smaller in final size, these worms nevertheless reached adulthood and were able to reproduce, suggesting that the observed phenotype did not affect developmental or reproductive indices.

TABLE 3. Effect of Mixtures on the FET Test with Zebrafish and the FETAX

	Time	Endpoint	Chemical mixture		
			Mix14 10×EQS	Mix14 1×EQS	Mix19 1×EQS
FET	72 h	Number of defected embryos	a	-	-
	96 h	Number of hatched embryos	a	-	-
	120 h	Number of defected embryos	a, c	-	a
		Head deformities	a	-	-
		Absence of gas bladder	a	-	-
		Underdeveloped embryos	a	-	a, c
FETAX	96 h	Total number of malformed embryos	a	a	a
		Incomplete gut coiling	a	-	a
		Tail malformation	a	-	a

a: endpoint significantly different from SC (chi-square test, $p < 0.05$); c: endpoint significantly different from SC (ANOVA followed by Fisher LSD *post hoc* test).

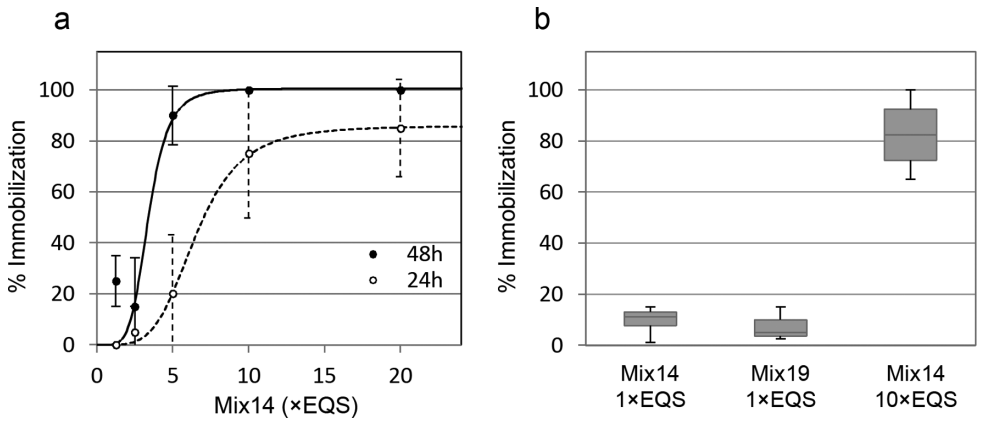


FIG. 3. Acute immobilization in *D. magna*. (a) Dose response of Mix14 in EQS equivalent concentrations, for immobilization at 24-h exposure (open symbols) and 48-h exposure (closed symbols). The lines represent the fit of non-linear regression model to the data for the calculation of the EC_{50}. Error bars represent the standard deviation, $n = 4$. (b) Combined immobilization data from three different laboratories for Mix14 (at 1× and 10×EQS) and Mix19 (at 10×EQS).

Nuclear Receptors Binding Activity

The activity of four different human receptors was assessed in this study with respect to the tested mixtures, i.e., ER, AR, peroxisome proliferator-activated receptor (PPAR), and pregnane X receptor (PXR). No activity was measured associated with the binding to the AR, PPAR in all concentrations tested, whereas PXR-mediated activity was measured only at concentrations of the mixture >50×EQS (Table 2).

Four *in vitro* methods, ER-CALUX, MELN, YES, and a competition assay with recombinant wtERα^{LBD} detected estrogenic activity of the mixtures close to the EQS concentration (Fig. 5). The model compound 17β-estradiol was used as a reference compound (EC_{50} values shown in Fig. 5) with the three ER-mediated transactivation assays yielding EC_{50} values that were similar to those previously reported (Leusch *et al.*, 2010). Estrogenic activity was detected at lower concentrations of the mixtures for the ER-CALUX, followed by the MELN assay, the recombinant ERα competition assay, and finally the YES assay (Fig. 5).

In addition, the *in vivo* EASZY test was performed using transgenic zebrafish larvae. In this test, Mix14 induced GFP expression in a dose-dependent manner, which was significant at 4×EQS (Fig. 6), whereas for Mix19, tested only up to 0.4×EQS, no effect was observed.

Molecular Biomarkers

Among the bioluminescent *E. coli* reporters, the sensor elements exhibiting the lowest detection thresholds for Mix14 were the *zntA* and *arsR* gene promoters, indicating the presence of heavy metals at concentrations higher than 6.2×EQS (Supplementary fig. 1). In Mix19, the *micF* gene promoter (indicator of chemical-induced oxidative stress) and *cydA* (indicator of respiratory inhi-

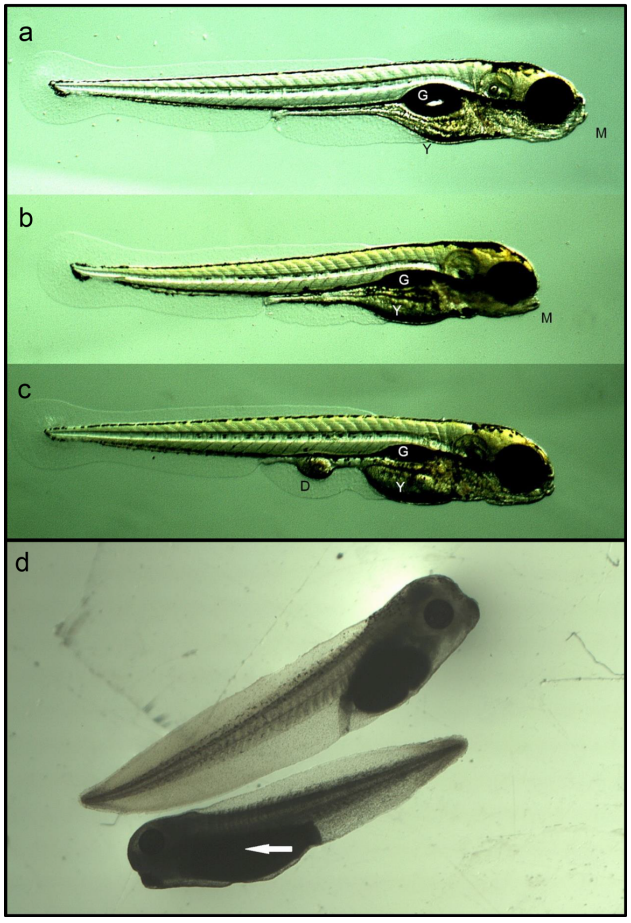
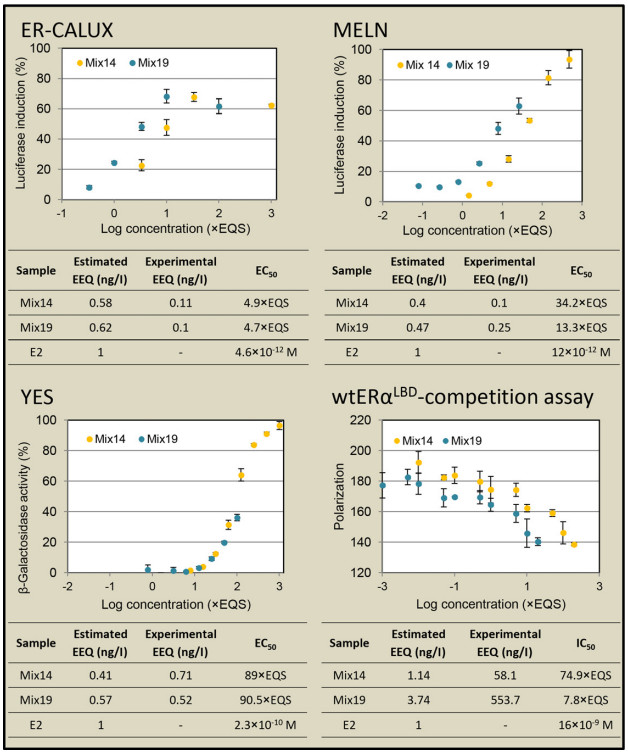


FIG. 4. Embryos of *Danio rerio* from the FET (a)–(c) and *Xenopus laevis* from FETAX (d). (a) Control fish embryo 120-h post fertilization. (b) Embryo exposed to Mix14 at 10×EQS for 120 h - typical underdeveloped (smaller) embryo with non-inflated gas (swimming) bladder (G), deformed head especially at the mouth region (M), and not fully consumed yolk (Y). (c) Embryo from the same exposure as in panel (b) with highlighted deformation nearby the anal region (D), non-inflated gas bladder (G), and not fully consumed yolk (Y). (d) Control 96-h embryo of *X. laevis* (upper individual) compared with underdeveloped and malformed embryo exposed for 96 h to MIX19 1xEQS (the arrow shows the incomplete intestine coiling, which was the most frequent malformation observed).



Sample	Estimated EEQ (ng/l)	Experimental EEQ (ng/l)	EC_{50}
Mix14	0.58	0.11	4.9×EQS
Mix19	0.62	0.1	4.7×EQS
E2	1	-	4.6×10^{-12} M

Sample	Estimated EEQ (ng/l)	Experimental EEQ (ng/l)	EC_{50}
Mix14	0.4	0.1	34.2×EQS
Mix19	0.47	0.25	13.3×EQS
E2	1	-	12×10^{-12} M

Sample	Estimated EEQ (ng/l)	Experimental EEQ (ng/l)	EC_{50}
Mix14	0.41	0.71	89×EQS
Mix19	0.57	0.52	90.5×EQS
E2	1	-	2.3×10^{-10} M

Sample	Estimated EEQ (ng/l)	Experimental EEQ (ng/l)	IC_{50}
Mix14	1.14	58.1	74.9×EQS
Mix19	3.74	553.7	7.8×EQS
E2	1	-	16×10^{-9} M

FIG. 5. Estrogenic activity measurement using *in vitro* bioassays. Dose-dependent estrogenic activity of Mix14 and Mix19 was measured via ER-activated luminescence induction using the ER-CALUX and the MELN system, the β-galactosidase activity using the YES test, and the competition assay using the recombinant wtERα^{LBD}. The EC_{50} values are shown, calculated from the fit to the data measured with the two mixtures and of E2 in the test, as well as the estimated and experimental EEQ concentrations. The error bars represent the standard deviation, $n = 3$.

bition) were induced above 0.16× and 5×EQS, respectively (Supplementary fig. 1).

In addition, a transgenic *C. elegans* strain, carrying the red fluorescent protein reporter gene under the promoter of the glutathione-S-transferase *gst-38*, was responsive to Mix19. GST is a protein involved in phase II detoxification and its induction was significant ($p < 0.05$) in Mix19 at 1×EQS, but not in Mix14, even at 10×EQS (Supplementary fig. 2).

Finally, the expression of several genes was modified in HeLa cells following exposure to the mixtures (Supplementary fig. 3). The highest regulation was found for the *IL6* gene with an increase by 4-fold in Mix19 and by 2.5-fold in Mix14 at 1×EQS. The other regulated genes showed a decreased expression (<2-fold decrease) in Mix14 (at 1× and 10×EQS), but not in Mix19, and included the *AR*, *mt2A*, *GSTK1*, *IL8*, and *p53* genes (Supplementary fig. 3). None of the tested genes responded to the mixtures in the ZFL cells. In LMH cells, only *IL8* showed a small downregulation following exposure to Mix14 at 10×EQS.

Additional bioassays performed in the exercise either displayed no effect with the mixtures or measured an effect only at concentrations higher than 10×EQS (Table 2). Some widely used bioassays did not detect an effect of the mixtures at low concentrations. This was the case of the acute toxicity bioassay with *Vibrio fischeri*, which was tested in four different laboratories, with a measured EC_{50} around 400× and 200×EQS for Mix14 and Mix19, respectively.

DISCUSSION

In the last few years, concern over the impact of chemical mixtures on human and ecosystem health has been highlighted by the scientific community and brought to the attention of the European Commission (SCHER, SCENIHR and SCCS, 2012).

The exercise described here employed chemical mixtures at concentrations of the individual compounds believed to be safe and studied the hazard to wildlife organisms of different trophic levels. Artificial mixtures were produced as reference solutions to ensure that the chemical composition and concentrations were known, and in this way facilitate a direct association between chemical and biological effect. Such cause-effect relationships would likely be harder to reach with complex environmental samples, although this is definitely an important matter to address in the future.

By using a battery of ecotoxicity bioassays, ranging from gene-expression tests to whole organism bioassays, we demonstrate biologically relevant effects of chemical mixtures where each contaminant exists at or in some cases considerably below

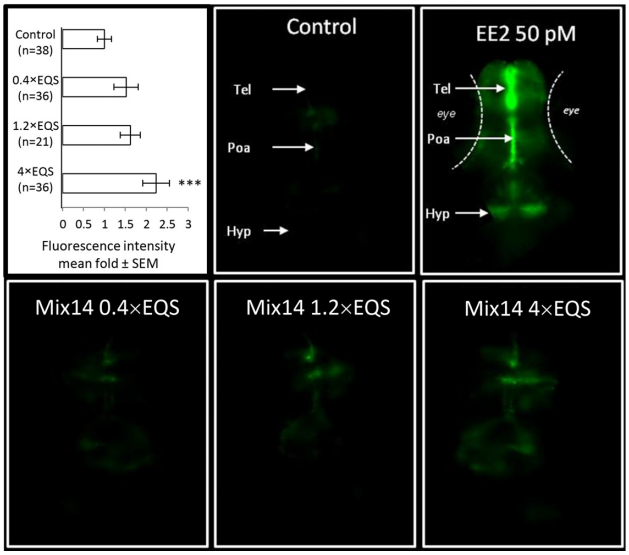


FIG. 6. *In vivo* estrogenic activity of Mix14 as shown by induction of GFP in 96-hpf-old transgenic cyp191ab-GFP zebrafish larvae. Exposure was done at different concentrations of Mix14, during 96 h from fertilization, under static condition, after which fluorescence imaging on living zebrafish was performed. GFP was expressed in various brain regions in radial glial cells. Dorsal view, magnification X10, Tel: telencephal; Poa: preoptic area; Hyp: inferior lobe of hypothalamus. EE2 50pM was used as positive control. The mean fluorescent intensity is shown in the graph, indicating the number of larvae imaged for each condition (*n*), ***$p < 0.001$. EE2 led to a 26-fold induction.

the EQS concentration. Effects of the mixtures at 1×EQS were observed across a wide range of taxa that included bacteria, algae, nematodes, fish and amphibians. These results seriously question the present paradigm for assessing the safety of chemicals to the environment and demonstrate that regulatory safety concentrations (EQS) may not provide sufficient protection when multiple chemicals are present.

The interpretation of the toxicity results measured in our artificial mixtures with respect to environmental samples could be a matter of discussion. Most of the chemical pollutants in environmental samples are usually found at concentrations considerably below the safety limits for toxicological effects, and concentrations exceeding the EQS values of priority pollutants are reported for only a minority of the monitored samples. A summary of a literature search on EQS exceedances from surface water monitoring data in Europe in recent years can be found in Supplementary table 1. WFD EQS exceedances (in some countries) concern usually only a small number of "ubiquitous" substances [e.g., mercury, cadmium, tributyltin, brominated diphenylethers, some polyaromatic hydrocarbons (PAHs), nickel, and Di(2-ethylhexyl)phthalate (DEHP)].

On the other hand, the number of chemicals present in environmental samples likely exceeds the 14 or 19 included in the artificial mixtures of this exercise. When multiple components in a sample, even at low concentrations, affect the same pathway, their combined toxicity can usually be described by the concentration addition concept and may induce significant toxicity to aquatic organisms (Broderius, 1990). This was confirmed in this study for the algae toxicity elicited by the four herbicides in the mixture (diuron, atrazine, isoproturon, and simazine), acting as PSII inhibitors, and the endocrine disruptor compounds (E2, 4-nonylphenol and bisphenol A) binding to the ER and activating the expression of reporter genes.

A less predictable hazard may arise from combinations of chemicals from different classes and with different modes of action. This is the case for the well-known heavy metal modulation of cytochrome P450 1A1 (CYP1A1) expression and activity, responsible for xenobiotic metabolism and activation (Anwar-Mohamed *et al.*, 2009). Another example is the inhibition by several contaminants of cellular efflux pumps, which are multixenobiotic resistance transporters, thus potentiating the cellular accumulation and toxicity of other chemicals. This mechanism has been reported in echinoid larvae (Anselmo *et al.*, 2012) as well as in zebrafish embryo (Fischer *et al.*, 2013).

The fact that the correlation between ecological and chemical indicators has not been straightforward in the implementation of the WFD, further substantiates the need for complementary indicators. The assessment of biological effects in key trophic organisms could play this part in linking ecological and chemical assessment by providing the combined toxicity from all chemicals present.

This study shows that co-occurring chemicals can elicit an effect in some ecologically relevant and surrogate organisms in a manner that may imbalance the entire ecosystem. The concentrations selected for each chemical in the mixtures were that of the AA-EQS, a safety threshold under European legislation aiming to protect the environment from chronic toxicity effects. However, the mixture at AA-EQS in this study was able to induce effects in both chronic and acute toxicity tests. Even stronger toxicological effects were visible when the mixtures were tested at concentrations corresponding to the maximum allowed concentration (MAC-EQS), as indicated by the responses in several of the bioassays.

At the lower trophic level, the study showed that the mixtures at EQS equivalent concentrations affected the bacteria-phytoplankton composition in a marine microcosm, with a significant reduction in the phytoplankton community and an increase in the bacteria population. The increase in bacterial growth rate might be due to fast selection of bacteria that are capable of utilizing selected pollutants or dissolved organic carbon released by decaying phytoplankton. Unfortunately, no measurements of dissolved organic compounds were performed simultaneous with the treatments to assess this possibility. An imbalanced composition of bacteria/plankton population would likely influence the ecosystem functioning (food wed, biodiversity, ecosystem services) (Naeem *et al.*, 2000).

No effect was observed at the AA-EQS equivalent concentration of the mixtures at the single species level for the three microalgae (*P. subcapitata*, *C. reinhardtii*, and *T. pseudonana*), indicating that this value is sufficiently protective when considering only four herbicides with a similar mode of action. However, at concentrations of the mixture corresponding to the MAC-EQS, an effect was measured for the PSII inhibition endpoint.

Going up in the trophic levels, other endpoints for which an effect was observed close to EQS concentrations included the acute immobilization of *D. magna* and effects on toxicity and development of fish and frog embryos. Several of the substances in the mixtures have been described as embryotoxic or teratogenic. These include the pharmaceuticals sulfamethoxazole and carbamazepine (Richards and Cole, 2006), chlorpyrifos (Bonfanti *et al.*, 2004), atrazine (Fort *et al.*, 2004), the polyaromatic hydrocarbons BaP (Fort *et al.*, 1989), and fluoranthene (Hatch and Burton, 1998), E2 and bisphenol A (Saili *et al.*, 2013). However, the effects of these substances have been reported only at concentrations exceeding those currently detected in surface waters and the ways they interfere with developmental processes is poorly understood. Their combined action cannot directly explain the

observed toxicity of the mixtures to fish and frog embryos in this study. Developmental effects and daphnia immobilization are general endpoints that may be triggered by a multitude of substances, molecular targets, and intercalating events. They represent a bigger challenge in linking the observed effect from the mixture to specific substances.

Diverse and unpredictable combinatorial effects of mixtures have been well documented, when the individual substances appear safe when tested alone, including for endocrine disrupting chemicals (EDCs) with other compounds (Fagin, 2012).

Additional responses of the mixtures at concentrations close to EQS values were measured in this exercise by estrogen-receptor mediated *in vitro* and *in vivo* bioassays. Several chemical substances released into the environment are able to mimic the action of natural hormones by binding to the ER and may show estrogenic activity, thereby influencing the sexual function and differentiation in aquatic organisms. Some of the substances included in the mixtures are known ligands for the ER, including the natural estrogen 17β-estradiol, 4-nonylphenol, bisphenol A, and possibly triclosan although with lower potency (Svobodová *et al.*, 2009; Torres-Duarte *et al.*, 2012). It is possible that also other substances in the mixtures may bind to the ER in an agonist or antagonist way. Binding of different compounds in the mixtures to the ER without activation of the downstream pathway could explain the highest experimental EEQ in the wtERα^{LBD} competition assay, with respect to the estimated EEQ. A difference between estimated and experimental EEQ was also observed in the ER-CALUX and MELN assays and may be the result of a mixture antagonistic effect, although this requires further investigation. Binding of several molecules to hERα is well known and proven also by co-crystallization of the receptor (Baker, 2011). The binding can occur in an agonist or antagonist way. This suggests a wide flexibility of the ligand binding domain to accommodate chemically different structures into its active site.

The *in vitro* tests used in this study are suitable assays for monitoring of estrogenic activity in water samples, and interestingly, the estrogenic activity was further confirmed in intact fish embryos as measured by the brain-specific upregulation of the ER-mediated cyp191ab expression during early and critical developmental stages. The rising interest in bioassays as alternative tools for the detection of estrogens in water close to the European regulatory limits lies in the fact that EQS values of estrogenic compounds of concern (E2 and EE2) are below the analytical limits of quantification of most routine chemical methods (Loos, 2012).

We could show that exposure to mixtures of dissimilarly acting substances at concentrations considered environmentally acceptable can exert significant effects on the biota. In this exercise, the bioassays showed i) general comparability among the laboratories for the same assay, ii) complementarity covering several trophic levels of the ecosystem, and iii) potential for the future implementation in water management as holistic approaches for the ecological risk assessment of chemicals under realistic conditions.

Chemical monitoring alone cannot assess the quality status of water impacted by anthropogenic mixtures. Bioassays can be included in the workflow, and their selection should be based on the outcome of a risk assessment of the specific water body, taking into account the known sources of pollutants (e.g., agriculture, industry, household, hospital, etc.), expected concentrations but also considering the methods cost, technical time, and concentration range applicability. In any case, there is no "one size fits all" bioassay that could provide the toxicological potency of every mixture toward all aquatic organisms in all water bodies, but rather a battery of bioassays that should be selected as "fit for purpose". Whether the focus is on low concentration of pollutants such as those found in most fresh and marine waters, or higher concentration of pollutants, e.g., in wastewater treatment plant effluents, different batteries of bioassay can be selected to provide a snapshot of the ecosystem health.

Furthermore, the use of tailor-made reference mixtures with rather-characterized modes of acting chemicals, as described in this study, could i) aid the "quantification" of the observed effects in terms of toxicity units, ii) allow intercalibration among laboratories using the same bioassay, and iii) help establishing a threshold for "no observed mixture effect" in future regulatory applications.

In conclusion, the present study highlights an urgent need to revise tools and paradigms used to assess the safety of chemicals to the environment. Bioassays as part of a multi-tier approach to water quality monitoring can fill the gap between chemical and ecological assessments for a more holistic characterization of water quality.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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